Tree Physiology 28, 1761–1772

Could the differences in O_3 sensitivity between two poplar clones be related to a difference in antioxidant defense and secondary metabolic response to O_3 influx?

DANIELA DI BACCIO,[1,2] ANTONELLA CASTAGNA,[3] ELENA PAOLETTI,[4] LUCA SEBASTIANI[1] and ANNAMARIA RANIERI[3]

[1] *Scuola Superiore Sant'Anna, Piazza Martiri della Libertà 33, I-56127 Pisa, Italy*

[2] *Corresponding author (dibaccio@sssup.it)*

[3] *Department of Agricultural Chemistry and Biotechnology, University of Pisa, Via del Borghetto 80, I-56124 Pisa, Italy*

[4] *Institute of Plant Protection, National Research Council of Italy, Via Madonna del Piano 10, I-50019 Sesto Fiorentino, Italy*

Received June 1, 2008; accepted July 17, 2008; published online October 1, 2008

Summary Increasing global background concentrations of tropospheric ozone (O_3) are expected to affect both crops and forest ecosystems negatively. The phytotoxic effects of O_3 are mainly associated with the O_3-induced production of reactive oxygen species (ROS) in excess of the ability of the plant to maintain ROS below the tolerance threshold. It is the balance between O_3 uptake and cellular antioxidant potential that determines O_3 effects on vegetation. The greater sensitivity to ambient O_3 exposure (60 nl l^{-1} O_3, 15 days, 5 h a day) of poplar clone Eridano (*Populus deltoides* × *maximowiczii*) compared with clone I-214 (*P.* × *euramericana*) was reflected in a lower photosynthetic efficiency, higher stomatal conductance and hydrogen peroxide (H_2O_2) accumulation and more pronounced leaf tissue membrane injury in Eridano than in I-214. We checked if the differences in clonal responses to O_3 fumigation were related to differing capacities for antioxidant defense and phenylpropanoid metabolism and found that the increases in foliar ascorbate and phenolic concentrations and phenylpropanoid metabolism in Eridano were insufficient to counteract H_2O_2 accumulation and the consequent oxidative stress. This was probably because the higher influx of O_3 into Eridano leaves compared with I-214 leaves resulted in a lower potential detoxification capacity per unit of O_3 influx.

Keywords: antioxidant potential, ascorbate, Eridano, glutathione, hydrogen peroxide, I-214, ozone, phenylpropanoid metabolism.

Introduction

Ground-level ozone (O_3) is one of the most ubiquitous and damaging air pollutants affecting vegetation (Ashmore 2005). Since the late 1980s, international agreements have reduced peak ozone concentrations in many urbanized regions of the world, but there is evidence of an increase in global background tropospheric concentrations (Bytnerowicz et al. 2007), which is expected to negatively impact vegetation. In contrast to the sudden increase in O_3 concentration, typical of single-pulse experiments, in nature O_3 concentration peaks at least once a day over a period of several weeks. Acute exposure to high O_3 concentrations for a short time generally leads to a hypersensitive response similar to that elicited by the incompatible plant–pathogen interaction (Sandermann et al. 1998, Diara et al. 2005, Kangasjärvi et al. 2005). Long-term exposure to relatively low O_3 concentrations causes reductions in photosynthesis and growth, and an acceleration of leaf senescence (Matyssek and Sandermann 2003, Paoletti and Grulke 2005, Gielen et al. 2007).

Inside the leaf, O_3 phytotoxicity is mainly due to the generation of reactive oxygen species (ROS), including free radicals such as superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical ($OH^{\cdot}$), and peroxides such as hydrogen peroxide (H_2O_2) (Kangasjärvi et al. 2005). Although $O_2^{\cdot-}$ and H_2O_2 are among the less reactive ROS, they are as dangerous as the more reactive species because they can diffuse into any cell compartment. Oxidative damage is believed to occur when ROS production exceeds the plant's ability to maintain the ROS concentration below a tolerance threshold (Matyssek and Sandermann 2003). To counteract excess ROS concentrations, plants have evolved efficient enzymatic and non-enzymatic antioxidant mechanisms (Dixon and Paiva 1995, Noctor and Foyer 1998).

Among the antioxidant metabolites, ascorbate (AsA) is recognized for its ability to scavenge significant amounts of O_3-induced ROS in the leaf apoplast (Ranieri et al. 2000*a*, Conklin and Barth 2004); however, the relationship between the O_3 sensitivity of a plant and its apoplastic AsA concentration remains controversial (Ranieri et al. 1999, Conklin and Barth 2004, D'Haese et al. 2005). Van Hove et al. (2001) demonstrated that apoplastic AsA concentrations were insufficient to protect poplar leaves against damage by ambient O_3 concentrations (maximum concentrations of 50–60 nl l^{-1}), and that apoplastic AsA was replenished quickly with AsA from the mesophyll cells, where the antioxidant concentrations were

10–30 times higher than in the apoplast. Low apoplastic AsA concentrations were also reported by Moldau et al. (1997) and Plöchl et al. (2000). The role of AsA as an antioxidant is strictly dependent on the cell's ability to maintain it in a reduced state. This usually occurs through oxidation of reduced glutathione (GSH) in the so called Halliwell–Asada cycle, which operates in cytosol, chloroplasts, mitochondria and peroxisomes (Jiménez et al. 1997, Noctor and Foyer 1998). Although glutathione has many functions in plants, including a role in signal transduction (Noctor 2006), it is known mainly as a central component of the antioxidant defense system in most aerobic organisms, including plants.

Phenolic compounds have many functions among which are to serve as lignin precursors and oxygen radical scavengers (Grace 2005). Antioxidant properties have been attributed to phenolic compounds because the electron reduction potential of the phenolic radical is lower than the electron reduction potential of oxygen radicals, and also because phenoxyl radicals are generally less reactive than oxygen radicals (Rice-Evans et al. 1997, Grace and Logan 2000, Grace 2005). Exposure to O_3 generally stimulates phenylpropanoid metabolism (Cabané et al. 2004).

There is a growing consensus that exposure indices, such as AOT40, are insufficient to predict the effects of O_3 on vegetation and that flux- or uptake-based approaches are needed to account for the physiologically effective O_3 dose absorbed by a plant (Matyssek et al. 2007). Cell fate in an O_3-polluted environment depends on exposure, uptake and biological responses (Reich 1987), so a meaningful tool to estimate plant susceptibility to O_3 should take into account both O_3 flux through stomata and cellular biochemical defenses (Wieser et al. 2002, Tausz et al. 2007).

We analyzed some of the main defense mechanisms involved in the O_3 response in an O_3-tolerant poplar clone (I-214) and an O_3-sensitive clone (Eridano) exposed to O_3 at a concentration typical of ambient air in urbanized areas (60 nl l^{-1} O_3, 15 days, 5 h a day). Specifically, we measured the concentrations and redox states of ascorbate and glutathione and the activities and gene expression levels of some related enzymes. We tested whether the foliar concentrations of phenolics and several enzymes of the phenylpropanoid biosynthetic pathway were differentially affected by O_3 in the selected clones. Hydrogen peroxide accumulation and changes in leaf gas exchange parameters and chlorophyll a fluorescence were assessed as indices of ongoing O_3-induced stress. To evaluate whether clonal differences in O_3 sensitivity were related to differences in potential antioxidant capacity relative to O_3 influx rather than to changes in antioxidant concentrations, we assessed the concentrations of ascorbate, glutathione and phenolic compounds relative to stomatal O_3 flux.

Materials and methods

Plant material and ozone fumigation

Cuttings of two poplar clones (*P.* × *euramericana* clone I-214, O_3-tolerant, and *Populus deltoides* × *maximowiczii* clone Eridano, O_3-sensitive) were planted in 4-l plastic pots filled with 1:1 (v/v) commercial soil:expanded clay mix and grown for 2 months (spring 2005) outdoors under a shade net (mean daylight 500–600 μmol CO_2 m^{-2} s^{-1}) at Pisa, Italy (43°43′ N, 10°23′ E).

Uniform plants (about 50 cm high) with 10 fully expanded leaves were randomly selected and assigned to a control group or an O_3-fumigation group. Plants were adapted to the growth chamber (0.48 m^3) conditions for 48 h at a day/night temperature of 20/17 °C, relative humidity (RH) of 60–85% and a 14-h photoperiod at a photosynthetic photon flux (PPF) at plant height of 530 μmol m^{-2} s^{-1} from incandescent lamps. Plants (three per clone) in the O_3-treatment group were then fumigated for 15 days with 60 nl l^{-1} O_3 (5 h a day, from 0800 to 1300 h), and plants (three per clone) in the control group were supplied with charcoal-filtered air under the same conditions. Ozone was generated by passing pure oxygen through a Fisher 500 air-cooled generator (Fisher Labor und Verfahrenstechnik, Meckenheim, Germany) and the O_3 concentration of the fumigation chamber was continuously monitored with a UV analyzer (Model 8810, Monitor Labs, San Diego, CA). During O_3 fumigation and the administration of charcoal-filtered air, the temperature in the growth chambers was maintained at 20 ± 1°C, RH at 85 ± 5% and PPF at plant height was 530 μmol m^{-2} s^{-1}. A second set of cuttings transplanted 2 weeks after the first was used to replicate the fumigation experiment, which was carried out by interchanging the control and fumigation chambers.

