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The impact of elevated ozone and carbon dioxide on young *Acer saccharum* seedlings

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The effects of high O_3 (200 nl l^{-1} during the light period) and high CO_2 (650 µl l^{-1} CO_2, 24 h a day) alone and in combination were studied on 45-day-old sugar maple (*Acer saccharum* Marsh.) seedlings for 61 days in growth chambers. After 2 months of treatment under the environmental conditions of the experiment, sugar maple seedlings did not show a marked response to the elevated CO_2 treatment: the effect of high CO_2 on biomass was only detected in the leaves which developed during the treatment, and assimilation rate was not increased. Under high O_3 at ambient CO_2, assimilation rate at days 41 and 55 and Rubisco content at day 61 decreased in the first pair of leaves; total biomass was reduced by 43%. In these seedlings large increases (more than 2-fold) in glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) activity and in anaplerotic CO_2 fixation by phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) were observed, suggesting that an enhanced reducing power and carbon skeleton production was needed for detoxification and repair of oxidative damage. Under high O_3 at elevated CO_2, a stimulation of net CO_2 assimilation was observed after 41 days but was no longer observed at day 55. However, at day 61, the total biomass was only reduced by 21% and stimulation of G6PDH and PEPC was less pronounced than under high O_3 at ambient CO_2. This suggests that high CO_2 concentration protects, to some extent, against O_3 by providing additional carbon and energy through increased net assimilation.

Introduction

CO_2 concentration has increased from 280 µl l^{-1} in 1880 to approximately 370 µl l^{-1} at present. A doubling of the present CO_2 concentration is expected by the end of the 21st century (Conway et al. 1988). Many studies have reported an increase of the photosynthetic rate under high CO_2 (Vivin et al. 1996, Saxe et al. 1998). This often results in an increased biomass production (Allen 1990, Ceulemans and Jiang 1995) suggesting a fertilizing effect of increasing CO_2 concentrations on plants (Drake et al. 1997). However, in the long term, ribulose-1,5 *bis*phosphate carboxylase/oxygenase (Rubisco) activity and content may decrease (Van Oosten et al. 1992, Drake et al. 1997). This phenomenon, known as photosynthesis acclimation to elevated CO_2, may be due to a nutrient supply limitation or a source-sink limitation (Brown 1991, El Kohen et al. 1993, Ceulemans and Mousseau 1994). This will lead to an accumulation of non-structural carbohydrates and a subsequent feedback inhibition of Rubisco synthesis (Van Oosten and Besford 1996).

Tropospheric O_3 is one of the most damaging gaseous pollutants that affects vegetation (Kickert and Krupa 1990). Its concentration increases by 1 or 2% a year (Bojkov 1988, Volz and Kley 1988). Exposure of plants to O_3 can cause a significant reduction in productivity as well as important economic losses at relatively low concentrations (30–40 nl l^{-1}) in sensitive species (Heck et al. 1988, Heagle 1989). O_3 penetrates within the leaves through open stomata and diffuses to the cells through

Abbreviations – A, net CO_2 assimilation rate; Ca, ambient CO_2 concentration; Ci, intercellular CO_2 concentration; g, stomatal conductance; G6PDH, glucose 6-phosphate dehydrogenase; GR, glutathione reductase; NL, new leaves; NR, nitrate reductase; OL, old leaves; PEPC, phosphoenolpyruvate carboxylase; Rubisco, ribulose 1,5-*bis*phosphate carboxylase/oxygenase; RuBP, ribulose 1,5-*bis*phosphate.

the air spaces, giving rise to very toxic free radicals (Reich 1987, Kangasjärvi et al. 1994, Sandermann et al. 1998, Dizengremel 2001). This often leads to a decrease in the photosynthetic rate and growth (Reich and Amundson 1985, Darrall 1989, Heath 1994, Mikkelsen 1995, Pääkkönen et al. 1995, Stockwell et al. 1997), even before foliar injuries are apparent (Davis and Skelly 1992, Heath and Taylor 1997). A decrease of Rubisco activity and content is often observed in plants under O_3 exposure (Pell et al. 1994, Lütz et al. 2000, Pelloux et al. 2001). Photochemical reactions may be impaired, limiting the ATP and NADPH supplies for CO_2 fixation (Shimazaki 1988). While the effects of oxidative stress on the photosynthetic process have been widely investigated, very few studies have dealt with the catabolic pathway responses (Dizengremel and Citerne 1988). Stimulation of glycolysis and dark respiration have been observed under O_3 exposure (Dizengremel and Pétrini 1994). In C_3 plants, the CO_2-fixing enzyme phosphoenolpyruvate carboxylase (PEPC), which has anaplerotic function such as the replenishment of the tricarboxylic acid cycle (Latzko and Kelly 1983), was recently shown to present an increased activity in response to ozone stress, in order to supply the repair mechanisms with carbon skeletons (Landolt et al. 1997, Sehmer et al. 1998, Fontaine et al. 1999, Dizengremel 2001). Stimulation of glucose 6-phosphate dehydrogenase (G6PDH), the first enzyme of the pentose-phosphate pathway, has also been observed during oxidative treatment (Dizengremel et al. 1994). This pathway may be involved in the production of energy and carbon skeletons necessary for detoxification and repair processes.

In a future high CO_2 atmosphere, seedlings will have to face high O_3 episodes during the summertime. In some studies on plants exposed to high CO_2 in combination with high O_3, it was shown that the fertilization effect of high CO_2 enhanced the capacity of plants to tolerate oxidative stress (Rao et al. 1995, Schwanz et al. 1996, Volin et al. 1998, Kytöviita et al. 2001). However, other studies failed to conclusively demonstrate that CO_2 protects plants against oxidative stress (Polle et al. 1993, Barnes et al. 1995, Kull et al. 1996). Whereas PEPC and G6PDH activities are usually reported to increase under oxidative stress, these enzyme activities were found to decrease under elevated CO_2 atmosphere (Van Oosten et al. 1992). Only a few studies reported the response of these enzymes to a combination of high CO_2 and high O_3 (Sehmer et al. 1998).

