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Ozone-induced accumulation of carbohydrates changes enzyme activities of carbohydrate metabolism in birch leaves

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SUMMARY

Birch cuttings (*Betula pendula* Roth) were grown in a sand-culture system with two concentrations (0·05, HF and 0·005 %, LF) of fertilizer containing macronutrients and micronutrients, and were exposed to 90/40 nl l^{-1} O_3 (day/night) and < 3 nl l^{-1} O_3 (control) for one growing season in the field fumigation chambers at Birmensdorf (Switzerland). Leaves of different ages were analysed for gas exchange, contents of chlorophyll, protein, and for metabolites as well as enzyme activities of carbohydrate metabolism.

Ozone reduced net photosynthesis and chlorophyll contents in mature leaves of both fertilization treatments, whereas that of protein was only reduced in high-fertilized plants (HF). However, net photosynthesis, chlorophyll, and protein increased in young leaves of low-fertilized plants (LF). The effects of ozone on enzyme activities of carbohydrate metabolism were most pronounced in leaves of LF plants. Specific activities of the sucrose-cleaving enzymes, sucrose synthase and alkaline invertase, were induced, whereas acid invertase was unchanged. Extractable activity of sucrose phosphate synthase, which is a key enzyme of sucrose synthesis, was reduced. Levels of fructose 2,6-bisphosphate, an inhibitor of sucrose synthesis, were increased in leaves of O_3/LF plants, but reduced in O_3/HF plants. In addition, activities of enzymes involved in starch metabolism, ADP-glucose pyrophosphorylase and starch phosphorylase, were lowered in ozone-treated samples and the ratio of ATP : ADP was increased.

It is concluded that chronic ozone exposure leads to an inhibition of sucrose synthesis and favours sucrose degradation. This effect is modulated by the nutrient status of the plants, indicating higher O_3 tolerance in HF plants. Furthermore, as the metabolic responses in the ozone-treated samples resemble very closely those observed under end-product inhibition of photosynthesis, we assume that the O_3 effect is mainly due to reduced photosynthate export.

Key words: Ozone, regulation of carbohydrate metabolism, birch (*Betula pendula* Roth), carbon allocation/partitioning, sugar accumulation.

INTRODUCTION

Ozone is a widespread air pollutant and affects vigour of sensitive plants at increased ambient concentrations. The most important consequence of ozone-induced injury is a reduction of productivity which has been observed after fumigation of crops and trees. Although the exact mechanism of the injury process is still poorly understood, changes in carbon allocation and a decline of net photosynthesis are well documented and can affect plant growth (for reviews see Darrall, 1989; Hampp, 1992; Heath, 1994; Matyssek *et al.*, 1995; Schmieden & Wild, 1995). In many fumigation experiments reduced C export and an accumulation of carbohydrates in

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Abbreviations: AGPase, ADP-glucose pyrophosphorylase; F26BP, fructose 2,6-bisphosphate; HF, high-fertilized; LF, low-fertilized; PV(P)P, polyvinyl(poly)pyrrolidone; SPS, sucrose phosphate synthase.

mature leaves were found (Ito, Mitsumori & Totsuka, 1985; Küppers & Klumpp, 1988; Luethy-Krause & Landolt, 1990; Bücker & Ballach, 1992; Matyssek *et al.*, 1992; Willenbrink & Schatten, 1993; Landolt *et al.*, 1994). Accumulation of carbohydrates or disturbances of C flux can lead to regulatory changes of metabolism: A feedback control of sucrose synthesis reduces activities of key enzymes such as sucrose phosphate synthase and fructose 1,6-bisphosphatase, and induces enzyme activities of sucrose breakdown (sucrose synthase and invertase) thus favouring glycolysis (for review see Koch, 1996). Furthermore, reduced utilization of photosynthetic end-products can reduce the size of the photosynthetic apparatus (Krapp, Quick & Stitt, 1991). Thus, a decrease in net photosynthesis in leaves after chronic ozone exposure could result from an accumulation of carbohydrates as a consequence of an inhibited sugar export.

