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Differential ozone sensitivity interferes with cadmium stress in poplar clones

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Abstract

Information on plant responses to combined ozone and cadmium stresses are scarce and limited to herbaceous species. In this research, two poplar clones (I-214 and Eridano), differently sensitive to O_3, were grown for 5 weeks in pots supplied with 0, 53.5, and 160.5 mg(Cd) kg^{-1}(soil d.m.) and then exposed to 15-d O_3 fumigation (0.06 mm^3 dm^{-3}, 5 h a day). The effects of the two stressors, alone or in combination, on Cd, Ca, Fe, and Zn accumulation in above- and below-ground organs, photosynthesis, leaf pigments, and accumulation of H_2O_2 and NO were investigated. Cadmium induced a reduction in stomatal conductance and a significant accumulation of H_2O_2 and NO in both clones and negatively affected the carotenoid content in I-214. Ozone, on the other hand, counteracted Cd accumulation in the above-ground organs and significantly increased the xanthophyll de-epoxidation state indicating photoinhibition in O_3-treated plants. Surprisingly, O_3 alone or in combination with Cd decreased H_2O_2 accumulation in I-214. The NO production was generally stimulated by Cd, whereas it decreased following O_3 exposure in I-214. The overall data indicate that Cd and O_3 induced clone specific responses. Moreover, when they were applied in combination, antagonistic rather than synergistic effects were observed.

Additional key words: carotenoids, hydrogen peroxide; nitric oxide; net photosynthetic rate; *Populus* spp., stomatal conductance, xanthophyll cycle.

Introduction

Ground-level ozone pollution is one of the most ubiquitous and damaging stressful factors affecting vegetation (Ashmore 2005). International agreements have gradually reduced peak O_3 concentrations in many urbanized regions, but new evidence showed an increase in global background tropospheric O_3 concentrations (Bytnerowicz *et al.* 2007). Several studies showed that acute exposure of plants to high O_3 concentration 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), whereas prolonged exposures to relatively low O_3 concentration causes reductions in photosynthesis and growth and an acceleration of leaf senescence (Matyssek and Sandermann 2003, Gielen *et al.* 2007, Wittig *et al.* 2007, Bagard *et al.* 2008, Di Baccio *et al.* 2008).

Cadmium is a trace pollutant. Although Cd is not essential for plants, it is easily taken up by roots. In general, 70 - 85 % of the absorbed Cd in many plants

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Abbreviations: A - anteraxanthin; P_{Nmax} - maximum photosynthetic rate; Chl - chlorophyll; c_i - internal CO_2 concentration; cPTIO - 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-FM DA - amino-5-methylamino-2′,7′-difluorescein diacetate; DEPS - de-epoxidation index; g_s - stomatal conductance; PPFD - photosynthetic photon flux density, ROS - reactive oxygen species; SNP - sodium nitroprusside; V - violaxanthin; VPD - vapour pressure deficit; Z - zeaxanthin.
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remains in roots (Wu 1990), where it can accumulate up to micromolar concentration before it begins altering some functions and ultrastructure (Ederli *et al*. 2004). In some plant species this metal is relatively mobile and it can be translocated from root tips, where it may cause browning, to shoots (Uraguchi *et al.* 2009), causing chlorosis, inhibition of water and nutrient uptake, photosynthesis, respiration, and growth (Sanità di Toppi and Gabbrielli 1999). In general, Cd has been shown to interfere with the uptake, transport, and use of several elements such as calcium, magnesium, phosphorous, and potassium (Das *et al.* 1997), and by inhibition of root iron (Fe)(III) reductase. Cd also leads to Fe(II) deficiency (Alcantara *et al.* 1994). Cd injury has been attributed to several factors, *i.e.* blocking of essential functional groups in biomolecules (Schützendübel *et al.* 2002), displacement of metal ions from biomolecules (Rivetta *et al.* 1997), increase in reactive oxygen species (ROS) accumulation (Van Assche and Clijsters 1990, Iannone *et al.* 2010, He *et al.* 2011), as well as by indirectly activating NADPH oxidases in membranes (Romero-Puertas *et al.* 2004), and/or reducing activity/content of enzymatic and non-enzymatic antioxidants (Sandalio *et al.* 2001, Ranieri *et al.* 2005, Scebba *et al*. 2006, Wójcik *et al*. 2011).

ROS are known to trigger a complex sequence of events leading to activation of MAP kinase cascade which in turn affects the synthesis of compounds such as ethylene, salicylic and jasmonic acids involved in modulation of the plant responses to the imposed stress (Kangasjärvi *et al*. 2005). However, when ROS production exceeds the plant scavenging ability, oxidative damage can occur (Castagna and Ranieri 2009).

Nitric oxide is a crucial player in the regulation of many plant physiological processes including stomatal closure, growth, and development (Neill *et al.* 2002, Wendehenne *et al.* 2004, Baudouin 2011), and its interaction with other signal molecules modulates O_3-induced responses and cell death in particular (Neill *et al.* 2002, Ahlfors *et al.* 2009, Di Baccio *et al*. 2011a). Both cytotoxic and cyto-protecting properties of NO have been described in plants (Beligni and Lamattina 2001). The cross-communication of NO with either pro-oxidant or anti-oxidant molecules critically modulates the fate of the cell (Wendehenne *et al.* 2004).

Poplar has been shown to be a suitable model to study the physiological and molecular effects of O_3 (Diara *et al.* 2005, Di Baccio *et al.* 2008, Renaut *et al.* 2009) and heavy metal pollution (Sebastiani *et al.* 2004, Di Baccio *et al.* 2009, 2010, 2011b, Gaudet *et al.* 2011). However, the concomitant effects of O_3 and Cd on poplar physiology (including gas exchange, antioxidant activity, pigment concentrations, *etc*.) have never been tested. To the best of our knowledge, studies on the possible interactions between Cd and O_3 pollution in plants have been limited to herbaceous plants (Di Cagno *et al.* 1999, 2001, Li *et al.* 2011). In poplar, O_3 and Cd stresses seem to share common pathways (Diara *et al.* 2005, Di Baccio *et al.* 2008, Kieffer *et al.* 2009, Pietrini *et al.* 2010a) and affect synergistically or antagonistically plant responses when are concomitant.