Each analysis was made on a fully expanded young leaf (2nd to 6th leaf from the apex), or on a pooled sample of leaves from the same plant (for assays of enzymatic activities and RNA isolation), collected from three individual plants of each clone and treatment. Leaves were homogenized in liquid nitrogen, divided into aliquots and stored at –80 °C until analyzed, unless specified otherwise. Freshly excised leaves were used to determine fresh mass and area measured with a scanner and image analysis software (SCION IMAGE, release 4.0.2, Scion Corporation, MD). Dry mass was measured after drying leaf samples for 72 h at 80 °C. Specific leaf area (SLA) was determined as one-side leaf area (m^2) per leaf DM (kg).

Gas exchange and chlorophyll fluorescence

Measurements were carried out at the end of the 15-day O_3 exposure period on 2–3 leaves per plant (4th to 6th leaf from the apex) at 1200–1400 h. Photosynthesis at saturating photosynthetic active radiation (PAR) (A_{max}, μmol CO_2 m^{-2} s^{-1}), internal CO_2 concentration (C_i, ppm), transpiration (T, mmol H_2O m^{-2} s^{-1}), and stomatal conductance to water vapor (g_s, mmol H_2O m^{-2} s^{-1}) were measured with an open infrared gas-exchange system (CIRAS-1 PP-Systems, Herts, U.K.), equipped with a Parkinson broadleaf chamber. The system controlled leaf temperature (20 °C), leaf-to-air vapor pressure difference (2.0 ± 0.2 kPa), saturating PAR (1500 μmol m^{-2} s^{-1}) and CO_2 concentration (360 μl l^{-1}). Instantaneous water-use efficiency, WUE (mmol CO_2 mol^{-1} H_2O), was calculated as A/T.

Concurrent with gas exchange measurements, chlorophyll a fluorescence of photosystem II (PSII) was measured on the

adaxial surface of interveinal leaf regions with a fluorometer (Handy PEA, Hansatech Instruments., Norfolk, U.K.). Before measurements, leaves were dark-adapted for 40 min. In the dark-adapted state, initial fluorescence (F_o, when all PSII reaction centers were open) was measured. After F_o determination, maximum fluorescence (F_m, when all PSII reaction centers were closed) was measured by applying a 1-s saturating actinic light pulse (3000 μmol m^{-2} s^{-1}). Maximum quantum yield for electron transport by open PSII centers (photochemical efficiency) was calculated as $F_v/F_m = (F_m - F_o)/F_m$ (Schreiber and Bilger 1987).

Cell viability measurement

Cell viability was assessed by Evans Blue dye, which is excluded from cells with intact membranes. Three fresh leaves per plant were vacuum infiltrated for 1 h with 0.25% Evans Blue solution, rinsed with distilled water to remove excess dye and photographed (Castagna et al. 2007).

Histochemical detection of hydrogen peroxide

Hydrogen peroxide accumulation was visualized by staining with 3,3′-diaminobenzidine (DAB) which reacts with H_2O_2 to form a red-brown complex. Three fresh leaves per plant were vacuum infiltrated (−65 kPa, three cycles of 1 min each) with 0.1% DAB in 10 mM MES pH 6.5. After a 45-min incubation in ambient laboratory light, leaves were destained with 96% ethanol at 40 °C and stored in 50% ethanol (Castagna et al. 2007).

Determination of H_2O_2

Leaf tissue was frozen in liquid nitrogen and ground. Then 200 μl of 20 mM potassium-phosphate buffer, pH = 6.5, was added to 30 mg of ground frozen tissue. After centrifugation, 25 μl of the supernatant was used to measure foliar H_2O_2 production following reaction with 10-acetyl-3,7-dihydrophenoxazine (Amplex Red reagent) to produce the red-fluorescent oxidation product resofurin (excitation/emission = 530/590 nm) (Mohanty et al. 1997). We used the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes), according to Shin et al. (2005) and the manufacturer's recommendations. The assay has a detection limit of 50 nM H_2O_2. Values were expressed as μmol H_2O_2 mg^{-1} fresh mass by reference to an H_2O_2 standard curve.

Determination of AsA and DHA leaf concentration

Leaf samples frozen in liquid nitrogen were extracted in 5% metaphosphoric acid (1:2 w/v) and centrifuged at 24,000 *g* for 20 min at 4 °C. After neutralization with 1.5 M KOH, an aliquot of supernatant was added to 10% (w/v) trichloracetic acid (TCA) and 5 M NaOH. The mixture was centrifuged at 12,000 *g* for 2 min, and 150 mM phosphate buffer (pH 7.4) was added to the supernatant. The total amount of ascorbate acid (AsA) plus dehydroascorbate (DHA) (AsA + DHA) in each sample was quantified as described by Ranieri et al. (2000*a*). Values were expressed as μmol AsA g^{-1} FM by reference to a standard curve of AsA (Sigma).

Determination of reduced and oxidized glutathione

Leaf samples frozen in liquid nitrogen were extracted in 5% (w/v) TCA containing 5% insoluble polyvinylpolypyrrolidone (PVPP) and centrifuged at 14,000 *g* for 15 min at 4 °C. Total reduced glutathione (GSH) + oxidized glutathione (GSSG) in the supernatant were determined spectrophotometrically by an enzymatic recycling procedure in which GSH is sequentially oxidized by 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, i.e., Ellman's reagent) and reduced by NADPH in the presence of glutathione reductase, as described by Di Baccio et al. (2004). 5-Thio-2-nitrobenzoic acid formation was monitored at 412 nm for 1 min at 25 °C. Total glutathione was calculated from a standard curve in which GSH equivalents were plotted against the rate of change in absorbance at 412 nm. Oxidized glutathione was determined after derivatization of GSH by 2-vinylpyridine by reference to a GSSG standard curve. Reduced glutathione was determined by subtracting GSSG, as GSH equivalents, from the total glutathione concentration. Values were expressed as nmol GSH g^{-1} FM.

Calculation of the potentially available amount of antioxidants per unit of stomatal O_3 flux

The potentially available leaf antioxidant concentration per unit of O_3 flux was calculated for each plant as: $AA = A/F_{O_3}$, where AA represents the available amount of the antioxidant per unit of O_3 flux, A is antioxidant concentration expressed on a leaf area basis, and F_{O_3} (nmol m^{-2} s^{-1}) is stomatal O_3 flux (Wieser et al. 2002, Tausz et al. 2007). We calculated F_{O_3} as: $F_{O_3} = O_a(g_{O_3})$, where g_{O_3} is stomatal conductance of water vapor multiplied by 0.613 and O_a is ambient O_3 concentration (nl l^{-1}). Because internal leaf O_3 concentration approaches zero (Laisk et al. 1989) and the mesophyll resistance to O_3 is typically small (cf. Runeckles 1992), we ignored internal leaf O_3 concentration when calculating O_3 flux.

Enzyme extraction and activity

Ascorbate peroxidase (APX, E.C. 1.11.1.11) was extracted from leaf samples frozen in liquid nitrogen by homogenizing with 10% (w/w) PVPP in 100 mM Tricine-KOH buffer (pH 8.0), 20 mM $MgCl_2$, 50 mM KCl, 10 mM EDTA, 50 mM Na ascorbate, 0.1% (v/v) Triton X-100, 1 mM dithiothreitol (DTT), and 0.50 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 12,000 *g* for 30 min at 4 °C, the supernatant was dialyzed against diluted extraction buffer. The APX activity was determined from the decrease in absorbance at 290 nm resulting from the oxidation of ascorbic acid during the first 30 s of the reaction (Diara et al. 2005), and expressed as μmol of ascorbic acid oxidized mg^{-1} protein min^{-1}. To discriminate between APX and POD activities, 50 mM *p*-chloromercuribenzoate, known to inactivate APX, was added to the enzymatic reaction mixture (Diara et al. 2005).

Glutathione reductase (GR, E.C. 1.6.4.2) was extracted by homogenizing 200-mg aliquots of frozen leaf powder with 1.5 ml of 0.1 M phosphate buffer (KH_2PO_4/K_2HPO_4) containing 5 mM ascorbate, 0.5% (v/v) Triton X-100 (pH 7.8) and 100 mg of PVPP. After centrifugation at 14,000 *g* for 20 min at

4 °C, the supernatant was collected and immediately assayed. The GR activity was determined by following the GSSG-dependent NADPH oxidation at 340 nm for 10 min at 25 °C, as described by Di Baccio et al. (2005), and expressed as μmol of GSSG reduced mg^{-1} protein min^{-1}.

Shikimate dehydrogenase (SKDH, E.C. 1.1.1.25) was extracted from freshly collected leaf samples by grinding in liquid nitrogen with 50% (w/w) PVPP in 0.1 M K-phosphate buffer (pH 7.4), 0.5 mM DTT, 2 mM L-cysteine, 2 mM EDTA and 8 mM 2-mercaptoethanol. After centrifugation at 19,000 *g* for 20 min at 4 °C, the supernatant was collected and immediately assayed for SKDH activity by following the reduction in $NADP^+$ at 340 nm for 5 min, as described by Cabané et al. (2004). Activity was expressed as μmol of NADPH oxidized mg^{-1} protein min^{-1}.

L-Phenylalanine ammonium lyase (PAL, E.C. 4.3.1.5) was extracted by grinding fresh leaves in liquid nitrogen with 10% (w/w) PVPP followed by homogenization in 50 mM borate buffer (pH 8.5) containing 5 mM 2-mercaptoethanol and centrifuging at 20,000 *g* for 20 min at 4 °C. Enzymatic activity was determined by measuring the production of *trans*-cinnamic acid at 290 nm, as described by Cabané et al. (2004). Activity was expressed as μmol of *trans*-cinnamic acid produced mg^{-1} protein h^{-1}.

