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Early responses to cadmium of two poplar clones that differ in stress tolerance

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ABSTRACT

Soil cadmium (Cd) contamination is becoming a matter of great global concern. The identification of plants differentially sensitive to Cd excess is of interest for the selection of genotype adaptive to grow and develop in polluted areas and capable of ameliorating or reducing the negative environmental effects of this toxic metal. The two poplar clones I-214 (*Populus* × *canadensis*) and Eridano (*Populus deltoides* × *maximowiczii*) are, respectively, tolerant and sensitive to ozone (O_3) exposure. Because stress tolerance is mediated by an array of overlapping defence mechanisms, we tested the hypothesis that these two clones differently sensitive to O_3 stress factor also exhibit different tolerance to Cd. With this purpose, an outdoor pot experiment was designed to study the responses of I-214 and Eridano to the distribution of different Cd solutions enriched with $CdCl_2$ (0, 50 and 150 μM) for 35 days. Changes in leaf area, biomass allocation and Cd uptake, photosynthesis, chlorophyll fluorescence, leaf concentration of nutrients and pigments, hydrogen peroxide (H_2O_2) and nitric oxide (NO) production and thiol compounds were investigated. The two poplar clones showed similar sensitivity to excess Cd in terms of biomass production, photosynthesis activity and Cd accumulation, though physiological and biochemical traits revealed different defence strategies. In particular, Eridano maintained in any Cd treatment the number of its constitutively wider blade leaves, while the number of I-214 leaves (with lower size) was reduced. H_2O_2 increased 4.5- and 13-fold in I-214 leaves after the lowest (L) and highest (H) Cd treatments, respectively, revealing the induction of oxidative burst. NO, constitutively higher in I-214 than Eridano, progressively increased in both clones with the enhancement of Cd concentration in the substrate. I-214 showed a more elevated antioxidative capacity (GSH/GSSG) and higher photochemical efficiency of PSII (F_v/F_m) and de-epoxidation degree of xantophylls-cycle (DEPS). The glutathione pool was not affected by Cd treatment in both clones, while non-protein thiols and phytochelatins were reduced at L Cd treatment in I-214. Overall, these two clones presented high adaptability to Cd stress and are both suitable to develop and growth in environments contaminated with this metal, thus being promising for their potential use in phytoremediation programmes.

Abbreviations: A, antheraxanthin; A_{max}, photosynthetic rate measured at light saturation; C_a, ambient CO_2 concentration; C_i, intercellular CO_2 concentration; DEPS, de-epoxidation index; F_o, minimum chlorophyll fluorescence yield in 'dark-adapted' leaves; F_m, maximum chlorophyll fluorescence yield in 'dark-adapted' leaves; F_v/F_m, ratio of variable and maximal fluorescence; g_s, stomatal conductance to H_2O; ΦPSII, quantum efficiency of PSII photochemistry; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; LAR, leaf area ratio; LMA, leaf mean area; LMR, leaf mass ratio; LN, leaf number; MLA, mean leaf area; NO, nitric oxide; NPQ, non-photochemical quenching; NPTs, non-protein thiols; O_3, ozone; PCs, phytochelatins; qP, photochemical quenching; ROS, reactive oxygen species; SLA, specific leaf area; SMR, stem mass ratio; V, violaxanthin; Z, zeaxanthin.

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Introduction

Soil pollution with heavy metals and their accumulation in biotic systems as a consequence of human activities are becoming matters of growing global concern. Together with lead, mercury, chromium, nickel, zinc, arsenic and selenium, cadmium (Cd) represents a real threat for human health and the environment (Lado et al., 2008). Of all non-essential heavy metals, Cd has attracted most attention in soil science and plant nutrition due to the mobility in the soil–plant system and high toxicity to human health (McLaughlin and Singh, 1999). Cadmium is a trace metal naturally present in the environment, occurring as a minor constituent of phosphate rock ore, and it is released into the environment by many industrial activities, waste incinerators, urban traffic and as by-product of phosphate fertilizers (Sanità di Toppi and Gabbrielli, 1999). Cadmium accumulation in soil can result in a decrease of microbial activity, biodiversity and soil fertility with consequent damages on growth and development of crops up to animal and human health through the food chain (Sanità di Toppi and Gabbrielli, 1999; DalCorso et al., 2008; Baize et al., 2009).

Excess Cd in plants causes leaf roll and chlorosis, growth inhibition both in shoots and roots, early senescence and eventually plant death (Sanità di Toppi and Gabbrielli, 1999; Benavides et al., 2005). These effects are due to direct or indirect interferences with a series of physiological, biochemical and molecular processes, including photosynthesis, transpiration, water and nutrients balance, enzymatic activities, functions of transporter, antioxidants and several biomolecules, gene expression and regulation (Benavides et al., 2005; DalCorso et al., 2008). Despite the numerous studies on plant responses to Cd in the last decades, the mechanisms of Cd toxicity and, consequently, the plant capacity to tolerate Cd excess are not completely understood yet. However, we know that heavy metals and Cd accumulation in plants depends on physical–chemical characteristics of soils (pH, organic matter and water contents, cation exchange capacity) and biotic factors such as plant species, root activity and rizosphere-associated microrganisms (McLaughlin and Singh, 1999; Greger and Landberg, 2008; Baize et al., 2009). In particular, many plant species or cultivars differ in their Cd sensitivity and uptake capacity (Hart et al., 2006; Baize et al., 2009; Polle et al., 2013). The identification of properties (physiological processes, biochemical patterns and morphological traits) that make plants more or less sensitive to Cd excess in the environment is still an ongoing objective for the selection of genotype adaptive to grow and develop in polluted areas and/or capable to ameliorate or reduce the negative effects of this pollutant (Sebastiani et al., 2004, 2014; Greger and Landberg, 2008; Durand et al., 2010; Castagna et al., 2013; Cocozza et al., 2014).