To prove this hypothesis we used two poplar clones differing in their sensitivity to O_3. We assayed gas exchange parameters, pigment contents, H_2O_2 and NO accumulation in leaves, and Ca, Fe, Zn, and Cd uptake and accumulation in roots, stem, and leaves. Moreover, we studied whether plant functions were differently influenced by the two stress factors alone or in combination.

Materials and methods

Woody cuttings (30 cm in length) of two poplar clones, *P.* × *canadensis* I-214 (O_3-tolerant), and *Populus deltoides* × *maximowiczii* Eridano (O_3-sensitive), known for their differential responses to O_3 in terms of development of leaf injuries following acute O_3 exposure (Ranieri *et al*. 1999, 2000, Diara *et al*. 2005), were planted in plastic pots filled with soil:sand:silt:clay mix (93.5:1.75:4.75; organic carbon of 2.34 %; inorganic carbon of 0.096 %).

Cuttings were irrigated with half-strength Hoagland solution with 0, 50, or 150 μM $CdCl_2$ to reach the final Cd amounts in the substrate: 53.5 (L Cd) and 160.5 (H Cd) mg(Cd) kg^{-1}(soil d.m.). After 5 weeks (from cutting plantation) of outdoor cultivation (from 10 April to 15 May) under a shade net (mean photosynthetic photon flux density, PPFD of 500 - 600 μmol m^{-2} s^{-1}) plants were randomly selected and, for each clone and each Cd treatment, assigned to a control (charcoal-filtered air) or an O_3-fumigation group. Forty plants in total were adapted to the growth chamber 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 PPFD of 530 μmol m^{-2} s^{-1} (incandescent lamps). Nine plants per clone (three per each Cd treatment) were then fumigated for 15 d with 0.06 mm^3 dm^{-3} O_3 (5 h a day, from 08.00 to 13.00; referred to as O_3-treated samples) or supplied with charcoal-filtered air under the same conditions (referred to as C samples).

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 analyser (model *8810*, *Monitor Labs*, San Diego, CA, USA). During O_3 fumigation, the temperature in the growth chambers (C and O_3-treatment) was 20 ± 1 °C, RH at 85 ± 5 %, and

PPFD at plant height of 530 μmol m^{-2} s^{-1}.

All measurements were made on fully expanded young leaves (leaf plastochron index, LPI 4 - 6; Dickmann 1971) from three individual plants of each clone-treatment combination (n = 3). Leaf disks collected for pigment, H_2O_2, and NO analyses were stored at -80 °C until analysed.

The available form of Cd present in the substrates used for cutting cultivation was extracted for 60 min with 0.05 M EDTA, 0.5 M ammonium acetate, pH 4.65 (Lakanen and Erviö 1971). After centrifugation at 15 000 *g* for 10 min, the supernatant was collected and filtered through a *Whatman No. 41* filter paper (*Whatman International*, Maidstone, UK). The amount of Cd in the native substrates (before $CdCl_2$ addition) was negligible. After harvesting, leaves, stem, and roots were washed in distilled water. For root samples, the procedure was repeated until complete removal of substrate particles. This plant material was then oven-dried, ground to powder with liquid nitrogen, and stored under vacuum until use. Samples were digested in concentrated H_2SO_4, and hydrogen peroxide drops were added until their complete clarification. Before analyses, all the extracted samples were diluted with ultra-pure water.

The concentrations of Cd, Ca, Fe, and Zn were determined by atomic absorption spectrophotometer (*Perkin-Elmer Analyst 100*, Waltham, MA, USA) equipped with *Perkin-Elmer Intensitron*™ lamps.

Gas exchange was measured with a photosynthesis system *LI 6400* (*LI-COR*, Lincoln, NE, USA) equipped with CO_2 control module and light source consisting of blue-red light-emitting diodes (model *6400-02B*). All measurements were carried out on control and treated plants at the end of the 15-d O_3 exposure. Maximum net photosynthetic rate (P_{Nmax}), stomatal conductance (g_s), and internal CO_2 concentration (c_i) were measured at a leaf temperature of 25 °C, vapour pressure deficit (VPD) of about 1.2 kPa and saturating PPFD of 800 μmol m^{-2} s^{1}).

Leaf pigment analysis was carried out according to the method reported by Castagna *et al*. (2001). Leaf disks of known area (1.13 cm^2) were punched from leaves previously utilized for gas exchange measurements (in the morning), frozen in liquid nitrogen, and stored at -80 °C until use. Leaf disks were ground in a mortar with liquid nitrogen and homogenized in 100 % HPLC-grade acetone in the presence of sodium ascorbate under dimmed light and filtered through 0.2-μm filters (*Sartorius Stedim Biotech*, Göttingen, Germany) and immediately analysed. The HPLC separation was performed using a *Zorbax ODS* column (5 μm particle size, 250 × 4.6 mm Ø; *Agilent Technologies*, Milan, Italy) and pigments were eluted using the following gradient of solvent A (acetonitrile/methanol, 75/25, v/v) and solvent B (methanol/ethylacetate, 68/32, v/v): 100 % A for the first 15 min, followed by a 2.5-min linear gradient to 100 % B which continued isocratically until the end of the cycle. The column was allowed to re-equilibrate in 100 % solvent A for 10 min before the next injection. The separation cycle was 32 min with a flow rate of 1 cm^3 min^{-1}. Pigments were detected by their absorbance at 445 nm. To quantify the pigment content, known amounts of pure standards (*Sigma-Aldrich*, Milan, Italy) were injected into the HPLC system. The degree of de-epoxidation (DEPS) was calculated according to the equation: DEPS = [(A/2) + Z]/(V + A + Z) × 100 (where A - anteraxanthin; Z - zeaxanthin; V - violaxanthin).

