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RESEARCH PAPER

The alternative respiratory pathway allows sink to cope with changes in carbon availability in the sink-limited plant *Erythronium americanum*

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Abstract

Mechanisms that allow plants to cope with a recurrent surplus of carbon in conditions of imbalance between source and sink activity has not received much attention. The response of sink growth and metabolism to the modulation of source activity was investigated using elevated CO_2 and elevated O_3 growth conditions in *Erythronium americanum*. Sink activity was monitored via slice and mitochondrial respiratory rates, sucrose hydrolysis activity, carbohydrates, and biomass accumulation throughout the growth season, while source activity was monitored via gas exchanges, rubisco and phosphoenolpyruvate carboxylase activities, carbohydrates, and respiratory rates. Elevated CO_2 increased the net photosynthetic rate by increasing substrate availability for rubisco. Elevated O_3 decreased the net photosynthetic rate mainly through a reduction in rubisco activity. Despite this modulation of the source activity, neither plant growth nor starch accumulation were affected by the treatments. Sucrose synthase activity was higher in the sink under elevated CO_2 and lower under elevated O_3, thereby modulating the pool of glycolytic intermediates. The alternative respiratory pathway was similarly modulated in the sink, as seen with both the activity and capacity of the pathway, as well as with the alternative oxidase abundance. In this sink-limited species, the alternative respiratory pathway appears to balance carbon availability with sink capacity, thereby avoiding early feedback-inhibition of photosynthesis in conditions of excess carbon availability.

Key words: Alternative pathway, bulbous plant, carbohydrate, carbon allocation, carbon metabolism, *Erythronium americanum*, respiration, sink limitation.

Introduction

During growth, source tissues are photosynthetically active and export synthesized carbohydrates to photosynthetically less active or inactive sink tissues, such as roots, fruits, tubers or bulbs (Dickson, 1991). Sinks are thus characterized by a net importation of carbohydrates, which are used for growth, maintenance, and storage. The ability of a sink organ to import assimilates, also referred to as sink strength, is determined by both sink size and sink activity (Farrar, 1993). Strengths of the different sinks define, in turn, carbon (C) allocation patterns at the whole plant level. C allocation patterns change throughout the season, following plant developmental stages, but many abiotic factors can also modify C allocation pattern through changes in the amount of C fixed by the plant.

C allocation in plants with storage organs has often been studied through modulation of the source activity by altering either light levels (Kehr *et al.*, 1998) or gas concentrations (i.e. increasing level of CO_2 and O_3, Andersen, 2003; Balaguer *et al.*, 1995). Stimulation of source activity translates into higher photosynthetic rates, leading to an increased supply of carbohydrates. Excess C is usually allocated to the sinks, leading to more accumulation of

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reserves in the storage organs (Balaguer *et al.*, 1995). This is the case for source-limited plants. In sink-limited species, photosynthetic rates are modulated more extensively by sink C-demand than by abiotic factors (Sawada *et al.*, 2003), leading to complex physiological and biochemical controls of source activity. A decrease of assimilate utilization by sinks generally leads to the accumulation of sucrose or starch in leaves, which then decreases photosynthesis through feedback inhibition (Paul and Foyer, 2001). However, sugar accumulation in either source or sink organs can also stimulate respiration (Amthor, 1991) and avoid feedback inhibition of different C metabolism pathways. Indeed, as respiration is central to all C metabolism pathways, it could play a key role in C exchanges between source and sink.

Increasing respiratory rates can cause an over-reduction of some of the electron transport chain components (Turrens, 2003). In the terminal step of the respiratory process, electrons can either pass along the phosphorylating cytochrome pathway or the non-phosphorylating alternative oxidase pathway. It has been suggested that the alternative pathway has the ability to use excess ubiquinone electron pools, thereby avoiding over-reduction of the electron transport chain, which could lead to the synthesis of reactive oxygen species (ROS) (Moller, 2001; Rich and Bonner, 1978). This homeostatic regulation is linked to the hypothesis that the alternative pathway acts as an 'energy overflow' conduit for the cytochrome pathway (Lambers, 1982). The main factors that determine electron partitioning between the cytochrome and alternative pathways are the ratio of reduced ubiquinone to total ubiquinone pools (Wagner *et al.*, 1998), the amount and redox state of alternative oxidase (AOX) proteins (Umbach and Siedow, 1993), the presence of α-keto acids (Millar *et al.*, 1993; Umbach *et al.*, 1994), and the availability of ADP and Pi (Juszczuk *et al.*, 2001). The levels of these specific metabolites can vary with developmental stage and environmental conditions. However, modulation of the different respiratory pathways has only been well-documented for source organs (Gonzàlez-Meler *et al.*, 2001; Millenaar and Lambers, 2003). Information is scarce for sink organs, especially in relation to source activity.

The present study attempted to modulate the source activity in *Erythronium americanum* Ker Gawl. (trout lily) and to study its impact on both source and sink C metabolism. Source activity was modulated using different environmental gas conditions: elevated CO_2 to increase C fixation and elevated O_3 to reduce rubisco activity. Due to its simple morphology, *E. americanum* is very close to a theoretical source–sink model and, thus, is an interesting biological model in which to study whole-plant C allocation. Indeed, 90% of individuals in this species have only one leaf and only one bulb (Blodgett, 1910), corresponding to one source and one strong sink. *Erythronium americanum* is an abundant spring geophyte of North American maple forests, whose epigeous development begins early in the spring and takes place over a short period, from snow melt to canopy closure (Muller, 1978; Taylor and Pearcy, 1976). Carbohydrate storage in the bulb is renewed during this period and when completed, appears to induce leaf senescence (Lapointe, 2001). Thus, plant growth becomes rapidly sink-limited as the bulb reaches its final size. Source activity was monitored via gas exchanges, rubisco and phosphoenolpyruvate carboxylase (PEPc) activities, respiratory rates, and sugar concentrations, whereas sink activity was monitored via respiratory rates, sucrose hydrolysis activity, carbohydrate concentrations, and plant biomass. This study should help unravel some of the regulatory mechanisms that allow sink-limited species to cope with changes in C availability.

Materials and methods

Plant material and growing conditions

Bulbs of *E. americanum* were collected in September in a maple forest near Saint-Augustin-de-Desmaures (QC, Canada; 46° 48′ N, 71° 23′ W). Bulbs of similar biomass (0.35–0.40 g fresh weight) were selected and planted in plastic pots containing Turface (calcined clay granules, Applied Industrial Materials Corp., Buffalo Grove, IL, USA) as substrate. Plants were kept in a cold chamber for 5 months of cold stratification at 4 °C and then randomly transferred into eight phytotron chambers. Four different gas treatments were applied to these chambers: control, with 390 mmol mol^{-1} of CO_2 and 0 nmol mol^{-1} of O_3 (charcoal-filtered air); elevated CO_2, with 1000 mmol mol^{-1} of CO_2; elevated O_3, with 80 nmol mol^{-1} of O_3; and elevated CO_2+O_3, with 1000 mmol mol^{-1} of CO_2 and 80 nmol mol^{-1} of O_3. Other growth parameters were held constant: photoperiod, 14 h; air temperature, 18/14 °C day/night; relative humidity, 75%; and photon flux density (*PPFD*), 350 μmol m^{-2} s^{-1}. Ambient air in each chamber was analysed continuously by an ozone analyser (O341M, Environment SA, Paris, France) and a CO_2 analyser (WMA2 PPsystems, Stotfold, UK). Plants were watered daily and fertilized weekly with 10% Hoagland's solution to ensure optimal growth (Lapointe and Lerat, 2006).

Gas exchange measurements

Net CO_2 photosynthetic rate (*Pn*) was measured under phytotron conditions (CO_2, temperature, and RH) using a Li−Cor 6400 Portable Photosynthesis System (Li−Cor Inc, Lincoln, NE, USA). Light conditions during the measurements were constant at 350 μmol m^{-2} s^{-1}, with air flow at 200 μmol s^{-1}. Measurements were randomized between treatments from 10 h to 13 h. *Pn* was measured on six plants per treatment (three per chamber) at leaf unfolding (t_1, day 5), at the initiation of leaf senescence (t_8, day 21), at complete leaf senescence (t_9, day 28), and every 2 d from t_1 to t_8.

Plant growth measurements

Three plants per chamber were harvested on the same days that *Pn* was measured, plus one extra harvest at the time

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plants were moved to the chamber (t_0). Leaves, bulbs, and roots were dried for 24 h at 70 °C and weighed separately.

Enzyme extraction and assays

Measurements were done on four plants per chamber that were harvested 4, 8, 12, 16, and 20 d after transfer to the growth chamber. Leaves and bulbs from each plant were separated and immediately frozen in liquid nitrogen. Tissues were then stored at –80 °C until extraction. Frozen leaf and bulb tissues (300 mg FW) were ground in liquid nitrogen with a mortar and pestle. Crude enzyme extractions were performed at 4 °C according to Fontaine *et al.* (2002). Enzymatic activities were determined spectrophotometrically (ELX 808 Microplate reader, Bio-Tek instruments, St-Quentin-en-Yvelines, France) in coupled reactions by monitoring NADH oxidation at 340 nm. Measurements of rubisco and PEPc activities were carried out according to Fontaine *et al.* (2002). Cytoplasmic invertase was assayed in 200 µl of 50 mM HEPES–NaOH (pH 7) containing 2.8 mM ATP, 1.2 mM NADP, 12 U ml^{-1} hexokinase (EC 2.7.1.1), 3.5 U ml^{-1} phosphoglucoisomerase (EC 5.3.1.9), 1.75 U ml^{-1} glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and 10 µl of enzymatic extract. The reaction was initiated by adding 100 mM sucrose. A similar medium with 2 mM UDP was used for the Susy assay. Susy activity was estimated by difference between the reactions with and without UDP. Controls without substrate addition were run with all assays.