As sessile organisms, plants withstand a plethora of stress conditions during their life cycle in the natural and cultivated environment, including abiotic and biotic stresses; for this reason they have developed unique strategies for responding to environmental changes, monitoring their surroundings and adjusting their metabolic systems to maintain homeostasis (Pastori and Foyer, 2002). Many plant species are naturally less sensitive to some stresses in comparison with other plants, and this behaviour is one of the most relevant factors that drive the distribution and survival of vegetation worldwide. For example, in *Sedum alfredii* species Cd hyperaccumulator and non-hyperaccumulator ecotypes have been characterized (Sun et al., 2013), and it is well known that *Silene vulgaris* species includes Cd-sensitive and Cd-tolerant ecotypes. Two poplar clones with well known different sensitivity to ozone (O_3) stress (Diara et al., 2005; Rizzo et al., 2007) evidenced different responses to industrial heavy metal-enriched organic waste pollution (Sebastiani et al., 2004; Tognetti et al., 2004) and, in particular, to elevated Zn levels (Fernàndez et al., 2012), or to the combination of high Cd and O_3 concentrations (Castagna et al., 2013). Several authors support the thesis that different plant species make use of common pathways and components in the stress-response relationship, showing that plants that can tolerate a particular stress factor also exhibit co-tolerance to other stresses (Pastori and Foyer, 2002; Singh et al., 2010). Polle et al. (2013), for example, tested the tolerance capacity of *Populus euphratica* (salt tolerant species) and *Populus* × *canescens* (salt sensitive species) to Cd stress. *P. euphratica* was more sensitive to Cd than *P.* × *canescens*, but the former accumulated 10-times more Cd in the leaves than the latter.

Previous studies revealed that the two poplar clones I-214 (*Populus* × *canadensis*) and Eridano (*Populus deltoides* × *maximowiczii*) are differentially sensitive to O_3 stress, both under acute and chronic exposure (Diara et al., 2005; Di Baccio et al., 2008). Indeed, to our knowledge no study has ever tested how I-214 and Eridano poplar clones respond to high level of the single Cd stress factor in the substrate.

The aim of the work was to verify if the two hybrid poplars I-214 and Eridano have different sensitivity to Cd pollution; with this purpose, woody cuttings of both clones were rooted and young plants grown in a substrate contaminated with different Cd levels in an outdoor pot experiment. In addition to Cd uptake and distribution in leaves, stems and roots, growth and gas exchange parameters, chlorophyll fluorescence, pigment and the main cation and anion concentrations, H_2O_2 and nitric oxide (NO) production and the concentration of thiols in leaves were investigated. Moreover, the performance of the two poplar clones under Cd stress during early and more sensitive phase of life cycle (rooting and first weeks after leaf full expansion) is of great importance to evaluate the tolerance and the potential of fast growing tree species for their use in soil reclamation.

Materials and methods

Plant materials and Cd treatments

Woody cuttings of two poplar clones, I-214 (*Populus* × *canadensis*) and Eridano (*Populus deltoides* × *maximowiczii*), were planted in 4 L-plastic pots filled with a sandy soil:peat substrate mix (2:1, v:v) with pH (H_2O) 5.8. To simulate field conditions, the pot experiment was conducted outdoors under a waterproof roofing with a shade net (mean daily photosynthetic photon flux density of 500–600 μmol photons m^{-2} s^{-1}) at the experimental station of the University of Pisa (43°43′ N, 10°23′ E). After 2–3 days of acclimation, twenty four uniform cuttings (12 for each clone) were selected for the experiments and irrigated with half-strength Hoagland nutrient solution (pH 6.5) added with 0, 50 and 150 μM Cd concentrations in order to reach the following final total Cd amounts in the pot substrate, respectively: 0 mg Cd kg^{-1} soil DM or ppm (control), 54.3 ppm (the lowest Cd concentration, L Cd) and 163.0 ppm (the highest Cd concentration, H Cd). Cadmium was supplied as $CdCl_2$ totally dissolved in the nutrient solution and distributed every week in each pot, never exceeding the field capacity; the plants were grown in these conditions for 35 days. Each plant possessed 1–5 shoots (mean value: 2.2), which were maintained until the end of the experiment. During the experiment no growth restriction due to limited pot volume was observed. Meteorological data for the experimental period (March–May) were as follows: average air temperature, 13.2 ± 4.0 °C; relative humidity, 71 ± 10%.

Sample collection and growth analysis

After 35 days of Cd treatment, poplar plants (n = 4) of I-214 and Eridano from each Cd treatment were harvested, the roots washed free of growth substrate particles and all the organs (leaves,

stems, woody cuttings and roots) were sampled separately. The fresh mass (FM) of total leaves, stems and roots were determined, as well as the leaf area and the length of stems. Leaf discs and samples collected from individual plants of each clone and treatment were immediately plunged in liquid nitrogen (N_2) and then stored frozen (−80 °C) for pigment and biochemical analyses. The remaining plant material was oven dried at 60 °C until constant weight for the determination of dry mass (DM). In the measurement of the whole plant dry mass, woody cutting contribution was never considered. Leaf area was measured using scanner and image analysis software (Scion Image 4.0.2, Scion Corporation, Frederick, MD, USA). The length of stem was calculated as the average of measured lengths of single shoot for each plant. The specific leaf area (SLA), leaf mass ratio (LMR), leaf area ratio (LAR) and stem mass ratio (SMR) were derived as previously described (Di Baccio et al., 2009).

Cadmium determinations

In leaves, stems and roots of the two poplar clones the concentration of Cd was determined after digestion in concentrated HNO_3 by atomic absorption spectrophotometer (AAS, model 373; Perkin Elmer, Norwalk, CT, USA). Results were expressed on a dry mass basis, Cd mg kg^{-1} DM (ppm). The Cd content (uptake) in each organ was calculated by multiplying the Cd concentration (mg kg^{-1} DM) per the DM (kg) of the corresponding biomass.

In order to evaluate the Cd accumulation, the bioconcentration factor (BCF) was calculated as the ratio of the Cd concentration in leaves (BCF_L), stems (BCF_S) or in roots (BCF_R) to the Cd concentration in soil (Wu et al., 2010). The translocation factor was calculated as the ratio of the Cd content in plant shoot (leaves + stems) to the Cd content in roots (Pietrini et al., 2010).