Cinnamyl alcohol dehydrogenase (CAD, E.C. 1.1.1.195) was extracted by grinding freshly collected leaf samples in liquid nitrogen with 10% (w/w) PVPP in 0.1 M K-phosphate buffer (pH 7.4), 0.5 mM DTT, 2 mM EDTA and 8 mM 2-mercaptoethanol and centrifuging at 19,000 *g* for 20 min at 4 °C. The CAD activity was measured by following the oxidation of coniferyl alcohol to its corresponding aldehyde at 400 nm for 5 min at 30 °C, as described by Babar Ali et al. (2005). The activity was expressed as μmol of coniferylaldehyde mg^{-1} protein h^{-1}.

Soluble protein concentration in leaf extracts was determined by the protein-dye binding method of Bradford (1976), with bovine serum albumine (BSA) as standard.

RNA isolation, reverse transcription and real-time PCR

Total RNA isolation from poplar leaves and reverse transcription were performed as described by Di Baccio et al. (2005). Real-time PCR was performed on an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) with the Applied Biosystems Custom TaqMan® gene expression small-scale assay, according to the manufacturer's protocol. Each reaction was performed on 5 μl of 1:25 (w/v) dilution of the first cDNA strands, in a total reaction of 20 μl. β-Tubulin was used as the housekeeping gene. Fluorescent reporter molecules and specific primers (Table 1) for each gene analyzed (*GR_{chl}*, *GR_{cyt}* and γ-*ECS* plus β-*tubulin*) were designed and made by Applied Biosystems. Thermal cycling conditions were: incubation at 95 °C for 10 min to activate the Taq Gold DNA polymerase, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. For each gene analyzed, real-time-PCR products were quantified by generating standard curves at known concentrations, where fluorescence signal intensities (corresponding to cDNA concentrations) were plotted versus Ct (threshold cycle) values on a log scale. The correlation coefficient ranged from 0.95 to 0.99. The efficiency of the primer sets was evaluated by performing real-time PCR on several dilutions of a mix of the different first strands. All determinations were made in quadruplicate wells using the same 96-well plate for the three target genes plus the housekeeping gene for all clone × O_3 combinations. The results were normalized to the constitutive β-*tubulin* gene expression level and expressed as $\log_{10}$(ng mRNA).

Determination of total phenol concentration and antioxidant activity

Phenols were extracted from 400 mg of fresh leaf tissues (pooled from three leaves per plant) by homogenization in 2.5 ml of 70% methanol for 2 h. The mixture was centrifuged twice for 5 min at 2000 *g* and the supernatant used for the Folin-Ciocalteau assay (modified from Singleton and Rossi 1965). A 30-μl aliquot of each sample was mixed with 0.150 ml of 2 N Folin-Ciocalteau reagent (Sigma Chemical Co.), 0.60 ml of 10% Na_2CO_3 and 2.22 ml of distilled water in a 3.0 ml final volume reaction. After a 3-h incubation at room temperature in the darkness, absorbance at 750 nm was measured. Gallic acid (GA) was used to prepare a standard curve, and assay results were expressed as mg of GA equivalents per g of leaf fresh mass.

Antioxidant activity of leaf phenolic extracts was measured as Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) equivalent antioxidant capacity (TEAC), as described by by Re et al. (1999). The assay is based on the ability of putative antioxidants to reduce 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cations ($ABTS^{\ddagger}$). Radical production was maximal after 6 h and stable for up to 2 days. The percentage inhibition of $ABTS^{\ddagger}$ absorbance by the leaf phenolic extract was recorded at 734 nm and antioxidant activity was calculated by reference to a Trolox standard curve and expressed as μmol Trolox per g of fresh mass.

Table 1. Primer sets for real-time PCR analyses. Abbreviations: γ-ECS, glutamylcysteine synthetase; GR_{chl}, chloroplastic glutathione reductase; and GR_{cyt}, cytoplasmic glutathione reductase.

Enzyme	Forward primer	Reverse primer
γγ-ECS	5′-GCTGGCCTTGCTTTGCA-3′	5′-CATCAGTGAAAGGTGAGTTTGCA-3′
GR_{chl}	5′-CGATGTTCATGTATTCATAAGGCAGAAA-3′	5′-TCCTCTTAGAGACATCTGTTCTGCAA-3′
GR_{cyt}	5′-GTTGGGAAGTGAACGAGAAAGTG-3′	5′-CCCAGCATTAGATAGTAACCTCTTGTAAATT-3′
β-Tubulin	5′-TAAATCCGGTCACCGATTTC-3′	5′-TCCATGCTCAGCGCAAAC-3′

Statistical analysis

The experiment was a completely randomized block design with six replicates (3 plants per clone × 2 fumigation experiments, $n = 6$ for each clone). The effects of clone (I-214 and Eridano), O_3 treatments (charcoal-filtered air and ozone fumigation) and the interaction between clones and O_3 treatments (clone × O_3) were evaluated by two-way analysis of variance (ANOVA). For measurements of enzymatic activities and RNA isolation, several young leaves from the same plant were pooled and each extraction and assay was repeated four times with different plants. Data shown in tables and figures are means ± SE for each clone × O_3 treatment combination. The significance level was set at $P = 0.05$. Separation of means was performed by Tukey's test at the 0.05 significance level.

Results

Visible injury and cell viability

At the end of the 15-day O_3 fumigation there was no visible injury to young fully expanded leaves (2nd to 6th leaf from the apex) of either clone. Mature leaves of Eridano displayed small necrotic lesions, and the oldest leaves of I-214 underwent premature detachment, suggesting accelerated senescence (data not shown). Ozone exposure caused a greater increase in the area stained with Evans Blue dye in young leaves of Eridano than in young leaves of I-214, indicating greater membrane injury in Eridano (Figure 1).

Leaf traits, gas exchange and chlorophyll a fluorescence

No differences in foliar fresh mass/dry mass ratio (FM/DM) or specific leaf area (SLA) were observed between clones or between treatments (Table 2). Mean leaf area (2nd to 6th leaf from the apex) was higher in Eridano than in I-214, but the O_3 treatment had no effect on leaf area in either clone. Photosynthesis at saturating PAR (A_{max}), g_s, T, C_i and WUE were influenced by clone, O_3 exposure and their interaction (Table 2). In I-214, O_3 exposure did not significantly affect gas exchange parameters. In Eridano, A_{max} decreased 39% in response to O_3 treatment, whereas g_s, T and C_i increased by 50, 54 and 20%, respectively, compared with control values. Although WUE was similar in control and O_3-treated I-214 plants, O_3 reduced WUE by 63% in Eridano plants (Table 2). The PSII photochemical efficiency (F_v/F_m) was higher in Eridano than in I-214, and was reduced by O_3 in both clones (Table 2).

Hydrogen peroxide

In situ reaction with DAB revealed H_2O_2 accumulation in O_3-treated leaves of Eridano, whereas only a few red-brown spots were present in I-214 leaves exposed to O_3 (Figure 2A). Quantification of H_2O_2 with the fluorescent probe Amplex Red confirmed these results and revealed that O_3 fumigation caused a 10-fold increase in H_2O_2 concentration in Eridano leaves compared with control leaves, whereas H_2O_2 concentration in I-214 leaves was unaffected by O_3 treatment (Figure 2B).

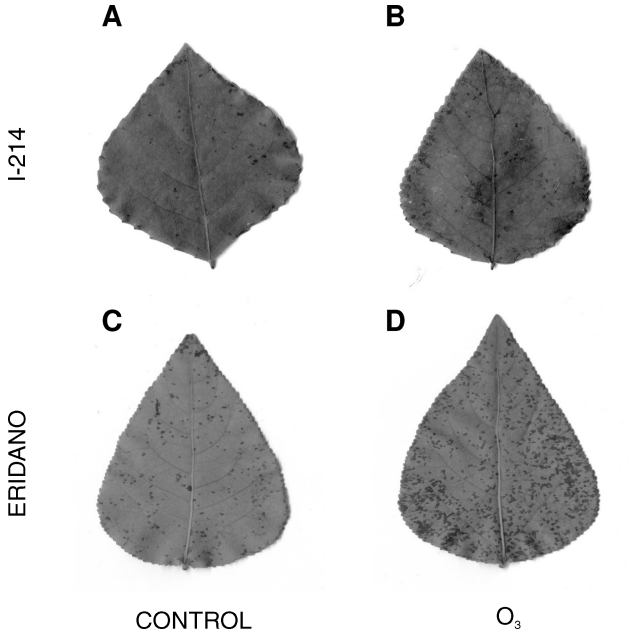


Figure 1. Integrity of cell plasma membranes. determined by the Evans Blue staining method, in hybrid poplar clones I-214 (A, B) and Eridano (C, D) exposed for 15 days (5 h a day, from 0800 to 1300 h) to charcoal-filtered air (A, C) or 60 nl l^{-1} O_3 (B, D). Heavy staining is indicative of damaged cell membranes.

Antioxidant metabolites and enzymes

Foliar AsA concentration was significantly affected by both clone and O_3 treatment and their interaction (Table 3). In Eridano, AsA concentration increased 21% in response to O_3 treatment, whereas there was no significant change in I-214. Total ascorbate (AsA + DHA) concentration was higher in Eridano than in I-214 and increased with O_3 exposure independently of clone. Dehydroascorbate (DHA) concentration was unaffected by any factor, whereas the ascorbate redox state (AsA/(AsA + DHA)) was dependent on clone, being generally higher in Eridano than in I-214 (Table 3).