In addition to ozone, sucrose synthesis and transport can be influenced by other factors such as developmental state of the leaves (Turgeon, 1989), diurnal changes (Gerhardt, Stitt & Held, 1987) and supply of nutrients, especially of nitrogen and magnesium (Wikström & Ericsson, 1995 and literature cited therein).

Therefore, it was the aim of this study to investigate regulatory responses of carbohydrate metabolism in birch leaves to long-term ozone exposure in relation to the nutritional and developmental status of the leaves. For this purpose, birch plants were treated with moderately elevated ozone concentrations (40–90 nl l^{-1}) and two different regimes of nutrient supply.

MATERIALS AND METHODS

Birch plants (*Betula pendula* Roth) were grown from cuttings in the Birmensdorf field fumigation chambers (Landolt, Pfenninger & Lüthy-Krause, 1989; Saurer *et al.*, 1995) in a sand-culture system with two different concentrations of a commercial fertilizer (high fertilization (HF), 0·05 %; or low fertilization (LF), 0·005 %; corresponding to 6·5 mM N or 0·65 mM N, respectively) which contained macronutrients and micronutrients balanced in an optimal range (see Landolt *et al.*, 1997). Plants in both fertilization treatments were exposed to charcoal-filtered air (controls) or charcoal-filtered air enriched by ozone (O_3): 40 nl l^{-1} O_3 from 2100 to 0700 hours and 90 nl l^{-1} O_3 from 0700 to 2100 hours in the fumigation chambers, from early May until mid-September. Details of plant culture and the fumigation system were described by Landolt *et al.* (1989) and Saurer *et al.* (1995).

Gas exchange of leaves of different age classes from the main axis was measured shortly before harvest using an open gas-exchange system (Walz, Effeltrich, Germany) as described by Matyssek *et al.* (1991). The rate of net photosynthesis was calculated according to von Caemmerer & Farquhar (1981) and was based on the one-sided leaf area which was determined with the Delta-T area meter MK2.

In order to evaluate the effect of leaf age on C metabolism every second (LF) or third (HF) leaf was harvested from five trees per treatment. Leaves were separately quick-frozen in liquid nitrogen. Effects of changing environmental factors such as irradiance and temperature on parameters of carbohydrate metabolism were minimized by reducing the time of sampling (1100–1300 hours). Homogenization and lyophilization of individual leaves were as described by Hampp, Rieger & Outlaw (1990). Chlorophyll was extracted in 95 % *N*,*N*-dimethylformamide and determined photometrically according to Inskeep & Bloom (1985).

Fructose 2,6-bisphosphate was extracted in a buffer containing 100 mM β-mercaptoethanol at pH 10 and 5 % polyvinylpolypyrrolidone (PVPP), and assayed through the activation of pyrophosphate fructose 6-phosphate kinase according to Wingler *et al.* (1994). Extraction and determination of ATP and ADP were based on the method described by Hampp (1985). About 3 mg of leaf material were extracted in 10 % perchloric acid, containing 5 % PVPP. Ten μl of the neutralized extract were incubated with 85 μl of reconstituted luminescence reagent, and ATP-dependent light emission was recorded in a microplate luminometer (Dynatech, Denkendorf, Germany).

Extracts of protein and enzymes were prepared in a buffer system containing Tris/borate (300 mM/100 mM, pH 7·6, 4 °C, Guttenberger, Schaeffer & Hampp, 1994) supplemented with 1 mM β-mercaptoethanol, 0·85 % BSA, 5 % polyvinylpyrrolidone (PVP; sucrose phosphate synthase, SPS), 1 mM β-mercaptoethanol, 3 % PVP (sucrose synthase), 3 % PVP (acid invertase), 13 % PVPP (alkaline invertase, ADP-glucose pyrophosphorylase (AGPase), and starch phosphorylase). Extractable activities of sucrose phosphate synthase, sucrose synthase, acid (pH 3·8) and alkaline invertase (pH 6·8) were measured using a microplate photometer according to Egger & Hampp (1993). ADP-glucose pyrophosphorylase and starch phosphorylase were determined as described by Egger *et al.* (1996).