The total Cd content in the growth substrate at the end of the experiment was determined by AAS (model 373; Perkin Elmer, Norwalk, CT, USA) after digestion of air-dried and sieved samples (≤2 mm) with HNO_3:HCl mixture (1:3, v:v). The amount of Cd in the native substrates (before $CdCl_2$ addition) was negligible.

Gas exchange and chlorophyll fluorescence measurements

Gas exchange was measured with a portable 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 (LI-6400-02B). Net photosynthesis (A), stomatal conductance to H_2O (g_s) and intercellular CO_2 concentration (C_i) were measured on the third and fourth fully expanded leaves from the apex (Leaf Plastochron Index, LPI, 4–5) of each plant shoot. All gas exchange measurements were conducted on May 19th on three replicate plants for each clone-treatment combination. Leaf chamber temperature and humidity were adjusted to maintain a leaf-to-air vapour pressure difference of about 1.3 kPa, with irradiance maintained at a previously determined saturating value of 800 μmol m^{-2} s^{-1}.

Contemporary to gas exchange determinations, chlorophyll fluorescence emissions in 30-min dark-adapted leaves were measured with a portable pulse amplitude modulation fluorometer (PAM-2000, Walz, Effeltrich, Germany) to estimate the effect of treatments on PSII (photosystem II) efficiency. Background fluorescence signal (F_o), maximum fluorescence (F_m) and potential quantum yield of PSII photochemistry [$F_v/F_m = (F_m - F_o)/F_m$] were determined. Other fluorescence parameters were measured on light-adapted leaves using the equations of Genty et al. (1989) (quantum yield of PSII electron transport, $\Phi PSII = (F'_m - F)/F'_m$; photochemical quenching, $qP = (F'_m - F)/(F'_m - F'_o)$, and non-photochemical quenching, $NPQ = 1 - (F'_m - F'_o)/(F_m - F_o)$, where F is the steady state fluorescence, F'_o the minimal fluorescence, F'_m the maximal fluorescence for light-adapted leaves).

Photosynthetic pigment analysis

Pigment concentration and pattern were determined according to the method previously reported (Castagna et al., 2013). Leaf discs of known area (1.13 cm^2) were punched from leaves previously utilized for gas exchange measurements, frozen in liquid N_2 and stored at −80 °C until use.

Frozen samples were homogenized under dimmed room light in 100% HPLC-grade acetone with 1 mM Na-ascorbate, filtered through 0.2-μm filters (Sartorius Stedim Biotech, Goettingen, Germany) and immediately analyzed. The analysis was performed by HPLC (HPLC P4000, Thermo Fisher Scientific, Waltham, MA, USA) using a non-endcapped column (Zorbax ODS column, Chrompack, Raritan, NJ, USA). Pigments were eluted using 100% solvent A (acetonitrile/methanol, 75/25, v/v) for the first 15 min, followed by a 2.5-min linear gradient to 100% solvent B (methanol/ethylacetate, 68/32, v/v), which continued isocratically until the end of the cycle. The separation cycle was 32 min with a flow rate of 1 mL min^{-1}. The column was allowed to re-equilibrate in 100% solvent A for 10 min before the next injection.

Pigments were detected by their absorbance at 445 nm, and quantified by injecting known amounts of pure standards (Sigma–Aldrich, Milan, Italy) into the HPLC system. The de-epoxidation index (DEPS index) was calculated according to the following equation: $[(A/2) + Z]/(V + A + Z) \times 100$ (A, anteraxanthin; Z, zeaxanthin; V, violaxanthin).

Leaf cations and anions

The concentrations of leaf K, Ca, Mg, Fe and Zn were determined by AAS (*Perkin-Elmer Analyst 100*, Waltham, MA, USA) equipped with *Perkin-Elmer Intensitron*™ lamps. Immediately after harvesting, samples were washed in distilled water, oven-dried, ground to powder and stored under vacuum until use. Samples were digested in concentrated H_2SO_4 and hydrogen peroxide drops were added until complete clarification of samples.

Leaf anion (chloride, sulphate, phosphate and nitrate) concentration was determined by ion chromatography (D-X-100 ion chromatograph, Dionex) on water extracts of lyophilized material. A 25 mg sample was incubated in 5 mL of water for 30 min at 60 °C, followed by centrifugation to recover the supernatant. The procedure was repeated twice and the pooled supernatants were evaporated to dryness and resuspended in 2 mL of water.

Hydrogen peroxide and nitric oxide determinations

Leaf H_2O_2 production was measured fluorimetrically using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes), according to Di Baccio et al. (2008) and to the manufacturer's recommendations. The assay is based on the reaction of 10-acetyl-3,7-dihydrophenoxazine (Amplex Red reagent) with H_2O_2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product resofurin. Leaf tissue (30 mg) was frozen in liquid N_2 and ground, added to 200 μl of 20 mM K-P buffer, pH 6.5. After centrifugation, the supernatant was incubated with 50 μM Amplex Red reagent and 0.1 U mL^{-1} HRP at 25 °C for 30 min under dark conditions. The resofurin fluorescence (Ex/Em = 530–590 nm) was quantified with a fluorescence/absorbance microplate reader (Victor^{3TM}, Perkin Elmer, Monza, Italy). Values were expressed as μmoles of H_2O_2 per milligramme of FM.

Leaf NO accumulation was determined using the DAF-FM DA (4-amino-5-methylamino-2′,7′-difluorescein diacetate) fluorescent dye (Molecular probes), according to Di Baccio et al. (2012)

and to the manufacturer's recommendations. Leaf discs (5 mm diameter) were treated with 10 μM DAF-FM DA dissolved in 0.25 M Na-P buffer pH 7.4 (loading buffer), in a final reaction volume of 200 μL. After incubation (dark, 60 min, 25 °C), DAF-FM/NO reactive (benzotriazole derivative) fluorescence (Ex/Em = 495/515 nm) was measured with a fluorescence/absorbance microplate reader (Victor^{3TM}, Perkin Elmer, Monza, Italy).

Determination of thiols

Nitrogen-frozen leaf samples were extracted in 5% (w/v) trichloracetic acid containing 5% insoluble polyvinylpolypyrrolidone (PVPP) and centrifuged at 14,000 × g_n for 15 min at 4 °C.