Leaf production of H_2O_2 was measured fluorimetrically using the *Amplex Red* hydrogen peroxide/ peroxidase assay kit (*Molecular Probes*, *Invitrogen*, Carlsbad, CA, USA), according to Di Baccio *et al.* (2008) and the manufacturer's recommendations. Leaf tissue (30 mg) was frozen in liquid nitrogen and ground. Then 0.2 cm^3 of 20 mM potassium-phosphate buffer (pH 6.5) was added to 30 mg of ground frozen tissue. After centrifugation, 0.025 cm^3 of the supernatant was incubated (at 25 °C under dark for 30 min) with 50 μM 10-acetyl-3,7-dihydrophenoxazine (*Amplex Red* reagent) to measure foliar H_2O_2 production by the formation of the red-fluorescent oxidation product resorufin (excitation/ emission 530/590 nm). The resorufin fluorescence was quantified with a fluorescence/ absorbance microplate reader (*Victor*^{3TM}, *Perkin-Elmer*, Monza, Italy). The assay has a detection limit of 50 nM H_2O_2.

Leaf NO accumulation was determined according to Di Baccio *et al*. (2011a) using the DAF-FM DA (4-amino-5-methylamino-2′,7′-difluorescein diacetate) fluorescent dye (*Molecular Probes*, Leiden, The Netherlands) which is cell-permeable and passively diffuses across cellular membranes (Kojima *et al*. 1998). Leaf discs (5 mm diameter) were treated with 10 μM DAF-FM DA dissolved in 0.25 M sodium phosphate buffer, pH 7.4 (loading buffer), in a final reaction volume of 0.2 cm^3. After incubation (dark, 25 °C, 60 min), the fluorescence (495/515 nm ratio) of DAF-FM/NO complex (benzotriazole derivative) was measured with a fluorescence/absorbance microplate reader (*Victor*^{3TM}). Background fluorescence of the probe and the leaf sample was determined in 10 μM DAF-FM DA solution without leaf disk and in the leaf disk put in the loading buffer without DAF-FM DA, respectively. As negative and positive NO controls, the NO quencher 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (cPTIO) and the NO donor sodium nitroprusside (SNP) were used, respectively. To ascertain the specificity of the NO signal, we also simultaneously infiltrated leaf disks with SNP (160 μM and 1 mM) and cPTIO (200 μM).

The experiment setup was in a completely randomized design (n = 3). The effects of clone, Cd concentration in the substrate, O_3 fumigation, and of their interactions were evaluated by three-way *ANOVA*. Separation of means was performed by Tukey's test at the 0.05 significance level. Statistical analysis was conducted using *Statistica 6.0* (*StatSoft*, Tulsa, OK, USA).

Results

The two poplar clones did not differ in their ability to take up Cd and to translocate it to the above-ground organs, the clone factor being not significant in the roots as well as in leaves and stem according to the three-way *ANOVA* (Table 1). As expected, poplar growth in Cd-enriched soils led to a significant increase in Cd content in any organ analysed (Table 1). Cadmium content in shoots was also significantly affected by Cd × O_3 interaction (Table 1). In particular, control (not fumigated) samples exhibited a progressive increment in Cd content in both leaves and stem as soil Cd concentration increased (Table 1); whereas, in O_3-treated organs, the metal accumulation showed a step increase in L Cd treated samples and did not further augment at the highest Cd treatment, somewhat showing a trend to decrease (particularly in Eridano). Accordingly, O_3 exposure led to a 47 and 49 % decrease of Cd accumulation in stem and leaves, respectively, in H Cd treated samples in comparison to the corresponding non-fumigated controls.

Eridano generally showed a higher ability to accumulate Ca and Fe in both below- and above-ground organs in comparison to I-214, the only exception being Ca content in the stem (Table 1). Ca content in roots and leaves was about 41 and 35 % higher in Eridano than in I-214, respectively (Table 1). Similarly, Fe content in roots, stem, and leaves was 68, 71, and 97 % higher in Eridano than in I-214, respectively. Conversely, Eridano accumulated less Zn than I-214 but only in the leaves (Table 1). Content of measured cations in the different organs of the two poplar clones were not significantly correlated with Cd content, the only exception being Ca × Cd correlation in the stem (r = 0.376; P = 0.028; data not shown).

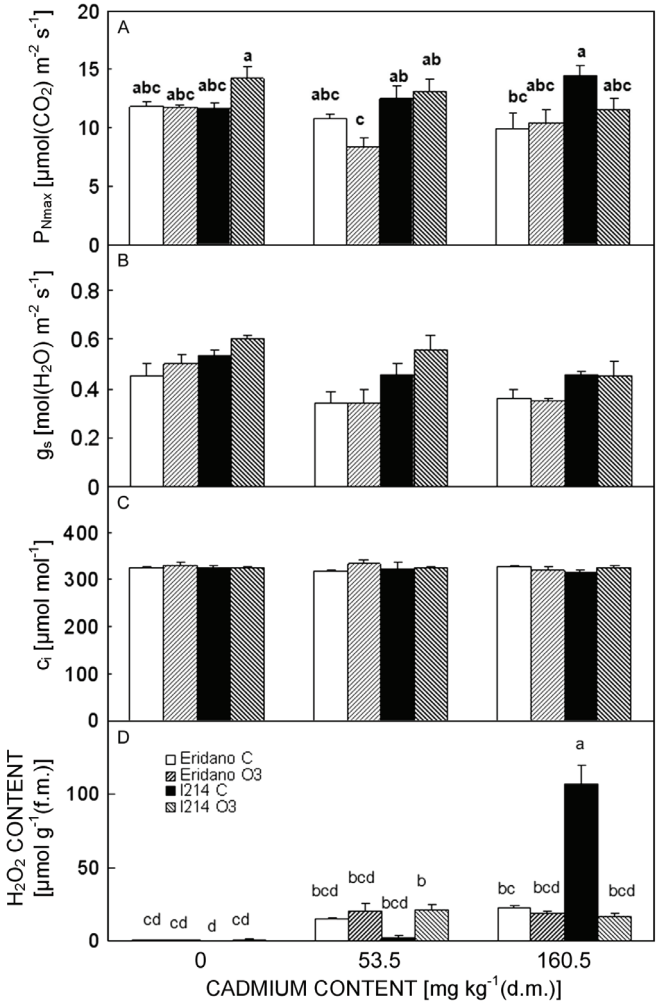

Fig. 1. Net photosynthetic rate at saturating irradiance (*A*), stomatal conductance (*B*), internal CO_2 concentration (*C*), and hydrogen peroxide (*D*) in leaves of the hybrid poplar clones I-214 and Eridano cultivated at different Cd concentrations in the soil and exposed for 15 d (5 h a day) to charcoal-filtered air (C) or 0.06 mm^3 dm^{-3} O_3. Data represent the mean of 3 replicates ± SE and values having different letter are significantly different according to Tukey HSD test.