To avoid interferences from phenolic compounds within the leaf extract, protein was determined according to Bradford (1976) after precipitation in saturating $(NH_4)_2SO_4$-solution. Extraction and determination of metabolites and enzymes were checked for linearity with respect to leaf d. wt per extract volume and extract volume per assay.

The statistical significance of effects of ozone treatments was tested by comparing leaves of corresponding positions using the Kruskal & Wallis test of ranks (STSC Statgraphics Software Package, Rockville, USA).

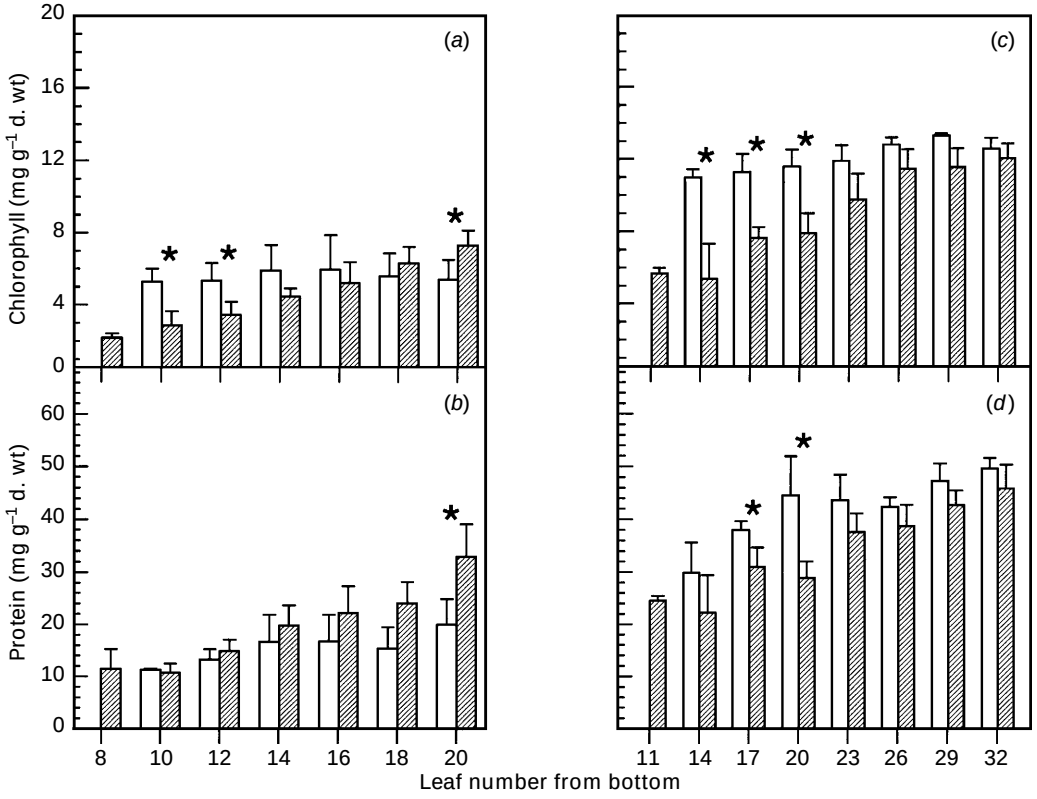

Figure 1. Contents of chlorophyll and protein in leaves of ozone-fumigated (▨) and control plants (□) of two fertilization treatments. (*a*, *b*) Low fertilization. (*c*, *d*). High fertilization. Old leaves are indicated by low numbers. Data represent means ±SD. $*P \leqslant 0{\cdot}05$.

