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Original article

Response to an ozone gradient of growth and enzymes implicated in tolerance to oxidative stress in *Acer saccharum* (Marsh.) seedlings

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Abstract – Two-year-old sugar maple (*Acer saccharum* Marsh.) seedlings were exposed in open top chambers to an extensive gradient of O_3 (0 to 300 nL.L^{-1}) during 85 days under two light environments (20% and 80% of full sun at noon on a sunny day). The growth of truncated seedlings (with one flush of leaves) and episodic seedlings (with two flushes) was decreased as O_3 increased, especially the growth of the second flush which developed under the oxidative treatment. Visible leaf injuries developed during the season under high O_3 concentrations. Survivalist growth strategy of sugar maple, as seen by the root/shoot ratio, together with the enzymatic stimulations of glucose 6-phosphate dehydrogenase, phosphoenolpyruvate carboxylase and glutathione reductase allowed the seedlings to tolerate the O_3 doses. However, at the end of the season, the cumulative oxidative stress in the second flush of the episodic seedlings exposed to concentrations over 150 nL.L^{-1} O_3 was too large and exceeded the capacity of seedlings for detoxification and repair.

carboxylation / detoxification / growth / oxidative stress / sugar maple seedlings

Résumé – Réponse de la croissance et des enzymes impliquées dans la tolérance au stress oxydatif chez des semis d'érable à sucre *Acer saccharum (Marsh.)* exposés à un gradient d'ozone. Des semis d'érable à sucre (*Acer saccharum* Marsh.) de deux ans sont exposés en chambre à ciel ouvert à un large gradient d'O_3 (0 to 300 nL.L^{-1}) pendant 85 jours sous deux environnements lumineux (20 ou 80 % de plein soleil, journée ensoleillée à midi). Avec l'augmentation des concentrations d'O_3, on observe une réduction de la croissance des semis ayant un ou deux flushs de feuilles. La réduction de croissance est particulièrement importante pour le deuxième flush de feuilles qui se développe pendant le traitement. Des dommages foliaires apparaissent durant la saison et sous fortes concentrations d'O_3. La stratégie de croissance de survie de l'érable à sucre, montré par le rapport racine/tige, de même que les stimulations enzymatiques de la glucose 6-phosphate déhydrogenase, la phosphoénolpyruvate carboxylase et la glutathion réductase permettent une tolérance aux doses d'O_3 reçues. Cependant, à la fin de la saison, le stress oxydatif cumulatif dans le deuxième flush des semis exposés à des concentrations d'O_3 supérieures à 150 nL.L^{-1} est trop fort et excède la capacité de détoxification et réparation des semis.

carboxylation / detoxification / croissance / stress oxydatif / érable à sucre

1. INTRODUCTION

Tropospheric ozone is one of the most damaging phytotoxic pollutants [24], and annual biomass losses of forest species can reach 33% depending on the species and the O_3 concentration [20]. O_3 is formed by the photochemical reaction between anthropogenic and biogenic nitrogen oxides (NOx), and volatile organic compounds (VOCs) in polluted air masses. In contrast to other gaseous anthropogenic pollutants such as SO_2, tropospheric O_3 concentration is increasing, probably due to the increase in the levels of NO_x and VOC emissions [21]. Increase in global tropospheric O_3 concentration during the 21st century was projected by a range of global emission scenarios studied by the IPCC 2001 assessment. In the northern hemisphere, near-surface O_3 concentrations are estimated to be increase by about 5 ppb by 2030 and about – 4 to over 20 ppb by 2100, depending on the scenarios [40].

Over the last several decades, surface O_3 concentrations have been closely monitored in North America by the US Environmental Protection Agency and Environment Canada. The Windsor-Québec region (along the St-Laurence River, Québec, Canada) receives high O_3 concentrations from the large industrial and urban regions of the Great Lakes [15]. Several studies have already reported that during the summer, hourly ozone episodes frequently reach 150 nL.L^{-1}, with maximum values of 200 nL.L^{-1} [15, 44, 55].

Sugar maple (*Acer saccharum* Marsh.) is found extensively in the Windsor-Québec region and is of major economic importance for the production of timber and sap. It is a shade tolerant, slow growing species [3]. Mature sugar maple trees have been described as a fixed growth species but young and vigorous sugar maple seedlings may have an episodic growth strategy with production of a second flush of leaves [6, 18].

Following management of sugar maple stands, sugar maple seedlings may be exposed to contrasting light regimes [54]. In

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plantations, seedlings can be exposed to high irradiance levels [57, 59]. Asthon et al. [1] have reported that the leaves of young maple seedlings have a great capacity to adapt to different irradiance environments. Topa et al. [52] reported that leaves grown in shade show a greater susceptibility to O_3 than leaves grown in higher light environments, which is mainly due to the structural differences of the sun and shade leaves [2, 58].

Several studies have shown that sugar maple can be affected by O_3 [7,26,51,52], although it has been classified as a tolerant species to O_3 [30, 35, 41]. No significant effect on growth was observed in sugar maple seedlings after an exposure to 2 times ambient O_3 over two growing seasons (seasonal 24-h mean in air-ambient was 31 ppb O_3 and 66 ppb O_3 in 1990 and 1991 respectively; [42]). Similarly, Laurence et al. [30] found that gas exchange parameters were not affected by O_3 after an exposure to 2 times ambient concentration during three growing seasons. However, decreases in biomass accumulation, photosynthetic rate and Rubisco content have been observed in response to higher O_3 concentrations (200 $nL.L^{-1}$ during 61 days, [16]).

The increased activity of the ascorbate-glutathione detoxication pathway and an increased concentration of antioxidants contribute to the scavenging of toxic oxygen species derived from ozone [14, 36, 37]. Catabolic pathways such as dark respiration, glycolysis and the pentose phosphate pathway have been reported to show increased activity under oxidative stress in some tree species [9, 10, 12, 16, 45].

We still lack knowledge on the tolerance of sugar maple to O_3 and on the detoxification and repair capacities of sugar maple seedlings under an extensive gradient of O_3 in contrasting light environments. Thus, the aim of this study was to evaluate (1) the growth and (2) the response of major enzymes of the catabolic pathways of sugar maple seedlings exposed to an extensive gradient of O_3, from 0 to 300 $nL.L^{-1}$.