Carbohydrate concentrations

The leaf and bulb of three plants per chamber were analysed for starch, sucrose, and glucose-fructose concentrations at 0, 4, 8, 12, 20, and 24 d after transfer to the growth chambers. Carbohydrates were estimated according to Blakeney and Mutton (1980). Frozen tissues were lyophilized for 24 h and weighed before maceration in a solution of methanol, chloroform, and water (12:5:3 by vol.) for 20 min at 65 °C. The mixture was ground with a Polytron (Kinematica, Lucerne, Switzerland) and centrifuged at 3500 rpm for 10 min at 4 °C. Starch contained in the pellet was gelatinized in boiling water for 90 min and then hydrolysed at 55 °C for 60 min in the presence of amyloglucosidase. The supernatant was analysed before and after invertase digestion to estimate reducing sugars and sucrose concentrations, respectively. Finally, all reducing sugars were quantified colorimetrically at 415 nm after reaction with *p*-hydroxybenzoic acid hydrazide (Sigma Chemical Co., St Louis, MO, USA).

Slice respiration measurements

Respiration was recorded on leaves and bulbs of six plants per treatment at 4, 8, 12, 16, and 20 d following transfer to the growth chambers. A slice of fresh tissue was infiltrated, according to Jolivet *et al.* (1990), in a medium containing 100 mM mannitol, 10 mM HEPES, 10 mM MES (pH 6.6), and 0.2 mM $CaCl_2$. Slice respiration was measured polarographically in the same medium, using a Clarke-type electrode (Rank Brothers Ltd., Cambridge, England). The alternative pathway was inhibited by adding 10 mM salicylhydroxamic acid (SHAM, resuspended in methoxy-ethanol) and the cytochrome pathway was inhibited by adding 1 mM potassium cyanide (KCN). Addition of SHAM and KCN together was used to record residual respiration (v_{res}). Residual respiration was constant, around 6.1% of the total respiration, for both leaf and bulb tissues, regardless of the treatment or date of harvesting. In accordance with Bahr and Bonner (1973), it is postulated that the cytochrome pathway runs at a saturating rate under the experimental conditions ($V_{cyt}=v_{cyt}$). Total respiratory rate (V_T) was measured in the absence of inhibitors, whereas the activity of the cytochrome pathway (v_{cyt}) was measured in the presence of SHAM as $v_{cyt}=V_{T+SHAM}-v_{res}$, where V_{T+SHAM} corresponds to the respiratory rate when SHAM was added first. The capacity of the alternative pathway (V_{alt}) was measured in the presence of KCN as $V_{alt}=V_{T+KCN}-v_{res}$., where V_{T+KCN} represents the respiratory rate when KCN was added first. The activity of the alternative pathway (v_{alt}) was estimated as $v_{alt}=V_T-v_{cyt}-v_{res}$. The engagement of the alternative pathway (ρ') was determined as the ratio of v_{alt} to V_{alt}. The participation of the alternative pathway (P) was determined as the ratio of v_{alt} to V_T.

Isolation of mitochondria

Fresh bulbs (10 g) were cut in 100 ml of cold extraction medium containing 0.35 M mannitol, 30 mM MOPS buffer (pH 7.4), 7 mM cysteine, 2 mM EGTA, 2.5 mM $MgCl_2$, 0.2% BSA (w/v), and 0.3% PVP (w/v). The bulbs were homogenized for 4× 1 s at full speed in a blender (Moulinex, Ecully, France). The homogenate was filtered through a 60 µm nylon net. The bulb fragments retained in the net were reblended in the mixer with 100 ml of the extraction medium. Homogenization and filtration were repeated as described above. The two successive filtrates were pooled and centrifuged according to Gerard and Dizengremel (1988). The mitochondrial pellet was resuspended in washing medium containing 0.35 mM mannitol, 10 mM MOPS buffer (pH 7.2), and 0.1% BSA w/v. Membrane integrity was measured spectrophotometrically at 550 nm, as the oxidation of reduced cytochrome *c* by washed intact versus burst mitochondria (Krippner *et al.*, 1996). As mitochondrial isolation required much more material (10 g) than respiration on slices of tissue, it was only done for material harvested on day 16 for each treatment.

Mitochondrial respiration

Oxygen uptake was followed polarographically with a Clark-type electrode (Rank Brothers Ltd., Cambridge, England) on isolated mitochondria at 16 d. Respiratory studies were performed in a reaction medium containing 0.35 M mannitol, 5 mM $MgCl_2$, 10 mm KC1, 0.1% BSA (w/v), and 10 mM phosphate buffer (pH 7.2). The oxidation of

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succinate (20 mM) was measured in the presence of 200 μM ATP at pH 7.2. The oxidation of malate (30 mM) was followed at pH 6.7 in MES buffer, and at pH 7.8 in TRIS-HCl buffer, as these two pHs were optimal for NAD-malic enzyme and malate dehydrogenase activity, respectively. The oxidation of malate at pH 7.8 was carried out in the presence of glutamate (2 mM) and NAD (400 μM) as a co-factor of malate dehydrogenase. The oxidation of NADH (1 mM) was followed at pH 7.2. Mitochondrial protein concentrations were determined according to Bradford (1976). KCN (1 mM) in aqueous solution and SHAM (700 μM) in methoxy-ethanol were used as inhibitors of the cytochrome and alternative pathways, respectively. V_T, v_{cyt}, v_{alt}, ρ', and P were estimated in the same manner as for slice respiration measurements. Moreover, ADP/O ratio was estimated as the molar ratio of ADP added to the mitochondria to oxygen consumed following complete utilization of the ADP. This ratio represents the efficiency of oxidative phosphorylation. Respiratory control (*RC*) was determined as the ratio of state 3 (i.e. when ADP is in excess) to state 4 (i.e. when ADP is limiting) and represents the control exerted by ATP synthase on the electron transport chain.

AOX immunoblot

The amount of AOX was estimated by the Western blot method on the same isolated mitochondrial extracts used for respiration measurements. Mitochondrial proteins were extracted in 62.5 mM TRIS (pH 6.8) buffer containing 10% v/v glycerol, 2% v/v SDS, 0.005% v/v bromophenol blue, and 28 mM β-mercaptoethanol. The amount of total protein was fixed at 180 μg and separated by SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane using 48 mM TRIS buffer containing 39 mM glycine, 0.04% SDS, and 20% v/v methanol. AOX monoclonal antibodies were used as primary antibodies and anti-mouse IgG fragments conjugated with peroxidase were used as secondary antibodies. The bands were revealed on the autoradiograms using SuperSignal Ultra Chemiluminescent Substrate. Densitometric analysis was performed to quantify the intensities of the bands corrected for the background using ImageJ software (NIH ImageJ, NIH, Bethesda, MD).

Statistical analysis

All variables were analysed by three-way ANOVA (Statistix 8.2, Analytical Software, Tallahassee, FL, USA) testing CO_2 (Elevated versus Low), O_3 (Elevated versus Low), and time (7–10 harvest dates depending on the variable) as fixed effects, except for mitochondrial respiration and AOX immunoblots for which several plants had to be pooled to get enough material for one measurement per treatment. In this case, no ANOVA could be performed and only technical repetitions were carried out. Each of the four treatments was assigned to one of the eight chambers, for a total of two repetitions per treatment (n=2 for most variables). A new series of plants were grown under the same conditions the following year. Plant growth variables were recorded each year to ensure the repeatability of growth conditions over the two years (n=4). Other variables were measured either in the first or the second year due to time constraints and the number of plants per chamber needed for each type of measurements. Significant effects were determined for P <0.05. *A posteriori* multiple comparisons tests were performed using Fisher's LSD. Pearson product–moment correlations (r) were also carried out on cumulative amount of C fixed per plant, Susy activity and bulb respiratory rate for each harvest date and each treatment.

Results

Net photosynthetic rate and leaf carboxylase activities

Net photosynthetic rate increased quickly from leaf unfolding (5 d) to reach maximum rates at 9 d (Fig. 1), after which *Pn* decreased continuously until complete leaf senescence at day 28. When averaged over the whole life of the leaf, *Pn* under elevated CO_2 was 59% higher than under control conditions, whereas *Pn* under elevated O_3 was 29% lower, compared with the control (Table 1). Under elevated O_3, elevated CO_2 stimulated *Pn* by 25% until day 9, compared with the control. Subsequently, decreases in *Pn* under elevated CO_2+O_3 were faster than under ambient air. Thus, *Pn* values under elevated CO_2+O_3 no longer differed from those of the control after 15 d.

Under ambient air, leaf rubisco activity increased rapidly until day 16 and then decreased before the first visual sign

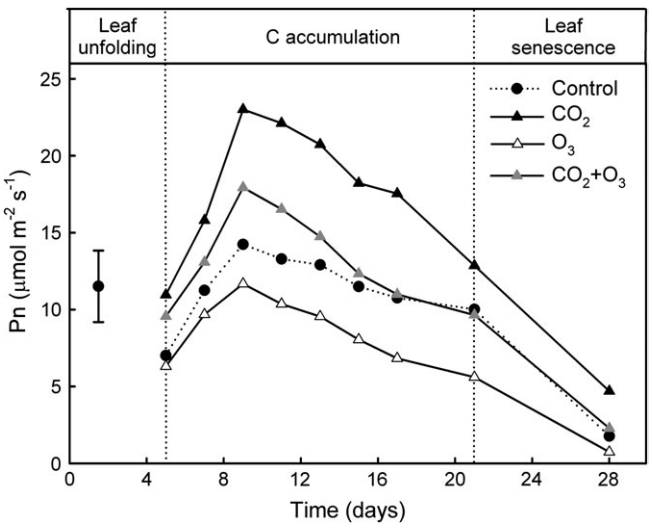


Fig. 1. Time-courses of net photosynthetic rate (*Pn*) in leaves of *E. americanum* grown under control, elevated CO_2, elevated O_3, and elevated CO_2+O_3 during the epigeous growth period. The last data points (28 d) correspond to complete leaf senescence. The standard error, estimated from the MSE term in the ANOVA, is shown with the grand mean (N=2). The two vertical dotted lines indicate the end of leaf unfolding and the beginning of leaf senescence, respectively.