Table 1. Cadmium, calcium, iron, and zinc content [μg g^{-1}(d.m.)] in roots, stem, and leaves of the hybrid poplar clones I-214 and Eridano cultivated with 0 (0 Cd), 53.5 (L Cd), or 160.5 (H Cd) mg(Cd) kg^{-1}(soil d.m.) and exposed for 15 d (5 h a day) to charcoal-filtered air (C) or 0.06 mm^3 dm^{-3} O_3. Data represent the mean of 3 replicates ± SE. The three-way *ANOVA P*-values for the effect of clone, Cd addition to soil, exposure to O_3, and their interactions are shown.

Parameter	Cd	I-214 control	I-214 ozone	Eridano control	Eridano ozone
Cd roots	0 Cd	6.49 ± 0.25	9.07 ± 1.92	5.27± 0.67	5.47 ± 2.61
	L Cd	58.36 ± 15.67	61.91 ± 9.57	56.90 ± 6.72	65.28 ± 17.93
	H Cd	85.03 ± 8.39	72.31 ± 12.72	84.72 ± 15.64	74.53 ± 17.46
Cd stem	0 Cd	1.65 ± 0.40	2.44 ± 0.71	1.60 ± 0.39	2.83 ± 0.81
	L Cd	7.86 ± 1.42	12.93 ± 2.17	11.29 ± 1.92	13.42 ± 2.79
	H Cd	18.91 ± 0.60	12.10 ± 3.05	16.73 ± 0.70	6.94 ± 1.03
Cd leaves	0 Cd	1.20 ± 0.02	1.96 ± 0.38	1.17 ± 0.03	1.97 ± 0.39
	L Cd	6.93 ± 2.03	8.38 ± 1.53	11.88 ± 2.42	10.87 ± 1.50
	H Cd	16.80 ± 4.18	13.18 ± 1.00	7.68 ± 0.49	7.66 ± 1.18
Ca roots	0 Cd	3593 ± 651	3265 ± 667	5199 ± 322	5045 ± 724
	L Cd	3511 ± 244	2214 ± 397	4458 ± 758	4502 ± 663
	H Cd	3337 ± 548	3664 ± 275	3129 ± 710	5347 ± 746
Ca stem	0 Cd	5324 ± 458	6121 ± 532	5235 ± 98	4881 ± 349
	L Cd	6000 ± 676	6557 ± 683	5437 ± 362	5763 ± 838
	H Cd	7080 ± 946	8735 ± 738	5182 ± 360	5647 ± 353
Ca leaves	0 Cd	5701 ± 385	5868 ± 684	7014 ± 647	6136 ± 536
	L Cd	5560 ± 1163	3121 ± 407	7623 ± 986	5217 ± 971
	H Cd	4715 ± 840	4084 ± 826	6374 ± 1068	6850 ± 279
Fe roots	0 Cd	1272 ± 206	1606 ± 131	2313 ± 485	2143 ± 215
	L Cd	2029 ± 469	1117 ± 213	2087 ± 532	3301 ± 491
	H Cd	1942 ± 213	1053 ± 206	2497 ±5865	2838 ± 674
Fe stem	0 Cd	24.00 ± 1.0	24.00 ± 5.0	51.00 ± 7.0	46.00 ± 2.0
	L Cd	31.00 ± 7.0	32.00 ± 8.0	54.00 ± 6.0	79.00 ± 27
	H Cd	38.00 ± 2.0	35.00 ± 2.0	37.00 ± 7.0	51.00 ± 4.0
Fe leaves	0 Cd	224.0 ± 30	83.00 ± 24	421.0 ± 248	626.0 ± 220
	L Cd	337.0 ± 62	190.0 ± 34	191.0 ± 176	617.0 ± 151
	H Cd	275.0 ± 78	121.0 ± 22	388.0 ± 88	180.0 ± 3.0
Zn roots	0 Cd	121.0 ± 20	150.0 ± 35	131.0 ± 47	115.0 ± 25
	L Cd	117.0 ± 16	79.00 ± 23	136.0 ± 48	171.0 ± 22
	H Cd	149.0 ± 32	180.0 ± 47	248.0 ± 71	133.0 ± 22
Zn stem	0 Cd	48.00 ± 6.0	46.00 ± 6.0	52.00 ± 6.0	45.00 ± 6.0
	L Cd	44.00 ± 7.0	50.00 ± 11	45.00 ± 3.0	50.00 ± 3.0
	H Cd	47.00 ± 6.0	39.00 ± 4.0	42.00 ± 3.0	44.00 ± 0.1
Zn leaves	0 Cd	157.0 ± 13	163.0 ± 10	115.0 ± 3.0	121.0 ± 7.0
	L Cd	10.03 ± 10	141.0 ± 49	100.0 ± 8.0	145.0 ± 22
	H Cd	137.0 ± 0.1	122.0 ± 14	104.0 ± 10	86.00 ± 6.0