2. MATERIAL AND METHODS

2.1. Growth of the seedlings and fumigation treatments

One hundred and forty four sugar maple (*Acer saccharum* Marsh.) seedlings (two years old) were potted in early May of 1996 in 16 L pots (the soil came from a nearby maple stand: sandy loam with more than 10% organic matter). All the seedlings were from the Berthierville Nursery (Ministère des Ressources Naturelles, Québec, Canada). On May 29, after the development of the first flush of leaves, seedlings were equally distributed into 6 open-top chambers. The chambers used were similar to those described by Heagle et al. [19] (without a rain cap) and were located at the Centre de Recherche Acéricole du MAPAQ in Tingwick, approximately 200 km east of Montréal (45° 54' N and 71° 57' W). Each chamber represented one of the 6 O_3 concentrations used in the gradient: (1) 0 $nL.L^{-1}$ O_3; (2) 50 $nL.L^{-1}$ O_3; (3) 100 $nL.L^{-1}$ O_3; (4) 150 $nL.L^{-1}$ O_3; (5) 200 $nL.L^{-1}$ O_3; (6) 300 $nL.L^{-1}$ O_3. Measured mean O_3 concentration in each chamber was 1 ± 2.46 $nL.L^{-1}$ O_3, 46 ± 12 $nL.L^{-1}$ O_3, 100 ± 19 $nL.L^{-1}$ O_3, 149 ± 15 $nL.L^{-1}$ O_3, 199 ± 21 $nL.L^{-1}$ O_3, 293 ± 27 $nL.L^{-1}$ O_3 respectively. Ozone treatments were administered from 6 a.m. to 8 p.m. for the entire growing season. The air entering the chambers was filtered with activated charcoal to remove pollutants prior to ozone enrichment. The ventilation rate was ~ 85 $m^3.min^{-1}$. O_3 concentrations were measured hourly in the centre of the chamber. Hourly control and feedback adjustments of the O_3 level were made using two UV-photometric O_3 analysers (Monitor labs Inc., model 8810, Englewood, Colorado) linked to a datalogger (Campbell scientific Inc., model CR10, Edmonton, Alberta). Ozone was generated from dried ambient air using an OREC auto control ozonator (Ozone research & Equipment Corporation, model 03SP38-ARW, Phoenix, AZ, USA) linked to the datalogger for feedback control. During the night, filtered air entered the chambers. Preliminary tests did not show any significant NOx increase in the out coming air. A more complete description of the chambers can be found in Renaud et al. [43].

To create contrasting light conditions, a neutral-density shade cloth was hung over half the area of each chamber. The irradiance was measured using a quantum sensor (Li-190SA, Li-COR, Lincoln, Neb.). At noon on a sunny day, the irradiance was 20% (350 $\mu mol.m^{-2}.s^{-1}$) and 80% (1500 $\mu mol.m^{-2}.s^{-1}$) of the sunlight intensity measured in the field, for the low and high light treatments respectively.

2.1.1. Morphological types of seedlings

Sugar maple is often considered to be a fixed growth species, characterized by an entirely preformed shoot in the bud [25]. However, young seedlings, saplings, and young and vigorous branches of mature sugar maple may have an episodic growth pattern [6, 18, 48]

In this study, budbreak occurred in mid May prior to setting the seedlings into the chambers. The two seasonal patterns of shoot growth were observed among the seedlings. Two-third of the seedlings had a "truncated" shoot growth pattern similar to that described by Canham et al. [6] as a "cessation of aboveground growth early in the growing season". These seedlings with one leaf flush will be referred to as "truncated" seedlings. The other third of the seedlings had an "episodic" growth strategy [6]: once in the chambers, a second flush of leaves occurred between the end of June and the end of July. These seedlings with two flushes will be referred to as "episodic" seedlings. The first flush of leaves will be hereafter referred to as the preformed flush, consisting of preformed leaves and the second flush as neoformed flush consisting of neoformed leaves as described by Gregory [18].

2.1.2. Harvesting of seedlings

Seedlings were randomly harvested from each chamber and each light treatment on June 28 (31 days of O_3 treatment), July 25 (57 days of O_3 treatment) and August 22 (85 days of O_3 treatment). Harvesting started at 13 h:00 and was completed within 4 h.

At days 31 and 57, we collected two truncated seedlings per chamber per light treatment for a total of 24 seedlings. A sub-sample from a single leaf for each seedling was used for in vivo measurement of nitrate reductase (NR) activity and another sub-sample was oven-dried at 65 °C for 4 days and weighed to determine water content. The rest of the leaves were immediately set on dry ice and kept at –80 °C for future enzymatic analysis.

At day 85, we collected two "truncated" seedlings per treatment per chamber and two episodic seedlings per treatment per chamber

when available. The cumulative effects of O_3 on the biomass were analyzed at this date. The two types of seedling were analyzed separately. Seedlings were immediately divided into roots, stems + petioles and leaves, which were weighed separately to estimate their fresh weight. The leaves and stems of the episodic seedlings were divided according to the different flushes. The projected leaf area of every leaf was measured using an area meter (Delta-T devices, Cambridge, England). Shoot length was measured in cm from root collar to terminal bud for truncated seedlings. For episodic seedlings, total shoot length was separated into two parts: from root collar to the last bud scar (previous year's growth + spring growth) and from bud scar to terminal bud (second flush of growth). The whole root, the whole stem of each flush and a sub-sample of preformed and neoformed leaves were oven-dried at 65 °C for 4 days and weighed to determine water content. A sub-sample from a single leaf was used for the measurement of in vivo NR activity. The rest of the leaves were immediately set on dry ice and kept at –80 °C for future enzymatic analysis.

2.2. Evaluation of visible foliar injury

Rapid visible foliar injury evaluations were done at day 57 and day 85. A 20% scale (O–20%; 21–40%; 41–60%; 61–80%; 81–100%) was used to evaluate the percentage of symptomatic leaves per seedling. We considered that a leaf was symptomatic if at least 2% of his area was injured.

2.3. Enzymatic analysis

2.3.1. In vivo nitrate reductase assay

At the field site, in parallel with the harvests and at all sampling dates, NR (E.C. 1.6.6.1.) activity was measured according to the method of Jaworski et al. [22] as modified by Truax et al. [53]. One hundred mg fresh weight of material from a single leaf was sampled from each flush of each seedling, cut into 2 × 2 mm pieces and incubated in 5 mL of 100 mM phosphate buffer (pH 7.5) containing 40 mM 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 containing one hundred mg fresh weight of leaf material without KNO_3 was prepared for each sample. The reaction was stopped by immersing the tubes for 5 min in boiling water. The colorimetric determination of NO_2^- was done by mixing 0.25 mL of incubation medium with 0.25 mL 0.02% N-(1-Naphtyl) ethylenediamine and 0.25 mL of sulfanilamide. After 30 min, the absorbance was read at 540 nm.