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Table 1. Results of three-way factorial ANOVA testing effects of elevated CO_2 and elevated O_3 treatments on *E. americanum* growth, gas exchanges, metabolites, enzyme activities and respiration over time

F-values are presented along with statistical differences: * P <0.05, ** P <0.01, *** P <0.001.

	CO_2	O_3	Time	$CO_2 \times O_3$	$CO_2 \times$time	$O_3 \times$time	$CO_2 \times O_3 \times$time
Leaf parameters							
Net photosynthesis	121.19 ***	53.66 ***	45.85 ***	4.49 *	1.96	1.53	0.09
Rubisco activity	1.68	36.07 ***	11.54 ***	0.80	1.06	7.29 ***	1.05
PEPc activity	1.76	77.95 ***	26.75 ***	8.16 *	0.14	6.93 **	1.11
Dry mass	0.03	0.09	20.50 ***	0.01	0.04	0.19	0.02
Sucrose	0.07	4.34	37.43 ***	0.35	0.16 *	2.24	0.41
Reducing sugars	0.12	0.40	0.93	0.25	0.13	0.30	0.50
Slice respiration	0.31	92.76 ***	426.16 ***	3.65	0.62	7.30 ***	0.77
Slice alternative respiration	3.87	152.43 ***	32.27 ***	13.47 **	0.54	6.14 **	0.10
Slice alternative capacity	0.06	342.85 ***	451.84 ***	70.02 ***	5.40 **	25.30 ***	5.87 **
Participation of the alternative pathway	3.87	152.43 ***	32.27 ***	13.47 **	0.54	6.14 **	0.10
Engagement of the alternative pathway	2.41	30.77 ***	–	0.43	–	–	–
Bulb parameters							
Dry mass	0.07	2.55	84.31 ***	0.22	0.12	0.29	0.33
Susy activity	38.43 ***	38.06 ***	84.97 ***	4.88 *	3.11 *	4.35 *	0.34
Invertase activity	0.15	4.29	375.35 ***	1.22	1.92	0.73	0.38
Starch	4.44	1.38	306.53 ***	0.00	0.62	1.02	0.21
Sucrose	2.19	0.17	165.57 ***	0.07	0.41	0.79	0.43
Reducing sugars	9.58 ***	6.30 *	112.01 ***	3.90	1.43	1.34	0.55
Slice respiration	359.49 ***	190.10 ***	843.79 ***	12.01 **	23.56 ***	19.22 ***	19.30 ***
Slice alternative respiration	305.05 ***	359.87 ***	24.44 ***	94.29 ***	15.70 ***	31.28 ***	20.11 ***
Slice alternative capacity	905.09 ***	796.56 ***	522.52 ***	416.53 ***	78.97 ***	86.45 ***	51.32 ***
Participation of the alternative pathway	305.05 ***	359.87 ***	24.44 ***	94.29 ***	15.70 ***	31.28 ***	20.11 ***
Engagement of the alternative pathway	39.19 **	57.42 **	–	8.24	–	–	–

of leaf senescence, which occurs at day 21 (Fig. 2A). Leaf rubisco activity tended to increase more rapidly under elevated CO_2 until day 12, but overall activity was not significantly different from that of the control (Table 1). O_3 treatment decreased leaf Rubisco activity by 22% from day 12 to 20, regardless of CO_2 concentration. However, leaf PEPc activity responded differently to O_3 stimulation depending on CO_2 concentration (Fig. 2B). Between days 12 and 20, O_3-induced increases in PEPc activity were 56% and 38% under ambient and elevated CO_2, respectively. Elevated CO_2 concentrations alone did not affect leaf PEPc activity compared with the control.

Plant biomass

Bulb biomass exhibited a large increase shortly after complete leaf unfolding (i.e. 5 d; Fig. 3). The maximum was reached at day 17, a few days before the initiation of leaf senescence (i.e. 21 d), after which time bulb biomass stopped increasing. No treatment significantly affected bulb growth kinetics or its final biomass, compared with the controls (Table 1). Bulb biomass represented 83±0.5% of total plant biomass at final harvest. Leaf and root biomass were fairly constant over time and similar among treatments.

Bulb sucrose hydrolysis activities

Invertase activity was high at the beginning of the season up to 8 d in all treatments, while Susy activity was minimum (Fig. 4). While invertase decreased progressively thereafter, Susy activity increased to maximum activity at 12 d. At 16 d, invertase activity reached minimum values around 20.1 nkat g^{-1} FW. Invertase activity was greater than Susy activity by 4-fold, suggesting that sucrose was mainly hydrolysed by invertase in the bulb of *E. americanum* (Fig. 4A). Elevated CO_2 conditions increased Susy activity by 21% from day 8 to the initiation of senescence, compared with the control, whereas elevated O_3 decreased Susy activity by 10% (Fig. 4B, Table 1). Susy activity was similar under elevated CO_2+O_3 and control conditions, and presented intermediate values between elevated CO_2 and elevated O_3 treatments. Elevated CO_2 and elevated O_3 did not affect invertase activity.

Sugar accumulation

Starch was absent from the leaf throughout the season in *E. americanum*. Sucrose concentrations in the leaf increased over time (Fig. 5A), whereas reducing sugar concentrations were constant, around 25 mg g^{-1} DW (Fig. 5B). Neither elevated CO_2 nor elevated O_3 treatments significantly affected leaf sucrose or reducing sugar concentrations (Table 1).

In the bulb, starch concentration exhibited a sigmoidal curve similar to that of the bulb mass, suggesting that the main constituent of bulb growth was starch accumulation. This result was confirmed by high final starch concentrations, which reached around 80% in the bulb, irrespective of the

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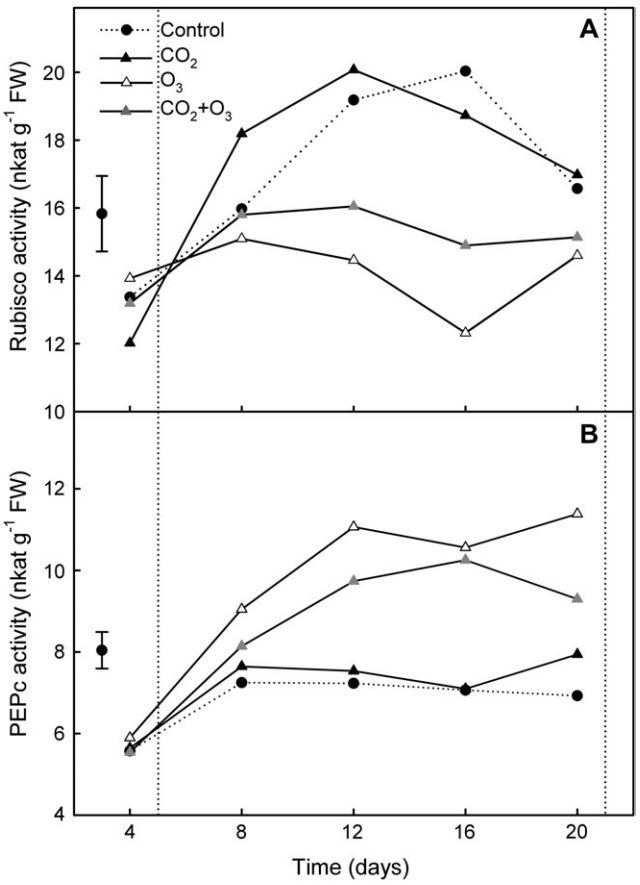


Fig. 2. Time-courses of Rubisco carboxylase activity (A) and PEPc activity (B) in leaves of *E. americanum* grown under control, elevated CO_2, elevated O_3, and elevated CO_2+O_3 during the epigeous growth period The standard error, estimated from the MSE term in the ANOVA, is shown with the grand mean (N=2). The two vertical dotted lines indicate the end of leaf unfolding and the beginning of leaf senescence, respectively.

treatment (Fig. 5C). Starch concentration tended to increase more quickly after 4 d under elevated CO_2 conditions than under the three other growth conditions (P=0.090). Neither elevated O_3 nor elevated CO_2+O_3 conditions affected starch accumulation kinetics. After 16 d, starch concentrations were similar among treatments until leaf senescence. Sucrose exhibited a similar decrease in concentrations under all treatments (Fig. 5D). Similarly, reducing sugar concentrations decreased sharply after 4 d (Fig. 5E). However, elevated CO_2 conditions maintained a 26% higher concentration from day 8 to complete leaf senescence, whereas elevated O_3 conditions slightly reduced soluble sugar concentrations compared with the control.

Leaf and bulb slice respiration

In the leaf, total O_2 consumption (V_T) increased until 12–16 d and decreased abruptly several days before leaf senescence (Fig. 6A). Elevated CO_2 curves were similar to the control curves (Table 1), whereas O_3 treatment increased total leaf respiration by 18% between day 8 and day 16 (i.e. during the fastest growth phase), independent of CO_2 concentration. During this period, leaf respiration inhibited by SHAM (v_{alt}) was 2-fold greater in O_3-treated plants than in control plants, leading to an increased participation of the alternative pathway in total respiration (v_{alt}/V_T), from 0.16 in the controls to 0.26 in both O_3 treatments (Fig. 6B). Moreover, alternative pathway respiration tended to be higher at elevated CO_2 concentrations than under control conditions, starting from day 8 (P=0.076). Elevated O_3 increased the total capacity of the alternative pathway (V_{alt}) by 59% compared with the controls (Figs 6C, 7A, C). Elevated CO_2 did not affect the capacity of the alternative pathway (Fig. 7B). Engagement of the alternative pathway (v_{alt}/V_{alt}) also increased under elevated O_3 compared with the control.