P value	clone	Cd	clone × Cd	O_3	clone × O_3	Cd × O_3	clone× Cd × O_3
Cd roots	0.979	<0.001*	0.970	0.834	0.899	0.529	0.973
Cd stem	0.586	<0.001*	0.058	0.197	0.333	<0.001*	0.692
Cd leaves	0.785	<0.001*	0.533	0.234	0.843	<0.001*	0.462
Ca roots	<0.001*	0.379	0.483	0.706	0.123	0.091	0.605
Ca stem	0.008*	0.089	0.886	0.424	0.660	0.707	0.968
Ca leaves	0.001*	0.082	0.112	0.101	0.615	0.053	0.314
Fe roots	<0.001*	0.548	0.774	0.955	0.057	0.735	0.092
Fe stem	<0.001*	0.168	0.152	0.330	0.300	0.550	0.530
Fe leaves	0.010*	0.439	0.229	0.963	0.051	0.183	0.198
Zn roots	0.317	0.129	0.460	0.578	0.389	0.634	0.156
Zn stem	0.838	0.474	0.990	0.884	0.895	0.480	0.717
Zn leaves	0.033*	0.155	0.263	0.336	0.961	0.112	0.976

Table 2. Stem length (cm), number of leaves and fresh mass and dry mass [g] of roots, stem and leaves of the hybrid poplar clones I-214 and Eridano cultivated with 0 (0 Cd), 53.5 (L Cd) or 160.5 (H Cd) mg(Cd) kg^{-1}(soil d.m.) and exposed for 15 d (5 h a day) to charcoal-filtered air (C) or 0.06 mm^3 dm^{-3} O_3. Data represent the mean of 3 replicates ± SE. The three-way *ANOVA P*-values for the effect of clone, Cd addition to soil, exposure to O_3, and their interactions are shown.

Parameter	Cd	I-214 control	I-214 ozone	Eridano control	Eridano ozone
Stem length	0 Cd	18.6 ± 1.8	19.6 ± 3.9	16.8 ± 2.8	18.8 ± 3.8
	L Cd	16.7 ± 3.5	15.9 ± 3.1	13.2 ± 1.4	15.1 ± 3.6
	H Cd	12.4 ± 1.9	10.3 ± 3.1	13.2 ± 2.5	14.4 ± 3.6
Leaves number	0 Cd	18.7 ± 0.7	14.0 ± 0.3	17.3 ± 0.5	17.0 ± 0.3
	L Cd	16.7 ± 0.5	12.0 ± 0.3	16.3 ± 0.8	15.3 ± 0.8
	H Cd	11.0 ± 0.3	7.70 ± 0.4	9.30 ± 0.2	8.70 ± 0.2
Root FM	0 Cd	2.69 ± 0.41	2.62 ± 0.11	2.22 ± 0.50	3.12 ± 0.15
	L Cd	2.31 ± 0.30	1.82 ± 0.44	2.01 ± 0.07	2.15 ± 0.45
	H Cd	1.35 ± 0.22	1.62 ± 0.34	2.41 ± 0.39	1.72 ± 0.37
Root DM	0 Cd	0.24 ± 0.03	0.17 ± 0.03	0.17 ± 0.05	0.26 ± 0.05
	L Cd	0.17 ± 0.04	0.13 ± 0.04	0.13 ± 0.01	0.18 ± 0.06
	H Cd	0.12 ± 0.02	0.14 ± 0.04	0.16 ± 0.03	0.15 ± 0.01
Stem FM	0 Cd	7.34 ± 0.86	6.54 ± 0.46	7.31 ± 0.40	8.05 ± 1.47
	L Cd	6.54 ± 1.01	6.19 ± 0.72	5.66 ± 0.58	5.45 ± 0.80
	H Cd	3.66 ± 0.60	3.75 ± 0.19	4.98 ± 1.13	5.08 ± 0.32
Stem DM	0 Cd	1.67 ± 0.27	1.60 ± 0.19	1.67 ± 0.29	1.84 ± 0.32
	L Cd	1.47 ± 0.24	1.20 ± 0.19	0.95 ± 0.08	1.12 ± 0.26
	H Cd	0.76 ± 0.10	0.76 ± 0.19	0.90 ± 0.15	0.94 ± 0.22
Leaves FM	0 Cd	10.1 ± 1.42	6.52 ± 0.39	13.8 ± 1.02	13.2 ± 0.68
	L Cd	8.59 ± 1.07	5.64 ± 0.97	10.9 ± 2.04	10.2 ± 1.26
	H Cd	6.33 ± 1.04	3.40 ± 0.47	8.41 ± 0.99	8.41 ± 1.25
Leaves DM	0 Cd	2.25 ± 0.35	1.41 ± 0.01	2.85 ± 0.11	2.88 ± 0.02
	L Cd	1.76 ± 0.18	1.25 ± 0.26	2.29 ± 0.45	2.16 ± 0.28
	H Cd	1.38 ± 0.17	0.73 ± 0.06	1.88 ± 0.09	1.80 ± 0.28

P value	clone	Cd	clone × Cd	O_3	clone × O_3	Cd × O_3	clone × Cd × O_3
Stem length	0.845	0.037*	0.526	0.757	0.501	0.899	0.963
Leaf number	0.260	<0.001*	0.383	<0.001*	0.002*	0.796	0.750
Root FM	0.309	0.004*	0.414	0.959	0.585	0.363	0.122
Roots DM	0.475	0.030*	0.934	0.786	0.107	0.991	0.197
Stem FM	0.381	<0.001*	0.177	0.879	0.552	0.944	0.762
Stem DM	0.543	<0.001*	0.399	0.541	0.159	0.585	0.604
Leaves FM	<0.001*	<0.001*	0.470	0.011*	0.049*	0.933	0.959
Leaves DM	<0.001*	<0.001*	0.598	0.011*	0.032*	0.967	0.747

Cadmium significantly influenced all the investigated growth parameters (Table 2). Leaf number as well as fresh and dry masses significantly decreased already at the lowest Cd concentration (10, 19, and 20 % for leaf number, fresh mass, and dry mass of L Cd samples, and 46, 39, and 38 % for leaf number, fresh mass, and dry mass of H Cd samples, respectively, Table 2). Ozone also reduced leaf number, fresh mass, and dry mass of I-214 (Table 2). This, as indicated by the significant O_3 × clone interaction, was probably due to the negative effect played by O_3 exposure on I-214 clone only, Eridano being unaffected by the pollutant (Table 2).