2.3.2. Enzyme extraction

A sub-sample of frozen leaf tissue (200 mg FW) was ground to a fine powder in liquid nitrogen using a mortar and pestle. The leaf powder was extracted with 4 mL of cold (4 °C) 0.1 M Hepes-KOH buffer (pH 7.5) containing 7% (w/w) polyethylene glycol 20 000, 2 mM dithiothreitol, 5 mM $MgCl_2$, 5 mM ethylene glycol-bis-(ß-aminoethyl ether)-N,N,N',N'-tetra acetic acid, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 9% (w/v) insoluble polyvinylpyrrolidone 25 000. The homogenate was centrifuged at 8800 × *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, G6PDH and GR activities.

2.3.3. Enzyme assays

Total ribulose 1,5-*bis*phosphate carboxylase oxygenase (Rubisco, E.C. 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 [31] as modified by Van Oosten et al. [56]. The assay medium consisted of 100 mM bicine buffer (pH 8) containing 20 mM $MgCl_2$, 25 mM $NaHCO_3$, 3.5 mM ATP, 0.25 mM NADH, 3.5 mM phospho-creatine, 80 nkat creatine phosphokinase (E.C. 2.7.3.2), 80 nkat 3-phosphoglycerate kinase (E.C. 2.7.2.3), 80 nkat glyceraldehyde 3-phosphate dehydrogenase (E.C. 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 mM ribulose 1,5-*bis*phosphate (RuBP) was added to start the reaction. A control without RuBP was prepared for each assay.

Phosphoenolpyruvate carboxylase (PEPC, E.C.4.1.1.31) activity was assayed by monitoring the decrease in absorbance at 340 nm in an assay system coupled with malate dehydrogenase (E.C. 1.1.1.37) at 30 °C. The assay medium was based on that of Tietz and Wild [50] and consisted of 112.5 mM Tris-HCl buffer (pH 8.5) containing 5 mM $MgCl_2$, 5 mM $NaHCO_3$, 0.2 mM NADH, 2 mM glucose 6-phosphate, 3 U/mL malate dehydrogenase and 30 µL crude extract in a final volume of 600 µL. The reaction was initiated by adding 4.4 mM phosphoenolpyruvate. The reference assay did not contain phosphoenolpyruvate.

Glucose 6-phosphate dehydrogenase (G6PDH, E.C. 1.11.49) activity was assayed at 30 °C by monitoring the increase in absorbance at 340 nm using a modification of the method of Pitel and Cheliak [39]. The assay medium contained 50 mM Hepes buffer (pH 7.6), 10 mM $MgCl_2$, 300 µM $NADP^+$ and 30 µL crude extract in a final volume of 600 µL. The reaction was initiated by adding 2 mM glucose 6-phosphate. A control without glucose 6-phosphate was used for each assay.

Glutathione reductase (GR, E.C. 1.6.4.2) was assayed according to the procedure of Smith et al. [46]. The assay was performed at 34 °C in a final reaction volume of 1 mL containing 0.1 M K-phosphate buffer (pH 7.5), 0,5 mM EDTA, 0,75 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), 0.1 mM NADPH and 25 µL of enzyme extract. The reaction was initiated by the addition of 2 mM GSSG. The formation of GSH was followed by the increase of A_{412} (ε = 7.2 $mM^{-1}.cm^{-1}$). Soluble proteins in crude extracts were assayed using the Bio-Rad DC method. Enzymatic extracts were precipitated with 20% TCA for 10 min at 4 °C and centrifuged at 8800 × *g* for 10 min. The pellets were dissolved in 1 N NaOH. Enzyme activities were expressed in nkat mg^{-1} protein.

2.4. Measurement of total phenolic compounds

Leaf powder (120 mg DW from the oven-dried leaf sub-sample used for water content determination) was homogenized in 4 mL of 50% (v/v) ethanol. The homogenate was incubated at 40 °C for 3 h and centrifuged at 8800 × *g* for 10 min at room temperature. The supernatant was recovered and evaporated to dryness under a stream of nitrogen. The residue was resuspended in 3 mL distilled water and the phenol content was assayed colorimetrically using the Folin-Denis reagent as described by Swain and Hillis [49]. Total phenolic compounds were determined by comparison with a standard curve generated with tannic acid as a reference.

Table I. Summary of the regression analysis between $diff_{HL-LL}$ for the biomass of leaves, stems and roots (g DW), foliar surface (cm^2), specific leaf mass (SLM, g/Mm^2), height (cm), root/shoot ratio and O_3 concentration ($nL.L^{-1}$) after 85 days of treatment.

		Seedlings with one flush				
	$Diff_{HL-LL}$	Parameter estimates (prob>|t|)		R^2	I†	B†
		Slope	Intercept			
	Leaves	0.070	0.470	0.58	0.4092	–0.00728
	Foliar surface	0.433	0.528	0.16	4.8251	–0.03665
	SLM	0.75	0.80	0.03	–2.4840	–0.01919
	Stems	0.057	0.250	0.64	1.2956	–0.01541
	Height	0.185	0.532	0.39	5.6857	–0.08140
	Roots	0.050	0.134	0.66	3.5493	–0.03160
	Root/shoot	0.182	0.177	039	0.3404	–0.00202
		Seedlings with two flushes				
	$diff_{HL-LL}$	Parameter estimates (prob>|t|)		R^2	I†	B†
		Slope	Intercept			
First flush	Leaves	0.988	0.929	8.10^{-5}	–0.1364	0.00012
	Foliar surface	0.692	0.818	0.06	–2.4027	0.02314
	SLM	0.317	0.441	0.32	–1.465	1.0980
	Stems	0.790	0.710	0.03	–0.6696	0.00263
	Height	0.774	0.858	0.03	–2.3500	0.02100
Second flush	Leaves	0.625	0.779	0.09	0.2074	–0.00203
	Foliar surface	0.394	0.357	0.25	8.8002	–0.04462
	SLM	0.627	0.878	0.09	–4.4760	0.0802
	Stems	0.863	0.707	0.01	–0.1192	–0.00030
	Height	0.698	0.962	0.06	–0.4625	–0.02125
	Roots	0.322	0.411	0.32	1.7093	–0.01178
	Root/shoot	0.688	0.727	0.06	–0.1933	–0.00125

† $Diff_{HL-LL} = I + B(O_3)$, where I is the intercept, B is the slope and O_3 is the O_3 concentration ($nL.L^{-1}$).

2.5. Statistical analyses

We established the influence of the light regimes on the responses of the different variables tested (biomass parameters, Rubisco, G6PDH, PEPC, GR and NR activities, soluble protein content, total phenolic compounds) to the ozone gradient. We calculated the difference between the value of a variable under high light and low light; the difference was reported as "$diff_{HL-LL}$" at each concentration of the O_3 gradient and at each harvest date. We then completed a series of regression analyses using O_3 as an independent variable and "$diff_{HL-LL}$" as a dependent variable for each measured parameters.