In the bulb, total respiration increased during the first 12 d of growth, after which a maximum value was maintained up to the initiation of leaf senescence (Fig. 6D). Respiratory rates were 29% higher under elevated CO_2 from day 12 onwards, whereas elevated O_3 decreased respiration by 10%, compared with ambient air (Table 1). Elevated CO_2+O_3 conditions did not significantly affect respiratory rates. Bulb respiration inhibited by SHAM was up to 2.8 times higher under elevated CO_2 conditions from day 12 onwards, compared with the control (Fig. 6E). This increase in alternative pathway respiration was responsible for 100% of the increase in total respiration under elevated CO_2 conditions. Elevated O_3 induced a 39% decrease in the activity of the alternative pathway, as averaged over day 12 to day 20, which was responsible for 81% of the decrease in respiratory rates under elevated O_3. Thus, the participation of the bulb alternative pathway was increased from 0.18 in the control to 0.37 under elevated CO_2 and decreased to 0.08 under elevated O_3 at 16 d. Elevated CO_2+O_3 significantly affected neither alternative respiratory pathway rates nor alternative respiratory capacity in the bulb, compared with the control (Figs 6E, F, 7E, H). The alternative pathway capacity (V_{alt}) in the bulb was 2.4 times higher under elevated CO_2 compared with the control, whereas it was slightly lower than the control under elevated O_3 (Figs 6F, 7F, G). Engagement of the alternative pathway increased from 0.84 in the control to 0.98 under elevated CO_2 and was 2-fold lower under elevated O_3, suggesting a full utilization of the AOX enzyme under elevated CO_2. Engagement of the alternative pathway under elevated CO_2+O_3 conditions (0.80) was similar to that of the control.

Bulb mitochondrial respiration

Mitochondria were extracted from material harvested at 16 d, where the largest differences between treatments were observed for bulb slice respiration (Fig. 6). The integrity of washed mitochondrial membrane was high and constant among treatments (78–82%, data not shown). The respiratory rate of the bulb mitochondria was the highest in state 3 (i.e. when ADP is available in excess), with succinate as the initial substrate of the electron transport chain (i.e. 99 nmol O_2 min^{-1} mg^{-1} protein; Fig. 8A), and lowest with NADH (i.e. 39 nmol O_2 min^{-1} mg^{-1} protein; Fig. 8B). Malate

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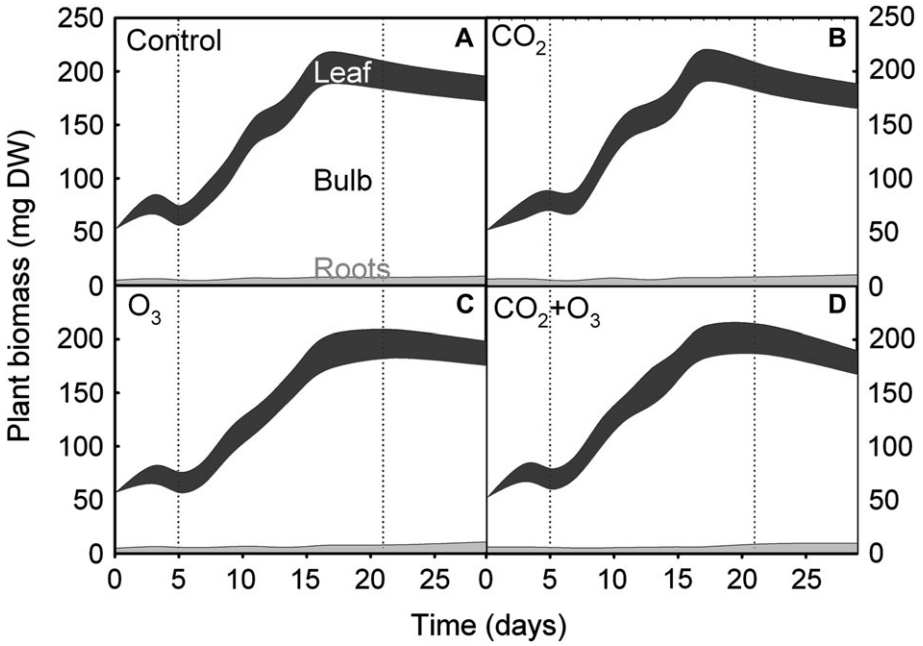


Fig. 3. Time-courses of plant biomass of *E. americanum* grown under control (A), elevated CO_2 (B), elevated O_3 (C) and elevated CO_2+O_3 (D) during the epigeous growth period. The last data points (28 d) correspond to complete leaf senescence. Leaf, bulb, and roots are presented in black, white, and grey, respectively (N=4). The two vertical dotted lines indicate the end of leaf unfolding and the beginning of leaf senescence respectively.

oxidation was 37% higher when malate dehydrogenase activity was induced at pH 7.8 (81 nmol O_2 min^{-1} mg^{-1} protein; Fig. 8C) than under malic enzyme activity at pH 6.7 (62 nmol O_2 min^{-1} mg^{-1} protein; Fig. 8D). Succinate oxidation was partially inhibited by KCN (1 mM) and cyanide-insensitive respiration, which represented 18% of total respiration, was completely blocked by SHAM (700 μM). Similarly, cyanide-insensitive respiration represented 19% and 24% of the total respiration during malate oxidation at pH 7.8 and pH 6.7, respectively. These results suggest that electron flow was mediated by both the cytochrome and the alternative pathways for all three substrates. On the other hand, KCN almost completely blocked NADH oxidation by the electron transport chain.

Malate oxidation at pH 6.7 exhibited treatment effects, on both respiratory rate and the alternative pathway, that were most similar to the treatment effects observed with slice respiration. In order to approximate the true physiological state and because of the central role played by malate in plant cell metabolism the behaviour of this substrate was therefore presented here to compare the four treatments. When SHAM was first added, it inhibited 15% of malate oxidation at pH 6.7 (i.e. mostly by malic enzyme), suggesting a rate (v_{alt}) of 9 nmol O_2 min^{-1} mg^{-1} protein for the alternative pathway (Table 2). Elevated CO_2 concentrations strongly stimulated malate oxidation from 68 nmol O_2 to 91 nmol O_2 min^{-1} mg^{-1} protein (V_T). This stimulation was linked to a 4.2-fold higher activity of the cyanide-insensitive pathway (v_{alt}) and a 2.4-fold higher alternative pathway capacity (V_{alt}). Elevated O_3 affected malate oxidation at pH 6.7. The alternative pathway activity was slightly lower, whereas the alternative pathway capacity was reduced by 44% under elevated O_3, compared with the controls. Elevated CO_2+O_3 conditions affected neither malate oxidation at pH 6.7 nor allocation of the mitochondrial electron flux to the alternative pathway or the cytochrome pathway. Moreover, as the activity of the cytochrome pathway was essentially constant under the different treatments (v_{cyt}), modulation of malate oxidation at pH 6.7 can be entirely explained by changes in the activity of the alternative pathway. Thus, the engagement and participation of the alternative pathway was much higher under elevated CO_2 compared with the control. Lower *RC* and a lower *ADP/O* ratio were recorded under elevated CO_2 compared to the control, supporting the idea of increased participation of the alternative pathway. Indeed, the alternative pathway reduces the H^+ gradient leading to reduced ATP synthesis. By contrast, *RC* increased under elevated O_3. *RC* and the *ADP/O* ratio were also slightly higher under elevated CO_2+O_3 than under control conditions.

Immunoblotted AOX protein

Western blot analysis of mitochondria isolated from bulbs indicated that, at 16 d, the amount of AOX protein was 1.4 times higher under elevated CO_2 (Fig. 9), and 3.4 times lower under elevated O_3, compared with the control. Under elevated CO_2+O_3 conditions, AOX content was slightly lower than in the control plants. On the Western blots, AOX from *E. americanum* appeared as a monomeric reduced form with a molecular mass of 39 kDa. This apparent molecular mass of AOX coincided with the predicted molecular mass of the mature enzyme (39.331 kDa).

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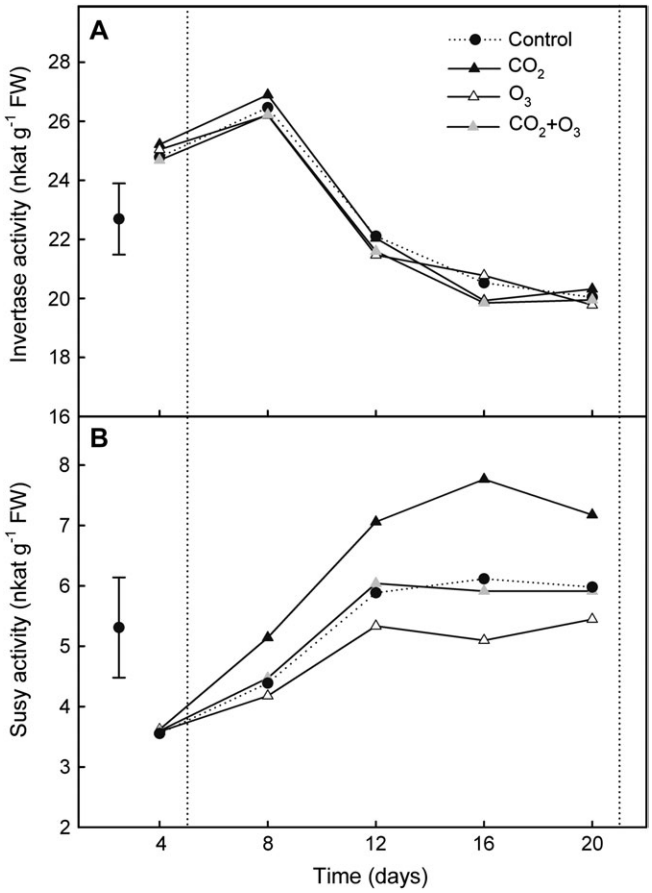


Fig. 4. Time-courses of cytoplasmic invertase activity (A) and sucrose synthase activity (B) in bulbs of *E. americanum* grown under control, elevated CO_2, elevated O_3, and elevated CO_2+O_3 during the epigeous growth period. The standard error, estimated from the MSE term in the ANOVA, is shown with the grand mean (N=2). The two vertical dotted lines indicate the end of leaf unfolding and the beginning of leaf senescence, respectively.