Net photosynthetic rate at saturating PPFD (P_{Nmax}) was significantly influenced by clone, Eridano showing a 19 % lower P_{Nmax} than I-214 (Fig. 1*A*). According to the three-way *ANOVA*, a significant effect of the triple interaction clone × Cd × O_3 was also observed. However, only marginal variations among the different treatments were detected within the same clone (Fig. 1*A*). The two clones also exhibited a constitutive difference in stomatal conductance (Fig. 1*B*). Similarly to P_{Nmax}, g_s showed 24 % lower values in Eridano as compared to I-214. Moreover, Cd treatment negatively affected g_s (with a reduction of 19 and 23 % in L Cd and H Cd treated plants, respectively). Conversely, c_i was not significantly influenced by any factor or by their interaction (Fig. 1*C*).

Chlorophyll *a* and *b* content was higher in I-214 than in Eridano leaves (16 and 14 % for Chl *a* and Chl *b*,

Table 3. Content of chlorophyll *a*, chlorophyll *b*, β-carotene, total xanthophylls, sum of V + A + Z [nmol cm^{-2}] and DEPS in the leaves of the hybrid poplar clones I-214 and Eridano cultivated with 0 (0 Cd), 53.5 (L Cd) or 160.5 (H Cd) mg(Cd) kg^{-1}(soil d.m.) and exposed for 15 d (5 h a day) to charcoal-filtered air (C) or 0.06 mm^3 dm^{-3} O_3. Data represent the mean of 3 replicates ± SE. The three-way *ANOVA P*-values for the effect of clone, Cd addition to soil, exposure to O_3, and their interactions are shown.

Parameter	Cd	I-214		Eridano	
		control	ozone	control	ozone
Chlorophyll *a*	0 Cd	18.72 ± 0.96	20.51 ± 0.74	14.08 ± 0.92	16.52 ± 1.02
	L Cd	16.02 ± 1.06	16.58 ± 0.75	15.31 ± 1.20	16.35 ± 1.22
	H Cd	20.48 ± 1.50	18.70 ± 2.34	16.61 ± 0.81	16.61 ± 1.24
Chlorophyll *b*	0 Cd	6.03 ± 0.35	6.79 ± 0.28	4.82 ± 0.32	5.56 ± 0.33
	L Cd	5.47 ± 0.45	5.37 ± 0.24	5.13 ± 0.45	5.77 ± 0.45
	H Cd	7.01 ± 0.47	6.33 ± 0.81	5.58 ± 0.24	5.67 ± 0.45
β-Carotene	0 Cd	2.44 ± 0.11	2.58 ± 0.08	1.74 ± 0.06	2.03 ± 0.08
	L Cd	2.00 ± 0.11	2.03 ± 0.07	1.99 ± 0.10	1.86 ± 0.12
	H Cd	2.04 ± 0.22	1.90 ± 0.13	2.02 ± 0.11	1.92 ± 0.20
Xanthophylls	0 Cd	5.57 ± 0.15	5.66 ± 0.20	3.85 ± 0.25	4.58 ± 0.24
	L Cd	4.53 ± 0.20	4.66 ± 0.09	4.09 ± 0.15	4.28 ± 0.23
	H Cd	4.50 ± 0.24	4.61 ± 0.42	4.45 ± 0.25	4.19 ± 0.28
V+A+Z	0 Cd	0.74 ± 0.08	0.77 ± 0.03	0.55 ± 0.06	0.66 ± 0.04
	L Cd	0.56 ± 0.05	0.72 ± 0.03	0.54 ± 0.05	0.57 ± 0.04
	H Cd	0.60 ± 0.03	0.60 ± 0.08	0.60 ± 0.02	0.58 ± 0.07
DEPS	0 Cd	19.21 ± 0.87	34.52 ± 2.77	34.52 ± 2.98	46.02 ± 2.15
	L Cd	23.14 ± 0.49	30.69 ± 8.82	24.93 ± 2.57	48.60 ± 3.11
	H Cd	26.71 ± 1.53	30.41 ± 1.30	25.22 ± 1.77	36.48 ± 5.44

P value	clone	Cd	clone × Cd	O_3	clone × O_3	Cd × O_3
Chlorophyll *a*	<0.001*	0.068	0.092	0.344	0.231	0.920
Chlorophyll *b*	0.005*	0.079	0.098	0.334	0.239	0.766
β-Carotene	0.002*	0.021*	0.003*	0.839	0.161	0.674
Xanthophylls	<0.001*	0.003*	0.003*	0.162	0.476	0.420
V+A+Z	0.012*	0.041*	0.182	0.104	0.348	0.401
DEPS	<0.001*	0.321	0.094	<0.001*	0.263	0.156

respectively). Neither Cd nor O_3 treatment influenced chlorophyll content (Table 3). Similarly, the two clones showed different content of β-carotene and xanthophyll cycle pigments (V+A+Z; Table 3). Plant growing in Cd-treated soils underwent a significant decrease in these pigments, in particular in I-214 (Table 3). A constitutive difference was also detected for degree of de-epoxidation (DEPS, Table 3) which was about 31 % higher in Eridano than in I-214. The presence of Cd did not affect this parameter whereas O_3 exposure led to a 47 % increase in DEPS (Table 3).

Leaf hydrogen peroxide content was significantly influenced by clone, Cd addition to soil, exposure to O_3, as well as by the interaction among the three factors (Fig. 1*D*). I-214 leaves accumulated in average about twice H_2O_2 than Eridano. Such a result was mainly due to the very high H_2O_2 content in H Cd leaves. Dramatic increase in H_2O_2 content induced by Cd (about 20-fold and 58-fold in L Cd and H Cd leaves, respectively, in comparison with 0 Cd plants) was ameliorated by O_3 fumigation (Fig. 1*D*). In O_3-exposed I-214 leaves, H_2O_2 accumulation reached a maximum at L Cd and did not further increase at H Cd (Fig. 1*D*).

Eridano accumulated about 25 % more NO as compared to I-214 (Table 4). Cd induced a similar increase in NO content at L Cd (about 2-fold) and H Cd (about 1.4-fold, Table 4). NO content decreased in I-214 following O_3 fumigation whereas in Eridano it was unaffected (Table 4).