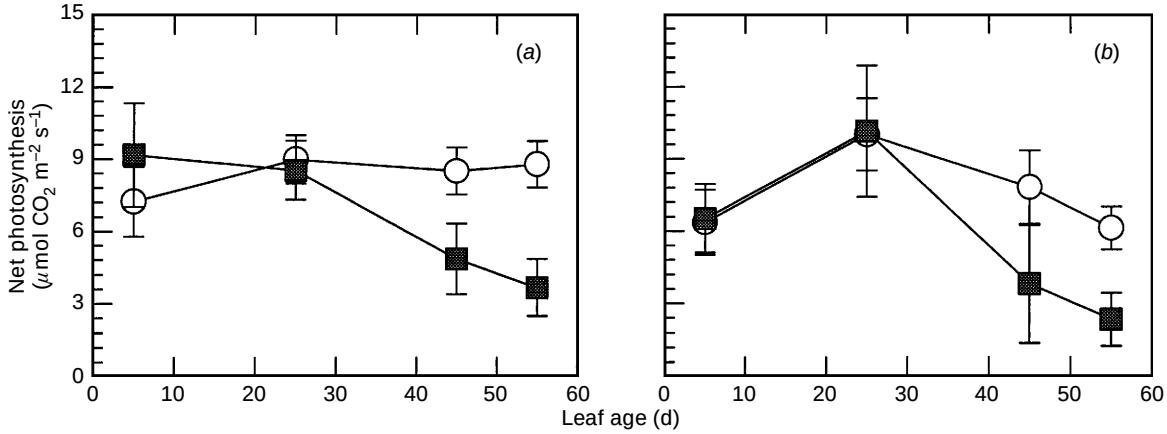

Figure 2. Net photosynthesis of leaves of different age classes of ozone-fumigated (■) and control plants (○) of two fertilization treatments. (*a*) Low fertilization. (*b*) High fertilization. Gas exchange was registered shortly before harvest at ambient CO_2 concentrations and saturating light (PAR > 750 μmol m^{-2} s^{-1}). Data represent means ±SD ($n = 4$–8).

RESULTS

Chlorophyll and protein were determined as basic parameters which allow the quantification of visible discoloration or serve as reference parameters for enzyme activities, respectively. High fertilization nearly doubled chlorophyll and protein contents of the leaves (Fig. 1). Independent of fertilization, ozone reduced chlorophyll in mature leaves compared with controls, whereas protein was only reduced in the HF treatment (Fig.1). In young leaves, however, chlorophyll and protein contents were almost unchanged (HF) or even higher (LF).

Ozone-induced changes of chlorophyll contents were closely correlated with the rate of net photosynthesis. In mature ozone-treated leaves of both nutrient regimes, net photosynthesis was less than that of controls, whereas it was greater in young leaves of the O_3/LF and no differences occurred in young HF-samples (Fig. 2).