Discussion

Modulation of the source strength

Net photosynthetic rate of *E. americanum* was modulated by CO_2 and O_3 concentrations, as has been shown in numerous other species (Ceulemans and Mousseau, 1994; Ainsworth and Long, 2005; Vandermeiren *et al.*, 2005). *Pn* was stimulated under elevated CO_2 concentrations. It is well known that elevated CO_2 conditions increase CO_2 availability as a substrate for Rubisco, thereby positively affecting the carboxylation/oxygenation ratio. However, the enzyme itself appeared unaffected since the same rates were observed for *in vivo* Rubisco activity in elevated CO_2 and in the control. By contrast, Rubisco activity was decreased under elevated O_3. Such decreases have previously been observed and appear to be due to an alteration of Rubisco structure and expression (Pelloux *et al.*, 2001). Elevated O_3 stimulated leaf PEPc activity in *E. americanum*. This enzyme supplies the anaplerotic pathway with C skeletons (Dizengremel, 2001). Thus, stimulation of PEPc activity could compensate for C loss induced by Rubisco alteration under elevated O_3. However, this compensation was partial since *Pn* were lower in O_3-treated plants than in control plants. Thus, we succeeded in both stimulating and inhibiting source activity by increasing the CO_2 and O_3 concentrations, respectively.

O_3 stimulated leaf respiration in *E. americanum*. Eighty per cent of this increased respiratory rate under elevated O_3 can be explained by the increased activity of the alternative pathway. Induction of AOX expression by O_3 has previously been described and has been related to protective mechanisms avoiding ROS production in mitochondria (Tosti *et al.*, 2006). Indeed, AOX protein prevents the over-reduction of the electron transport chain, especially the ubiquinone pool, thus alleviating ozone damage (Maxwell *et al.*, 1999). The higher respiratory rate was partly responsible for the lower leaf *Pn* in O_3-treated plants. On the other hand, elevated CO_2 did not affect leaf respiration. Respiration is often inhibited under elevated CO_2 concentrations, as demonstrated by Gonzalez-Meler *et al.* (1996). Moreover, these authors reported a direct inhibition of the cytochrome pathway by elevated CO_2. This inhibition suggests that a portion of the electrons was transferred from the cytochrome pathway to the alternative pathway, which could explain the slightly higher activity of the alternative pathway observed in leaves of *E. americanum* under elevated CO_2, but without any impact on total respiratory rates. Thus, leaf respiration seems to be modulated more by abiotic factors than by source or sink activity.

Source–sink imbalance

Neither starch nor soluble sugar accumulation in the leaf was observed over time under elevated CO_2, nor a reduction in these constituents under elevated O_3. Starch has often been described as a temporary storage sugar in the leaf which contributes to photosynthesis regulation (Goldschmidt and Huber, 1992). Twice-ambient CO_2 often leads to an increase in leaf carbohydrates; for example, Long and Drake (1992) reported an increase of 52% for the soluble sugar content and 160% for starch content. Therefore, it can safely be assumed that more C was translocated to the bulb under elevated CO_2, whereas the opposite occurred under elevated O_3, compared with the controls, since the leaf did modulate its carbohydrate content in response to changes in *Pn*.

Despite differences in the amount of C translocated to the bulb under the different growth conditions, biomass allocation patterns were similar among treatments. Biomass accumulation in the sink increases steadily during the epigeous growth phase, until it reached a maximum which precedes by a few days the first visual sign of leaf senescence. Numerous reports in the literature have been made regarding positive responses of plant growth to the elevated C supply under elevated CO_2 conditions, including for perennial organs (Daymond *et al.*, 1997; Donnelly *et al.*, 2001). However, a lack of effect on plant growth is possible in sink-limited plants (Arp, 1991; Woodward, 2002). Thus,

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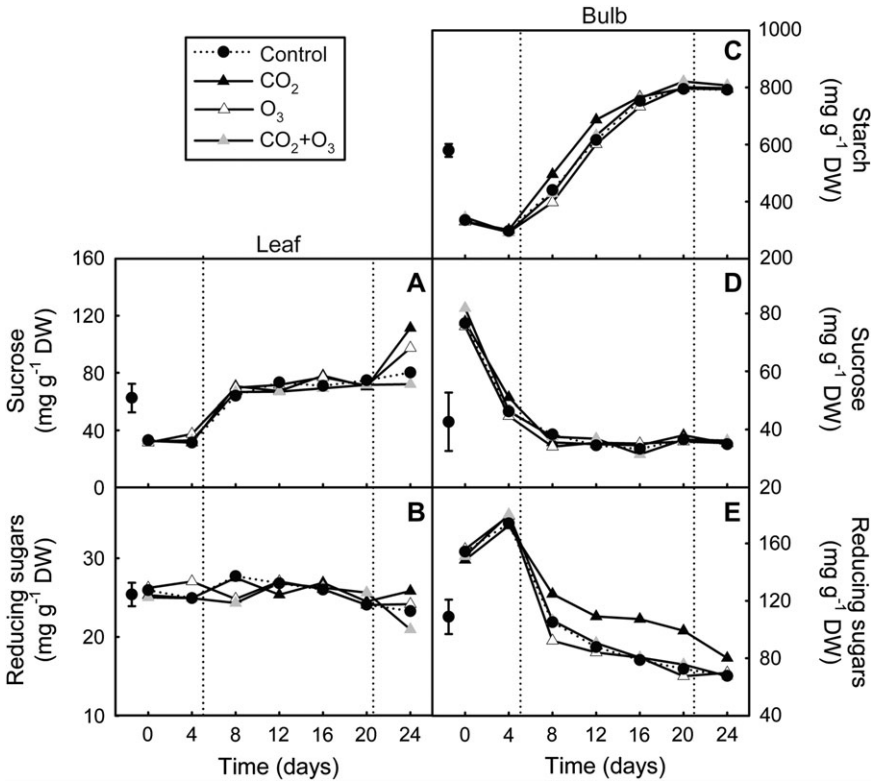


Fig. 5. Time-courses of sucrose (A, D), reducing sugar (B, E), and starch concentrations (C) in leaf (A, B), and bulb (C, D, E) of *E. americanum* grown under control, elevated CO_2, elevated O_3, and elevated CO_2+O_3 during the epigeous growth period. The standard error, estimated from the MSE term in the ANOVA, is shown with the grand mean (N=2). The two vertical dotted lines indicate the end of leaf unfolding and the beginning of leaf senescence, respectively.

despite CO_2 stimulation of the source activity, similar rates of biomass accumulation have been reported for onions from bulbing to bulb maturity (Daymond *et al.*, 1997) and for potatoes before tuber initiation (Conn and Cochran, 2006). Thus, storage organ growth cannot increase in sink-limited conditions, whatever the strength of the C source. In these studies, the inability to use the surplus C in the sink organs leads to a down-regulation of *Pn*, which did not occur in *E. americanum*.

Invertase was strongly activated at the beginning of bulb growth, whereas Susy became activated later in the season. Koch (2004) associated the activation of invertase with cell growth and division and Susy activation with cell differentiation and reserve accumulation. Similar sequential contributions of the different sucrose-cleaving enzymes, as a function of developmental stage, seem to take place in the bulb of *E. americanum*. During invertase activation (4–12 d), sink C-demand was high, as indicated by the kinetics of growth and starch accumulation in the bulb, which were maximum. This high demand could explain why neutral invertase activity was not affected by treatments. Sink limitation, i.e. growth and starch accumulation slow down, occurred later (from day 12 onward) when Susy activation took over. The rate of sucrose hydrolysis by Susy in the bulb was then stimulated by elevated CO_2 concentrations and inhibited by O_3 exposure. The activity of this enzyme was strongly correlated with the cumulative amount of CO_2 fixed by the leaf (Fig. 10A). Among the main carbohydrates stored in the sink, only reducing sugar concentrations were modulated by CO_2 and O_3 concentrations, being higher under elevated CO_2 and slightly lower under elevated O_3. It therefore seems that modulation of Susy activity was sufficient to maintain a constant sucrose concentration in the bulb of *E. americanum*, despite the variable amount of carbohydrates being translocated to the bulb. Susy activity and expression are stimulated by sucrose availability, whereas invertase activity is regulated by the hexose pool (Geigenberger *et al.*, 2004; Koch, 2004). Geigenberger *et al.* (2004), however, proposed a mechanism to drive carbon into starch synthesis and away from respiration in response to sucrose supply, based on redox-activation of ADP-glucose pyrophosphorylase by sucrose. In the present study, potential surpluses of sucrose under elevated CO_2 did not affect starch accumulation or bulb growth of *E. americanum*. Thus, it appears that the higher amount of C fixed under elevated CO_2 led to a higher flow of sucrose unloaded in the bulb, then to a higher concentration in glucose and fructose through a modulation of Susy. The opposite occurred when the amount of C fixed was lower, as was the case under elevated O_3, thus avoiding in both cases, an accumulation of sucrose in the bulb. Our results suggest that modulation of the activity of Susy avoided AGPase activation and, instead, modulated the pool of glycolytic intermediates available for respiration. Yet it is also