Most of the studies on the effects of pollutants on woody plants were done with young seedlings (2 to 4 years old). Recently, new experimental designs allowed the use of mature trees (branch bag experiment, Kellomäki and Wang 1997 or FACE, free-air CO_2 enrichment, Karnosky et al. 1999). Recently germinated seedlings have been used for only a few studies (Bazzaz et al. 1990, Noble et al. 1992). It now seems established that young seedlings (a few years old) are more sensitive to stress pollutants than mature trees which have a larger buffer capacity against stress due to their higher storage capacity (Saxe et al. 1998). However, it is of great importance to study the development of recently germinated seedlings of trees because in the near future they will grow in an atmosphere enriched in CO_2 and O_3.

Sugar maple (*Acer saccharum* Marsh.) is a widely distributed deciduous species in North America with a major economical interest. Sap production generates $140 million per year and sugar maple is a high quality timber. Maple forests in Québec receive high levels of O_3 during summer: common hourly episodes reach 150–200 $nl\,l^{-1}$ O_3 in June and July (Fuentes and Dann 1994). The aim of this study was to investigate the effect of high CO_2 and high O_3, alone and in combination, during the first growing season of sugar maple seedlings. Seedlings were 45 days old when exposed to the treatments for 61 days. The 106-day period represents the length of a normal growing season at 45° latitude in North America. Stomatal conductance, photosynthetic parameters (net CO_2 assimilation rate, activity and quantity of Rubisco) have been investigated. Since NO_3^- assimilation and PEPC activity are linked (Champigny 1995), we measured nitrate reductase (NR) activity and anaplerotic CO_2 fixation by PEPC. Finally, G6PDH activity has also been studied. The activities of several antioxidant enzymes on the same seedlings have been published in a recent paper by Niewiadomska et al. (1999).

Materials and methods

Growth of the seedlings and fumigation treatments

Sugar maple (*Acer saccharum* Marsh.) seeds from the Berthierville nursery (Québec, Canada) were soaked in water for 14 days and placed on moist sand in the dark and allowed to germinate according to the method of Charlebois (1993). Imbibition of the seeds lasted for 2 months. Once the roots were 3–10 cm long, emerging sugar maple seedlings (360) were planted in 512 cm^3 pots containing a commercial organic soil mixed with sugar maple forest topsoil containing native mycorrhizal inoculum (2:1).

Seedlings were set in the phytotronic growth chambers of the University of Nancy (France). Height phytotronic chambers are set within a large room and designed to receive the same temperature, light intensity, photoperiod and relative humidity. Initial testing of the setting ensured that all these parameters were perfectly controlled and kept the same in each phytotronic chamber (J. Banvoy, personal communication).

Ninety seedlings were set into each of four phytotronic growth chambers and allowed to acclimate for 1 month under artificial light (photosynthetic photon flux density of 250 $\mu mol\,m^{-2}\,s^{-1}$ above canopy, Son-T-AGRO Philips Bio & Hydro, Bordeaux, France). The temperature was maintained at 24 ± 2°C during the day (16 h photoperiod) and 20 ± 2°C during the night. The relative humidity was 75 ± 5%. Temperature, relative humidity, photoperiod and light intensity were kept constant in each phytotronic chamber.

After the 1-month acclimation period, the young seedlings had one pair of newly expanded true leaves, which

are hereafter referred to as the old leaves (OL). The length of the treatment period concurs with summer episodes of O_3 in Québec forest whereby high O_3 levels are commonly reached during the months of June and July (Gaucher 2001). The following treatments for a total period of 61 days were: (1) control: $10\,nl\,l^{-1}$ O_3 + $350\,\mu l\,l^{-1}$ CO_2; (2) high O_3: $200\,nl\,l^{-1}$ O_3 + $350\,\mu l\,l^{-1}$ CO_2; (3) high CO_2: $10\,nl\,l^{-1}$ O_3 + $650\,\mu l\,l^{-1}$ CO_2; and (4) high O_3 + high CO_2: $200\,nl\,l^{-1}$ O_3 + $650\,\mu l\,l^{-1}$ CO_2. Ozone, generated from pure oxygen (Air liquide, Nancy, France) with an ozone generator (model OZ 500, Fisher TM, Bonn, Germany) was delivered only during the light period. Pure CO_2 was delivered 24 h a day (Air liquide, Nancy, France). Ambient air in the chamber was analysed continuously by an ozone analyser (O341M, Environment S.A., Paris, France) and a CO_2 analyser (WMA2 PPsystems, Stotfold, UK). CO_2 concentration may fluctuate by 10% and O_3 by 12%. In each chamber, seedling position was changed every day. During the treatments, a second flush of two to four pairs of leaves developed for each seedling. These leaves are hereafter referred to as the new leaves (NL).

Harvest of the seedlings

Seedlings were harvested 4, 31 and 61 days after the treatments were initiated. Harvesting started at 1400 h and consisted of a complete defoliation of 10 randomly chosen seedlings for each treatment. The NL were collected separately from the OL. On day 31, the NL of the seedlings on the high O_3 and high O_3 + high CO_2 treatments were not collected because of insufficient leaf size. The leaves were collected without the petiole and weighed. Foliar discs were taken for in vivo nitrate reductase (NR, EC 1.6.6.1) assay and total chlorophyll content determination. The rest of the leaves were immediately frozen in liquid nitrogen and kept at −80°C for further analysis.

