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Effect of chronic O_3 fumigation on the activity of some Calvin cycle enzymes in two poplar clones

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Abstract

The effects of long-term exposure to ozone (O_3, 60 mm^3 m^{-3} for 5 h d^{-1}) on some Calvin cycle enzymes, in particular those modulated by the thioredoxin system, were studied in two poplar clones. These clones differ in sensitivity to O_3. In the I-214 clone, the first effects from O_3 treatment were seen after 40 d of fumigation, while the Eridano clone showed visible symptoms of damage after only 15 d of the treatment. Specific activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (E.C. 4.1.1.39) diminished in both the clones, while specific activity of phospho*enol*pyruvate carboxylase (E.C. 4.1.1.31) increased. Exposure to O_3 also caused a reduction in the specific activity of ribulose-1,5-bisphosphate kinase (E.C. 2.7.1.19) in both clones. At the end of the exposure to O_3, specific activity of glyceraldehyde 3-phosphate dehydrogenase (E.C. 1.2.1.13) increased in I-214 and remained similar to the control in Eridano, whereas specific activity of fructose-1,6-bisphosphate phosphatase (E.C. 3.1.3.11) was higher in Eridano and similar to the control in I-214.

Additional key words: fructose 1,6-bisphosphate phosphatase; glyceraldehyde-3-phosphate dehydrogenase; phospho*enol*pyruvate carboxylase; ozone; phosphoribulose kinase; ribulose-1,5-bisphosphate carboxylase/oxygenase.

Introduction

Ozone (O_3) is one of the major air pollutants which severely influence survival and yield of plants (Pell *et al.* 1997). Acute stress from exposure to high concentrations of O_3 for short periods of time generally produces visible injury. Chronic O_3 stress, resulting from exposure to low concentrations for a long period, produces few symptoms of damage but results in reduced growth (Heath and Taylor 1997).

Many authors report the negative effects of O_3 on photosynthesis (Heath 1994, Reichenauer *et al.* 1997, Plażek *et al.* 2000), and the reduction of photosynthesis is attributed to decreased stomatal conductance (Farage and Long 1995) and/or change in biochemical processes. The alterations in light harvesting (Schreiber *et al.* 1978, Pell *et al.* 1992), change in regeneration rate of RuBP, and/or changes in the activity of RuBPCO (Pell *et al.* 1992, Dizengremel *et al.* 1994, Lütz *et al.* 2000) may occur. Pell *et al.* (1992, 1994a) reported that the loss of RuBPCO is closely associated with the acceleration of leaf senescence and that a major loss of RuBPCO is reflected in enhanced degradation. Similar results are reported by Eckardt and Pell (1995) in *in vitro* and whole plant experiments.

Some investigations deal with the effects of O_3 on another carboxylase in C_3 plants, PEPC. This enzyme generally increases in O_3-stressed plants (Luethy-Krause *et al.* 1990, Fontaine *et al.* 1999, Lütz *et al.* 2000). A possible explanation for this rise is the fact that PEPC supplies the reducing power necessary for detoxification processes (Sehmer *et al.* 1998).

Up until now, there have been no reports on the effects of O_3 on other enzymes of the Calvin cycle. However, the enzymes of carbon assimilation are inhibited by other oxidative stresses such as low temperature (Sassenrath *et al.* 1991, Kingston-Smith *et al.* 1997). In the Calvin-Benson cycle there is also regulatory enzyme activity

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Abbreviations: ANOVA – analysis of variance; DTT – dithiothreitol; EDTA – ethylenediaminetetraacetic acid; FBP – fructose-1,6-bisphosphate phosphatase; F1,6BP – fructose-1,6-bisphosphate; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; LSD – least significant difference; PEP – phospho*enol*pyruvate; PEPC – phospho*enol*pyruvate carboxylase; PGK – phosphoglycerate kinase; PMSF – phenylmethyl sulfonyl fluoride; PPF – photosynthetic photon flux; PRK – ribulose-1,5-bisphosphate kinase; PVP – polyvinylpolypyrrolidone; Ru5P – 5-phosphoribulose; RuBP – ribulose-1,5-bisphosphate; RuBPCO – ribulose-1,5-bisphosphate carboxylase/oxygenase; SE – standard error; β-mesh – mercaptoethanol.
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which operates *via* thioredoxin-mediated reduction of specific sulfhydryl groups. Some of the Calvin-Benson cycle enzymes are inhibited by oxidation of their regulatory thiol groups.