Total (GSH + GSSG) and oxidized (GSSG) glutathione were determined spectrophotometrically in the supernatant 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. The 5-thio-2-nitrobenzoic acid formation was monitored at 412 nm at 25 °C for 1 min (Di Baccio et al., 2004). Values were expressed as nmol GSH cm^{-2}.

For total non-protein thiols (NPTs), 200 μL of the supernatant was mixed to 1.8 mL of 0.6 mM DTNB and 250 mM K-Pi buffer, pH 8.00. The concentration of NPTs was measured spectrophotometrically at the absorbance of 412 nm and expressed as GSH equivalents (Schäfer et al., 1997). Phytochelatins (PCs) were estimated from the difference between total NPTs and GSH (Hartley-Whitaker et al., 2001).

Experimental design and statistical analyses

The experiment was set up in a completely randomized design with four biological replicates ($n = 4$) for each poplar clone. A two-way analysis of variance (ANOVA) was applied to the data in order to evaluate the effect of poplar clone (I-214 and Eridano), Cd treatment (control, 0 Cd; the lowest Cd dose: L Cd, 54.3 mg Cd kg^{-1} soil DM; the highest Cd dose: H Cd, 163.0 mg Cd kg^{-1} soil DM) and the interaction between clone and treatment effects (Clone × Cd). Data shown in tables and figures are mean values ± SE for each clone–treatment combination. When necessary, the single and combined effects of the two factors on results (mean and mean group values) were shown. Statistical analysis was conducted using STATISTICA 6.0, StatSoft, Inc. (Tulsa, OK 74104, USA). The significance level of the F ratio (P) was shown and considered significant when ≤0.05. Separation of means was performed by LSD test, at the 0.05 significance level.

Results

Leaf, stem traits and growth analysis

Cadmium treatment and clone factors had significant effects both on the leaf number and area. The leaf number was higher in I-214 than in Eridano clone, while the mean leaf area (MLA) was more elevated in Eridano than in I-214 (Table 1). The leaf number was effectively reduced in I-214 at the lowest (L, −25%) and highest (H, −33%) Cd dose. The H Cd treatment decreased by 26% the MLA when compared to the control. The total leaf area per plant followed the same trend of the MLA in both clones (data not shown). The leaf biomass was affected by both L and H Cd treatments (−22 and −35%, respectively). The LMR and LAR were higher in Eridano than in I-214. The LMR was influenced by clone × Cd interaction, showing a general increase (12%) at H Cd (Table 1).

In stems and roots, the biomass allocation did not show significant differences between I-214 and Eridano clones (Table 2). The mean length of the plants (indicated as stem length) showed no differences between the two clones and among Cd treatments, while the mean stem biomass progressively decreased with the increasing of Cd concentration in the substrate. The SMR was lower in Eridano and reduced by H Cd treatment. The Cd exposure reduced the biomass production in both clones, with major effect at the H Cd treatment, as well as the leaf to roots ratio (Table 2).

Cd accumulation

The Cd concentration in leaves was higher in I-214 than in Eridano; in both clones, the leaf Cd concentration increased with increasing Cd addition to the substrate, although no significant interaction between the clone and Cd treatment factors was observed (Table 3). In stems of the two clones, the Cd concentration significantly increased at H Cd, while in roots it was higher at both L and H Cd treatments in comparison with the control plants

Table 1
Leaf traits of the two hybrid poplar clones I-214 and Eridano grown in pots filled with soil treated with Cd ($CdCl_2$): Cd 0, control; L Cd (50 μM, the lowest Cd concentration); H Cd (150 μM, the highest Cd concentration) for 35 days. Data were analyzed independently by two-way ANOVA to evaluate the clone effect (Clone), the Cd treatment effect (Cd) and the interaction of these two factors (Clone × Cd). Means within a column flanked by different letters are significantly different at $P \leq 0.05$ (LSD test). The mean values from the combination of Clone × Cd are the results of four ($n = 4$) biological replicates ± SE.

	LN	MLA	DM	FM/DM	SLA	LMR	LAR
Clone							
I-214	30.4 a	27.72 b	1.69	4.35	50.35	0.61 b	30.57 b
Eridano	13.8 b	77.14 a	1.97	4.70	53.85	0.65 a	34.87 a
Cd treatment							
Control (C)	26.3 a	53.71 a	2.23 a	4.44	50.30	0.60 b	30.09
L Cd	20.3 b	63.47 a	1.74 b	4.70	56.20	0.61 b	34.49
H Cd	19.7 b	39.86 b	1.45 b	4.46	50.16	0.68 a	34.10
Clone × Cd							
I-214C	37.7 ± 1.5 a	27.40 ± 0.79	2.06 ± 0.07	4.55 ± 0.42	50.54 ± 4.68	0.56 ± 0.01 b	28.15 ± 2.65
I-214 L Cd	28.3 ± 1.8 b	31.71 ± 5.55	1.61 ± 0.15	4.38 ± 0.37	55.32 ± 4.62	0.58 ± 0.02 b	31.76 ± 1.73
I-214 H Cd	25.3 ± 0.9 b	25.39 ± 1.54	1.37 ± 0.08	4.14 ± 0.06	46.83 ± 0.62	0.69 ± 0.02 a	32.20 ± 1.42
Eridano C	15.0 ± 0.6 c	80.02 ± 7.22	2.40 ± 0.23	4.33 ± 0.17	50.05 ± 1.92	0.64 ± 0.01 ab	32.04 ± 1.10
Eridano L Cd	12.3 ± 1.5 c	84.65 ± 5.75	1.82 ± 0.11	4.91 ± 0.28	56.79 ± 3.21	0.64 ± 0.02 ab	36.31 ± 2.23
Eridano H Cd	14.0 ± 0.6 c	61.56 ± 4.85	1.56 ± 0.12	4.95 ± 0.01	55.14 ± 0.04	0.69 ± 0.01 a	36.96 ± 0.52
Two-way ANOVA (P-values)							
Clone	<0.001	<0.001	0.065	0.128	0.261	0.005	0.020
Cd	<0.001	0.045	0.001	0.753	0.197	<0.001	0.075
Clone × Cd	0.002	0.216	0.842	0.190	0.384	0.017	0.971

Abbreviations: LN, leaf number; MLA, mean leaf area; DM, dry mass; FM, fresh mass; SLA, specific leaf area; LMR, leaf mass ratio; LAR, leaf area ratio. Units are cm^2 for area, g for DM, $m^2\ kg^{-1}$ for SLA, kg kg^{-1} for LMR and $m^2\ kg^{-1}$ for LAR.