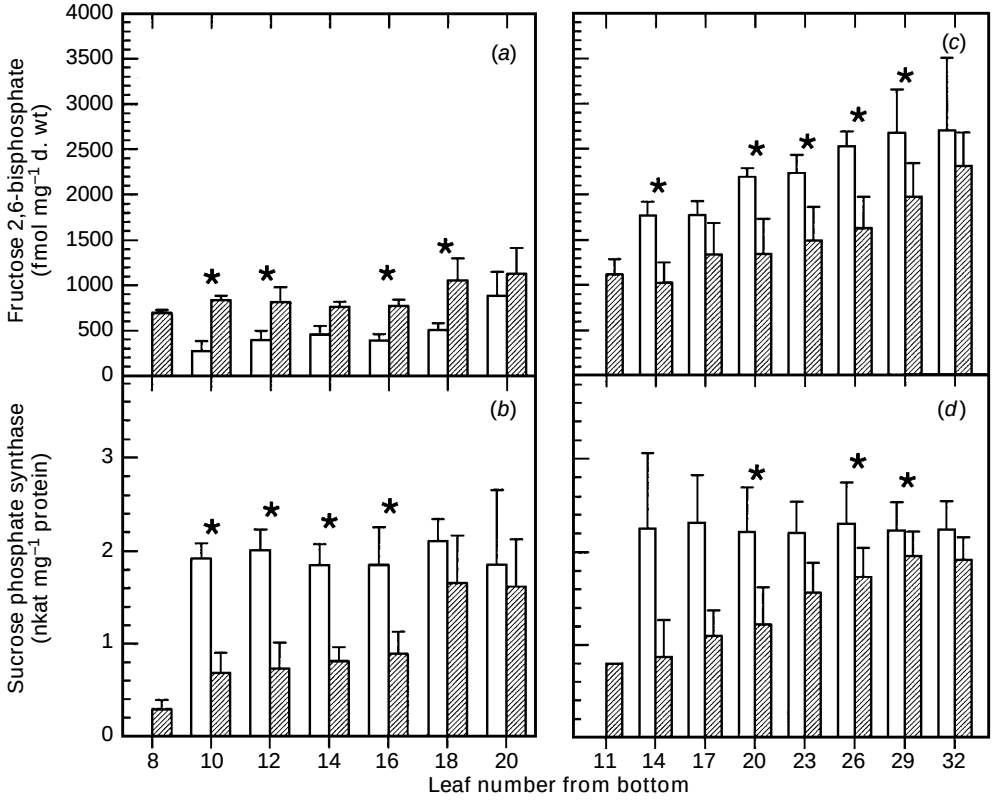

Figure 3. Contents of fructose 2,6-bisphosphate and specific activity of sucrose phosphate synthase in leaves of ozone-fumigated (▨) and control plants (□) of two fertilization treatments. (*a*, *b*) Low fertilization. (*c*, *d*) High fertilization. Old leaves are indicated by low numbers. Data represent means ± SE. * $P \leqslant 0.05$.

High fertilization increased the content of fructose 2,6-bisphosphate (F26BP), which is an inhibitor of fructose 1,6-bisphosphatase and thus affects sucrose synthesis. Effects of ozone on F26BP content were modulated by fertilization. In LF plants, ozone increased F26BP levels in leaves independent of age, whereas in HF plants, ozone decreased F26BP contents (Fig. 3*a*, *c*).

Specific activities of most enzymes were only slightly affected by fertilization. Extractable activity of SPS, a key enzyme of sucrose synthesis, declined in older ozone-treated leaves in both fertilization treatments (Fig. 3*b*, *d*).

By contrast, activities of sucrose synthase and alkaline invertase, both involved in cleavage of sucrose, increased in ozone-treated samples (Fig. 4). Acid invertase, on the other hand, was not affected by ozone, but increased in low-fertilized samples.

Like the enzyme activities, the ATP:ADP ratio, an indicator of cellular energy state, was only slightly affected by fertilization. Ozone induced an increase of the ATP:ADP ratio preferentially in older leaves which was most pronounced in the LF treatment (Fig. 4*d*, *h*). Enzymes involved in starch synthesis and degradation such as AGPase and starch phosphorylase were also affected by ozone (Fig. 5). Specific activity of both enzymes was slightly diminished in O_3/HF leaves, whereas in O_3/LF leaves the decrease was more pronounced.

DISCUSSION

The principal objective of this study was to investigate the extent to which different nutrient supplies might affect the O_3-tolerance of birch leaves. Fertilization of plants resulted in different growth rates, C allocation pattern and leaf nutrient contents. For N, 3·6% (HF) and 1·9% (LF) of d. wt were found (Landolt *et al*., 1997).

Leaf N concentrations in both treatments were in a range described for birch plants with different growth rates (Ingestad & McDonald, 1989) and leaves showed no signs of deficiency. Our data indicate that high fertilization not only led to an increase of leaf contents of chlorophyll and protein, but also affected ozone-induced changes of both parameters. Increased contents of chlorophyll and protein in young leaves of O_3/LF treatments suggest an increased N mobilization from old leaves. Evidence for an increased movement of N-compounds between senescing and young leaves in N-limited plants was also found after ozone exposure by Pell, Sinn & Johansen (1995). This could represent a compensation for reduced productivity of old leaves in order to stimulate primary production of newly formed leaves, since it was mirrored by the rate of net photosynthesis in our samples. But independent of fertilization, chlorophyll loss and the decrease of net photosynthesis in older leaves were similar in