Information on the activation state of light-regulated enzymes of the Calvin-Benson cycle is necessary for the understanding of photosynthetic control. Electron transport can operate efficiently only when the supply of NADP, ADP, and P_i, which are non-limiting factors of the redox potential known as power force, is low (Heber *et al.* 1986). Power force is low when reduction and assimilation of CO_2 by the Calvin cycle are rapid and this occurs when CO_2 supply is adequate and the light-modulated enzymes of the Calvin cycle are activated.

The poplar clone *Populus deltoides* × *maximowiczii* Eridano is sensitive to O_3 and usually produces easily visible symptoms of injury after exposure to acute O_3 treatment (Ranieri *et al.* 1996). On the other hand, *Populus* × *euramericana* I-214 is a tolerant clone and no symptoms generally arise. In past experiments, we characterised these two clones (Soldatini *et al.* 1998, Guidi *et al.* 2001), with particular reference to the effects of O_3 at physiological level. Our conclusions were that in the clone I-214 active mechanisms of feedback regulation play a key role in the response to O_3. In the sensitive clone Eridano, these mechanisms are absent.

The aim of the present experiments was to determine the involvement of thioredoxin-modulated Calvin cycle enzymes, RuBPCO and PEPC, in the reduction of the CO_2 fixation process in poplar clones exposed to chronic O_3 fumigation.

Materials and methods

Plants and exposure to O_3: Experiments were conducted with two poplar clones (*Populus deltoides* × *maximowiczii* Eridano and *P.* × *euramericana* I-214), designated as ozone resistant (I-214) and ozone sensitive (Eridano). This designation was based on visible lesion formation in response to a single acute dose of ozone (Ranieri *et al.* 1996). Plants were obtained from cuttings which were grown in plastic pots (20×20×30 cm) containing a steam-sterilised soil : peat : perlite (1 : 1 : 1, vol.) mixture and kept for 2 months in a cooled green-house during the summer. Plants were transferred to fumigation chambers after complete expansion of the 10th leaf.

Exposure was made during the spring-summer of 2000 in a controlled-environment *Perspex* apparatus placed inside a walk-in growth chamber that was continuously ventilated with charcoal-filtered air (two complete air changes per min). Temperature was maintained constantly at 20±1 °C and relative humidity at 85±3 %. A photosynthetic photon flux (PPF) of about 500 μmol m^{-2} s^{-1} at plant height was provided by incandescent lamps. O_3 was produced by electric discharge with an air-cooled generator (*Fischer 500*, Zürich, Switzerland), supplied with pure oxygen, and was mixed with the inlet air entering the chambers. The O_3 concentrations at plant height were continuously monitored with a photometric *ML8810* analyser (*Monitor Labs*, San Diego, USA). The exposure regime was in the form of square wave, 61±8 mm^3 m^{-3}, from 09:00 to 14:00 (solar time) each day (5 h d^{-1}). Control plants were exposed to charcoal-filtered air, in which O_3 concentration was lower than 2 mm^3 m^{-3}.

Sampling of the leaves was carried out at the start (before the plants were put into growth chambers) and at the end of the experiment (after the O_3 fumigation) for both the clones. The duration of fumigation for the two clones was linked to the appearance of visible symptoms of injury. The different times of fumigation, 15 d for Eridano and 40 d for I-214, was due to the fact that the two clones showed the first signs of visible symptoms after these time periods. During the O_3 treatment, plants were maintained in well-watered conditions following normal horticultural practice. Control plants were maintained under the same experimental conditions as O_3-treated plants but exposed to charcoal-filtered air.