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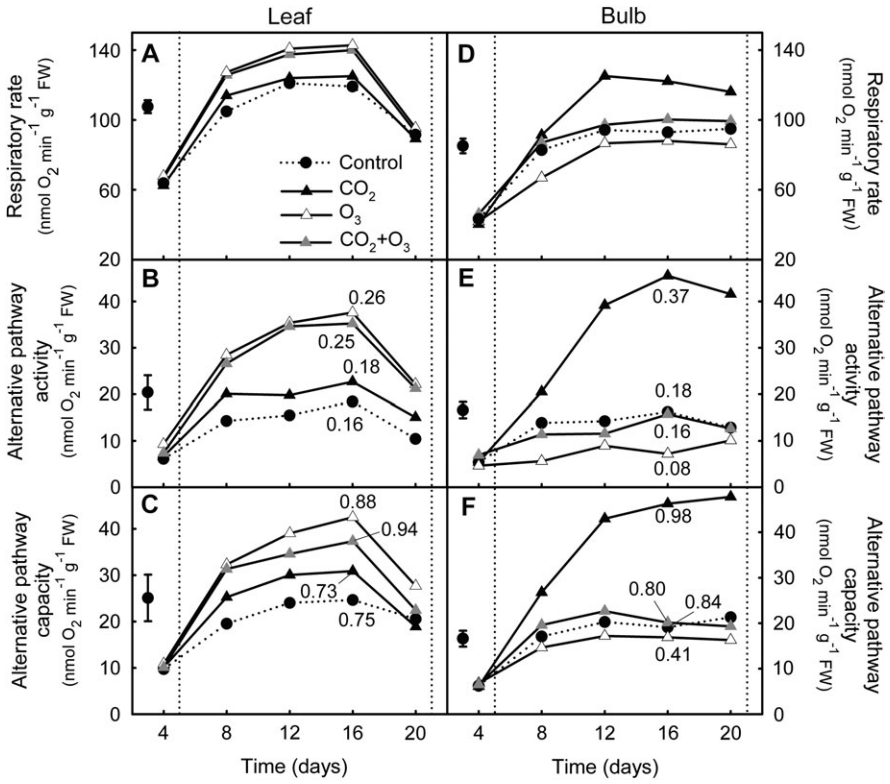


Fig. 6. Time-courses of total respiratory rate (A, D), alternative pathway respiratory rate (B, E), and alternative pathway capacity (C, F) in leaf (A, B, C) and bulb (D, E, F) of *E. americanum* grown under control, elevated CO_2, elevated O_3, and elevated CO_2+O_3 during the epigeous growth period. Participation (B, E) and the engagement of the alternative pathway (C, F) are indicated on the graphs for day 16. The standard error, estimated from the MSE term in the ANOVA, is shown with the grand mean (N=2). The two vertical dotted lines indicate the end of leaf unfolding and the beginning of leaf senescence, respectively.

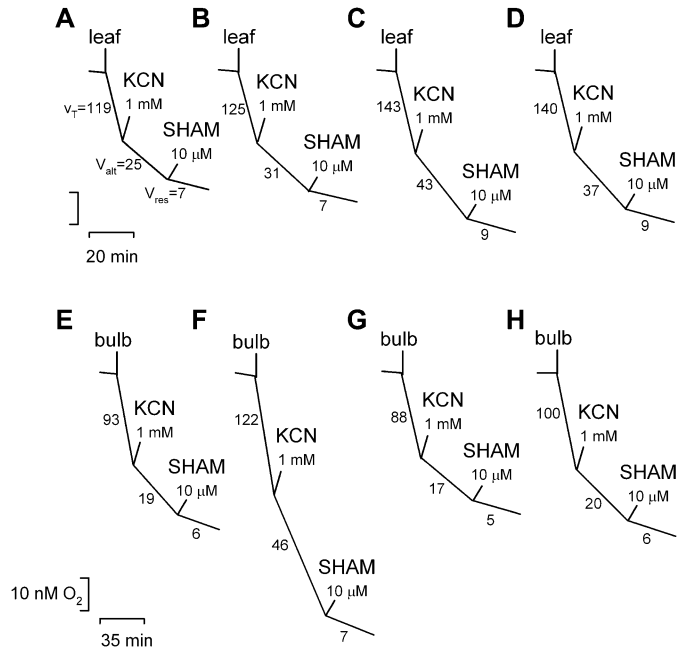


Fig. 7. Representative traces of respiratory activities of leaf (A, B, C, D) and bulb (E, F, G, H) slices from *E. americanum* grown under control (A, E), elevated CO_2 (B, F), elevated O_3 (C, G), and elevated CO_2+O_3 (D, H) during the epigeous growth period. The oxidation rates, expressed in nmol O_2 min^{-1} mg^{-1} protein, are indicated on the different traces.

possible that AGPase was already maximal under the O_3 treatment and that, therefore, the surplus of sucrose under all other growth conditions stimulated Susy activity and, sequentially, respiration.

The role of the alternative respiratory pathway

It seems that the bulb of *E. americanum* is unable to modulate its storage capacity in response to changes in the source activity, probably because growth in this species is sink-limited (Lapointe, 2001). Therefore, the different amounts of C translocated to the bulb must induce changes in C metabolism to counterbalance the constant growth rate of the perennial organ. O_2 consumption by the bulb mitochondrial electron transport chain was stimulated under elevated CO_2 and a reduction under elevated O_3 conditions. Bulb respiratory rate is also strongly correlated with the cumulative amount of C fixed in the leaves (Fig. 10B) and with Susy activity (Fig. 10C). Shugaev and Sokolova (2001) reported a higher respiratory rate in non-differentiated stolons of potato where starch synthesis was low and glucose and fructose concentrations were high, compared with newly formed tubers. Storage organs seem to be able to use the respiratory process to remove the surplus of carbohydrates that cells cannot immediately store. Alternatively, reduced availability of sugars can induce a reduction in respiratory rates without affecting

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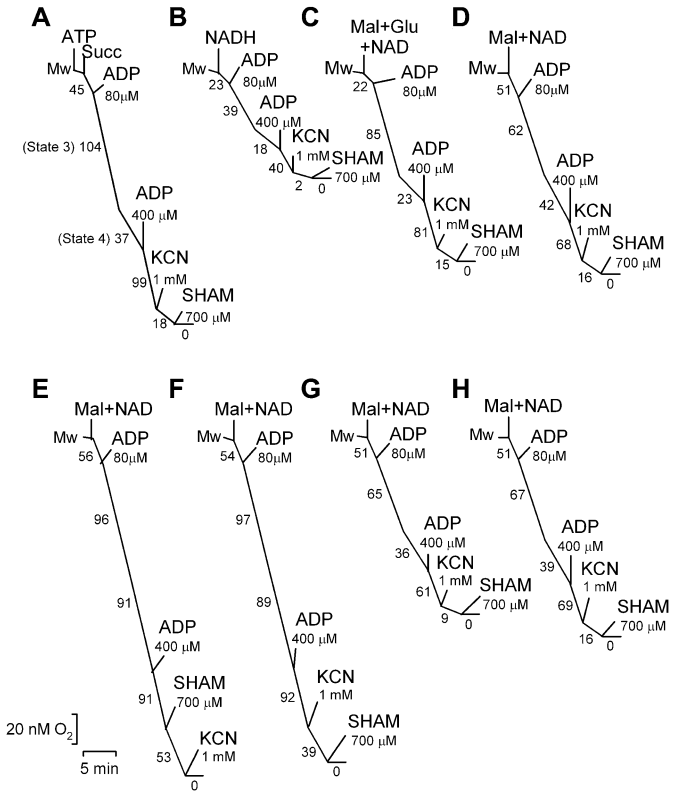


Fig. 8. Representative traces of respiratory activities of washed mitochondria isolated from *E. americanum* bulbs grown under control (A, B, C, D), elevated CO_2 (E, F), elevated O_3 (G), and elevated CO_2+O_3 (H) during the epigeous growth period. Activities were recorded in presence of succinate (A), NADH (B) and malate (C–H) as substrates. Malate was oxidized in the presence of 2 mM glutamate and 400 μM NAD at pH 7.8 (C) and without glutamate at pH 6.7 (D–H). Respiratory activities with SHAM added first (E) and with KCN added first (F) were presented. Mitochondrial preparations used in each series of measures contained 200 μg of proteins. The oxidation rates, expressed in nmol O_2 min^{-1} mg^{-1} protein, are indicated on the different traces.

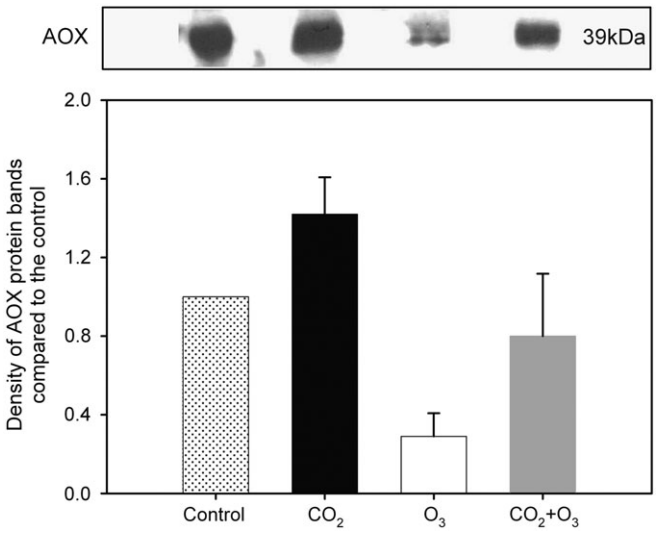


Fig. 9. A representative Western blot of AOX protein detection (above) and relative amount of AOX protein (below) in mitochondria isolated at 16 d from bulbs of *E. americanum* grown under control, elevated CO_2, elevated O_3, and elevated CO_2+O_3. Each value represents the mean of four technical repetitions ±SE.