These analyses showed that the responses of these variables to the ozone gradient were not different for the two light regimes, except in 2 cases (phenol content and NR activity). In most cases, the slope and the intercept of the regressions were not different from zero (Tabs. I, II and III). Therefore, we pooled the data from both light intensities for the analysis of the effect of the O_3 gradient, except for the phenol content of the truncated seedlings at day 57, which was significantly different under low and high light (Tab. II) and the NR activity of the first flush of the episodic seedlings at day 85, where there was an interaction between O_3 and light (Tab. III).

We chose the regression approach to assess the quantitative relationships between our measured variables and the O_3 gradient. With six levels of ozone, regression is the most powerful way of analyzing the data [47]. A series of linear regressions using the six different O_3 concentrations as the independent variable and the pooled data (biomass parameters, Rubisco, G6PDH, PEPC, GR and NR activities, soluble protein content, total phenolic compounds) as the dependent variable were completed at each harvest day. On two occasions data were not pooled, for the phenol content at day 57 and the NR activity of the first flush of the episodic seedlings at day 85. On day 85, a second-order regression was used to examine the response of G6PDH and PEPC activities, because these data responses were not linear. All regression analyses were performed using the statistical software JMP 3 (SAS Institute Inc.) The level of significance was set at 0.05.

3. RESULTS

3.1. The effects of light and ozone on biomass

Light treatments had no effect on biomass parameters (Figs. 1 and 2). Biomass accumulation of leaves, stems, roots and foliar surface area of the two morphologically different groups of seedlings, shoot length of the second flush of episodic seedlings decreased with increasing O_3 (Figs. 1

Table II. Summary of the regression analysis between $diff_{HL-LL}$ for soluble proteins, Rubisco, GR, G6PDH, PEPC, NR, phenol content and O_3 concentration for seedlings with one flush at each harvest day.

$Diff_{HL-LL}$	Day of harvest	Parameter estimates (prob>|t|)		R^2	I†	B†
		Slope	Intercept			
Proteins	31	0.257	0.507	0.30	0.8673	0.00948
	57	0.247	0.213	0.31	–3.0503	0.01682
	85	0.925	0.804	0.002	0.7933	0.00181
Rubisco	31	0.240	0.395	0.32	–0.3178	0.00278
	57	0.697	0.143	0.04	1.1231	0.00156
	85	0.307	0.087	0.25	1.6619	–0.00521
GR	31	0.790	0.680	0.02	–0.0315	0.00012
	57	0.606	0.148	0.07	0.1069	–0.00020
	85	0.959	0.602	0.001	0.0380	–0.00002
G6PDH	31	0.432	0.444	0.16	–0.0976	–0.00061
	57	0.267	0.817	0.29	–0.0362	0.00114
	85	0.403	0.984	0.18	–0.0024	0.00060
PEPC	31	0.578	0.809	0.08	0.0710	–0.00100
	57	0.123	0.099	0.48	0.3182	–0.00175
	85	0.957	0.493	0.001	0.0921	–0.0004
NR	31	0.401	0.996	0.18	0.0034	0.00360
	57	0.107	0.088	0.52	0.8901	0.00494
	85	0.626	0.682	0.06	0.0430	–0.00031
Phenols	31	0.847	0.362	0.014	30.8707	0.03716
	57	0.263	0.025	0.30	36.9461	–0.08331
	85	0.770	0.682	0.05	–31.7380	0.11430

† $Diff_{HL-LL} = I + B(O_3)$, where I is the intercept, B is the slope and O_3 is the O_3 concentration ($nL.L^{-1}$).

Table III. Summary of the regression analysis between $diff_{HL-LL}$ for soluble proteins, Rubisco, GR, G6PDH, PEPC, NR, phenol content and O_3 concentration for the heterophyllous seedlings after 85 days.

$Diff_{HL-LL}$	Flush	Parameter estimates (prob>|t|)		R^2	I†	B†
		Slope	Intercept			
Proteins	First	0.173	0.175	0.51	6.7360	–0.03758
	Second	0.776	0.794	0.03	1.0680	–0.00646
Rubisco	First	0.286	0.666	0.36	0.3482	–0.00524
	Second	0.404	0.455	0.24	0.9658	–0.00608
GR	First	0.289	0.970	0.35	–0.0042	0.00075
	Second	0.280	0.293	0.52	0.2680	–0.00206
G6PDH	First	0.328	0.652	0.31	–0.1620	0.00210
	Second	0.840	0.997	0.01	0.0012	0.00039
PEPC	First	0.929	0.850	0.01	–0.0753	–0.00019
	Second	0.544	0.464	0.13	–0.1491	–0.00067
NR	First	0.020	0.015	0.86	0.1113	0.00050
	Second	0.060	0.061	0.88	0.3081	0.00110
Phenols	First	0.726	0.472	0.07	30.7590	–0.07544
	Second	0.487	0.716	0.26	15.7376	–0.16955

† $Diff_{HL-LL} = I + B(O_3)$, where I is the intercept, B is the slope and O_3 is the O_3 concentration ($nL.L^{-1}$).

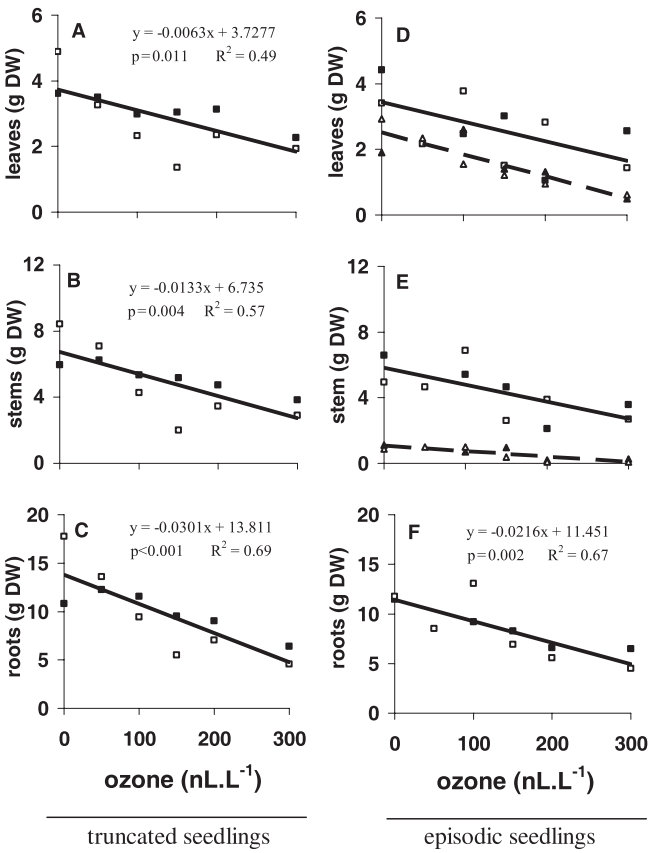

Figure 1. Biomass of leaves, stems, roots (g DW) of the truncated (A, B, C) and episodic (D, E, F) seedlings after 85 days under increasing O_3 concentrations (data from first flush grown under low light (■) and high light (□), — regression line; data from second flush grown under low light (▲) and high light (△), −− regression line). D: first flush: $y = -0.006x + 3.44$, $R^2 = 0.36$, $p = 0.05$; second flush: $y = -0.0067x + 2.5142$, $R^2 = 0.77$, $p < 0.001$. E: first flush: $y = -0.0104x + 5.8252$, $R^2 = 0.47$, $p = 0.02$; second flush: $y = -0.0033x + 1.0701$, $R^2 = 0.71$, $p = 0.001$.