Enzyme extraction: All analyses were performed on the youngest fully expanded leaf of each plant. Sampling was carried out during the irradiation period to ensure that the enzymes were fully activated. Leaflets were removed and frozen in liquid N_2 and maintained at –80 °C. Leaf sections were ground in a mortar and pestle pre-cooled with liquid nitrogen. Three cubic cm of ice-cold extraction buffer was added to the mixture. The medium contained 50 mM Tris-HCl (pH 7.8), 2 mM Na_2EDTA, 5 mM mercaptoethanol (β-mesh), 1 mM PMSF, and 10 kg m^{-3} PVP. The homogenate was centrifuged at 12 000×*g* for 10 min. The supernatant was used to determine enzyme activities and protein contents. All extraction steps were performed at 4 °C.

Enzyme activities were measured spectrophotometrically (*Biochrom 4060*, *Pharmacia*, Cambridge, England) with coupled reactions monitoring NADH oxidation or reduction at 340 nm (ε = 6.23 mM^{-1} cm^{-1}) and 25 °C with different aliquots of reaction mixture. Since protein content was not very variable, specific activity of the enzymes was calculated on protein basis.

RuBPCO activity was measured according to Sawada *et al.* (1990) with some modifications. The reaction mixture contained: 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 30 mM $NaHCO_3$, 5 mM β-mesh, 83 nkat PGK (E.C. 2.7.2.3), 83 nkat GAPDH, 17 nkat glycerol-3-phosphate dehydrogenase (E.C. 1.1.1.8), 100 nkat triose-phosphate isomerase (E.C. 5.3.1.1), 0.15 mM NADH, and 5 mM ATP. Assays were initiated by the addition of 0,5 mM (final concentration in the cuvette) RuBP.

The reaction mixture for PEPC contained: 50 mM

Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 10 mM $NaHCO_3$, 33 nkat MDH, and 0.3 mM NADH. Assays were initiated by the addition of 4 mM (final concentration in the cuvette) PEP (Tietz and Wild 1991).

The reaction mixture for GAPDH contained: 50 mM Tris-HCl (pH 7.8), 2 mM Na_2EDTA, 10 mM β-mesh, 10 mM $MgCl_2$, 2 mM 3-phosphoglyceric acid (3PGA), 83 nkat 3PGK, 3 mM ATP, and 0.15 mM NADPH. GAPDH assay was initiated by the addition of NADPH (Trost *et al.* 1993).

Stromal FBP activity was measured according to Gray *et al.* (1996) in the assay buffer containing 100 mM Tricine-NaOH (pH 8.8), 0.5 mM Na_2EDTA, 50 mM DTT, 17 nkat glucose-6-phosphate dehydrogenase, 33 nkat glucose-6-phosphate isomerase, 0.5 mM $NADP^+$, and 10 mM $MgCl_2$. The reaction was initiated by adding 0.4 mM fructose 1,6-bisphosphate (final concentration in the cuvette).

The analysis of PRK activity was carried out according to Wara-Aswapati *et al.* (1980). The reaction mixture contained: 50 mM Tris-HCl (pH 7.8), 2 mM DTT, 10 mM $MgCl_2$, 50 nkat pyruvate kinase (E.C. 2.7.1.40), 83 nkat lactate dehydrogenase (E.C. 1.1.1.27), 83 nkat ribose-5-phosphate isomerase, 5 mM ribose-5-phosphate, 0.1 mM NADH, 40 mM KCl, and 2 mM ATP. The reaction was initiated by adding 50 mm^3 of extract.

Protein determinations were performed according to Lowry *et al.* (1951) using bovine serum albumin as standard.

Statistical analysis: All experiments were repeated twice and a minimum of ten plants per treatment was used in each experiment. Samples for enzymatic activity were taken from three plants. Comparison of the means was made by ANOVA test, followed by the least significant difference (LSD) test.