Table 2
Stem traits and biomass allocation of the two hybrid poplar clones I-214 and Eridano grown in pots filled with soil treated with Cd ($CdCl_2$): Cd 0, control; L Cd (50 μM, the lowest Cd concentration); H Cd (150 μM, the highest Cd concentration) for 35 days. Elaboration of data and statistical analysis are as in Table 1.

	Stem length	Stem DM	SMR	Root DM	Whole plant DM	Leaf/root
Clone						
I-214	15.2	0.97	0.33 a	0.17	2.84	11.41
Eridano	12.4	0.91	0.29 b	0.16	3.05	11.96
Cd treatment						
Control (C)	14.9	1.25 a	0.34 a	0.24 a	3.72 a	9.84 b
L Cd	14.7	0.94 b	0.33 a	0.16 b	2.83 b	11.15 b
H Cd	11.6	0.58 c	0.27 b	0.10 b	2.13 c	14.44 a
Clone × Cd						
I-214C	17.1 ± 4.4	1.37 ± 0.12	0.37 ± 0.03	0.27 ± 0.05	3.70 ± 0.14	8.66 ± 2.46
I-214 L Cd	17.4 ± 5.9	1.04 ± 0.18	0.37 ± 0.02	0.15 ± 0.02	2.81 ± 0.34	10.52 ± 0.22
I-214 H Cd	11.0 ± 2.5	0.53 ± 0.05	0.27 ± 0.02	0.09 ± 0.005	2.00 ± 0.08	14.75 ± 1.31
Eridano C	12.7 ± 2.2	1.13 ± 0.11	0.30 ± 0.01	0.22 ± 0.02	3.75 ± 0.35	11.02 ± 0.23
Eridano L Cd	12.0 ± 2.9	0.86 ± 0.03	0.30 ± 0.02	0.16 ± 0.02	2.84 ± 0.14	11.57 ± 0.81
Eridano H Cd	12.5 ± 1.5	0.65 ± 0.02	0.28 ± 0.01	0.11 ± 0.005	2.33 ± 0.15	13.97 ± 0.48
Two-way ANOVA (*P*-values)						
Clone	0.397	0.245	0.024	0.723	0.467	0.465
Cd	0.679	<0.001	0.009	0.002	<0.001	0.024
Clone × Cd	0.654	0.190	0.073	0.454	0.764	0.548

Abbreviations: SMR, stem mass ratio. Units are cm for stem length, g for DM and kg kg^{-1} for SMR.

(Table 3). Also the BCF index was affected by Cd exposure in leaves (BCF_L) and roots (BCF_R), with any significant difference between I-214 and Eridano. The BCF was higher at L Cd level in leaves and roots, and in the latter it reached one unit (Table 3).

In I-214 and Eridano, the Cd content in all plant tissues and its distribution in shoot/root portions were affected by the level of Cd treatments in pot substrate (Fig. 1). Similar trend was observed for the whole plant Cd accumulation, with the highest Cd uptake at the H Cd treatment (33.3 and 32.5 μg in I-214 and Eridano, respectively). At L Cd treatment the total plant Cd content was 22 and 32% lower than at H Cd in I-214 and Eridano, respectively (Fig. 1D). The translocation factor was elevated and of the same amount in I-214 treated with L and H Cd doses (2.3 and 2.5, respectively). In Eridano under H Cd treatment the shoot to roots Cd content reached the maximum value (3.5), which was 2.4-fold more elevated than at the L Cd treatment (Fig. 1E). The clone × Cd interaction was never affected.

Gas exchange parameters and chlorophyll fluorescence

In both I-214 and Eridano the photosynthetic activity and the g_s were markedly reduced by the Cd treatment (Fig. 2A and B). The intercellular CO_2 concentration was not influenced by the clone or Cd treatment factors (Fig. 2C). The F_v/F_m was relatively higher in I-214 than in Eridano, but it was unaffected by Cd treatment, although the *P* value was only slightly higher (0.054) than the significance limit (Table 4). This was due to a relatively lower value of the basal fluorescence in the dark (F_o) in I-214 than Eridano. The interaction of clone × Cd factors influenced the ΦPSII and qP (Table 4). In I-214 the L and H Cd treatments decreased ΦPSII and qP (−7 and −11%, respectively). These two parameters were reduced of the same extent also in Eridano exposed to L Cd treatment, but in this case ΦPSII and qP did not significantly differ from Eridano control. Non-photochemical quenching (NPQ) was not affected by clone, Cd or clone × Cd (Table 4).

Photosynthetic pigment composition

Chlorophyll (Chl) *a* and *b* concentrations and, consequently, the total Chl amount were influenced by the clone factor, resulting higher in I-214 than in Eridano (Table 5). Violaxanthin (V) and antheraxanthin (A) were affected by clone and Cd treatment interaction. Violaxanthin was constitutively higher in Eridano

Table 3
Cadmium concentration (μg g^{-1} plant tissue dry mass, or ppm), bioconcentration factors for leaves, stem and roots in the two hybrid poplar clones I-214 and Eridano grown in pots filled with soil treated with Cd ($CdCl_2$): Cd 0, control; L Cd (50 μM, the lowest Cd concentration); H Cd (150 μM, the highest Cd concentration) for 35 days. Elaboration of data and statistical analysis are as in Table 1.