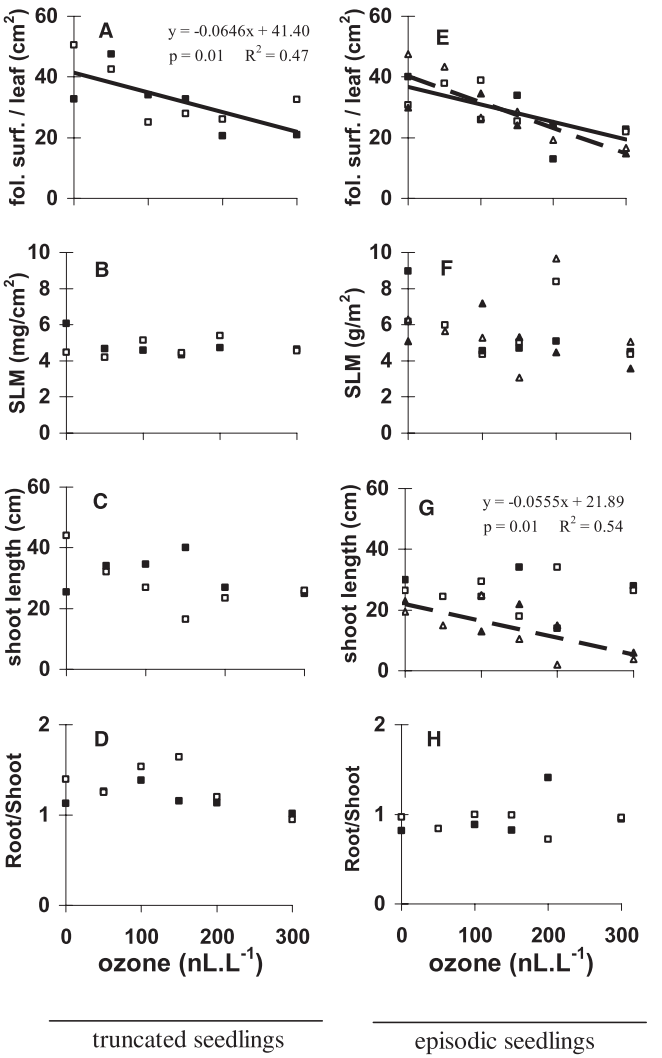

Figure 2. Foliar surface per leaf (cm^2), specific leaf mass per leaf (SLM, g/m^2), shoot length (cm), root/shoot ratio of the truncated (A, B, C, D) and episodic seedlings (E, F, G, H) after 85 days of treatment under increasing O_3 concentrations (data from first flush grown under low light (■) and high light (□), — regression line; data from second flush grown under low light (▲) and high light (△), −− regression line). E: first flush: $y = -0.0572x + 36.667$, $R^2 = 0.49$, $p = 0.01$; second flush: $y = -0.0852x + 40.18$, $R^2 = 0.73$, $p = 0.0007$.

and 2). The specific leaf mass (SLM) of both morphological types of seedling was not influenced by O_3 (Fig. 2).

The truncated seedlings allocated a large proportion of their biomass to the root system (with a root/shoot ratio above one) whereas the episodic seedlings, which allocated biomass for the development of the neoformed flush, had a root/shoot ratio less than one. Irradiance levels did not modify the root/shoot ratio of either group of seedlings (data not shown), nor did increasing O_3 level: both shoot growth and root growth decreased with increasing O_3, leading to a constant root/shoot ratio (Fig. 2).

3.2. Visible leaf injury

The different types of leaf injury usually associated with O_3 were observed mostly on the first flush. Stipples appeared uniformly on the upper leaf surface and have a purple-brown coloration. Some brown bifacial necrotic spots were observed. At day 57, no foliar injury was observed on seedlings exposed at 0 and 50 $nL.L^{-1}$ O_3(data not shown). Half of the seedlings exposed at 200 $nL.L^{-1}$ $O_3$and 85% of the seedlings exposed at 300 $nL.L^{-1}$ O_3 have more than 80% of symptomatic leaves (i.e. leaves with at least 2% of leaf area injured). At day 85, we still did not observe foliar injury on seedlings exposed to 0 and 50 $nL.L^{-1}$ O_3. More than 70% of the seedlings exposed at 200 $nL.L^{-1}$ $O_3$and 95% of the seedlings exposed at 300 $nL.L^{-1}$ O_3 have more than 80% of symptomatic leaves.