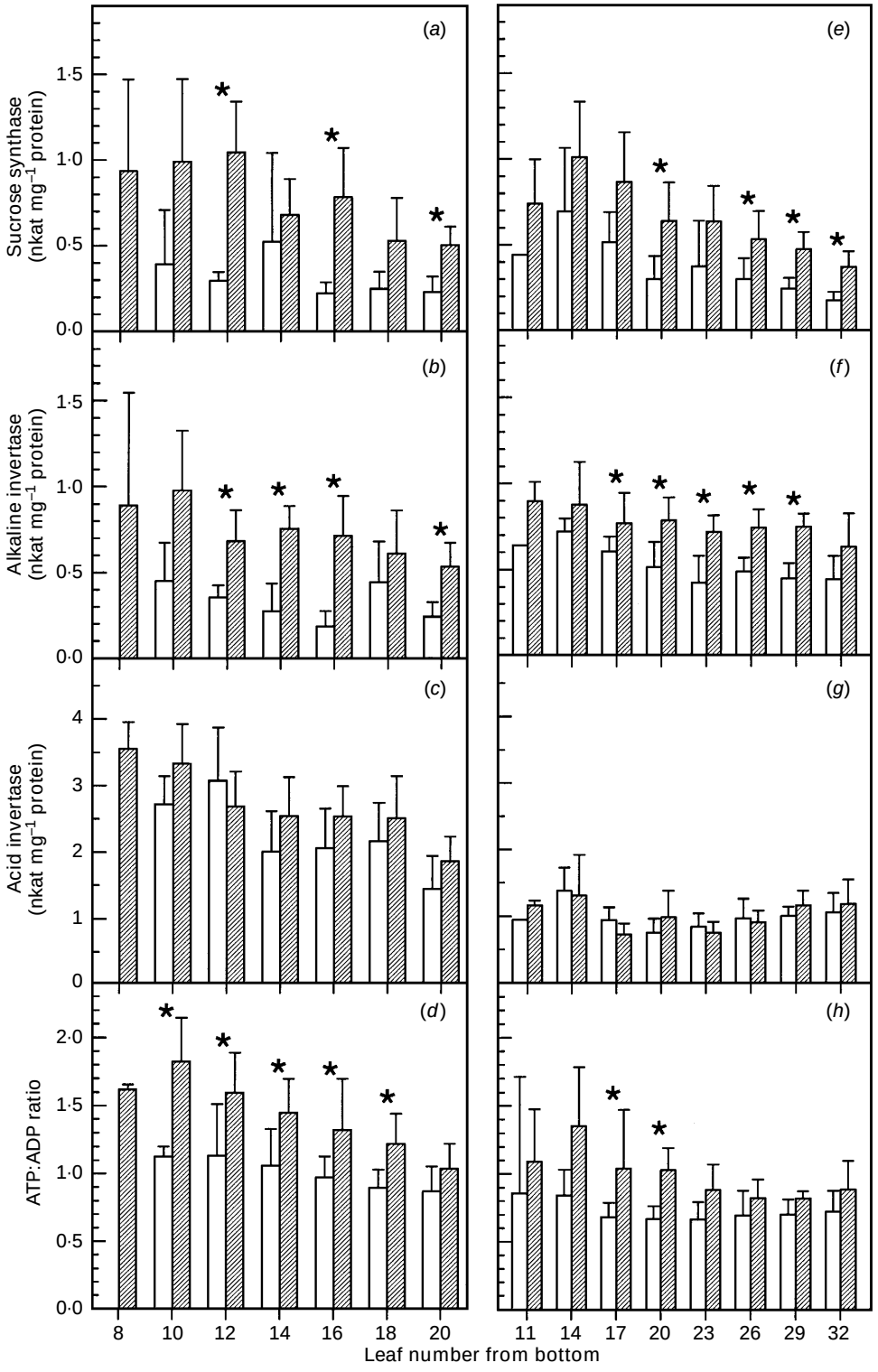

Figure 4. Specific activities of sucrose synthase, alkaline, and acid invertase, and ratios of ATP : ADP in leaves of ozone-fumigated (▨) and control plants (□) of two fertilization treatments. (*a–d*) Low fertilization. (*e–h*) High fertilization. Old leaves are indicated by low numbers. Data represent means ± SD. * $P \leqslant 0.05$.

both nutrient regimes. This correlates with the occurrence of visible symptoms (Landolt *et al.*, 1997) and is a typical effect of ozone on older leaves which had been exposed to ozone for a long time (Günthardt-Goerg *et al.*, 1993; Nie, Tomasevic & Baker, 1993).