Discussion

Plant responses to heavy metals (and Cd in particular) or to O_3 stress have been extensively studied, though little is known about the effects of the two stress factors acting in combination, with the exception of few papers dealing

Table 4. Leaf relative nitric oxide (NO) content [expressed as relative fluorescence units - RFU - by a fluorescence microplate reader] of the hybrid poplar clones I-214 and Eridano cultivated with 0 (0 Cd), 53.5 (L Cd) or 160.5 (H Cd) mg(Cd) kg^{-1}(soil d.m.) and exposed for 15 d (5 h a day) to charcoal-filtered air (C) or 0.06 mm^3 dm^{-3} O_3. Data represent the mean of 3 replicates ± SE. The three-way *ANOVA P*-values for the effect of clone, Cd addition to soil, exposure to O_3, and their interactions are shown.

Parameter	Cd	I-214 control	I-214 ozone	Eridano control	Eridano ozone
NO	0 Cd	499 ± 51	630 ± 106	417 ± 75	1421 ± 123
	L Cd	2669 ± 286	1318 ± 188	2591 ± 301	2647 ± 566
	H Cd	2136 ± 96	1517 ± 17	1975 ± 223	1849 ± 216

P value	clone	Cd	clone × Cd	O_3	Cd × O_3	clone × Cd × O_3
NO	0.020*	<0.001*	0.318	0.299	0.005*	0.432

with herbaceous species (Czuba and Ormrod 1974, Di Cagno *et al.* 1999, 2001, Li *et al.* 2011).

In the present experiment, plant growth in soil with the highest Cd dose resulted in a significant decrease of plant height, fresh mass, and dry mass of roots and stem (Table 2). At the leaf level, the negative effect of Cd was evident already at the lowest Cd concentration (Table 2). In the present experiment, O_3 exposure (15 d) induced a premature leaf shedding at the base of the stem in I-214 (O_3-tolerant) whereas few small necrotic lesions appeared on the surface of these oldest leaves in Eridano (O_3-sensitive; data not shown). In I-214, the premature shedding of basal leaves has been already found in response to water deficit (Giovannelli *et al*. 2007).

Cd accumulation in the above-ground organs following plant growth on Cd-treated soil (Table 1) indicates that both poplar clones were able not only to take up but also translocate the metal similarly as observed Tognetti *et al.* (2004). However, in both clones considerable amounts of Cd remained in the roots (Table 1) which can act as a barrier (storing compartment) limiting Cd diffusion to the above-ground organs (Sebastiani *et al.* 2004, Cocozza *et al.* 2008, 2011, López-Climent *et al*. 2011).

Interestingly, the combined Cd × O_3 effects were found to influence leaf and stem Cd content more at lower than at higher Cd content, with a limiting influence of O_3 on the metal accumulation at the highest Cd level in the substrate (160.5 mg(Cd) kg^{-1}(soil d.m.). Since Cd transport to the above-ground organs occurs *via* the xylem, a reduction in g_s as a consequence of O_3 exposure and so water transport could be a possible explanation for such a result. However, if O_3-induced reduction in g_s was a major reason for reduced Cd uptake, also the translocation of other minerals in the xylem stream should have been affected which was not the case of the present experiment. Indeed, a significant decrease in g_s was observed when Cd was the only stress factor (Fig. 1*B*). It is, therefore, reasonable to hypothesise that other mechanisms may participate. Nevertheless, the negative effect of O_3 on Cd translocation to the above-ground organs may represent an advantage for the plant limiting metal accumulation in the photosynthetically active tissues. However, the detrimental effect of O_3 on metal translocation from roots to shoots may pose serious limitations to the effective application of poplar clones in phytoremediation programs, particularly in the light of the foreseen worldwide increase in background O_3 concentration (Ashmore 2005, Bytnerowicz *et al.* 2007).

Cd is a non-essential element and therefore no Cd specific uptake mechanism is supposed. Cd might interact with uptake of essential cations (Clemens 2001, Hall and Williams 2003) disturbing mineral nutrition. However, in the present experiment, no changes were observed in Zn, Fe, and Ca content following treatment with Cd-polluted substrate (Table 1); a positive but week correlation between Ca and Cd concentrations was detected only in the stem. Contradictory results regarding the influence of Cd in shoot micronutrient concentrations have been published (Yang *et al.* 1996, Larbi *et al.* 2002, Dong *et al.* 2006, Lopez-Millan *et al.* 2009) underlying differences among species as well as the dependence on metal concentrations. In an experimental screening of metal accumulation in roots of four poplar (including I-214) and two willow clones, Cocozza *et al*. (2011) found a positive relationship between Cd and Ca contents in roots suggesting that the accumulation of Cd did not interfere with Ca absorption. Cd may affect water and ion channels and transport of osmotically active compounds (Perfus-Barbeoch *et al.* 2002) and so development of water stress. Disturbances in the plant water status might be particularly relevant in semi-arid environments, where poplar plantations are subjected to recurrent seasonal water shortage.

The two poplar clones showed constitutive differences in net photosynthetic rate, P_{Nmax} (Fig. 1*A*) and g_s (Fig. 1*B*), Eridano exhibiting lower values than I-214. Nevertheless, plants grown in Cd-treated substrate or exposed to O_3, as well as plants subjected to the combined effects accomplished the maintenance of