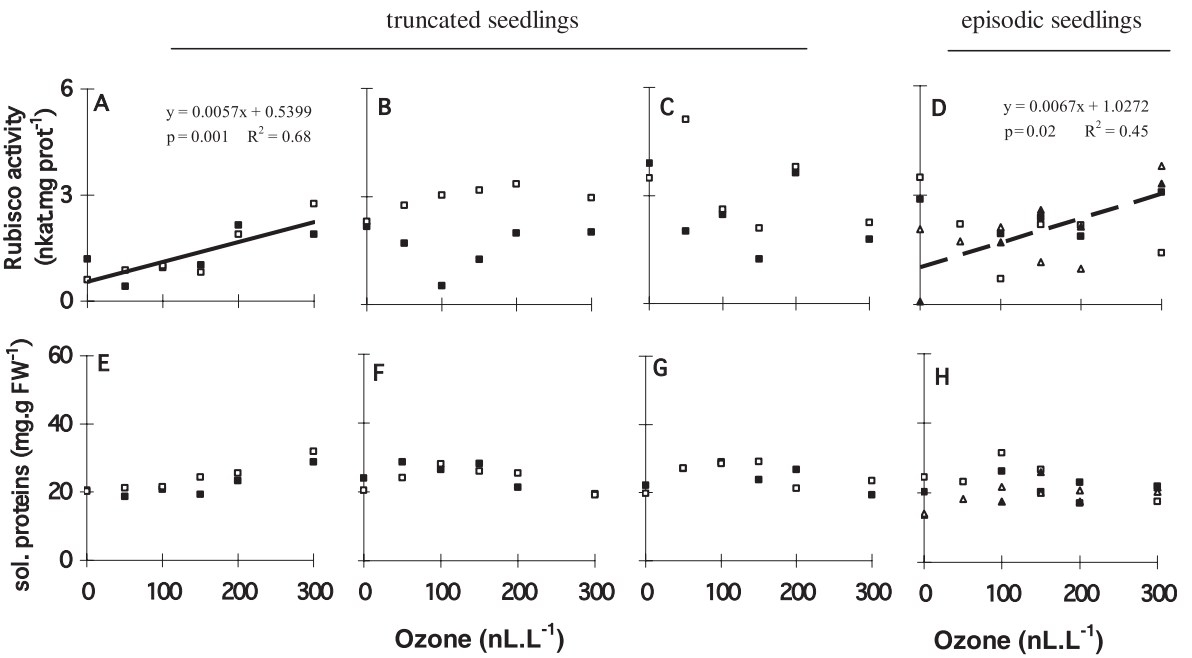

Figure 3. Rubisco activity (nkat.mg prot^{-1}) and soluble protein content (mg.gFW^{-1}) for the truncated seedlings after 31 days (A, E), 57 days (B, F) and 85 days (C, G) and the episodic seedlings (D, H) under increasing O_3 concentrations (data from first flush grown under low light (■) and high light (□), — regression line; data from second flush grown under low light (▲) and high light (△), – – regression line).

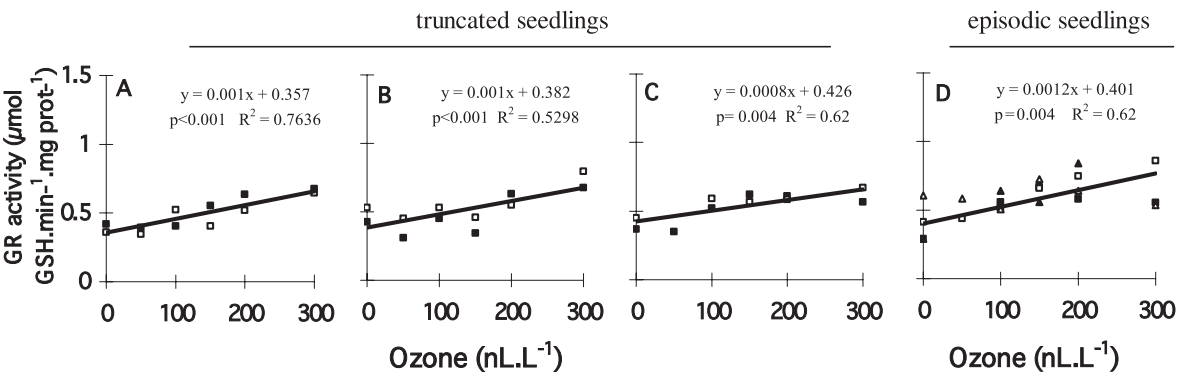

Figure 4. GR activity (µmol GSH.min^{-1}.mg prot^{-1}) for the truncated seedlings after 31 days (A), 57 days (B) and 85 days (C) and the episodic seedlings (D) under increasing O_3 concentrations (data from first flush grown under low light (■) and high light (□), — regression line; data from second flush grown under low light (▲) and high light (△), — regression line).

3.3. Effect of light and ozone on enzymatic responses

When the results were expressed on a foliar surface basis or on a protein basis the same variation in the response to the treatment was observed. The Rubisco activity of the truncated seedlings was increased by exposure to O_3 on day 31 but was not affected by O_3 on days 57 and 85 (Figs. 3A–3C). After 85 days of treatment, the Rubisco activity of the neoformed flush increased with increasing O_3 (Fig. 3D). The soluble protein content was constant during the whole growing season and was not affected by O_3 (Figs. 3E–3H).

The O_3 treatment led to a significant stimulation in the GR activity of preformed leaves of both seedling types throughout the growing season (Figs. 4A–4D).

G6PDH activity was highly stimulated by increased O_3 levels at each harvest day (Figs. 5A–5D). However, with time, the level of activity decreased under the higher concentrations of O_3: after 85 days, non-linear relationship was significant and the activity of G6PDH in the neoformed flush of the episodic seedlings decreased when O_3 concentration exceeded 150 nL.L^{-1} (Fig. 5D).

Anaplerotic fixation of CO_2 by PEPC increased in response to O_3 in both groups of seedlings (Figs. 5E–5H) during the growing season. The level of activity decreased in the neoformed flush of the episodic seedlings when the O_3 concentration exceeded 150 nL.L^{-1} (Fig. 5H). After 85 days the Rubisco/PEPC ratio in the truncated seedlings grown in the absence of O_3 was 5. At 300 nL.L^{-1} O_3 in the