Total ascorbate peroxidase (APX) activity depended on clone and O_3 and their interaction (Table 3). Ascorbate peroxidase (APX) activity in control leaves was higher in Eridano than in I-214. Ozone fumigation reversed the clonal pattern of APX activity, resulting in a 53% increase in I-214 and a 26% decrease in Eridano (Table 3).

Reduced (GSH) and total (GSH + GSSG) glutathione concentrations were unaffected by O_3 treatment, but they differed significantly between clones (Table 4). The constitutive concentrations of the reduced and total forms of glutathione were 3.1- and 2.4-fold higher, respectively, in I-214 than in Eridano. Neither clone nor O_3 treatment affected GSSG concentrations. Because of clonal differences in GSH and GSH + GSSG concentrations, the redox state of glutathione (GSH/(GSH + GSSG)) was affected by clone, but not by O_3 and there was no clone × O_3 interaction (Table 4). Glutathione reductase (GR) activity was also significantly influenced by genotypic differ-

Table 2. Effects of O_3 treatment on leaf fresh mass/dry mass ratio, mean leaf area, specific leaf area, gas exchange and chlorophyll a fluorescence in leaves (2nd to 6th leaf from the apex) of the hybrid poplar clones I-214 and Eridano after exposure for 15 days (5 h a day, from 0800 to 1300 h) to charcoal-filtered air or 60 nl l^{-1} O_3. Abbreviations: FM, fresh mass; DM, dry mass; LA, leaf area; SLA, specific leaf area; A_{max}, photosynthesis at saturating light; g_s, stomatal conductance; T, transpiration; C_i, internal CO_2 concentration; WUE, instantaneous water-use efficiency; F_v/F_m, maximum quantum yield for electron transport by open PSII centers. Values are means ± SE for each clone and O_3 treatment combination ($n = 6$). Data were analyzed by two-way ANOVA to evaluate the clone (clone) and ozone treatment (O_3) effects and their interaction (clone × O_3). Where the clone × O_3 interaction was significant, different letters indicate statistically different means following Tukey's test ($P = 0.05$).

Parameter	Clone				P		
	I-214		Eridano		Clone	O_3	Clone × O_3
	C	O_3	C	O_3			
FM/DM ratio	3.96 ± 0.17	4.00 ± 0.10	4.14 ± 0.20	4.27 ± 0.03	0.151	0.571	0.757
LA (cm^2 $leaf^{-1}$)	11.94 ± 1.67	9.64 ± 1.93	19.47 ± 3.21	20.16 ± 2.74	0.006	0.754	0.561
SLA (cm^2 g^{-1} DM)	54.9 ± 2.4	51.7 ± 4.6	57.2 ± 9.0	56.9 ± 2.7	0.538	0.777	0.813
A_{max} (μmol CO_2 m^{-2} s^{-1})	5.40 ± 0.42 b	4.87 ± 0.44 b	7.74 ± 0.72 a	4.70 ± 0.47 b	0.049	0.002	0.025
g_s (mmol H_2O m^{-2} s^{-1})	120.00 ± 7.15 c	129.14 ± 11.82 bc	187.29 ± 25.74 b	280.14 ± 12.97 a	< 0.001	0.004	0.015
T (mmol H_2O m^{-2} s^{-1})	1.43 ± 0.09 c	1.42 ± 0.19 c	2.12 ± 0.21 b	3.27 ± 0.21 a	< 0.001	0.002	0.002
C_i (ppm)	274.14 ± 3.00 b	280.86 ± 6.41 b	266.00 ± 8.51 b	319.00 ± 3.66 a	0.017	< 0.001	0.001
WUE (mmol CO_2 mol^{-1} H_2O)	3.58 ± 0.15 a	3.92 ± 0.45 a	3.97 ± 0.33 a	1.47 ± 0.12 b	0.002	0.001	< 0.001
F_v/F_m	0.833 ± 0.002	0.826 ± 0.003	0.843 ± 0.002	0.827 ± 0.004	0.027	< 0.001	0.114

ences, constitutive activity being 79% higher in I-214 than in Eridano, but in both clones it was unaffected by O_3 treatment and there was no clone × O_3 interaction (Table 4).

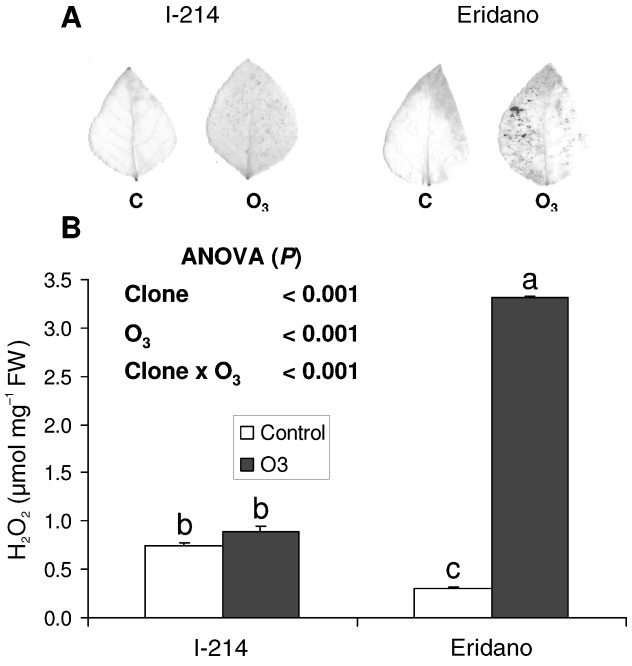


Figure 2. Histochemical localization of O_3-induced H_2O_2 accumulation by DAB staining (A) and H_2O_2 quantification with the fluorescent probe Amplex Red (B) in leaves of hybrid poplar clones I-214 and Eridano exposed for 15 days (5 h a day, from 0800 to 1300 h) to charcoal-filtered air (open bar) or 60 nl l^{-1} O_3 (filled bar). Values are means ± SE for each clone and O_3 treatment combination ($n = 6$). Data were analyzed by two-way ANOVA to evaluate the clone (clone) and ozone treatment (O_3) effects and their interaction (clone × O_3). Where the clone × O_3 interaction was significant, different letters indicate statistically different means following Tukey's test ($P = 0.05$).

Expression levels of GR and γ-ECS

Transcript levels of all the genes analyzed (Figure 3) were affected by clone. Transcripts of the chloroplastic and cytosolic GR isoforms (GR_{chl} and GR_{cyt}, respectively) were generally 4 and 2.5 times higher in I-214 than in Eridano, respectively, independently of O_3 treatment. Ozone fumigation increased GR_{cyt} transcript levels in I-214 leaves by 30% compared with the control value, resulting in an about 3-fold higher expression level compared with O_3-treated leaves of Eridano. Expression of GR_{cyt} was influenced by the interaction of clone and O_3 treatment (Figure 3). Transcript of γ-glutamylcysteine synthetase (γ-ECS) were about twice as abundant in I-214 than in Eridano, but in both clones they were unaffected by O_3 exposure (Figure 3).

Leaf total phenol concentration and antioxidant activity

Foliar total phenolic concentrations were significantly influenced by clone and O_3 treatments as well as by their interaction (Table 5). The constitutive foliar total phenolic concentration was 50% higher in Eridano than in I-214. Ozone fumigation caused a 90% increase in foliar total phenolic concentration in Eridano compared with a 68% increase in I-214 (Table 5). The antioxidant activities of foliar phenolic extracts, measured as the ability of phenolics to scavenge pre-formed $ABTS^+$ radicals, were similar in control plants of both clones. Following O_3 exposure, both clones exhibited an increase in antioxidant ability relative to controls, although the increase was significantly higher in Eridano than in I-214 (64 versus 18%) (Table 5).

Enzymes involved in phenylpropanoid pathway

In response to O_3 treatment, shikimate dehydrogenase (SKDH) activity increased by 36 and 62% in I-214 and Eridano, respectively (Figure 4A). The effects of clone, O_3

Table 3. Ascorbate (AsA), dehydroascorbate (DHA), total (AsA + DHA) ascorbate concentrations (μmol g^{-1} FM), the ratio of oxidized to total ascorbate (AsA/(AsA + DHA)) and ascorbate peroxidase (APX) activity (μmol AsA mg^{-1} protein min^{-1}) in leaves (2nd to 6th leaf from the apex) of the hybrid poplar clones I-214 and Eridano exposed for 15 days (5 h a day, from 0800 to 1300 h) to charcoal-filtered air or 60 nl l^{-1} O_3. Values are means ± SE for each clone and O_3 treatment combination ($n = 6$). Data were analyzed by two-way ANOVA to evaluate the clone effect (clone), the ozone treatment effect (O_3) and the interaction of these factors (clone × O_3). Where the clone × O_3 interaction was significant, different letters indicate statistically different means following Tukey's test ($P = 0.05$).