Table 2. Respiratory activities and parameters of *E. americanum* bulb mitochondria isolated at 16 d from control and treated plants

The substrate was malate (30 mM) at pH 6.7. Total respiratory rate (V_T), the activity of the cytochrome pathway (v_{cyt}), the activity of the alternative pathway (v_{alt}), the capacity of the alternative pathway (V_{alt}), *ADP/O* ratio, the respiratory control (*RC*), the engagement of the alternative pathway (ρ′) and the participation of the alternative pathway (*P*) are presented (see Materials and methods for description of these variables). Values are the mean of three to four technical repetitions ±SE and expressed in nmol O_2 min^{-1} mg^{-1} protein.

	V_T	v_{cyt}	v_{alt}	V_{alt}	*ADP/O*	*RC*	ρ′	*P*
Control	68±6	58±4	9±3	16±6	2.34±0.3	1.48±0.4	0.57	0.13
Elevated CO_2	91±4	53±1	38±4	39±3	1.98±0.2	1.07±0.2	0.97	0.42
Elevated O_3	61±5	56±6	6±4	9±5	2.21±0.4	1.81±0.1	0.66	0.10
Elevated CO_2+O_3	69±11	59±8	11±6	16±7	2.67±0.3	1.69±0.2	0.69	0.16

bulb growth rates, as seen under elevated O_3. This result suggests that respiration was already stimulated under control conditions due to the incapacity of the sink to use all sugars for bulb growth.

The use of inhibitors to estimate cytochrome and alternative pathway activities has been criticized (Millar *et al.*, 1995), as it could underestimate the activity of the alternative pathway if the cytochromic pathway is not saturated before the inhibitors are added. Measurements of the capacity of the alternative pathway, on the other hand, are not subject to the same criticism. In the present study, the effects of the treatments on the alternative pathway activity were supported by similar effects on the alternative pathway capacity, in both bulb slices and mitochondrial extracts. Furthermore, the amount of AOX protein in the mitochondrial extracts varied in accordance with the activity and capacity of the alternative pathway. The development of a sink limitation during the season suggests a biological system almost saturated with carbohydrates. The difference between v_{cyt} and V_{cyt} should therefore be minimum or nul. We are confident that, in the present study, the differences observed between treatments for the activity of the alternative pathway were not biased by the use of inhibitors.

It appears that the increase in bulb respiration could be entirely explained by an increased activity of the alternative pathway in elevated CO_2-grown plants. On the other hand, the decrease in respiration induced by the O_3 treatment was partially due to a reduction in the activity of the alternative pathway (80%). This modulation of alternative pathway respiration was also strongly correlated with the cumulative amount of C fixed in the leaves (Fig. 10D). In isolated mitochondria, the activity of the alternative pathway also entirely explained the increase in the respiratory rate under elevated CO_2 conditions in response to carbohydrate

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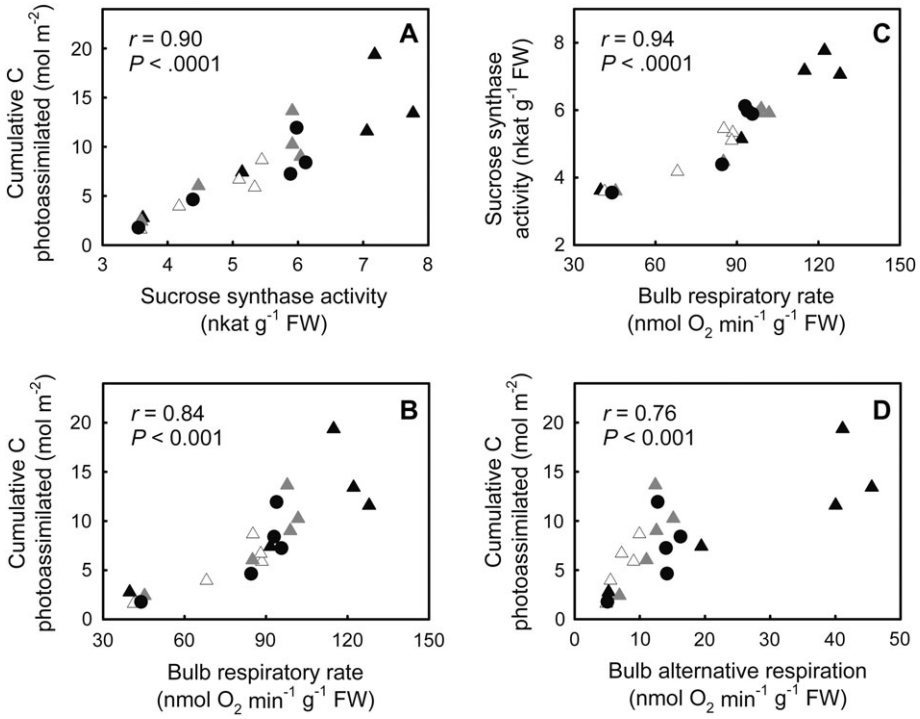


Fig. 10. Relationship between the cumulative amount of carbon fixed by the leaves and sucrose synthase activity (A), respiratory rate of bulb slice (B), and the alternative respiratory rates of bulb slice (D); and between sucrose synthase activity and respiratory rate in bulb slices (C) of *E. americanum*. Control (black circles), elevated CO_2 (black triangles), elevated O_3 (white triangles), and elevated CO_2+O_3 plants (grey triangles). Pearson correlation coefficients and associated *P*-values are indicated.

availability. Numerous studies have suggested that the alternative pathway could be a mechanism for energy overflow (Lambers, 1982), but few have tried to demonstrate it. The mitochondrial respiration data revealed a strong affinity of the alternative pathway for malate oxidation by NAD-malic enzyme in *E. americanum* bulbs. Moreover, this pathway increased under elevated CO_2 conditions and decreased under elevated O_3 conditions. NAD-malic enzyme may promote the reduction of the disulphide bond of the AOX protein, leading to the more active form of the enzyme (Vanlerberghe *et al.*, 1995). Furthermore, pyruvate produced by NAD-malic enzyme is a well-known activator of the reduced AOX (Millar *et al.*, 1993). Thus, NAD-malic enzyme activity and its subsequent organic acid production could play an important role in stimulating the consumption of excess carbohydrate by activation of the alternative respiratory pathway. In accordance with this hypothesis, both the capacity of the alternative pathway and the abundance of AOX were stimulated under elevated CO_2, and repressed under elevated O_3. Sieger *et al.* (2005) reported a modulation of AOX abundance and capacity in tobacco cells growing under limiting macronutrient conditions, and concluded that the alternative pathway was stimulated to correct the imbalance between carbohydrate supply and demand, thus controlling anabolism and growth. Stimulation of the alternative pathway would allow consumption of excess C, avoiding early senescence induced by a reduction in C sink demand.

The capacity of the alternative pathway was strongly stimulated by CO_2 and Western blot analysis also revealed a stimulation of AOX abundance, although of smaller amplitude. On the other hand, the capacity of the alternative pathway was slightly inhibited by ozone, whereas AOX abundance was strongly inhibited. Alternative oxidase exists as a covalent and a non-covalent dimer, the former being the less active form of the enzyme (Umbach and Siedow, 1993). The present results suggest that CO_2 mainly promotes the proportion of the more active form (i.e. non-covalent dimer), leading to high capacity of the alternative pathway, whereas O_3 mainly depletes the total amount of AOX protein. It has also been shown that only the more active form is susceptible to pyruvate activation (Umbach *et al.*, 1994). This would agree with the strong stimulation of activity of the alternative pathway by elevated CO_2 and of malate consumption by NAD-malic enzyme that was observed with the mitochondrial preparations. Both activation and expression of the AOX thus appear to be modulated as a function of C availability within the bulb.

In this study, the response of sink C metabolism to the modulation of source activity was examined in *E. americanum*. Despite the increase in *Pn* under elevated CO_2 and decrease under elevated O_3, neither bulb growth nor starch accumulation was affected. Susy activity responds to the amount of sucrose translocated to the bulb. This modulation of the enzyme could prevent AGPase activation by sucrose content unless AGPase was already at a maximal rate. In addition, the bulb alternative pathway—activity, capacity, and AOX content—was modulated in response to the amount of C fixed. This modulation was stronger when malate was oxidized as the mitochondrial substrate by the

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malic enzyme. It is hypothesized that the production of pyruvate by malic enzyme modulates the activity of the AOX, allowing respiration to burn more C in excess conditions and less in more limited C conditions. The regulation of the glycolytic intermediate pool by Susy and of the alternative pathway by pyruvate production adjusts starch accumulation in rhythm with sink growth capacity. In this sink-limited species, sink C metabolism is modulated in response to changes in C availability, whereas source C metabolism mostly appeared to respond to growth conditions. It would be interesting to investigate the kinetics of starch synthesis and remobilization as a function of C availability and sink capacity to determine if other key enzymes are modulated by sink capacity.

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References

Ainsworth EA, Long SP. 2005. What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. *New Phytologist* **165,** 351–372.

Amthor JS. 1991. Respiration in a future, higher-CO_2 world. *Plant, Cell and Environment* **14,** 13–20.

Andersen CP. 2003. Source–sink balance and carbon allocation below ground in plants exposed to ozone. *New Phytologist* **157,** 213–228.

Arp WJ. 1991. Effects of source–sink relations on photosynthetic acclimation to elevated CO_2. *Plant, Cell and Environment* **14,** 869–875.