Carbohydrate metabolism

Contents of soluble sugars (sucrose, glucose, and fructose) were enhanced in older leaves of the O_3/LF treatment (see Landolt *et al.*, 1997). In addition, higher starch contents were found along minor veins

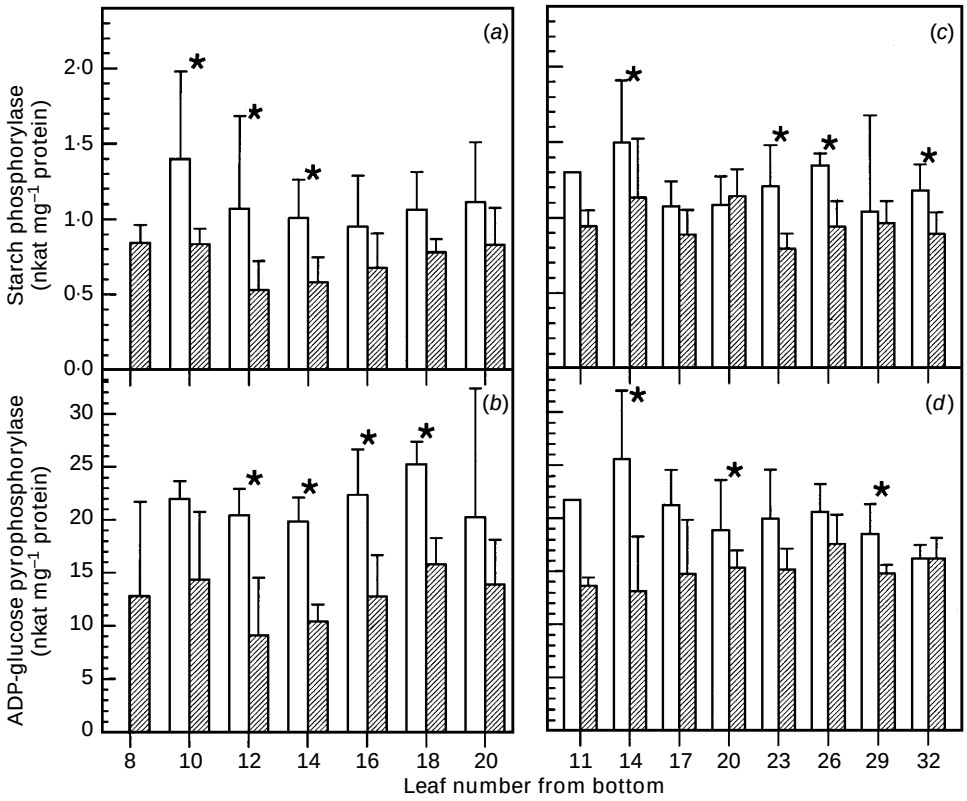

Figure 5. Specific activities of starch phosphorylase and ADP-glucose pyrophosphorylase in leaves of ozone-fumigated (▨) and control plants (□) of two fertilization treatments. (*a*, *b*) Low fertilization. (*c*, *d*) High fertilization. Old leaves are indicated by low numbers. Data represent means ±SD. *$P \leqslant 0.05$.

of O_3/LF and O_3/HF leaves (cf. Matyssek *et al.*, 1992). Together with a reduced growth rate, this gives evidence for a reduction of C export from source leaves.

When end-products of photosynthesis accumulate in leaves, sucrose synthesis is reduced by a feedback control. The initial step of this shift is thought to be the inactivation of SPS (for review see Stitt, 1991; Huber, Huber & McMichael, 1992). SPS is a highly regulated enzyme and the inactivation can involve protein phosphorylation as well as reduction of the amount of enzyme protein. Inactivation of SPS would result in a rise of cytosolic F6P, which in turn would increase the level of F26BP (Stitt, 1990). The increased F26BP would inhibit the cytosolic fructose 1,6-bisphosphatase and thereby restrict C flow to sucrose. Consequently, more C would be retained in the chloroplast as starch. This situation seems to characterize our O_3/LF samples, where a reduction of extractable specific activity of SPS was correlated with higher carbohydrate contents and increased F26BP levels, indicating a shift of C partitioning towards starch synthesis.