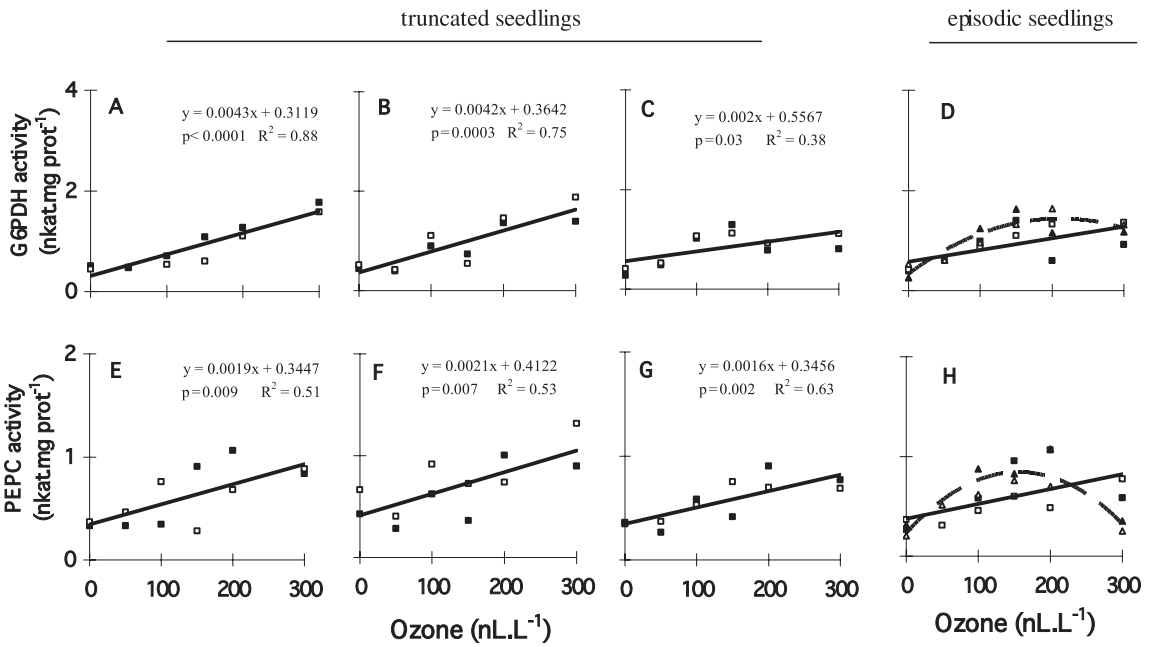

Figure 5. G6PDH activity (nkat.mg prot^{-1}) and PEPC activity (nkat.mg prot^{-1}) for the truncated seedlings after 31 days (A, E), 57 days (B, F) and 85 days (C, G) and the episodic seedlings (D, H) under increasing O_3 concentrations (data from first flush grown under low light (■) and high light (□), — regression line; data from second flush grown under low light (▲) and high light (△), – – regression line). D: first flush: $y = 0.0023x + 0.5751$, $p = 0.026$, $R^2 = 0.43$; second flush: $y = -2.10^{-5}x^2 + 0.0105x + 0.3356$, $p < 0.001$, $R^2 = 0.83$. H: first flush: $y = 0.00145x + 0.377$, $p = 0.017$, $R^2 = 0.29$; second flush: $y = -3.10^{-5}x^2 + 0.0078x + 0.2312$, $p = 0.001$, $R^2 = 0.82$.

truncated seedlings, as PEPC activity increased two-fold over the seedlings grown in absence of O_3 and Rubisco activity was not modified, the Rubisco/PEPC ratio decreased to 2.5.

The NR activity of the seedlings grown in the absence of O_3decreased more than two-fold during the growing season. In the truncated seedlings there was no change in the NR activity under O_3 (Figs. 6A–6C). On day 85, the first flush of the episodic seedlings has a higher NR activity under high light than under low light, whereas the effect of O_3 was not significant (Fig. 6D, note the distribution of the white and black squares). In the second flush, no effect of light was observed but NR activity decreased with increasing O_3 (Fig. 6D).

On day 57, the total phenolic content was higher under high irradiance than under low irradiance (Fig. 6F, note the distribution of the white and black squares). O_3 had no effect on the phenol content under high irradiance or low irradiance (Fig. 6F). On day 85, the total phenol content decreased in both flushes of leaves in response to O_3 (Fig. 6H).

4. DISCUSSION

The growth rate of the seedlings was similar under both light environments used in our experiment. Sugar maple is a shade tolerant species [3] and its assimilation rate is maximal at low levels of irradiance. The carbon gain of sugar maple has been shown to increase more between 1% and 10% of full sunlight than between 10 and 100% [11]. The light environments in our experiment were comparable to the levels found in large gaps (low irradiance treatment) and in plantations (high irradiance treatment). We conclude that the assimilation rates of the seedlings were not limited by light availability, resulting in a similar total carbon gain and a similar growth rate for seedlings in both light treatments. Leaf morphology, structure and thickness are known to differ with increasing irradiance levels [2, 51]. However, in our study, specific leaf mass values were similar at both irradiance levels. This indicates that all the leaves had a similar morphology. The SLM values are in the range of those found by Fortin and Mauffette [13] for the sun leaves of sugar maple, which confirmed that both light environments represent sunny conditions for the growth of sugar maple seedlings.

In the absence of O_3, episodic seedlings had a higher growth than truncated seedlings. However, these seedlings were not able to sustain episodic growth under high O_3, thus, after 85 days at 300 nL.L^{-1}, both types of seedlings had a similar total biomass. Increasing oxidative stress limited new carbon skeletons production, less of which were then available for the development of new sink tissues. The root/shoot ratio was not affected by increasing O_3 in truncated nor episodic seedlings. The seedlings showed a reduced accumulation of biomass in below and above ground tissues in the same proportion so that root/shoot ratios remained constant across the O_3 gradient. This may be considered to be a conservative strategy which enhances the survivorship of the seedlings of slow-growing species [5].

Some enzymatic stimulation was also involved in this conservative strategy. The Rubisco activity was generally not

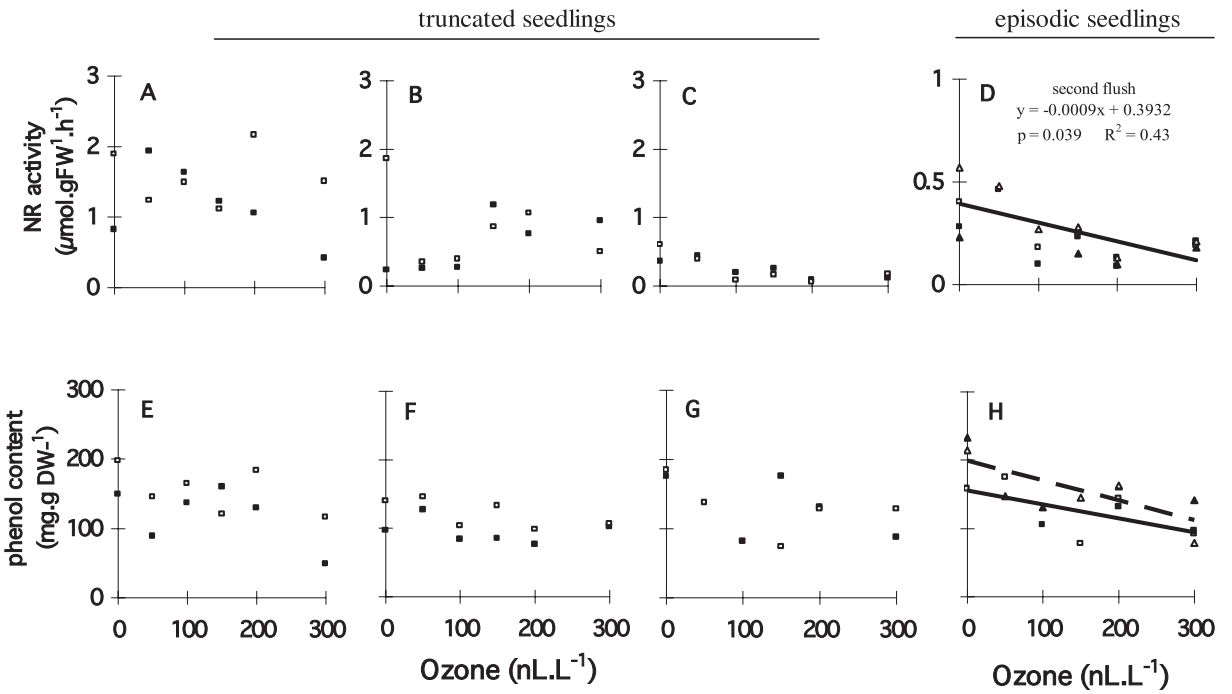

Figure 6. NR activity (µmol NO_2.g $FW^{-1}.h^{-1}$) and phenol content (mg.g DW^{-1}) for the truncated seedlings after 31 days (A, E), 57 days (B, F) and 85 days (C, G) and the episodic seedlings (D,H) under increasing O_3 concentrations (data from first flush grown under low light (■) and high light (□), — regression line; data from second flush grown under low light (▲) and high light (△), – – regression line). H: first flush: $y = -0.2041x + 155.85$, $p = 0.04$, $R^2 = 0.46$; second flush: $y = -0.2903x + 199.19$, $p = 0.02$, $R^2 = 0.55$.