Parameter	Clone				*P*		
	I-214		Eridano		Clone	O_3	Clone × O_3
	C	O_3	C	O_3			
AsA	3.40 ± 0.25 c	3.56 ± 0.15 c	4.80 ± 0.06 b	5.80 ± 0.09 a	< 0.001	0.002	0.016
DHA	0.79 ± 0.23	1.16 ± 0.18	0.77 ± 0.18	0.73 ± 0.24	0.287	0.426	0.331
AsA + DHA	4.19 ± 0.42	4.73 ± 0.12	5.57 ± 0.14	6.53 ± 0.21	< 0.001	0.009	0.410
AsA/(AsA + DHA)	0.82 ± 0.03	0.76 ± 0.03	0.86 ± 0.03	0.89 ± 0.03	0.015	0.564	0.174
APX	0.53 ± 0.004 c	0.81 ± 0.02 a	0.68 ± 0.01 b	0.51 ± 0.03 c	< 0.001	0.006	< 0.001

treatment and their interaction were significant for phenylalanine ammonia-lyase (PAL) and cinnamyl alcohol dehydrogenase (CAD) activities (Figures 4B and 4C). In I-214 leaves PAL activity, which was constitutively higher in Eridano than in I-214, was enhanced ninefold by O_3 fumigation compared with the control value, whereas O_3 had no effect on PAL activity in Eridano (Figure 4B). Similarly, CAD activity was constitutively higher in Eridano than in I-214, and showed 2.6- and 1.9-fold increases in response to O_3 in I-214 and Eridano, respectively (Figure 4C).

Potential antioxidant availability per unit of stomatal O_3 flux

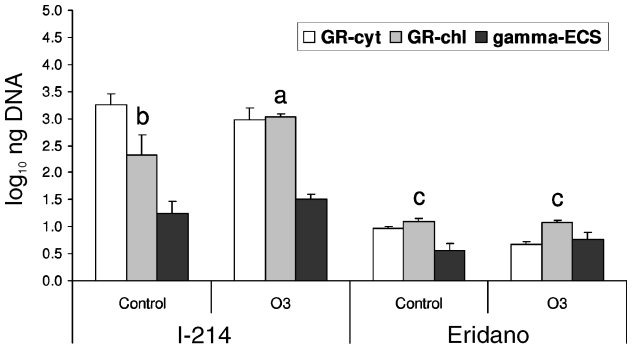
Calculation of the potentially available amount of antioxidants per unit of stomatal O_3 flux in control and O_3-treated plants allowed us to estimate whether the clones differed in constitutive detoxification ability per O_3 flux and how much the presence of ozone affected this capacity. Although the clones did not differ in constitutive AA_{AsA}, they reacted differently to the presence of O_3. In I-214, this index remained unchanged following O_3 exposure, whereas in Eridano it decreased significantly by 32% (Table 6). Thus, although the absolute concentration of reduced ascorbate in O_3-treated Eridano leaves was 63% higher than in O_3-treated I-214 leaves (Table 3), the concentration was 33% lower in Eridano than in I-214 when normalized by stomatal O_3 flux (AA_{AsA}) (Table 6).

The constitutive GSH concentration was 210% higher in I-214 than in Eridano (Table 3) and, when corrected by stomatal O_3 flux (AA_{GSH}), the GSH antioxidant capacity of I-214 was more than four times higher than in Eridano (Table 6). Moreover, although both clones displayed a significant decrease in AA_{GSH} following O_3 exposure, the decrease was more pronounced in Eridano than in I-214 (–31 versus –15%), resulting in AA_{GSH} being five times higher in I-214 than in Eridano at the end of the 15-day O_3 treatment (Table 6).

Potentially available phenols per unit of stomatal O_3 flux (AA_{PHE}) was similar in control leaves of I-214 and Eridano (Table 6). However, following O_3 exposure, AA_{PHE} significantly increased by 64% in I-214, whereas it was unchanged in Eridano. Thus, despite the 70% greater absolute phenolic concentration measured in O_3-fumigated leaves of Eridano compared with O_3-fumigated leaves of I-214 (Table 5), AA_{PHE} was 32% lower in Eridano leaves than in I-214 leaves (Table 6).

Table 4. Reduced (GSH), oxidized (GSSG) and total (GSH + GSSG) glutathione concentrations (nmol g^{-1} FM), the ratio of oxidized to total glutathione ratio (GSH/(GSH + GSSG)) and glutathione reductase (GR) activity (μmol GSH mg^{-1} protein min^{-1}) in leaves (2nd to 6th leaf from the apex) of the hybrid poplar clones I-214 and Eridano exposed for 15 days (5 h a day, from 0800 to 1300 h) to charcoal-filtered air or 60 nl l^{-1} O_3. Values are means ± SE for each clone and O_3 treatment combination ($n = 6$). Data were analyzed independently by two-way ANOVA to evaluate the clone effect (clone), the ozone treatment effect (O_3) and the interaction of these factors (clone × O_3).

Parameter	Clone				*P*		
	I-214		Eridano		Clone	O_3	Clone × O_3
	C	O_3	C	O_3			
GSH	169.25 ± 27.52	151.40 ± 21.25	54.63 ± 5.60	56.03 ± 3.54	< 0.001	0.655	0.602
GSSG	35.29 ± 7.20	39.39 ± 1.07	29.51 ± 1.60	37.99 ± 1.33	0.371	0.135	0.579
GSH + GSSG	204.53 ± 29.38	190.79 ± 20.20	84.15 ± 7.13	94.02 ± 4.59	< 0.001	0.918	0.537
GSH/(GSH + GSSG)	0.83 ± 0.03	0.79 ± 0.03	0.65 ± 0.01	0.60 ± 0.01	< 0.001	0.076	0.738
GR	0.034 ± 0.002	0.033 ± 0.003	0.019 ± 0.002	0.015 ± 0.002	< 0.001	0.223	0.428



	Clone	O_3	Clone x O_3
GR-chl	< 0.001	0.063	0.962
GR-cyt	< 0.001	0.049	0.039
γ-ECS	< 0.001	0.120	0.850

Figure 3. Expression of GR_{chl} (open bar), GR_{cyt} (gray bar) and γ-*ECS* (black bar) mRNAs in leaves of hybrid poplar clones I-214 and Eridano exposed for 15 days (5 h a day, from 0800 to 1300 h) to charcoal-filtered air or 60 nl l^{-1} O_3. Values are means ± SE for each clone and O_3 treatment combination ($n = 4$). Gene expression was analyzed by real-time RT-PCR and the results were standardized to the constitutive β-*tubulin* gene expression level. Data were analyzed by two-way ANOVA to evaluate the clone (clone) and ozone treatment (O_3) effects and their interaction (clone × O_3).Where the clone × O_3 interaction was significant, different letters indicate statistically different means following Tukey's test (P = 0.05). Abbreviations: γ-ECS, glutamylcysteine synthetase; GRchl, chloroplastic glutathione reductase; and GRcyt, cytoplasmic glutathione reductase.

Discussion

Moderate O_3 exposure generally produces little or no visible injury in plants but results in biochemical and physiological changes that lead to reduced growth (Matyssek and Sandermann 2003, Paoletti and Grulke 2005). In accordance with this behavior, neither the I-214 nor the Eridano poplar clone, which are known to differ in the degree of visible injury caused by acute O_3 exposure (Ranieri et al. 1999, 2000*b*, Diara et al. 2005), developed leaf lesions at the end of the 15-day O_3 exposure, with the exception of some of the oldest leaves of Eridano, which exhibited small necrotic lesions (data not shown). Despite the absence of visible symptoms, young leaves of Eridano suffered pronounced membrane injury, as revealed by the many areas stained blue with Evans Blue dye, indicative of ongoing oxidative stress (Figure 1).

Leaf FM/DM ratio, mean leaf area and SLA were unaffected by the O_3 treatment. Although mean leaf area was higher in Eridano than in I-214, when expressed per unit dry mass there was no difference between clones. The differential O_3 sensitivity of the clones was apparent in the marked decrease in A_{max} observed in O_3-fumigated plants of Eridano (Table 2). Previous studies have demonstrated that the photosynthetic apparatus of Eridano is more sensitive to O_3 than that of I-214, as indicated by greater decreases in photosynthetic electron transport rate and pigment concentration, as well as by major alterations in protein–pigment complexes of both photosystems in Eridano (Ranieri et al. 2001). These findings and the observation of an O_3-induced increase in intercellular CO_2 concentration are indicative of reduced carboxylation efficiency by the Calvin cycle enzymes resulting in decreased photosynthesis in Eridano. Stomatal closure was not responsible for the observed decrease in A_{max} because g_s increased following O_3 exposure (Table 2). The higher O_3 influx due to increased g_s must have increased the oxidative load on the photosynthetic machinery. An uncoupling between A and g_s has been reported in plants exposed to O_3 (Matyssek et al. 1991, Paoletti et al. 2007) and has been attributed to loss of stomatal control. As a consequence of increased g_s, transpiration increased in O_3-treated Eridano (Table 2) (Paoletti and Grulke 2005).

A higher g_s implies a higher flux of O_3 to the intercellular space (Sandermann and Matyssek 2004), which may account for the observed impairments in CO_2 fixation and the decrease in A_{max}. A reduction in carbon fixation is a common response of plants to O_3 (Pell et al. 1992, Fares et al. 2006). The altered photosynthetic process observed accords with the decrease in WUE in Eridano exposed to O_3 (Table 2). Increased transpiration in O_3-treated Eridano also decreased WUE, suggesting that O_3 has negative effects on the water balance of this O_3-sensitive clone (Paoletti and Grulke 2005).