An increase in sucrose-cleaving activity, enhanced glycolytic reactions, and a loss of enzymes of starch mobilization were reported from tissues where carbohydrate contents were increased artificially. Sucrose synthase activity increased when exogenous sucrose was supplied to root tips of maize (Koch *et al.*, 1992). In tobacco, the increase of sucrose synthase activity was paralleled by a rise in alkaline invertase activity, whereas acid invertase was unaffected (Paul & Stitt, 1993). High invertase activities in our O_3/LF leaves could explain elevated hexose contents (Landolt *et al.*, 1997). Together with the increased activity of sucrose synthase, these data could indicate that C flow would be enhanced towards glycolysis. This could stimulate respiration, as found in samples of the same experiment (Maurer, 1995). An increased ATP:ADP ratio could result from stimulated respiration, but, because metabolites were extracted from leaf homogenates, it is also possible that chloroplast metabolism contributed to the increased ratio. Interestingly, feeding glucose to spinach leaves via the transpiration stream increased respiration rates and elevated ratios of ATP:ADP (Krapp *et al.*, 1991).

In recent studies, fumigation of Norway spruce with O_3 and SO_2 led to elevated ratios of ATP:ADP (Hampp, Einig & Egger, 1990) which were observed also in needles of declining trees of the same species (Weidmann *et al.*, 1990). Therefore, it could be assumed that the increase in the ATP:ADP ratio might express ozone-induced repair activities as suggested for spruce (Weidmann *et al.*, 1990).

In addition to the key enzymes of sucrose metabolism and glycolysis, enzymes of starch metabolism of photosynthetic tissues have also been characterized as carbohydrate-responsive. Krapp & Stitt (1994) found a decrease of starch phosphorylase

activity in the chloroplast, whereas cytosolic activity of this enzyme was unaffected. Likewise, the overall activity of AGPase was reduced. All these sugar-modulated responses in plant tissues were similar to our findings in the O_3/LF treatment, indicating that regulation of carbohydrate metabolism in our samples might have adapted to elevated sugar contents or reduced C export rates of ozone-treated leaves.

However, in HF-treated plants, soluble sugars contents of leaves were not influenced by ozone, whereas starch contents increased and plant growth rate decreased (Landolt *et al*., 1997). In these leaves of O_3/HF plants, the maximal extractable activity of the enzymes (sucrose-P synthase, sucrose synthase, alkaline invertase, starch phosphorylase, and ADP-glucose pyrophosphorylase) reacted like those in O_3/LF plants. If this response were mediated by the carbohydrate status of the leaf, our data suggest that C flux rather than steady-state sugar level could be the critical signal. Similar conclusions were drawn from analysis of maize mutants with high-sugar kernels and transgenic, sugar-storing potatoes where little or no changes in expression of genes otherwise affected by sugars was found (for review see Koch 1996).

In contrast to enzyme activities, ozone affected F26BP levels in HF and LF plants in different ways. According to its regulatory properties, decreased levels of F26BP should favour sucrose synthesis. The metabolic context of this response is difficult to interpret at the moment as F26BP levels are generally higher in HF compared with LF plants. Interestingly, studies of fertilization studies of Norway spruce with N exhibited similar elevated concentrations of F26BP (Wingler *et al*., 1994, Wallenda *et al*., 1996). Thus, our data imply that the nutrient status can interact with carbohydrate metabolism and might influence its response to pollutants. This should be taken into account when parameters of carbohydrate metabolism are used to evaluate ozone effects.

In conclusion, our data suggest that ozone induced alterations of activities of carbohydrate-responsive enzymes. Therefore, it is reasonable to assume that the chronic ozone effect on photosynthesis, as was found in mature leaves in our experiment, could result from regulatory changes of carbohydrate metabolism due to an accumulation of photosynthetic end-products which is a consequence of ozone-induced limitation of assimilate export. This can lead to a long-term inhibition of photosynthesis (Krapp *et al*., 1991; Schäfer, Simper & Hofmann, 1992). Reports of altered C allocation before reductions in photosynthesis (McLaughlin & McConathy, 1983) and the accumulation of soluble carbohydrates in leaves after ozone fumigation (Bücker & Ballach, 1992; Landolt *et al*., 1994) support this view.

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