Biomass measurements

Biomass measurements were done with 20 seedlings per chamber for day 31 and day 61. OL, NL, stems and roots were sampled separately, oven-dried at 70°C for 4 days and weighed. Relative growth rate (RGR) was calculated using the following equation: (ln DW at day 61 − ln mean DW at day 0)/61 and was expressed in mg g^{-1} day^{-1}.

In vivo nitrate reductase assay

NR activity was measured according to the method of Jaworski 1971) modified by Truax et al. (1994). One foliar disc ($0.95\,cm^2$) was sampled from each seedling, cut in 2 × 2 mm pieces and incubated in 2.5 ml of 100 m*M* phosphate buffer (pH 7.5) containing 40 m*M* KNO_3 and 1.5% 1-propanol. Each sample was vortexed for 2 min to help tissue infiltration by the incubation solution. The test tubes were sealed and incubated for 1 h at 30°C. A blank without KNO_3 was done for each seedling. The enzymatic reaction was stopped by immersing the tubes for 5 min in boiling water. The colorimetric determination of the reaction was done by mixing 0.25 ml of incubation medium with 0.25 ml 0.02% *N*-(1-naphthyl) ethylenediamine and 0.25 ml of sulphanilamide. After 30 min, the absorbance at 540 nm was read.

Enzyme extraction

Frozen leaf tissue (200 mg FW) was ground to a fine powder with mortar and pestle in liquid nitrogen. The leaf powder was extracted with 3 ml of cold (4°C) 0.1 *M* HEPES-KOH buffer (pH 7.5) containing 7% (w/w) polyethylene glycol 20 000, 2 m*M* dithiothreitol, 5 m*M* $MgCl_2$, 5 m*M* ethylene glycol-bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 10% (v/v) glycerol, 1 m*M* phenylmethylsulphonyl fluoride, 9% (w/v) insoluble polyvinylpyrrolidone 25 000. The homogenate was centrifuged at 11 000 *g* for 10 min at 4°C. The supernatant was collected and used as a crude enzyme extract for the determination of total Rubisco, PEPC, and G6PDH activities. Measurements were done for 6 seedlings in each chamber for each harvest day.

Enzyme assays

Enzymatic activities were determined spectrophotometrically (Beckman DU 640). Total ribulose 1,5-*bis*phosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) activity was measured spectrophotometrically at 340 nm in a coupled reaction at 30°C according to the method of Lilley and Walker (1974) modified by Van Oosten et al. (1992). The assay medium consisted of 100 m*M* bicine buffer (pH 8) containing 20 m*M* $MgCl_2$, 25 m*M* $NaHCO_3$, 3.5 m*M* ATP, 0.25 m*M* NADH, 3.5 m*M* phospho-creatine, 80 nkat creatine phosphokinase (EC 2.7.3.2), 80 nkat 3-phosphoglycerate kinase (EC 2.7.2.3), 80 nkat glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) and 30 µl crude extract in a final volume of 600 µl. The mixture was pre-incubated for 15 min at 30°C and 0.5 m*M* ribulose 1,5-*bis*phosphate (RuBP) was added to start the reaction. Control without RuBP was done for each assay. Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) activity was assayed by monitoring the decrease in absorbance at 340 nm in an assay system coupled with malate dehydrogenase (EC 1.1.1.37) at 30°C. The assay medium was based on that of Tietz and Wild (1991) and consisted of 112.5 m*M* Tris-HCl buffer (pH 8.5) containing 5 m*M* $MgCl_2$, 5 m*M* $NaHCO_3$, 0.2 m*M* NADH, 2 m*M* glucose 6-phosphate, $3\,U\,ml^{-1}$ malate dehydrogenase and 30 µl crude extract in a final volume of 600 µl. The reaction was initiated by adding 4.4 m*M* phosphoenolpyruvate. The reference assay did not contain phosphoenolpyruvate. Glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) activity was assayed in the light at 30°C by monitoring the increase in absorbance at 340 nm following a modification of the method of Pitel and Cheliak (1985). The assay medium contained 50 m*M* HEPES buffer (pH 7.6), 10 m*M*

$MgCl_2$, 300 μM $NADP^+$ and 30 μl crude extract in a final volume of 600 μl. The reaction was initiated by adding 2 m*M* glucose 6-phosphate. Control without glucose 6-phosphate was done for each assay. Soluble proteins in crude extracts were assayed with the Bradford Coomassie blue method (Bradford 1976). Enzyme activities were expressed in nkat mg^{-1} protein.

Rubisco content

Total soluble proteins for all time points of the experiment were slot blotted (100 ng per slot) in three duplicate sets. The nitrocellulose membrane (Amersham, Uppsala, Sweden) was hybridized with anti-Rubisco antibodies from spinach (provided by Dr B. Ranty, Toulouse, France). The hybridization signal was developed using the chemiluminescent ECL detection system (Amersham). The membrane was exposed to hyperfilm ECL (Amersham). Hybridization signals were quantified using the Bio-Rad Imaging Densitometer (GS-690 Imaging Densitometer, Bio-Rad Laboratories, Marnes la Coquette, France). Averages of duplicate hybridization signals were used.

CO_2 assimilation, conductance and sugar analysis

Net CO_2 assimilation rate (A) was measured at growth light using a Li-Cor 6200 portable photosynthesis system (Li-Cor Inc, Lincoln, NE, USA) at 41 and 55 days after the start of fumigation treatments. Stomatal conductance (g) was calculated according to the equations of Von Caemmerer and Farquhar (1981). The measurements were taken on 9–13 OL and 4–5 NL seedlings in each growth chamber. Soluble sugars were estimated on 9 roots and 16 OL, NL and stems for each treatment at day 61. Tissues were freeze-dried and ground to a fine powder. Ten mg of the sample was weighed and 0.5 ml of 70% methanol was added to it three times, giving a final volume of 1.5 ml methanol. Afterwards, the samples were centrifuged for 10 min at 15 000 *g*. The spectrophotometric method of Savouré (1980) was used to measure carbohydrate concentrations. A 100-μl aliquot of the supernatant was added to 400 μl of distilled water and 1.5 ml of anthrone 1.5% (P/V). The solution was vortexed and incubated for 8 min at 100°C in a water bath. The solutions were then cooled and the A_{625nm} was read. Sugar concentrations were determined by comparison with a standard curve of known glucose concentrations.