Chlorophyll a fluorescence measurements showed that the efficiency of excitation capture of PSII of dark-adapted leaves (F_v/F_m) was negatively affected by O_3 treatment, with a slight effect on PSII photochemistry (Table 2). One-way ANOVA

Table 5. Leaf total phenolic concentration, expressed as mg of gallic acid (GA) equivalents per g fresh mass, and Trolox equivalent antioxidant activity (TEAC) of foliar phenolic extracts (2nd to 6th leaf from the apex), expressed as μmol Trolox per g of fresh mass, in hybrid poplar clones I-214 and Eridano exposed for 15 days (5 h a day, from 0800 to 1300 h) to charcoal-filtered air or 60 nl l^{-1} O_3. Values are means ± SE for each clone-O_3 treatment combination ($n = 6$). Data were analyzed by two-way ANOVA to evaluate the clone (clone), and ozone treatment (O_3) effects and their interaction (clone × O_3). Where the clone × O_3 interaction was significant, different letters indicate statistically different means following Tukey's test ($P = 0.05$).

Parameter	Clone				*P*		
	I-214		Eridano		Clone	O_3	Clone × O_3
	C	O_3	C	O_3			
Total phenolics (mg g^{-1} FM)	5.57 ± 0.48 c	9.35 ± 0.21 b	8.37 ± 0.44 b	15.90 ± 0.47 a	< 0.001	< 0.001	0.002
TEAC (μmol g^{-1} FM)	17.12 ± 0.67 c	20.14 ± 0.65 b	16.09 ± 0.52 c	26.37 ± 1.06 a	< 0.001	< 0.001	0.003

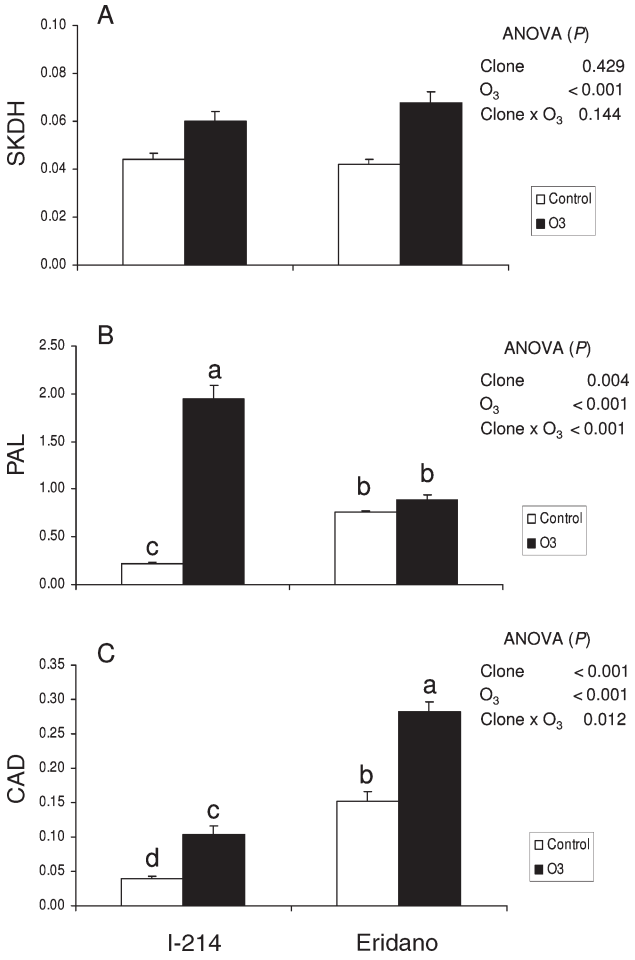


Figure 4. Shikimate dehydrogenase (SKDH, μmol $NADP^+$ reduced mg^{-1} protein min^{-1}; A), phenylalanine ammonia-lyase (PAL, μmol *trans*-cinnamic acid produced mg^{-1} protein h^{-1}; B) and cinnamyl alcohol dehydrogenase (CAD, μmol coniferylaldehyde mg^{-1} protein h^{-1}; C) activities in leaves of hybrid poplar clones I-214 and Eridano exposed for 15 days (5 h a day, from 0800 to 1300 h) to charcoal-filtered air (open bar) or 60 nl l^{-1} O_3 (filled bar). Values are means ± SE for each clone and O_3 treatment combination ($n = 6$). Data were analyzed by two-way ANOVA to evaluate the clone (clone) and ozone treatment (O_3) effects and their interaction (clone × O_3). The significance of the *P*-value is shown. Where the clone × O_3 interaction was significant, different letters indicate statistically different means following Tukey's test ($P = 0.05$).

applied to I-214 and Eridano separately revealed that F_v/F_m was reduced by O_3 exposure in both clones, but more so in Eridano than in I-214 (data not shown). A decrease in F_v/F_m, which is considered a measure of photoinhibition, has frequently been observed as a consequence of O_3 exposure (Loreto et al. 2004, Gielen et al. 2007, Paoletti et al. 2007).

Ozone phytotoxicity is believed to be due to ROS production following the breakdown of O_3 in the apoplast as well as by endogenous, active, self-propagating ROS generation or possibly because cellular ROS scavenging capacity is overwhelmed, or because of a combination of these factors (Matyssek and Sandermann 2003). Both DAB-staining and use of the specific fluorescent probe Amplex Red confirmed that O_3 caused greater H_2O_2-dependent oxidative stress in Eridano leaves than in I-214 leaves (Figure 2). Eridano is known to accumulate greater quantities of H_2O_2 than I-214 following a single pulse exposure to 150 ppb of O_3 (Diara et al. 2005). Accumulation of H_2O_2 is often observed in short- and long-term experiments with high O_3 concentrations, where an increase in H_2O_2 mediates some plant responses to O_3, such as protein phosphorylation, enzyme activation, gene expression, programmed cell death (PCD), and membrane denaturation with accumulation of lipid peroxidation end products such as MDA (Neill et al. 2002, Vranová et al. 2002, Diara et al. 2005, Loreto et al. 2004, Kangasjärvi et al. 2005). In our study, the marked overproduction of H_2O_2 observed in Eridano leaves at the end of the 15-days O_3-exposure was associated with oxidative stress, as demonstrated by pronounced membrane injury (Figure 1). However, the transient fluctuations in H_2O_2 concentrations during the O_3 exposure may have a signaling function.

The role of H_2O_2 as a signal elicitor or regulator molecule is unclear (Neill et al. 2002, Kangasjärvi et al. 2005). Reactive oxygen species, and H_2O_2 in particular, are not specific signal molecules, because they are not sensed through a direct receptor–ligand interaction, although changes in their concentrations may alter the redox balance of the cell, thus modifying activity of transcription factors, second messengers, enzymes involved in biochemical pathways, and redox sensitive molecules such as glutathione and ascorbate (Vranová et al. 2002, Kangasjärvi et al. 2005, Noctor 2006). For these reasons the

Table 6. Potentially available amount per unit of stomatal O_3 flux of reduced glutathione (AA_{GSH}, nmol GSH $nmol^{-1}$ s $O_3{}^{-1}$), total phenolics (AA_{PHE}, mg phenolics $nmol^{-1}$ s $O_3{}^{-1}$) and reduced ascorbate (AA_{AsA}, μmol ASC $nmol^{-1}$ s $O_3{}^{-1}$) in leaves of the hybrid poplar clones I-214 and Eridano exposed for 15 days (5 h a day, from 0800 to 1300 h) to charcoal-filtered air or 60 nl l^{-1} O_3. Values, which were calculated for each plant by dividing the mean antioxidant concentration on a leaf area basis by the stomatal O_3 flux, are means ± SE ($n = 3$). Data were analyzed by two-way ANOVA to evaluate the clone (clone) and ozone treatment (O_3) effects and their interaction (clone × O_3). Where the clone × O_3 interaction was significant, different letters indicate statistically different means following Tukey's test ($P = 0.05$).

Parameter	Clone				*P*		
	I-214		Eridano		Clone	O_3	Clone × O_3
	C	O_3	C	O_3			
AA_{AsA}	71.4 ± 1.5 a	71.9 ± 5.1 a	70.1 ± 1.5 a	47.9 ± 1.6 b	0.012	0.026	0.021
AA_{GSH}	3164.0 ± 59 a	2701.1 ± 177.7 b	768.4 ± 26.3 c	532.9 ± 36.5 d	< 0.001	< 0.001	0.029
AA_{PHE}	116.1 ± 1.6 b	190.9 ± 18.0 a	124.7 ± 5.8 b	129.4 ± 1.1 b	0.024	0.003	0.006

higher foliar H_2O_2 concentration in I-214 than in Eridano (Figure 2B) suggests that I-214 is more tolerant to alterations in cell redox balance induced by excess of ROS in the form of peroxides.

Because H_2O_2 is generated from various sources, plants have evolved efficient enzymatic and non-enzymatic antioxidant systems. Among them, APX plays a role in H_2O_2 reduction. Although constitutive APX activity was higher in Eridano than in I-214, APX activity in Eridano was inhibited by the O_3 treatment (Table 3), thus it contributed little to the H_2O_2 scavenging process in O_3-treated Eridano plants. Conversely, in I-214, there was a significant stimulation of APX activity following O_3 exposure that may explain, in part, the lower accumulation of H_2O_2 detected in this clone (Figure 2B).