affected by high O_3 concentrations and actually increased at the end of June and at the end of August in the younger leaves of the episodic flush under high O_3. This stimulation of CO_2 fixation by Rubisco in young foliar tissues may be a transient response to oxidative stress. A similar transient response was observed in a previous study where 45 day-old sugar maple seedlings were exposed to 200 $nL.L^{-1}$ O_3 in phytotronic chambers during 61 days [16]. However, in some other species decreased Rubisco activity and quantity is a more constant response to O_3 [12, 33, 38].

The scavenging of toxic O_3 derivatives is in part achieved by the ascorbate-glutathione detoxification cycle [14]. GR is one of the enzymes of this cycle. In the present study, GR activity increased two-fold in sugar maple seedlings in response to increasing O_3 stress. This enzyme ensures a high level of GSH regeneration and thus an efficient functioning of the detoxification cycle by keeping ascorbate in a functional reduced state. We measured a significant increase in the G6PDH activity with increasing O_3. G6PDH is a key enzyme of the oxidative pentose phosphate pathway, which may oxidize 5 to 20% of cellular glucose (the other 80 to 95% is oxidized by glycolysis). Stimulation of this enzyme allows an enhanced NADPH production. GR may consume 25 to 50% of the total NADPH produced during the day [4] and may use the NADPH produced by G6PDH. In the neoformed flush of the episodic seedlings, stimulation of G6PDH activity was not maintained until the end of the season (day 85) when O_3 exceeded 150 $nL.L^{-1}$. Therefore, NADPH production should also decrease with the reduction of G6PDH activity. However, GR activity was maintained at a high level suggesting that the enzyme used NADPH produced by the photochemical reactions of photosynthesis. At high O_3 concentrations the decrease in G6PDH may be due to a reduced availability of its substrate, glucose 6-phosphate, which in turn may be due to a larger allocation of the glyceraldehyde 3-P to glycolysis at the expense of starch synthesis and the pentose-phosphate pathway.

Stimulation of PEPC activity was observed as O_3 concentration increased. In the cytosol, PEPC produces the oxaloacetate needed for de novo synthesis of amino acids, which are used for protein synthesis [29]. Under oxidative stress, proteins may be degraded by O_3 and its by-products [28]. Thus, a large proportion of newly synthesized proteins are allocated to the repair processes [45]. The increased PEPC activity observed in our seedlings presumably supplied the repair processes via the anaplerotic pathway [45]. However, after 85 days, this stimulation was not maintained in the neoformed flush of the episodic seedlings when O_3 exceeded 150 $nL.L^{-1}$. This suggested a lower capacity for the production of amino- and organic acids and, consequently, a lower input to the repair processes at the end of the season.

The total phenol content is reported to increase in response to O_3 [8, 32] and phenolic compounds such as the precursors of lignin are known to be implicated in the repair processes of injured leaves [23]. However, phenol content of truncated seedlings remained unchanged with increasing O_3 and even decreased with increasing O_3 for episodic seedlings. Large progression of foliar symptoms observed at the end of the

season suggested that less precursors and less energy was allocated to repair of injured foliar tissues. As PEPC transformed PEP to OAA and as PEPC activity increased with increasing O_3, the PEP availability may have decreased. Thus the amount of PEP, which is a precursor of phenols, may be less available for the synthesis of phenolic compounds. Seedlings may partition PEP between the replenishment of the tricarboxylic acid cycle and the shikimate pathway. The allocation of PEP to the tricarboxylic acid cycle may have increased with increasing O_3 whereas its allocation to the shikimate pathway may have decreased.

The NR activity was decreased by more than two-fold during the growing season in the absence of ozone. During expansion and maturation of the leaves, N assimilation is mainly under the control of NR. After maturation, N assimilation decreases and recycled N from photorespiration or protein degradation constitutes the major source of N in the plant [27]. The high level of NR activity on days 31 and 57, together with the stimulation of the PEPC by O_3, may support the production of amino- and organic acids directed to repair processes. However, after 85 days, the NR activity in the seedlings was lower and decreased with increasing O_3. At that time in the season and with increasing oxidative stress, NH_3 may have been provided from photorespiration to allow the regeneration of amino acids derived from carbon skeletons provided by the PEPC, which still had a high activity. Only a few studies have measured the response of photorespiration to oxidative stress and contradictory results have been observed. Dizengremel and Pétrini [9] observed an increase in the photorespiration pathway in plants under pollutant stress. However, Manderscheid et al. [34] measured a decrease in glycolate oxidase activity (a peroxisomal enzyme of the photorespiratory pathway) in *Pinus taeda* needles exposed to air pollution.

As a shade tolerant, slow growing species [3], sugar maple has a low assimilation rate, leading to a compromise between maximizing aboveground growth and developing below ground growth to enhance the survivorship of seedlings [5,17]. Under oxidative stress, the stimulation of the enzymes implicated in energy, reducing power and carbon skeleton production in response to O_3 may be a part of the species-related survivalist strategy of growth for sugar maple, which allowed seedlings to tolerate the high O_3 levels of this experiment.

However, under high O_3, growth of neoformed flush of the episodic seedlings is reduced and foliar injuries of the seedlings are important. Moreover, PEPC and G6PDH activities were depressed after 85 days in the neoformed flush when exposed to more than 150 $nL.L^{-1}$ O_3. The cumulative oxidative stress was too large and possibly exceeded the capacity for detoxification and repair of the neoformed flush. The defensive capacity of these neoformed flush seems to collapse at the end of the season. As previously explained, this may lead to a decreased production of reducing power and carbon skeletons resulting in a less efficient repair and detoxification processes. The presence of a second flush did not confer an advantage to the episodic seedlings for detoxification or repair. Thus, the potentially more vigorous episodic seedlings did not tolerate the oxidative stress more efficiently than the truncated seedlings during one growing season.

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