Results

Clone I-214: At the beginning of fumigation, the specific activity of RuBPCO in the leaves of I-214 plants was about 83 mol(CO_2) s^{-1} kg^{-1}(protein) (values not shown) and it increased to reach a value of 8 666 and 650 mol(CO_2) s^{-1} kg^{-1}(protein) in control and ozonated plants, respectively, after 40 d (Fig. 1*A*). This means that O_3 fumigation resulted in a reduction in the specific activity of RuBPCO after 40 d of treatment in comparison to the control.

A value of about 583 mol(NADPH) s^{-1} kg^{-1}(protein) was recorded for specific activity of NADP-dependent GAPDH in the plants just before the beginning of the experiment. A decrease of about 90 % was observed in the controls after 40 d of their being kept in charcoal-filtered air chambers; in ozonated plants about 35 % reduction in the specific activity of this enzyme was observed (Fig. 1*A*). FBP showed a specific activity of about 1 000 mol(NADPH) s^{-1} kg^{-1}(protein) at the beginning of experi-

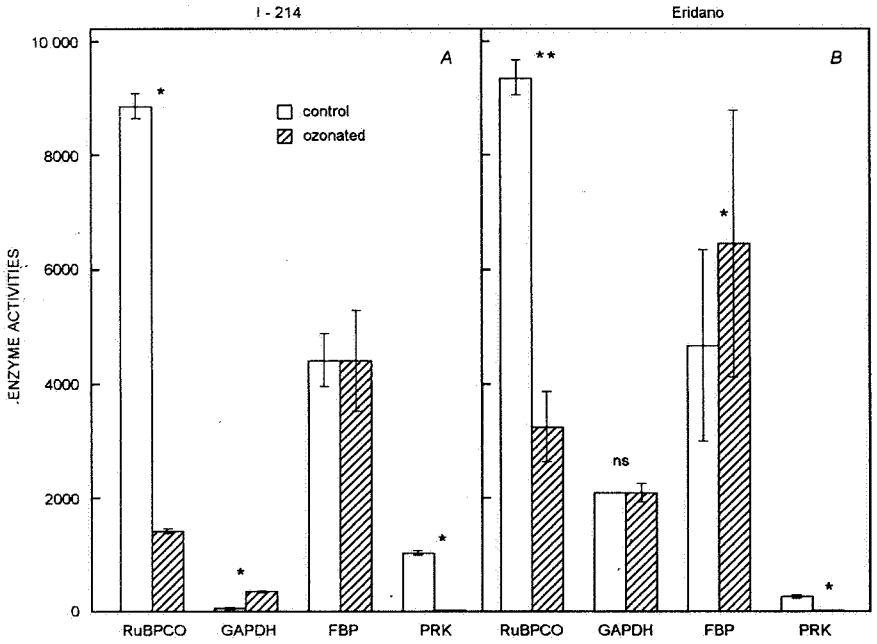

Fig. 1. Specific activity of RuBPCO [mol(CO_2) s^{-1} kg^{-1}(protein)], GAPDH [mol(NADPH) s^{-1} kg^{-1}(protein)], FBP [mol(NADPH) s^{-1} kg^{-1}(protein)] and PRK [mol(NADPH) s^{-1} kg^{-1}(protein)] in the youngest leaves of *Populus* × *euramericana* I-214 (*A*) and *Populus deltoides* × *maximowiczii* Eridano (*B*) before and after treatment with O_3 (60 mm^3 m^{-3} for 5 h d^{-1} for 40 d). Each value represents the mean of six replicates. For each mean the bar indicates SE. The significance of the differences between control and ozonated plants at the end of the fumigation period is reported (non-significant – ns, $p>0.05$; significant – * $p<0.05$, ** $p<0.01$).

mental period. At the end of the exposure period, an increase in FBP's specific activity was observed both in control and ozonated plants, *i.e.* a 4.5-fold increase (Fig. 1).