Ascorbate may be an important determinant of O_3 tolerance, although contradictory data have been reported (Conklin and Barth 2004, D'Haese et al. 2005 and references therein). Previously, we reported a lack of correlation between ascorbate concentrations and O_3 sensitivity in I-214 and Eridano treated with a single pulse of 150 ppb O_3 and established that extracellular ascorbate diminished O_3 flux to the leaf interior by only 4% in I-214 and 10% in Eridano (Ranieri et al. 1999). Because apoplastic ascorbate is quickly oxidized and the maintenance of high antioxidant activity depends on an intracellular supply of reduced ascorbate, in the present experiment we measured whole-cell ascorbate concentration to test whether this parameter correlated more closely with the difference in O_3 sensitivity between I-214 and Eridano. We found an increase in reduced ascorbate in Eridano leaves exposed to O_3 (Table 3) suggesting an active response of this clone to oxidative stress. It is therefore evident that a higher ascorbate concentration alone is insufficient to explain O_3 tolerance. Because the O_3 effect results from the combination of O_3 uptake and the ability of cells to scavenge O_3-derived ROS, we normalized the antioxidant concentration per unit of O_3 flux to leaves, as suggested by Wieser et al. (2002), to estimate the potential detoxification capacity. We found that I-214 and Eridano were similar in their constitutive ascorbate-dependent detoxification capacity per unit of O_3 influx (Table 6). However, following exposure, although the absolute concentration of reduced ascorbate was higher in Eridano leaves than in I-214 leaves (Table 3), the amount of ascorbate per unit of O_3 uptake was 33% lower in O_3-treated Eridano leaves than in O_3-treated I-214 leaves (Table 6). This means that, although Eridano increased its antioxidant capacity measured by the amount of reduced ascorbate, its response to O_3 influx was less effective than that of I-214. Thus, antioxidant concentration per unit of O_3 flux to leaves better reflected the differences in foliar H_2O_2 accumulation than absolute AsA concentration (Figure 2).

In both clones, the glutathione pool was unaffected by O_3 exposure (Table 4); however, there was a clonal difference in the pool size and redox state of glutathione, suggesting a higher antioxidant potential in I-214 leaves than in Eridano leaves. Clonal differences in GSH-dependent detoxificant capacity were further amplified when GSH concentration was normalized per unit of stomatal O_3 flux (Table 6). Although O_3 treatment had no effect on the absolute foliar GSH concentration in either clone (Table 4), GSH concentration was reduced by 14.6% in I-214 leaves and by 30.6% in Eridano leaves when related to stomatal O_3 flux (AA_{GSH}) (Table 6). In addition, the GSSG/GSH ratio, an indicator of general cellular redox balance, was more than twice as high in Eridano than in I-214, and increased 25.5% in response to the O_3 treatment (data not shown). These results indicate a greater sensitivity to oxidative stress in Eridano than in I-214.

The major factors controlling glutathione synthesis in plants are cysteine availability and γ-ECS activity (Noctor 2006). In our experiment, leaf *γ-ECS* gene expression paralleled the behaviour of the GSH pools—i.e., the *γ-ECS* transcript abundance was enhanced by O_3 exposure and was more than twice as high in I-214 as in Eridano (Figure 3).

Regeneration of GSH from GSSG is catalyzed by glutathione reductase (GR) at the expense of NADPH oxidation. In both clones, we found that GR activity was sufficient to maintain the glutathione pool in O_3-treated leaves (Table 4). Expression levels of the genes encoding the cytosolic and chloroplastic GR isoforms (GR_{chl} and GR_{cyt}) were higher in I-214 leaves than in Eridano leaves (Figure 3), corresponding to the elevated GR activity in I-214 (Table 4). The 30% O_3-induced increase in the transcript levels of GR_{cyt} detected in I-214 leaves (Figure 3), however, did not correspond to an enhancement in total GR activity (Table 4). Post-transcriptional regulation, post-translational oxidative stress interference or enzyme degradation may explain this discrepancy between GR_{cyt} transcript levels and GR activity (Pastori et al. 2000, Di Baccio et al. 2005). The constant glutathione redox state detected in both clones in response to O_3 treatment accords with the ability of various poplar lines (untransformed and transformed lines overexpressing genes involved in GSH metabolism) to maintain a constant glutathione redox potential under both acute and chronic O_3 response (Noctor et al. 1998, Foyer and Rennenberg 2000).

The O_3-dependent induction of the phenylpropanoid biosynthetic pathway in both clones was indicated by increases in foliar SKDH, PAL and CAD activities in response to the O_3 treatment, and was further confirmed by the total phenolic data (Table 5, Figure 4). Phenylpropanoids have numerous functions including providing protection against pathogens and abiotic stresses (Dixon and Paiva 1995, Booker and Miller 1998, Cabané et al. 2004). In poplar leaves (*Populus maximowiczii* × *P. trichocarpa*), higher PAL activity is associated with greater tolerance to O_3 (Koch et al. 1998). Similarly, we observed that O_3 had no effect on PAL activity in the O_3-sensitive Eridano clone, whereas PAL activity increased nine times in the O_3-tolerant I-214 clone following O_3 exposure (Figure 4B).

The effects of O_3 fumigation on foliar leaf phenolic concentrations and their antioxidant activity, expressed as Trolox equivalents (TEAC; Re et al. 1999), were similar in the two clones (Table 5), but with both parameters increasing more in response to O_3 fumigation in Eridano than in I-214 (Table 5). The higher TEAC value recorded in Eridano leaves than in

I-214 leaves following ozone treatment may be associated with the greater accumulation of phenolics capable of scavenging ROS in Eridano, or with the accumulation of specific phenolic molecules with a high antioxidant capacity. However, as observed for ascorbate, the increase in foliar phenol concentration, and consequently, of the putative antioxidant ability detected in Eridano was cancelled when these values were normalized per unit of stomatal O_3 flux. In contrast, in I-214, AA_{PHE} increased over the control value (Table 6), consistent with the high capacity of this clone to resist O_3 toxicity.

In conclusion, our data indicate that variation in ozone sensitivity between poplar clones I-214 and Eridano can be ascribed to an imbalance between O_3 uptake and the ability of cells to scavenge O_3-derived ROS. This imbalance can be described by an equation linking antioxidant concentrations and stomatal O_3 flux. Thus, although the O_3-sensitive clone Eridano generally increased its antioxidant defenses by increasing foliar ascorbate and phenolic concentrations, the greater stomatal O_3 flux in this clone compared with I-214 resulted in reduced photosynthetic efficiency and antioxidant capacity per unit of O_3 influx. As a consequence, H_2O_2 accumulation in leaves of Eridano was in excess of the toxicity threshold, resulting in pronounced membrane injury.

Acknowledgments

We acknowledge Dr. Giuseppe Nervo and Dr. Lorenzo Vietto (Poplar Research Institute, Casale Monferrato, Alessandria, Italy) for the supply of poplar clones. We thank Dr. Stanislav Kopriva (Department of Metabolic Biology, John Innes Centre, Norwich Research Park, Colney, Norwich, U.K.) for providing the sequences of cDNA fragments from poplar leaves that were used to design the fluorescent reporters and the primers for the genes analyzed by real-time PCR. The present research was supported by a grant from the Italian University and Research Ministry (PRIN Project 2005) and by funds of the University of Pisa and Scuola Superiore Sant'Anna, Pisa.

References

Ashmore, M.R. 2005. Assessing the future global impacts of ozone on vegetation. Plant Cell Environ. 28:949–964.

Babar Ali, M., E.J. Hahn and K.-Y. Paek. 2005. CO_2-induced total phenolics in suspension cultures of *Panax ginseng* C.A. Mayer roots: role of antioxidants and enzymes. Plant Physiol. Biochem. 43:449–457.

Booker, F.L. and J.E. Miller. 1998. Phenylpropanoid metabolism and phenolic composition of soybean [*Glycine max* (L.) Merr.] leaves following exposure to ozone. J. Exp. Bot. 49:1191–1202.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. 72:248–254.

Bytnerowicz, A., K. Omasa and E. Paoletti. 2007. Integrated effects of air pollution and climate change on forests: A northern hemisphere perspective. Environ. Pollut. 147:438–445.

Cabané, M., J.-C. Pireaux, E. Léger, E. Weber, P. Dizengremel, B. Pollet and C. Lapierre. 2004. Condensed lignins are synthesized in poplar leaves exposed to ozone. Plant Physiol. 134:586–594.

Castagna, A., L. Ederli, S. Pasqualini, A. Mensuali-Sodi, B. Baldan, S. Donnini and A. Ranieri. 2007. The tomato ethylene receptor LE-ETR3 (NR) is not involved in mediating ozone sensitivity: causal relationships among ethylene emission, oxidative burst and tissue damage. New Phytol. 174:342–356.

Conklin, P. and C. Barth. 2004. Ascorbic acid, a familiar small molecule intertwined in the response of plants to ozone, pathogens, and the onset of senescence. Plant Cell Environ. 27:959–970.

D'Haese, D., K. Vandermeiren, H. Asard and N. Horemans. 2005. Other factors than apoplastic ascorbate contribute to the differential ozone tolerance of two clones of *Trifolium repens* L. Plant Cell Environ. 28:623–632.

Di Baccio, D., F. Navari-Izzo and R. Izzo. 2004. Seawater irrigation: antioxidant defence responses in leaves and roots of a sunflower (*Helianthus annuus* L.) ecotype. J. Plant Physiol. 161:1359–1366.

Di Baccio, D., S. Kopriva, L. Sebastiani and H. Rennenberg. 2005. Does glutathione metabolism have a role in the defence of poplar against zinc excess? New Phytol. 167:73–80.