efficient photosynthetic activity. However, the significant negative effect of Cd on g_s was observed, accompanied by unvaried c_i. Similarly to our finding, the same poplar clones grown on soil amended with industrial waste containing a mix of different heavy metals (Zn, Cr, and Cu) did not exhibit changes in photosynthetic activity, although in that experiment, g_s increased over the control (Tognetti *et al.* 2004). The higher content of chlorophylls and carotenoids per leaf area in I-214 in comparison with Eridano (Table 3) was probably the main cause of the higher P_{Nmax} observed in I-214. Cd has been reported to induce negative effect on chlorophyll content due to inhibition of Δ-aminolevulinic acid dehydratase and protochlorophyllide reductase (Aravind and Prasad 2004, Gonçalves *et al.* 2009) or due to Cd-induced deficiency in micro-nutrients (Balsberg Påhlsson 1989, Van Assche and Clijsters 1990). Di Cagno *et al.* (2001) observed a strong decline in chlorophyll content in O_3-treated sunflower plants grown in presence of Cd even if the effect of two stresses was not additive. This was not the case of the two poplar clones which displayed unchanged chlorophyll content after Cd and O_3 treatments alone or in combination (Table 3). Interestingly, in the present experiment, only carotenoids were affected by Cd addition to the substrate and only in I-214 where β-carotene and total xanthophylls decreased (Table 3). It is anticipated that carotenoids were more sensitive than chlorophylls to Cd stress. Gonçalves *et al.* (2009) indeed found that, in Cd-exposed *Cucumis sativus* leaves, chlorophyll content decreased only at the highest Cd treatment (1 000 μM) whereas carotenoids were already affected at 400 μM Cd. One of the most impor-tant processes to avoid photoinhibition is the thermal dissipation of excess energy which is connected with the de-epoxidation of V to A and Z (Demmig-Adams and Adams 1996). In the present experiment, such a protective mechanism was differentially responsive to Cd or O_3, being unaffected by Cd, whereas a significant de-epoxidation occurred following O_3 exposure (Table 3). The absence of Cd-induced stimulation of the xanthophylls cycle, also reported in tomato (*Lycopersicon esculentum*) by López-Millan *et al.* (2009), suggests that Cd-treated plants were not suffering from photo-inhibition. In accordance to our finding, an activation of the xanthophylls cycle by O_3 stress has been frequently reported in both herbaceous and tree species (*e.g.* Elvira *et al.* 1998).

Both Cd and O_3 are well known inducers of oxidative stress. Cd produces disturbances in plant antioxidant balance by altering both ROS-scavenging and ROS producing enzymatic and non-enzymatic processes (Romero Puertas *et al.* 2004, Ranieri *et al.* 2005, Dong *et al.* 2006). Ozone, once entered the sub-stomatal cavity, spontaneously decomposes to ROS and/or reacts with a number of compounds present in the cell wall, apoplastic fluid and plasma membrane to originate ROS (reviewed by Castagna and Ranieri 2009). In the two poplar clones, H_2O_2 content was influenced by both stress factors alone or in combination. However, surprisingly, whereas Cd led to higher H_2O_2 accumulation, exposure to O_3 in I-214 at H Cd induced a decrease in its content (Fig. 2). One possible explanation is lower Cd accumulation detected in O_3-treated leaves of I-214 plants grown at the H Cd. Nevertheless, the H_2O_2 production in I-214 H Cd leaves was higher than at almost all other experimental conditions. This result is consistent with the highest Cd content registered in leaves (Table 1). It may be hypothesized that at the H Cd dose the oxidative stress induced by excess metal concentration overcomes defence mechanisms in I-214 (antioxidant enzymatic and non-enzymatic systems, phytochelatins, vacuole seques-tration, *etc.*). Optimization of pro- and/or anti-oxidant mechanisms and their activation probably occurred in Eridano. Gaudet *et al.* (2011) characterized the variability of responses to Cd in two *Populus nigra* genotypes (58-861 and Poli) which originate from sites with contrasting environmental conditions and concluded that the glutathione pathway was involved in the differential Cd tolerance of the two genotypes. These findings suggest new reason for specific screening of poplar clones (Pietrini *et al.* 2010b, Zacchini *et al.* 2011). The prolonged exposure to low O_3 concentration of Cd-stressed plants may have allowed I-214 plants to acclimate to the stress by enhancing antioxidant defences that, besides removing O_3-deriving ROS, also detoxified Cd-induced ones. However, since the present paper was not addressed to investigate the cell antioxidant systems, further specific investigation is needed to verify this hypothesis. Different behaviour between I-214 and Eridano under chronic O_3 stress was also observed in a previous experiment (Di Baccio *et al.* 2008), where Eridano only apparently increased its antioxidant defences in comparison with I-214 whereas its photo-synthetic efficiency and antioxidant capacity per unit of O_3 influx were reduced.

Nitric oxide, identified as an important messenger in plant defence signalling, was subsequently recognised as a crucial regulator of normal physiological processes (Gould *et al.* 2003). Our data showed a genotypic difference in NO production and a Cd-induced increase in leaf NO accumulation (Table 4). By contrast, Rodríguez-Serrano *et al.* (2006) observed a decrease in NO content in roots of pea (*Pisum sativum*) following 14 d of Cd exposure whereas a shorter treatment (24 h) was found to increase root NO accumulation (Bartha *et al.* 2005). It is, therefore, evident that a Cd on NO effects may vary depending on plant organ as well as on stress duration. When the two stresses were applied in combination, an increase of NO content over the control 0 Cd treatment was observed even if a lower value was detected in O_3-treated H Cd leaves in comparison with control L Cd leaves (Table 4). The cause might be the limited O_3 entry as a consequence of Cd-induced decrease in g_s.

Conclusions

It may be concluded that the combination of two stress factors, which share some common toxicity pathways, induced plant responses not always predictable on the basis of the known effects induced by the single factors. Cd was effective in reducing g_s and in promoting H_2O_2 and NO accumulation in both clones, and in decreasing carotenoid content in I-214. Ozone exposure, on the other hand, induced a significant increase in the xanthophyll de-epoxidation state. Fumigation with O_3 partially counteracted Cd accumulation in the above-ground organs. Surprisingly, O_3 exposure, alone and in combination with Cd, decreased H_2O_2 accumulation in I-214 but not in Eridano and also NO production was higher in leaves of Eridano than I-214. Overall O_3 and Cd at the applied doses interacted in antagonistic rather than additive way. Due to the complexity of plant reactions occurring in natural environments, this result should be confirmed under field conditions to better understand plant responses to multiple stress factors (*cf.* Marmiroli *et al.* 2011, Tognetti *et al.* 2011).

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