The specific activity of PRK rose approximately tenfold from about 58 mol($NADP^+$) s^{-1} kg^{-1}(protein). In the controls, significant increase in the specific activity of PRK was observed but remained unchanged in plants fumigated with O_3 (Fig. 1*A*).

Specific activity of PEPC was about 58 mol(CO_2) s^{-1} kg^{-1}(protein) at the beginning of the experiment and decreased in the control and to one fourth in ozonated plants. The difference between control and ozonated plants was significant (Fig. 2).

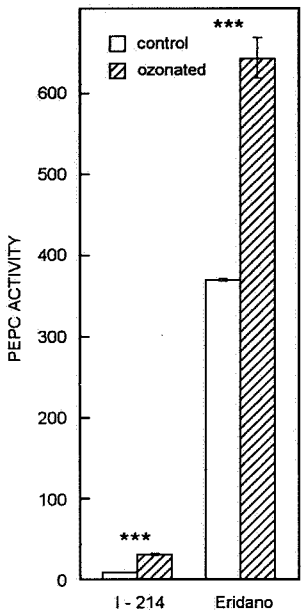

Fig. 2. The effect of treatment with O_3 (60 mm^3 m^{-3} for 5 h d^{-1}) on the activity of PEPC [mol(CO_2) s^{-1} kg^{-1}(protein)] in the youngest leaves of *Populus* × *euramericana* I-214 and *Populus deltoides* × *maximowiczii* Eridano. Each value represents the mean of six replicates. For each mean the bar indicates SE. The significance of the differences between control and ozonated plants at the end of the fumigation period is reported at the probability level $p<0.001$ (***).

Discussion

Different species and/or cultivars and clones show differential responses to O_3. The two clones used in this experiment showed very different responses to ozone, not only because one of them very quickly showed visible symptoms of injury, but also because they differed in their biochemical and time responses. The response of Eridano was very quick, while I-214 showed a slower response. In these conditions it is very difficult to compare the behaviour of these two clones to O_3 treatment directly. We can make some conclusive comparisons only for the period where the signs of visible symptoms were present in both clones (*i.e.* 40 d for I-214 and 15 d for Eridano).

As a result of fumigation, specific activity of RuBPCO decreased significantly in both clones. The decrease in RuBPCO activity was not associated with a reduction in soluble protein content, but, as also reported by other authors (Kangasjärvi *et al.* 1994), other stress-specific enzymes may be synthesised at the same time, leading to a stable total quantity of proteins. The reduction in RuBPCO activity upon O_3 fumigation in different tree species is a result widely reported in the literature

Table 1. Effects of chronic fumigation with O_3 (60 mm^3 m^{-3} for 5 h d^{-1}) on total soluble protein content [kg m^{-3}] in the youngest leaves of *Populus* × *euramericana* I-214 and *Populus deltoides* × *maximowiczii* Eridano. Each value represents the mean of six replicates (±SE). Means denoted by the same letter are not significantly different following the two-way ANOVA test for the factors of O_3 treatment and duration of exposure.

Clone	O_3 fumigation [d]	Control	O_3
I-214	0	1.10±0.03 c	1.11±0.03 c
	40	2.10±0.55 b	2.61±0.80 a
Eridano	0	0.93±0.22 a	0.93±0.19 a
	15	0.90±0.37 a	0.97±0.27 a

Total soluble protein content had increased by the end of the experiment in both control plants and in ozonated leaves (Table 1).

Eridano clone: The specific activity of RuBPCO had increased 3 times in controls when measured after 15 d, as compared to the beginning of the experiment [about 3 300 mol(CO_2) s^{-1} kg^{-1}(protein)]. In ozonated plants, RuBPCO activity remained similar to that at the beginning of the treatment (Fig. 1*B*).

NADP-dependent GAPDH activity decreased from 6 750 to 2 167 mol(NADPH) s^{-1} kg^{-1}(protein) in controls after 15 d of growth. A similar decrease was observed in ozonated plants (Fig. 1*B*).