Chlorophyll content

Chlorophyll *a* and *b* were calculated according to the method of Wellburn (1994). One foliar disc (0.95 cm^2) was sampled from each seedling, cut in 2 × 2 mm pieces and incubated at 65°C in 2.5 ml dimethylsulphoxide (DMSO) overnight in the dark. Absorbance was read at 480 nm, 649 nm and 665 nm and chlorophyll content was calculated according to Wellburn (1994).

Statistical analysis

To estimate between chamber variance, we realized a likelihood ratio test (Verbeke 1997). We collected 6 seedlings from each chamber 30 days after they were initially set in the chambers just 1 day before the initiation of fumigation. These seedlings were oven-dried at 70°C and weighed. The likelihood test allowed us to compare the between seedling variance within a chamber with the between seedling variance between the chambers (Table 1). The result of the test (*P*-value of 0.33) showed that between seedlings variance within one chamber was similar to between seedlings variance of seedlings located in different chambers. Thus, we can assume that chamber effect was minimal and that all the measured parameters would be due to the treatment effect.

Two-way ANOVAs were used to test the effect of CO_2, O_3 and their interactions on the enzymatic activities in the OL, NL, on biomass, assimilation, and soluble sugar content. When normality and homogeneity of variance were not obtained, data were transformed and ANOVAs were done with transformed data. Statistical analysis were performed using SAS version 6.12 (proc GLM routine, SAS, Cary, NC, USA) and JMPin 3.2.1 (Statistical Analysis System Inc., Cary, NC, USA). Treatments were considered significant if $P < 0.05$.

Results

Biomass

During the first month of treatment, all the seedlings developed a second flush of leaves (NL). High CO_2 showed a fertilization effect on day 61 in the NL (Fig. 1B): NL biomass was 63% higher than NL biomass of the control seedlings. The high O_3 treatment had a clear negative impact on seedling development, mainly on NL on day 31 (Fig. 1B). NL budbreak was delayed for about 10 days and foliar surfaces were reduced. Relative growth rate (RGR) was significantly lower than that of the control (21.36 ± 2.46 mg g^{-1} day^{-1} compared to 36.9 ± 1.81 mg g^{-1} day^{-1}). On day 61, the total biomass of the high O_3 seedlings was 43% lower than that of the control (Fig. 1A), NL and stems showing a reduced growth (Fig. 1B,D). Most of the leaves had visible damage, sometimes accompanied by desiccation. Under high O_3 + high CO_2, NL development was also delayed but the stress effects were less obvious. RGR was significantly higher

Table 1. Total biomass (mg DW, mean ± SE) of seedlings after 1 month of acclimation in the chambers. *P*-value of the likelihood ratio test result (to test that the between chamber variance hypothesis is 0).

Chambers	Treatments applied at day 1	Total biomass	Likelihood
1	control	160.1 ± 19.8	
2	high O_3	222.8 ± 31.1	$P = 0.33$
3	high CO_2	196.46 ± 17.31	
4	high $CO_2 + O_3$	183.08 ± 14.7	

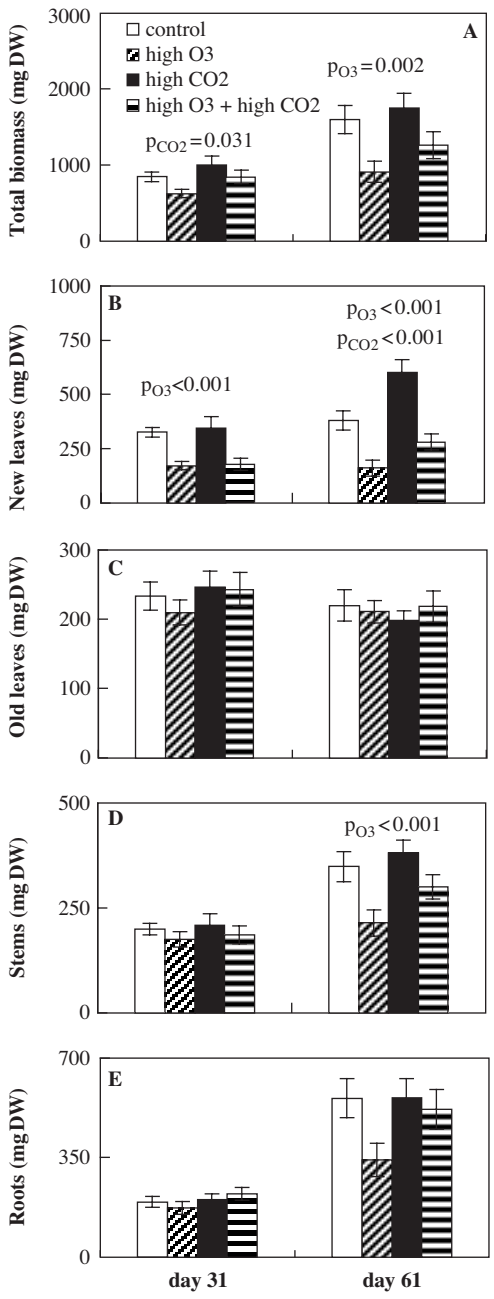

Fig. 1. Total biomass, biomass of the new leaves (NL), old leaves (OL), stems and roots (mg DW, mean ± SE) of sugar maple seedlings harvested at days 31 and 61. *P*-value of significant effects given by the ANOVA are indicated on the bars.

than under high O_3 ($29.88 \pm 2.73\,mg\,g^{-1}\,day^{-1}$ compared to $21.36 \pm 2.46\,mg\,g^{-1}\,day^{-1}$). On day 61, the total biomass of high O_3 + high CO_2 seedlings was intermediate between that of high O_3 and high CO_2 seedlings (Fig. 1A).