	Leaf	Stem	Roots	BCF_L	BCF_S	BCF_R
Clone						
I-214	6.10 a	6.78	44.37	0.087	0.088	0.72
Eridano	4.47 b	4.80	37.74	0.078	0.076	0.78
Cd treatment						
Control (C)	0.44 c	0.12 b	0.24 b	–	–	–
L Cd	5.87 b	4.25 b	56.23 a	0.108 a	0.078	1.04 a
H Cd	11.62 a	14.12 a	74.86 a	0.057 b	0.087	0.46 b
Clone × Cd						
I-214C	0.69 ± 0.04	0.08 ± 0.04	0.32 ± 0.08	–	–	–
I-214 L Cd	7.63 ± 0.64	4.57 ± 0.35	57.45 ± 20.12	0.14 ± 0.01	0.08 ± 0.01	1.06 ± 0.37
I-214 H Cd	12.69 ± 1.48	14.95 ± 4.33	79.71 ± 16.57	0.05 ± 0.02	0.09 ± 0.03	0.49 ± 0.10
Eridano C	0.20 ± 0.04	0.16 ± 0.04	0.16 ± 0.02	–	–	–
Eridano L Cd	4.70 ± 1.34	4.04 ± 2.20	55.43 ± 22.60	0.09 ± 0.03	0.07 ± 0.04	1.02 ± 0.42
Eridano H Cd	10.54 ± 0.89	12.89 ± 2.77	67.59 ± 17.06	0.06 ± 0.01	0.08 ± 0.02	0.41 ± 0.10
Two-way ANOVA (*P*-values)						
Clone	0.033	0.687	0.713	0.421	0.738	0.864
Cd	<0.001	<0.001	0.002	0.050	0.086	0.040
Clone × Cd	0.379	0.906	0.918	0.211	0.960	0.987

Abbreviations: BCF, bioconcentration factor; L, leaves; S, stems; R, roots.

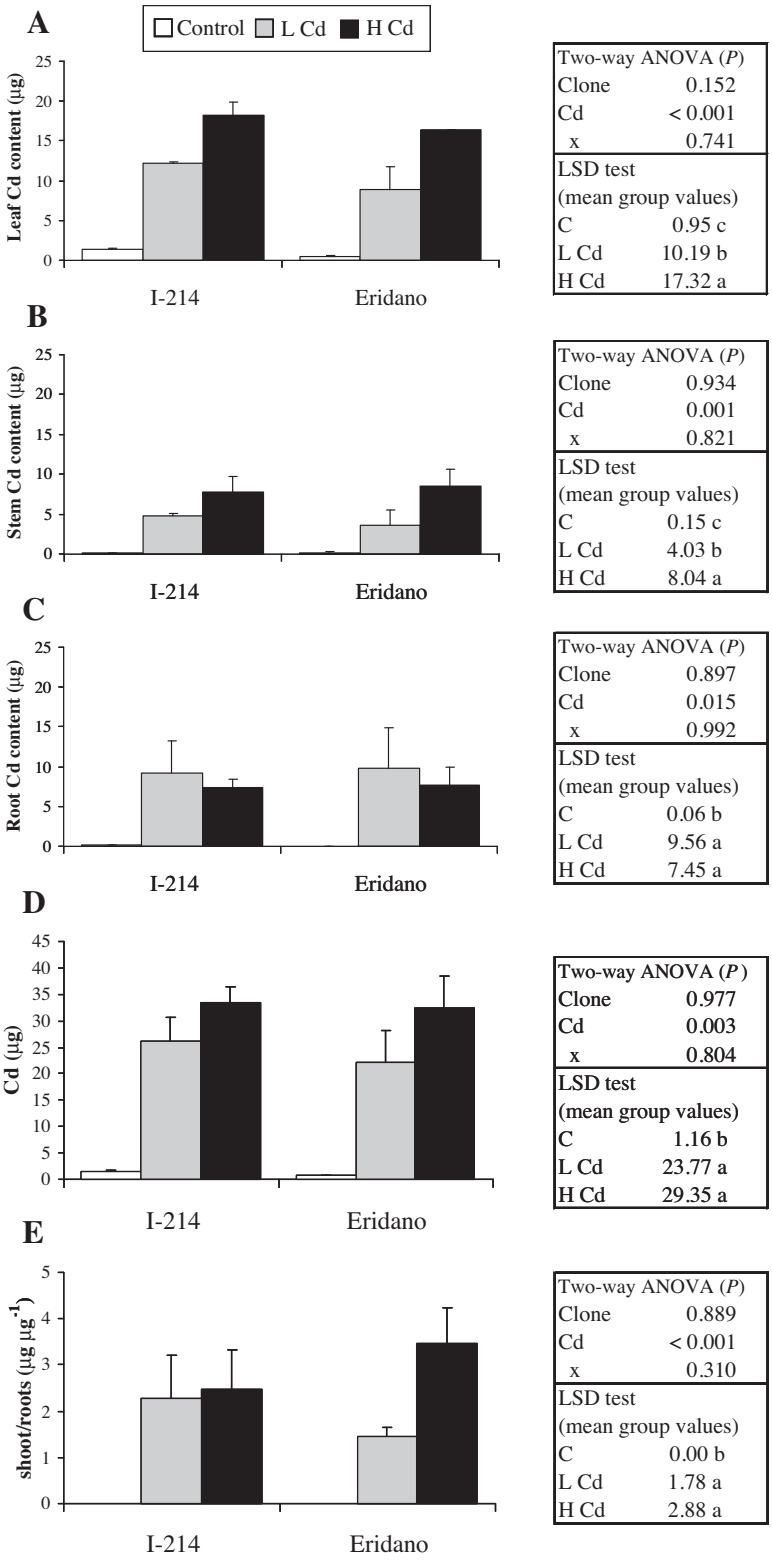

Fig. 1. Cadmium content or uptake (µg) in the leaves (A, 2nd–6th leaf from the apex), stems (B), roots (C), whole plants (D) and translocation factor (Cd shoot/root, E) of the two hybrid poplar clones I-214 and Eridano grown in pots filled with soil treated with $CdCl_2$: Cd 0, control; L Cd (50 µM Cd, the lowest Cd concentration); H Cd (150 µM Cd, the highest Cd concentration). Each value represents the mean of four ($n = 4$) biological replicates ± SE. Data were analyzed independently by two-way ANOVA to evaluate the clone effect (Clone), the Cd treatment effect (Cd) and the interaction of these two factors (x). The significance level of the F ratio (P) was indicated and considered significant when ≤0.05. Means flanked by different letters are significantly different at $P \leq 0.05$ (LSD test).

than in I-214, and in Eridano showed a slight decrease after Cd exposure while in I-214 a slight increase. In I-214, A increased at the L Cd treatment. These modifications altered the xanthophylls cycle pigments (V + A + Z), which were constitutively higher in Eridano (24.5%) than in I-214 and increased in I-214 (30%) under the L Cd treatment (Table 5). The degree of de-epoxidation (DEPS index) was only affected by the clone factor, being generally higher in I-214 (12.5%) than in Eridano (Table 5).