Stromal FBP activity was similar in controls when measured at the beginning and at the end of the experiment [5 500 and 4 830 mol(NADPH) s^{-1} kg^{-1} (protein)]. In ozonated plants a slight increase was observed (Fig. 1*B*).

At the beginning of the experiment, PRK showed specific activity of 1 250 mol($NADP^-$) s^{-1} kg^{-1}(protein). At the end of the O_3 fumigation period this had decreased to a value of about 83 mol($NADP^-$) s^{-1} kg^{-1}(protein).

PEPC in controls decreased after 15 d from 1 333 to 367 mol(CO_2) s^{-1} kg^{-1}(protein) and in ozonated plants from 633 mol(CO_2) s^{-1} kg^{-1}(protein) (Fig. 2).

Total soluble protein content did not change in this clone as a result of the O_3 treatment (Table 1).

(Pell *et al.* 1992, 1994b, Brendley and Pell 1998, Fontaine *et al.* 1999, Lütz *et al.* 2000).

Brendley and Pell (1998) point out the role of oxidative modification and enhanced degradation of RuBPCO after exposure to O_3. It is unlikely that O_3 directly oxidises the enzyme even if RuBPCO protein is highly sensitive to oxidative stress (Mehta *et al.* 1992). One possibility is that RuBPCO becomes degraded because of enhanced enzymatic proteolysis after O_3 exposure. Proteolysis occurs when proteins (and then RuBPCO) undergo chemical modification and become susceptible to proteases (Dalling 1987) and, as reported by Dann and Pell (1989), O_3 can enhance proteolysis of RuBPCO. Another possibility is that synthesis of RuBPCO is retarded (Reddy *et al.* 1993, Glick *et al.* 1995).

Other enzymes of the Calvin cycle have not yet been investigated for their sensitivity to O_3. In the I-214 clone, we found that chronic O_3 fumigation also caused a substantial reduction in the specific activity of PRK. The specific activity of GAPDH in I-214 was significantly increased following O_3 fumigation. Different behaviour was shown by the FBP that increased in the same way in the control and ozonated plants as compared to the beginning of the experiment. The picture is more complex than this, but it is certainly characterised by an alteration in the carboxylative and regenerative phases of the Calvin cycle. On the other hand, values derived from gas exchange analysis (not shown) indicated a substantial reduction in CO_2 fixation, unchanged stomatal conductance, but a strong and significant increase in intercellular CO_2 concentration. This confirms the involvement of the reactions of CO_2 photoassimilation in the reduction of photosynthetic rate.

Even though the time periods are different, reductions in RuBPCO and PRK were also found in the Eridano clone. A different response was observed in FBP: this enzyme did not change in either the control or in ozonated plants. GAPDH decreased by about 40 %, but did not alter in ozonated plants.

The results imply that the major targets of the effects of O_3, at the level of enzymatic reactions of the Calvin cycle, are RuBPCO and PRK. This explains previous results of gas exchange analysis (Farage *et al.* 1991, 1999, Heath 1994, Reichenauer *et al.* 1997). Thus we may conclude that in these two clones the response of photosynthetic apparatus to chronic fumigation with O_3 is substantially similar and characterised by a decrease in RuBPCO and PRK activities.

The specific activity of cytosole PEPC showed an opposite behaviour to RuBPCO activity. Indeed, the decrease of PEPC activity observed in control and ozonated plants was parallel to the increase in RuBPCO activity. PEPC activity in ozonated plants was significantly higher than in the control. This stimulation may provide the supply of reducing power that is used for detoxification as well as for energy and carbon skeletons involved in repair processes (Sehmer *et al.* 1998).

Despite the very different behaviour of the two poplar clones to O_3, our results evidence that many other enzymes of the Calvin cycle, besides RuBPCO, are affected by O_3 stress. The present work emphasises the importance of studying the activity of other Calvin cycle enzymes besides RuBPCO which is certainly the major target of O_3 exposure.

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