Bahr JT, Bonner WD. 1973. Cyanide-insensitive respiration. I. The steady states of skunk cabbage spadix and bean hypocotyl mitochondria. *Journal of Biological Chemistry* **248,** 3441–3445.

Balaguer L, Barnes JD, Panicucci A, Borland AM. 1995. Production and utilization of assimilates in wheat (*Triticum aestivum* L.) leaves exposed to elevated O_3 and/or CO_2. *New Phytologist* **129,** 557–568.

Blakeney AB, Mutton LL. 1980. A simple colorimetric method for the determination of sugars in fruit and vegetables. *Journal of the Science of Food and Agriculture* **31,** 889–897.

Blodgett FH. 1910. The origin and development of bulbs in the genus *Erythronium*. *Botanical Gazette* **50,** 340–373.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* **72,** 248–254.

Ceulemans R, Mousseau M. 1994. Effects of elevated atmospheric CO_2 on woody plants. *New Phytologist* **127,** 425–446.

Conn JS, Cochran VL. 2006. Response of potato (*Solanum tuberosum* L.) to elevated atmospheric CO_2 in the North American subarctic. *Agriculture, Ecosystems and Environment* **112,** 49–57.

Daymond AJ, Wheeler TR, Hadley P, Ellis RH, Morison JIL. 1997. The growth, development and yield of onion (*Allium cepa* L.) in response to temperature and CO_2. *Journal of Horticultural Science* **72,** 135–145.

Dickson RE. 1991. Assimilate distribution and storage. In: Raghavendra AS, ed. *Physiology of trees*. New York, USA: John Wiley & Sons, 51–85.

Dizengremel P. 2001. Effects of ozone on the carbon metabolism of forest trees. *Plant Physiology and Biochemistry* **39,** 729–742.

Donnelly A, Craigon J, Black CR, Colls JJ, Landon G. 2001. Elevated CO_2 increases biomass and tuber yield in potato even at high ozone concentrations. *New Phytologist* **149,** 265–274.

Farrar JF. 1993. Carbon partitioning. In: Hall DO, Scurlock JMO, Bolhar-Nordenkampf HR, Leegood RC, Long S, eds. *Photosynthesis and production in a changing environment: a field and laboratory manual*. London, UK: Chapman & Hall, 232–246.

Fontaine V, Pelloux J, Podor M, Afif D, Gerant D, Grieu P, Dizengremel P. 2002. Carbon fixation in *Pinus halepensis* submitted to ozone. Opposite response of ribulose-1,5-*bis*phosphate carboxylase/oxygenase and phosphoenolpyruvate carboxylase. *Physiologia Plantarum* **105,** 187–192.

Geigenberger P, Stitt M, Fernie AR. 2004. Metabolic control analysis and regulation of the conversion of sucrose to starch in growing potato tubers. *Plant, Cell and Environment* **27,** 655–673.

Gerard J, Dizengremel P. 1988. Properties of mitochondria isolated from greening soybean and lupin tissues. *Plant Science* **56,** 1–7.

Goldschmidt EE, Huber SC. 1992. Regulation of photosynthesis by end-product accumulation in leaves of plants storing starch, sucrose, and hexose sugars. *Plant Physiology* **99,** 1443–1448.

Gonzalez-Meler MA, Giles L, Thomas RB, Siedow JN. 2001. Metabolic regulation of leaf respiration and alternative pathway activity in response to phosphate supply. *Plant, Cell and Environment* **24,** 205–215.

Gonzalez-Meler MA, Ribas-Carbo M, Siedow JN, Drake BG. 1996. Direct inhibition of plant mitochondrial respiration by elevated CO_2. *Plant Physiology* **112,** 1349–1355.

Jolivet Y, Pireaux JC, Dizengremel P. 1990. Changes in properties of barley leaf mitochondria isolated from NaCl-treated plants. *Plant Physiology* **94,** 641–646.

Juszczuk IM, Wagner AM, Rychter AM. 2001. Regulation of alternative oxidase activity during phosphate deficiency in bean roots (*Phaseolus vulgaris*). *Physiologia Plantarum* **113,** 185–192.

Kehr J, Hustiak F, Walz C, Willmitzer L, Fisahn J. 1998. Transgenic plants changed in carbon allocation pattern display a shift in diurnal growth pattern. *The Plant Journal* **16,** 497–503.

Koch K. 2004. Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. *Current Opinion in Plant Biology* **7,** 235–246.

Krippner A, Matsuno-Yagi A, Gottlieb RA, Babior BM. 1996. Loss of function of cytochrome C in Jurkat cells undergoing Fas-mediated apoptosis. *Journal of Biological Chemistry* **271,** 21629–21936.

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Lambers H. 1982. Cyanide-resistant respiration: a non-phosphorylating electron transport pathway acting as an energy overflow. *Physiologia Plantarum* **55,** 478–485.

Lapointe L. 2001. How phenology influences physiology in deciduous forest spring ephemerals. *Physiologia Plantarum* **113,** 151–157.

Lapointe L, Lerat S. 2006. Annual growth of the spring ephemeral *Erythronium americanum* as a function of temperature and mycorrhizal status. *Canadian Journal of Botany* **84,** 39–48.

Long SP, Drake BG. 1992. Photosynthetic CO_2 assimilation and rising atmospheric CO_2 concentrations. In: Baker NR, Thomas H, eds. *Crop photosynthesis: spatial and temporal determinants. Topics in Photosynthesis research,* Vol. 12. Amsterdam, Netherlands: Elsevier, 69–103.

Maxwell DP, Wang Y, McIntosh L. 1999. The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. *Proceedings of the National Academy of Sciences, USA* **96,** 8271–8276.

Millar A, Atkin O, Lambers H, Wiskich J, Day D. 1995. A critique of the use of inhibitors to estimate partitioning of electrons between mitochondrial respiratory pathways in plants. *Physiologia Plantarum* **95,** 523–532.

Millar AH, Wiskich JT, Whelan J, Day DA. 1993. Organic acid activation of the alternative oxidase of plant mitochondria. *FEBS Letters* **329,** 259–262.

Millenaar FF, Lambers H. 2003. The alternative oxidase: *in vivo* regulation and function. *Plant Biology* **5,** 2–15.

Moller IM. 2001. Plant mitochondria and oxidative stress: electron transport, NADPH turnover and metabolism of reactive oxygen species. *Annual Reviews in Plant Physiology and Plant Molecular Biology* **52,** 561–591.

Muller RN. 1978. The phenology, growth and ecosystem dynamics of *Erythronium americanum* in the northern hardwood forest. *Ecological Monographs* **48,** 1–20.

Paul MJ, Foyer CH. 2001. Sink regulation of photosynthesis. *Journal of Experimental Botany* **52,** 1383–1400.

Pelloux J, Jolivet Y, Fontaine V, Banvoy J, Dizengremel P. 2001. Changes in rubisco and rubisco activase gene expression and polypeptide content in *Pinus halepensis* M. subjected to ozone and drought. *Plant, Cell and Environment* **24,** 123–131.

Rich PR, Bonner WD. 1978. The sites of superoxide anion generation in higher plant mitochondria. *Archives of Biochemistry and Biophysics* **188,** 206–213.

Sawada S, Sato M, Kasai A, Yaochi D, Kameya Y, Matsumoto I, Kasai M. 2003. Analysis of the feed-forward effects of sink activity on the photosynthetic source–sink balance in single-rooted sweet potato leaves. I. Activation of RuBPcase through the development of sinks. *Plant and Cell Physiology* **44,** 190–197.

Shugaev A, Sokolova S. 2001. The changes in the pathways of mitochondrial oxidation at the initial period of tuber development. *Russian Journal of Plant Physiology* **48,** 45–50.

Sieger SM, Kristensen BK, Robson CA, Amirsadeghi S, Eng EWY, Abdel-Mesih A, Moller IM, Vanlerberghe GC. 2005. The role of alternative oxidase in modulating carbon use efficiency and growth during macronutrient stress in tobacco cells. *Journal of Experimental Botany* **56,** 1499–1515.

Taylor RJ, Pearcy RW. 1976. Seasonal patterns of the CO_2 exchange characteristics of understory plants from a deciduous forest. *Canadian Journal of Botany* **54,** 1094–1103.

Tosti N, Pasqualini S, Borgogni A, Ederli L, Falistocco E, Crispi S, Paolocci F. 2006. Gene expression profiles of O_3-treated Arabidopsis plants. *Plant, Cell and Environment* **29,** 1686–1702.

Turrens J. 2003. Mitochondrial formation of reactive oxygen species. *The Journal of Physiology* **552,** 335–344.

Umbach AL, Siedow JN. 1993. Covalent and noncovalent dimers of the cyanide-resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. *Plant Physiology* **103,** 845–854.

Umbach AL, Wiskich JT, Siedow JN. 1994. Regulation of alternative oxidase kinetics by pyruvate and intermolecular disulphide bond redox status in soybean seedling mitochondria. *FEBS Letters* **348,** 181–184.

Vandermeiren K, Black C, Pleijel H, De Temmerman L. 2005. Impact of rising tropospheric ozone on potato: effects on photosynthesis, growth, productivity and yield quality. *Plant, Cell and Environment* **28,** 982–996.

Vanlerberghe GC, Day DA, Wiskich JT, Vanlerberghe AE, McIntosh L. 1995. Alternative oxidase activity in tobacco leaf mitochondria (dependence on tricarboxylic acid cycle-mediated redox regulation and pyruvate activation). *Plant Physiology* **109,** 353–361.

Wagner AM, Wagner MJ, Moore AL. 1998. *In vivo* ubiquinone reduction levels during thermogenesis in Araceae. *Plant Physiology* **117,** 1501–1506.

Woodward FI. 2002. Potential impacts of global elevated CO_2 concentrations on plants. *Current Opinion in Plant Biology* **5,** 207–211.

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