CO_2 assimilation and Rubisco

The net CO_2 assimilation rate was measured 41 and 55 days after fumigation had started. At ambient CO_2, high O_3 decreased significantly the net assimilation rate of OL by 58 and 56% on days 41 and 55, respectively (Table 2). The data showed that there was no significant stimulation of the assimilation rate in OL and NL of high CO_2 seedlings compared to the control (Table 2). However, under high O_3, high CO_2 stimulated the assimilation on day 41 in OL and NL. At day 61, the Rubisco content of OL estimated by slot-blot analysis was reduced by 17–25% in the three treatments compared to the control (Table 3). No effect of the treatments was observed on Rubisco activity at days 4, 31 and 61 in OL. In NL, the Rubisco activity at day 61 was increased under high O_3 compared to the control (Table 4).

G6PDH activity

In both types of leaves, G6PDH activity increased in response to the high O_3 treatment: on day 61 the activity was more than 2-fold higher in high O_3 seedlings than in the control (Tables 3 and 4). At day 61 in OL and NL under high O_3 + high CO_2 treatment, the stimulation response was less important than under high O_3 at ambient CO_2 (Tables 3 and 4).

PEPC activity

O_3 caused an increase of the PEPC activity in the OL at days 4 and 61 (Table 3). On day 61, PEPC activity in OL of high O_3 seedlings was more than 2-fold higher than in the control. At days 4 and 61, the presence of elevated CO_2 influenced the response of PEPC in OL under oxidative stress (Table 3).

NR activity

When the results were expressed on a foliar surface basis or on a FW basis the same variation in response to the treatments was observed. At days 31 and 61 NR activity in OL was higher than control under high O_3 whereas NR activity in NL was lower than control under high CO_2 at day 61 (Tables 3 and 4).

Soluble protein content

In OL, protein content under high CO_2 was lower than control at day 31 (Table 3). In NL, there was no significant difference in the soluble protein contents for all the treatments (Table 4).

Chlorophyll content

Minor changes observed between the total chlorophyll content expressed on a leaf area basis or on a fresh weight basis did not alter the interpretation of the results. No treatment effect was observed at days 4 and 31 in the OL. At day 61, total chlorophyll content was lower in the OL of the seedlings of the three treatments compared to the control seedlings whereas in NL, no treatment effect was observed (Table 5). Total chlorophyll

Table 2. Net CO_2 assimilation rate (A, μmol, CO_2 $m^{-2}s^{-1}$), ambient CO_2 concentration measured by Li-Cor (Ca), intercellular CO_2 concentration/Ca ratio (Ci/Ca), stomatal conductance to CO_2 (g, mmol. $m^{-2}s^{-1}$) (mean ± SD) for OL and NL of sugar maple seedlings measured at 41 and 55 under an irradiance of 250 μmol $m^2 s^{-1}$, at 24 ± 2°C and 75 ± 5% of relative humidity. Significance for net CO_2 assimilation rate of CO_2 treatment, the O_3 treatment and CO_2-O_3 interaction as determined by ANOVAs. ND = not determined.

			Old leaves			
Day	CO_2 ($\mu l l^{-1}$)	O_3 ($nl l^{-1}$)	A	Ca ($\mu l l^{-1}$)	Ci/Ca	g
41	350	10	1.95 ± 0.3	357.8 ± 11.7	0.65	14.44 ± 3.4
		200	1.14 ± 0.3	371.3 ± 4.9	0.817	17.13 ± 1.6
	650	10	2.21 ± 1.0	769.2 ± 13.3	0.778	12.97 ± 4
		200	2.56 ± 0.2	732.2 ± 4.6	ND	ND
55	350	10	1.97 ± 0.5	375.8 ± 31.6	ND	ND
		200	1.13 ± 0.4	379.6 ± 6.7	0.779	14.21 ± 2.7
	650	10	2.31 ± 1.0	747.2 ± 7.9	0.68	10.04 ± 3
		200	1.63 ± 0.7	689.2 ± 9.0	0.724	10.37 ± 5.1
			New leaves			
Day	CO_2 ($\mu l l^{-1}$)	O_3 ($nl l^{-1}$)	A	Ca ($\mu l l^{-1}$)	Ci/Ca	g
41	350	10	1.48 ± 0.0	358.9 ± 6.8	0.69	12.71 ± 1.1
		200	1.37 ± 0.7	371.3 ± 4.0	0.9	33.98 ± 4.9
	650	10	1.05 ± 0.2	759.7 ± 11.5	0.88	12.63 ± 2.7
		200	2.09 ± 0.4	737.1 ± 2.8	ND	ND
55	350	10	1.08 ± 0.1	380 ± 33.7	ND	ND
		200	1.05 ± 0.3	379.8 ± 6.8	0.81	20.02 ± 8.1
	650	10	1.2 ± 1.5	754.7 ± 25.5	0.84	8.23 ± 0.6
		200	1.45 ± 0.9	690.8 ± 4.9	0.87	15.11 ± 6.2

Significance of main and interaction effects of A (probability of $H = H_0$)

Day	Effect	OL	NL	Day	Effect	OL	NL
41	CO_2	0.308	0.174	55	CO_2	0.229	0.219
	O_3	**0.004**	0.711		O_3	**0.004**	0.637
	$CO_2 \times O_3$	**0.003**	**0.019**		$CO_2 \times O_3$	0.668	0.563

Table 3. Rubisco content, enzyme activities and protein content (mean ± SD) in OL at days 4, 31 and 61. Significance of these parameters for CO_2 treatment, O_3 treatment, time and their interactions as determined by ANOVAs.