Diara, C., A. Castagna, B. Baldan, A. Mensuali-Sodi, T. Sahr, C. Langebartels, L. Sebastiani and A. Ranieri. 2005. Differences in the kinetics and scale of signalling molecule production modulate the ozone sensitivity of hybrid poplar clones: the roles of H_2O_2, ethylene and salicylic acid. New Phytol. 168:351–364.

Dixon, R.A. and N.L. Paiva. 1995. Stress-induced phenylpropanoid metabolism. Plant Cell 7:1085–1097.

Fares, S., C. Barta, F. Brilli, M. Centritto, L. Ederli, F. Ferranti, S. Pasqualini, L. Reale, D. Tricoli and F. Loreto. 2006. Impact of high ozone on isoprene emission, photosynthesis and hystology of developing *Populus alba* leaves directly or indirectly exposed to the pollutant. Physiol. Plant. 128:456–465.

Foyer, C.H. and H. Rennenberg. 2000. Regulation of glutathione synthesis and its role in abiotic and biotic stress. *In* Sulfur Nutrition and Sulfur Assimilation in Higher Plants. Eds. C. Brunold, H. Rennenberg, L.J. De Kok, I. Stulen and J.C. Davidian. Paul Haupt, Bern, Switzerland, pp 127–153.

Gielen, B., M. Löw, G. Deckmyn, U. Metzger, F. Franck, C. Heerdt, R. Matyssek, R. Valcke and R. Ceulemans. 2007. Chronic ozone exposure affects leaf senescence of adult beech trees: a chlorophyll fluorescence approach. J. Exp. Bot. 58:785–795.

Grace, S.C. 2005. Phenolics as antioxidants. *In* Antioxidants and Reactive Oxygen Species in Plants. Ed. N. Smirnoff. Am. Soc. Plant Physiol., Beltsville, MD, pp 141–168.

Grace, S.C. and B.A. Logan. 2000. Energy dissipation and radical scavenging by the plant phenylpropanoid pathway. Phil. Trans. R. Soc. Lond. B 355:1499–1510.

Jiménez, A., J.A. Hernández, L.A. del Río and F. Sevilla. 1997. Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea (*Pisum sativum* L.) leaves. Plant Physiol. 114:275–284.

Kangasjärvi, J., P. Jaspers and H. Kollist. 2005. Signalling and cell death in ozone-exposed plants. Plant Cell Environ. 28:1021–1036.

Koch, J.R., A.J. Scherzer, S.M. Eshita and K.R. Davis. 1998. Ozone sensitivity in hybrid poplar is correlated with a lack of defense-gene activation. Plant Physiol. 118:1243–1252.

Laisk, A., O. Kull and H. Moldau. 1989. Ozone concentration in leaf intercellular air spaces is close to zero. Plant Physiol. 90: 1163–1167.

Loreto, F., P. Pinelli, F. Manes and H. Kollist. 2004. Impact of ozone on monoterpene emissions and evidence for an isoprene-like antioxidant action of monoterpenes emitted by *Quercus ilex* leaves. Tree Physiol. 24:361–367.

Matyssek, R. and H. Sandermann. 2003. Impact of ozone on trees: an ecophysiological perspective. Progr. Bot. 64:349–404.

Matyssek, R., M.S. Günthardt-Goerg, T. Keller and C. Ascheidegger. 1991. Impairment of gas exchange and structure in birch leaves (*Betula pendula*) caused by low ozone concentrations. Trees 5:5–13.

Matyssek, R., A. Bytnerowicz, P.-E. Karlsson, E. Paoletti, M. Sanz, M. Schaub and G. Wieser. 2007. Promoting the O_3 flux concept for European forest trees. Environ. Pollut. 146:587–607.

Mohanty, J.G., J.S. Jaffe, E.S. Schulman and D.G. Raible. 1997. A highly sensitive fluorescent micro-assay of H_2O_2 release from activated human leukocytes using a dihydroxyphenoxazine derivative. J. Immunol. Meth. 202:133–141.

Moldau, H., E. Padu and I. Bichele. 1997. Quantification of ozone decay and requirement for ascorbate in *Phaseolus vulgaris* L. mesophyll cell walls. Phyton 37:175–180.

Neill, S.J., R. Desikan and J. Hancock. 2002. Hydrogen peroxide signalling. Curr. Opin. Plant Biol. 5:388–395.

Noctor, G. 2006. Metabolic signalling in defence and stress: the central roles of soluble redox couples. Plant Cell Environ. 29: 409–425.

Noctor, G. and C.H. Foyer. 1998. Ascorbate and glutathione: keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49:249–279.

Noctor, G., A.C.M. Arisi, L. Jouanin, K.J. Kunert, H. Rennenberg and C.H. Foyer. 1998. Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. J. Exp. Bot. 49:623–647.

Paoletti, E. and N.E. Grulke. 2005. Does living in elevated CO_2 ameliorate tree response to ozone? A review on stomatal responses. Environ. Pollut. 137:483–493.

Paoletti, E., C. Nali and G. Lorenzini. 2007. Early responses to acute ozone in two *Fagus sylvatica* clones differing in xeromorphic adaptations: photosynthetic and stomatal processes, membrane and epicuticular characteristics. Environ. Monit. Assess. 128:93–108.

Pastori, G.M., P.M. Mullineaux and C.H. Foyer. 2000. Post-transcriptional regulation prevents glutathione reductase protein and activity in bundle sheath cells of maize. Plant Physiol. 122: 667–676.

Pell, E.J., N. Eckadt and A.J. Enyedi. 1992. Timing of ozone stress and resulting status of ribulose bisphosphate carboxylase/oxygenase and associated net photosynthesis. New Phytol. 120: 397–405.

Plöchl, M., T. Lyons, J. Ollerenshaw and J. Barnes. 2000. Simulating ozone detoxification in the leaf apoplast through the direct reaction with ascorbate. Planta 210:454–467.

Ranieri, A., A. Castagna, E. Padu, H. Moldau, M. Rahi and G.F. Soldatini. 1999. The decay of O_3 through direct reaction with cell wall ascorbate is not sufficient to explain the different degrees of O_3-sensitivity in two poplar clones. J. Plant Physiol. 150:250–255.

Ranieri, A., F. Petacco, A. Castagna and G.F. Soldatini. 2000*a*. Redox state and peroxidase system in sunflower plants exposed to ozone. Plant Sci. 159:159–167.

Ranieri, A., R. Serini, A. Castagna, C. Nali, B. Baldan, G. Lorenzini and G.F. Soldatini. 2000*b*. Differential sensitivity to ozone in two poplar clones. Analysis of thylakoid pigment-protein complexes. Physiol. Plant. 110:181–188.

Ranieri, A., D. Giuntini, F. Ferraro, C. Nali, B. Baldan, G. Lorenzini and G.F. Soldatini. 2001. Chronic ozone fumigation induces alterations in thylakoid functionality and composition in two poplar clones. Plant Physiol. Biochem. 39:999–1008.

Re, R., N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Rad. Biol. Med. 26:1231–1237.

Reich, P.B. 1987. Quantifying plant response to ozone: a unifying theory. Tree Physiol. 3:63–91.

Rice-Evans, C.A., N.J. Miller and G. Paganga. 1997. Antioxidant properties of phenolic compounds. Trends Plant Sci. 2:152–159.

Runeckles, V.C. 1992. Uptake of ozone by vegetation. *In* Surface Level Ozone Exposures and Their Effects on Vegetation. Ed. A.S. Lefohn. Lewis, Chelsea, MI, pp 157–188.

Sandermann, H. and R. Matyssek. 2004. Scaling up from molecular to ecological processes. *In* Molecular Ecotoxicology of Plants. Ed. H. Sandermann. Springer-Verlag, Berlin, pp 207–226.

Sandermann, H., D. Ernst, W. Heller and C. Langebartels. 1998. Ozone: An abiotic elicitor of plant defence reactions. Trends Plant Sci. 3:47–50.

Schreiber, U. and W. Bilger. 1987. Rapid assessment of stress effects on plant leaves by chlorophyll fluorescence measurement. *In* Plant Responses to Stress. Functional Analysis in Mediterranean Ecosystems. NATO ASI Series. Series G: Ecological Sciences Vol. 15. Eds. J.D. Tenhunen, F.M. Catarino, O.L. Lange and W.C. Oechel. Springer-Verlag, Berlin, pp 27–53.

Shin, R., R.H. Berg and D.P. Schachtman. 2005. Reactive oxygen species and root hairs in *Arabidopsis* root response to nitrogen, phosphorus and potassium deficiency. Plant Cell Physiol. 46: 1350–1357.

Singleton, V.L. and J.A. Rossi, Jr. 1965. Colorimetric of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vit. 16:144–158.

Tausz, M., N.E. Grulke and G. Wieser. 2007. Defence and avoidance of ozone under global change. Environ. Pollut. 147:525–531.

van Hove, L.W.A., M.E. Bossen, B.G. San Gabino and C. Sgreva. 2001. The ability of apoplastic ascorbate to protect poplar leaves against ambient ozone concentrations: a quantitative approach. Environ. Pollut. 114:371–382.

Vranová, E., D. Inzé and F. Van Breusegem. 2002. Signal transduction during oxidative stress. J. Exp. Bot. 53:1127–1236.

Wieser, G., K. Tegischer, M. Tausz, K.-H. Häberle, T.E.E. Grams and R. Matyssek. 2002. Age effects on Norway spruce (*Picea abies*) susceptibility to ozone uptake: a novel approach relating stress avoidance to defense. Tree Physiol. 22:583–590.