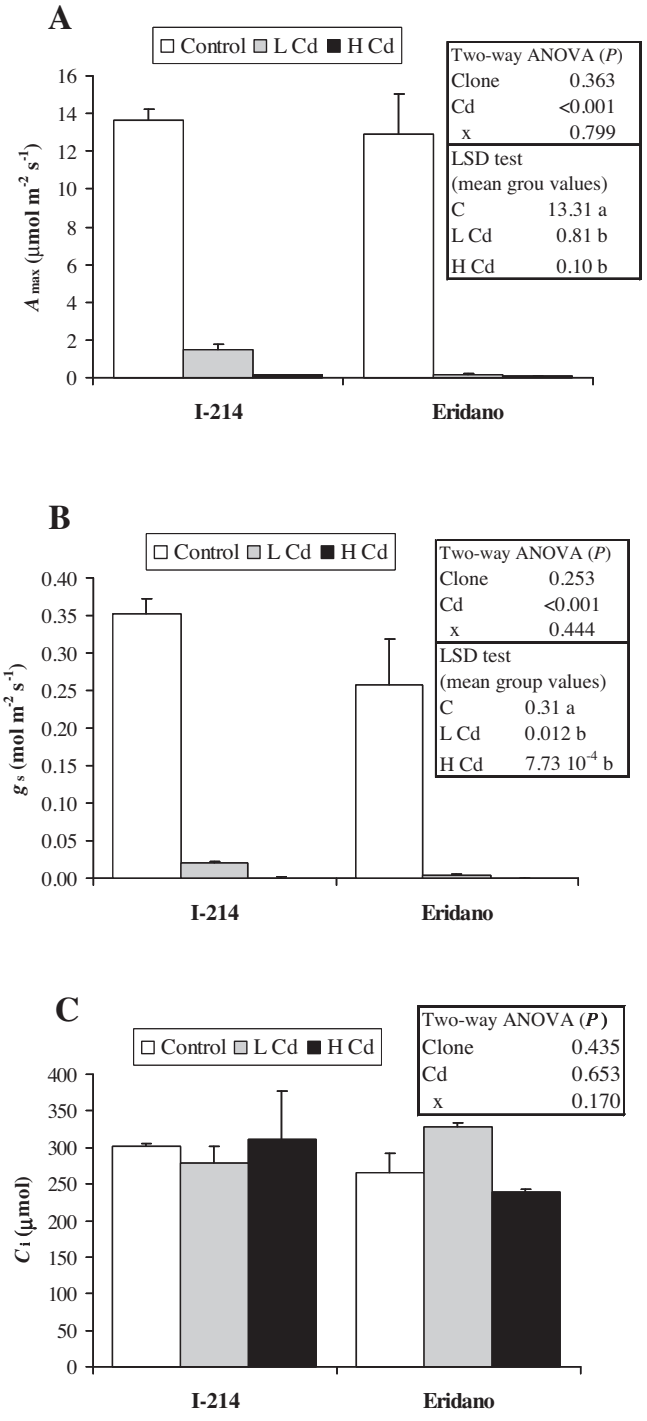

Fig. 2. Gas exchange parameters in the leaves (3rd–4th leaf from the apex) of the two hybrid poplar clones Eridano and I-214 grown in pots filled with soil treated with Cd ($CdCl_2$): Cd 0, control; L Cd (50 µM, the lowest Cd concentration); H Cd (150 µM, the highest Cd concentration) for 35 days. Each value represents the mean of three ($n = 3$) biological replicates ± SE. Analysis of data is as in Fig. 1. Abbreviations: A_{max}, photosynthetic rate measured at light saturation; g_s, stomatal conductance to H_2O; C_i and C_a, intercellular and ambient CO_2 concentration, respectively.

Leaf mineral composition

Leaves of I-214 were richer in NO_3, PO_4, SO_4, K and Zn compared to those of Eridano, which showed higher Ca concentration (Table 6). Cadmium treatment reduced the SO_4 and Mg concentration in leaves of I-214 at the H Cd treatment. The exposure to Cd decreased the leaf K and Ca amount in both clones. The concentrations of Cl and Fe in leaves were not affected by clone or Cd treatment (Table 6).

Leaf H_2O_2 and NO concentrations

The hydrogen peroxide (H_2O_2) concentration in leaves was affected by both clone and Cd factors, as well as by their interaction (Fig. 3A). In I-214, the H_2O_2 production was 4.5- and 13-fold higher after the L Cd and H Cd treatment, respectively, while no alterations were observed in Eridano.

The nitric oxide (NO) amount in leaves was higher in I-214 than in Eridano, and progressively increased with the increase of Cd treatment in both clones (Fig. 3B). Interestingly, in I-214 and Eridano leaves a good correlation between H_2O_2 or NO concentration and Cd content was observed (Fig. 3C and D).

Leaf non-protein thiols

In leaves of I-214 and Eridano, the glutathione pool (GSH, GSSG, GSH + GSSG and the relative content of GSH to GSSG form) was not affected by the clone and Cd factors and their interaction (Fig. 4A–C). The constitutive (control) leaf concentration of GSH and the GSH/GSSG ratio were higher in I-214 than in Eridano, while GSSG was naturally more elevated in Eridano (Fig. 4A–C). Cadmium and clone factors changed the NPTs and PCs amounts in leaves of I-214 and Eridano (Fig. 4D and E). Under L Cd treatment NPTs and PCs decreased in I-214 (1.8- and 2-fold lower, respectively). In Eridano, NPTs and PCs were affected by comparable reductions at both Cd treatments, although no significantly different compared to control (Fig. 4D and E).