Day	CO_2 ($\mu l l^{-1}$)	O_3 ($nl l^{-1}$)	Rubisco content (% of pre-treatment value)	Rubisco	G6PDH	PEPC	NR (μmol NO_2^- g $FW^{-1}.h^{-1}$)	Protein content (mg.g FW^{-1})
				(nkat.mg $prot^{-1}$)				
4	350	10	100	1.11 ± 0.49	1.42 ± 0.38	0.90 ± 0.20	3.15 ± 0.94	10.87 ± 2.33
		200	100	0.95 ± 0.58	1.51 ± 0.23	1.20 ± 0.30	2.48 ± 0.92	11.70 ± 1.30
	650	10	100	1.12 ± 0.41	1.18 ± 0.45	0.96 ± 0.16	3.53 ± 1.68	11.79 ± 1.87
		200	100	0.92 ± 0.42	1.50 ± 0.22	0.97 ± 0.26	3.60 ± 1.11	10.92 ± 1.58
31	350	10	128.78 ± 18.47	3.04 ± 0.55	1.55 ± 0.45	1.06 ± 0.24	0.74 ± 0.49	13.54 ± 2.62
		200	109.12 ± 9.13	3.14 ± 0.8	2.62 ± 0.71	1.47 ± 0.44	1.60 ± 0.81	13.90 ± 3.56
	650	10	98.38 ± 7.93	2.40 ± 0.53	1.51 ± 0.57	0.97 ± 0.29	1.12 ± 0.77	10.49 ± 2.85
		200	114.76 ± 18.99	2.60 ± 1.35	1.90 ± 0.55	1.58 ± 0.46	0.92 ± 0.49	11.2 ± 2.8
61	350	10	109.51 ± 6.06	2.56 ± 0.73	1.79 ± 0.15	0.75 ± 0.39	0.72 ± 0.45	9.37 ± 1.53
		200	82.54 ± 8.11	1.76 ± 1.25	4.83 ± 1.60	1.73 ± 0.61	1.35 ± 0.78	7.01 ± 2.63
	650	10	84.82 ± 7.65	2.19 ± 1.37	2.05 ± 0.46	0.99 ± 0.12	0.34 ± 0.45	7.57 ± 2.88
		200	90.75 ± 5.54	2.30 ± 0.92	2.78 ± 1.32	0.87 ± 0.40	1.03 ± 0.55	8.05 ± 2.21

Significance of main and interaction effects (probability of $H = H_0$)

Day	Effect	Rubisco content	Rubisco	G6PDH	PEPC	NR	proteins
4	CO_2	–	0.917	0.786	0.472	0.074	0.309
	O_3	–	0.900	0.150	**0.02**	0.467	0.235
	$CO_2 \times O_3$	–	0.886	0.796	0.06	0.369	0.206
31	CO_2	0.264	0.136	0.126	0.710	0.241	0.027
	O_3	0.877	0.707	**0.003**	0.096	**0.01**	0.665
	$CO_2 \times O_3$	0.118	0.908	0.142	0.578	**0.025**	0.886
61	CO_2	**0.007**	0.861	0.115	0.197	0.989	0.722
	O_3	**0.005**	0.544	**0.001**	**0.019**	**0.002**	0.384
	$CO_2 \times O_3$	**0.009**	0.425	**0.034**	**0.017**	0.884	0.192

Table 4. Enzyme activities and protein (mean ± SD) in NL at days 31 and 61. Significance for these parameters of the CO_2 treatment, the O_3 treatment and CO_3 treatment and CO_2-O_3 interaction as determined by ANOVAS. ND = not determined.

Day	CO_2 ($\mu l\,l^{-1}$)	O_3 ($nl\,l^{-1}$)	Rubisco	G6PDH	PEPC	NR ($\mu mol\ NO_2$ g $FW^{-1}.h^{-1}$)	Protein content (mg.g FW^{-1})
				(nkat.mg $prot^{-1}$)			
31	350	10	0.70 ± 0.28	1.17 ± 0.41	0.68 ± 0.21	1.32 ± 0.79	9.12 ± 4.10
		200	ND	ND	ND	ND	ND
	350	10	0.46 ± 0.23	1.47 ± 0.55	1.21 ± 0.65	1.00 ± 0.35	6.59 ± 1.55
		200	ND	ND	ND	ND	ND
61	350	10	0.57 ± 0.19	1.83 ± 0.21	0.99 ± 0.57	0.90 ± 0.38	9.93 ± 4.70
		200	1.60 ± 0.41	3.75 ± 0.64	2.01 ± 1.20	0.82 ± 0.37	11.46 ± 6.32
	650	10	1.07 ± 0.37	2.23 ± 0.68	1.51 ± 0.48	0.54 ± 0.20	7.72 ± 4.09
		200	0.83 ± 0.31	2.66 ± 0.70	2.34 ± 1.04	0.59 ± 0.32	11.24 ± 3.03

Significance of main and interaction effects (probability of $H = H_0$)

Day	Effect	Rubisco	G6PDH	PEPC	NR	proteins
31	CO_2	0.168	0.297	0.118	0.381	0.16
61	CO_2	0.087	0.263	0.229	**0.001**	0.588
	O_3	**0.003**	**0.001**	0.069	0.704	0.268
	$CO_2 \times O_3$	**0.006**	**0.022**	0.815	**0.033**	0.657

(chlorophyll *a* + chlorophyll *b*) showed a similar response to the treatments than chlorophyll *a* and chlorophyll *b* at each day, except for chlorophyll *a* content at day 31 which was significantly lower than the control (data not shown).

Soluble sugars

On day 61, both main effects were apparent in the OL (Table 6). In OL, high O_3 seedlings had less sugar than the seedlings of the three other treatments. This is consistent with the observed decrease of assimilation in these leaves at day 55. No effect of the treatments was observed in the other parts of the seedlings.

Discussion

High CO_2 effect

Young sugar maple seedlings did not show a marked response to the elevated CO_2 treatment for the duration of the experiment and under the environmental conditions used. After 61 days at high CO_2 level, the effect of high CO_2 on biomass was only detected in the NL. Assimilation rate and stomatal conductance were comparable to levels found with sugar maple seedlings of comparable age (Matthes-Sears and Larson 1990, Niinemets and Tenhunen 1997). Assimilation rate and Rubisco activity were not stimulated by high CO_2 level in OL and NL. Variable growth responses to high CO_2 for sugar maple seedlings have been reported in the literature. Bazzaz et al. (1990) showed that under $700\,\mu l\,l^{-1}$ CO_2, the biomass of 1-year-old sugar maple seedlings increased 2-fold compared to the control after 100 days of exposure. An explanation for this large response to CO_2 could be the use of a slow-release fertilizer (15:15:15 N-P-K) in a 1:1:1 sand-perlite-peat soil. Tschaplinski et al. (1995) measured an increase of the net CO_2 assimilation rate of 115% and a 3- to 4-fold increase in total plant DW of 1-year-old sugar maple seedlings exposed to $650\,\mu l\,l^{-1}$ CO_2 during 81 days. In our study, since no fertilizer was applied, we could not exclude that soil quality was diminished. Total chlorophyll content

Table 5. Total chlorophyll content (mg g FW^{-1}, mean ± SD) at days 4, 31 and 61 in OL and NL. Signifcane for total chlorophyll content of the CO_2 treatment, the O_3 treatment and CO_2–O_3 interaction as determined by ANOVA. – = no new leaves, ND = not determined.

Day	CO_2 ($\mu l\,l^{-1}$)	O_3 ($nl\,l^{-1}$)	Old leaves	New leaves	Day	Effect	Significance of main and interaction effects OL	NL
4	350	10	1.86 ± 0.47	–	4	CO_2	0.858	–
		200	1.96 ± 0.2	–		O_3	0.835	–
	650	10	1.82 ± 0.55	–		$CO_2 \times O_3$	0.984	–
		200	1.92 ± 0.38	–	–			
31	350	10	2.48 ± 0.61	2.91 ± 0.38	31	CO_2	0.075	0.297
		200	2.23 ± 0.38	–		O_3	0.314	ND
	650	10	2.03 ± 0.44	2.65 ± 0.55		$CO_2 \times O_3$	0.354	ND
		200	2.11 ± 0.45	–				
31	350	10	3.23 ± 0.67	2.21 ± 0.36	61	CO_2	**0.0001**	0.386
		200	1.26 ± 0.31	1.95 ± 0.78		O_3	**<0.0001**	0.977
	650	10	2.16 ± 0.55	2.47 ± 0.54		$CO_2 \times O_3$	**0.0001**	0.557
		200	1.67 ± 0.44	1.97 ± 0.39				

Table 6. Soluble sugars (mean ± SD) at day 61 for each part of the seedlings. Significance for soluble sugar content of the CO_2 treatment, the O_3 treatment and CO_2–O_3 interaction as determined by ANOVAS.

Day	CO_2 ($\mu l\,l^{-1}$)	O_3 ($nl\,l^{-1}$)	Soluble sugars ($mg\,g\,DW^{-1}$)			
			OL	NL	Stem	Roots
61	350	10	116.6 ± 25	54.3 ± 28	87.3 ± 19.2	41 ± 7.5
		200	84.2 ± 21.9	56.3 ± 20.6	77.9 ± 17.7	42.1 ± 8.8
	650	10	128.1 ± 38.1	56.4 ± 24.1	86.9 ± 16.9	42.9 ± 13.5
		200	116.2 ± 32.2	62.9 ± 25.2	95.3 ± 15.7	41.9 ± 12

Significance of main and interaction effects (probability of $H = H_0$)

Day	Effect	OL	NL	Stem	Roots
61	CO_2	0.005	0.499	0.204	0.815
	O_3	0.005	0.509	0.456	0.995
	$CO_2 \times O_3$	0.174	0.724	0.177	0.775

decreased in OL at day 61 under high CO_2. Reduced chlorophyll content is frequently reported under elevated CO_2 and may reflect a leaf nitrogen dilution or a reallocation to sink tissues (Ceulemans and Mousseau 1994, Ceulemans et al. 1997, Lütz et al. 2000). However in our experiment, the decrease of chlorophyll content, together with the decreased NR activity in NL at day 61, may more suggest a N limitation for the high CO_2 seedlings. This may have constrained physiological responses and could have suppressed the seedlings' responses to elevated CO_2. However, other studies reported the same slight effect of high CO_2 on carbon fixation rate and biomass accumulation in young sugar maple seedlings. Noble et al. (1992) reported that 15-day-old sugar maple seedlings exposed to $800\,\mu l\,l^{-1}$ CO_2 in a non-soil commercial potting mix and fertilized biweekly with a 20-20-20 General Purpose soluble fertilizer showed an increase of only 7% in growth after 85 days. In a recent study, Kruger et al. (1998) measured an increase of 9–11% in DW of 2-year-old sugar maple seedlings watered with a half Hoagland solution after an exposure to $645\,\mu l\,l^{-1}$ CO_2 for 70 days. The authors measured no significant effect of CO_2 on net assimilation on the first flush of the seedlings and a rarely significant reduction of the stomatal conductance. They concluded that the decrease in conductance was probably not responsible for the lack of photosynthetic CO_2 stimulation since the Ci:Ca ratio was always greater than 0.75. Kruger et al. (1998) believed that there were no mineral nutrition deficiencies or pot volume limitation. Also, there was no evidence of inhibition by end-products (sugar, starch), which may otherwise have explained the observed downregulation of photosynthesis and the slight growth response to elevated CO_2. Moreover, the authors confirmed these growth trends with data from an open-top-chambers experiment where sugar maple seedlings were planted in the ground.

High O_3 effect at ambient and elevated CO_2

Under high O_3 at ambient and elevated CO_2, growth of the seedlings is clearly affected by oxidative stress. At the end of the experiment under high O_3 at ambient and elevated CO_2, the Rubisco content in OL was lower than that of the control. Pell et al. (1994) suggested that in sugar maple, Rubisco protein content is low but stays at the same level during the entire life of the leaf. They predicted that oxidation and degradation of Rubisco occurred in the chloroplasts under oxidative stress and suggested that this loss is masked by a high turnover capacity in the leaves. This high turnover implies a high energy cost for the seedlings. However, in our study OL seemed unable to maintain the same high turnover rate, leading to a progressive loss of the Rubisco protein. Under high O_3 at ambient CO_2, net assimilation on day 55 and soluble sugar content on day 61 are decreased in the OL and may be a consequence of the loss of the Rubisco protein. At the end of the treatment, more than 50% of the chlorophyll content in the OL was photo-oxidized. The remaining chlorophyll content may be large enough to ensure the light capture and maintain the lower assimilation rate observed in the O_3-induced senescent older leaves. Under elevated CO_2 and O_3 the degradation of chlorophyll was in part reduced. By contrast, in NL of the seedlings a stable content of chlorophyll and constant or even higher assimilation rate and Rubisco activity were maintained during oxidative stress under both CO_2 levels compared to control. This may occur through a N-reallocation from source OL to younger leaves.

In a plant, detoxification processes are energy consuming. Glutathione reductase (GR, EC 1.6.4.2, one of the enzymes of the ascorbate-glutathione cycle, a detoxification pathway) may consume between 25 and 50% of total NADPH produced per day (Burke and Hatfield 1987). The NADPH produced by G6PDH, the key enzyme of the oxidative pentose-phosphate pathway, may be provided to the GR. We measured an increase of G6PDH activity under high O_3 and high O_3 + high CO_2 during the second month of the treatment. This enhanced production of NADPH may allow a high level of reduced glutathione regeneration by the GR and ensure an efficient functioning of the detoxification cycle in the sugar maple seedlings leaves. Dizengremel

et al. (1994) observed a stimulation of G6PDH activity by 65% over the control for an O_3 treatment of 300 $\mu l\,l^{-1}$. h in loblolly pine needles. A stimulation of the G6PDH activity may thus lead to the production of reducing power for detoxification, and for carbon skeletons necessary for the synthesis of secondary compounds and repair processes.

Under high O_3 and high O_3 + high CO_2, a stimulation of PEPC activity was observed in OL at days 4 and 61. PEPC fixes HCO_3^- in the cytosol and participates in the replenishment of the tricarboxylic acid cycle to provide oxaloacetate, malate or pyruvate (Melzer and O'Leary 1987). Increased activity of tricarboxylic acid cycle enzymes such as NAD dependent malic enzyme, fumarase, isocitrate dehydrogenase in parallel with a stimulation of the PEPC activity have been reported during oxidative stress (Dizengremel et al. 1994, Gérant et al. 1996). Sehmer et al. (1998) observed a stimulation of phosphofructokinase, PEPC and fumarase activities in Norway spruce needles after 84 days under 200 $nl\,l^{-1}$ of O_3. They concluded that glycolysis, the anaplerotic pathway as well as the tricarboxylic acid cycle participated in the production of energy and carbon skeletons for repair processes, thus helping the plants to cope with oxidative stress. Recently, Wohlfahrt et al. (1998) found a correlation between PEPC activity and the extent of damage of Norway spruce (*Picea abies* (L) Karst.) and silver fir (*Abies alba*) needles as well as with the level of proline in the needles. Proline is synthesized via the 2-oxoglutarate derived from the tricarboxylic acid cycle. The authors proposed that enhanced PEPC activity leads to enhanced proline content, which has often been associated with stress (Hare et al. 1999). In the O_3 fumigated OL, NR had a higher level of activity than the control at days 31 and 61. This result, together with the higher level of PEPC activity may support the production of amino and organic acids directed to the repair processes.

Under high O_3 + high CO_2, a slight stimulation of the assimilation rate compared to high CO_2 seedlings was measured at day 41 together with a growth advantage compared to high O_3 seedlings. These improvements may be attributed to high CO_2, which may to some extent protect against O_3 injury by providing additional carbon and energy through the increased net assimilation rate, which is stimulated by elevated CO_2 concentration. The stimulation of G6PDH and PEPC activities was in general less pronounced than under high O_3 at ambient CO_2, which confirms that the need for detoxification was less important than under high O_3 at ambient CO_2. However, this positive effect of high CO_2 may be transient since assimilation was decreased after 55 days of treatment.

Conclusions

After 2 months of growth in the environmental conditions of this experiment, high CO_2 concentration did confer a growth advantage to the new leaves which developed during the treatments. However, we did not measure any stimulating effect of elevated CO_2 on the CO_2 assimilation rate. O_3 largely decreased the growth of the seedlings. G6PDH and PEPC activity largely increased under O_3, contributing to efficient detoxification and reparation processes. Under high O_3 and elevated CO_2 additional carbon was available at the beginning of the treatment and the needs for detoxification were reduced compared to high O_3 seedlings. However, this did not confer a growth advantage compared to the seedlings exposed under high O_3.

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