Discussion

Cd effects on growth and the metal uptake

A general reduction of growth was observed in I-214 and Eridano poplar clones, but not chlorosis or leaf roll were detected (data not shown), latter appearance indicated as the main visible symptoms of Cd stress in plant (Benavides et al., 2005). Cadmium did not affect the number and expansion of Eridano leaves. On the contrary, both L and H Cd concentrations reduced the amount of I-214 leaves, which in any case maintained their mean area. Eridano leaves are constitutively wider than those of I-214 (Di Baccio et al., 2008), as confirmed by our results (Table 1), which revealed higher leaf area, LMR and LAR in Eridano than in I-214, apart from the Cd treatment. Despite Cd exposure caused a decrease of g_s, suggesting Cd interaction with water balance (Sanità di Toppi and Gabbrielli, 1999), the overall reduction of leaf DM was accompanied by an unchanged FM/DM. Indeed, both I-214 and Eridano plants exposed to Cd treatment appeared more stunting compared to controls, especially at the H Cd concentration, but they did not display leaf wilting or sign of dehydration (not shown), as observed in other poplar clones subjected to Cd stress (Polle et al., 2013). As in the leaves, Cd did not affect the water content of the stems (data not shown). In contrast, both Cd concentrations caused a severe reduction of the root DM, with disturbance of the water balance in presence of H Cd (data not shown). The two poplar clones showed similar responses to Cd stress in terms of biomass allocation and water balance, with a general and progressive decrease of the whole plant DM with the increasing Cd treatment, and impairment of the biomass distribution between shoots and roots only at H Cd.

Cadmium uptake in plants decreases in the order: roots > stems > leaves > fruits > seeds (Benavides et al., 2005). However, plant capacity of Cd translocation and accumulation is highly variable, depending on genotypic and environmental traits

Table 4
Chorophyll fluorescence parameters in the leaves (3rd–4th leaf from the apex, LPI 4–5) of two hybrid poplar clones I-214 and Eridano grown in pots filled with soil treated with Cd ($CdCl_2$): Cd 0, control; L Cd, 50 μM Cd; H Cd, 150 μM Cd. Each value represents the mean of four ($n = 4$) biological replicates ± SE. Data were analyzed independently by two-way ANOVA to evaluate the clone effect (clone), the Cd treatment effect (Cd) and the interaction of these two factors (×). The significance level of the F ratio (P) is shown. Means within a row flanked by the same letter are not significantly different at $P \leq 0.05$ (LSD test).

Clone	I-214			Eridano			ANOVA (P-value)		
Cd treatment	Control	L Cd	H Cd	Control	L Cd	H Cd	Clone	Cd	×
Parameter									
F_o	558 ± 5	557 ± 4	546 ± 21	613 ± 24	654 ± 27	659 ± 5	≤0.001	0.498	0.270
F_m	3074 ± 29	2933 ± 136	2800 ± 123	3039 ± 24	2976 ± 96	2924 ± 139	0.611	0.211	0.750
F_v/F_m	0.818 ± 0.003	0.808 ± 0.008	0.804 ± 0.007	0.798 ± 0.007	0.780 ± 0.003	0.773 ± 0.011	0.001	0.054	0.711
ΦPSII	0.769 ± 0.02 a	0.715 ± 0.02 b	0.687 ± 0.004 b	0.730 ± 0.01 ab	0.695 ± 0.02 b	0.728 ± 0.01 ab	0.630	0.019	0.048
qP	0.771 ± 0.02 a	0.717 ± 0.02 b	0.689 ± 0.004 b	0.731 ± 0.001 ab	0.697 ± 0.002 b	0.730 ± 0.01 ab	0.613	0.020	0.047
NPQ	0.408 ± 0.04	0.521 ± 0.06	0.519 ± 0.04	0.436 ± 0.03	0.468 ± 0.08	0.412 ± 0.03	0.292	0.363	0.405

Abbreviations: F_o = minimum chlorophyll fluorescence yield obtained with dark-adapted leaves; F_m = maximum chlorophyll fluorescence yield obtained with dark-adapted leaves; F_v/F_m = maximal quantum efficiency; ΦPSII = quantum efficiency of PSII photochemistry; qP = photochemical quenching; NPQ = non-photochemical quenching.

Table 5
Pigment concentrations (nmoles cm^{-2}) of leaves (3rd–4th leaf from the apex) of the two hybrid poplar clones I-214 and Eridano grown in pots filled with soil treated with Cd ($CdCl_2$): Cd 0, control; L Cd, 50 μM Cd; H Cd, 150 μM Cd. Each value represents the mean of four ($n = 4$) biological replicates ± SE. Analysis of data is as in Table 4.

Clone	I-214			Eridano			ANOVA (P-value)		
Cd treatment	Control	L Cd	H Cd	Control	L Cd	H Cd	Clone	Cd	×
Parameter									
Chlorophyll *a*	18.04 ± 0.61	19.61 ± 0.94	18.10 ± 0.55	18.25 ± 0.90	17.18 ± 0.33	16.97 ± 0.58	0.050	0.441	0.250
Chlorophyll *b*	6.10 ± 0.18	6.60 ± 0.26	6.06 ± 0.20	6.19 ± 0.18	5.88 ± 0.11	5.73 ± 0.21	0.050	0.196	0.129
Total Chl	24.14 ± 0.77	26.21 ± 1.19	24.16 ± 0.74	24.45 ± 1.08	23.06 ± 0.41	22.70 ± 0.76	0.049	0.365	0.149
Chl *a*/Chl *b*	2.96 ± 0.04	2.97 ± 0.05	2.99 ± 0.03	2.94 ± 0.06	2.92 ± 0.05	2.97 ± 0.06	0.449	0.746	0.954
Lutein	3.15 ± 0.11	3.27 ± 0.15	3.10 ± 0.10	3.11 ± 0.15	2.94 ± 0.08	2.96 ± 0.07	0.088	0.673	0.463
Neoxanthin	1.23 ± 0.04	1.33 ± 0.12	1.25 ± 0.04	1.38 ± 0.09	1.22 ± 0.04	1.29 ± 0.05	0.680	0.813	0.186
Violaxanthin	0.32 ± 0.02 b	0.39 ± 0.04 ab	0.36 ± 0.01 ab	0.42 ± 0.04 a	0.36 ± 0.02 ab	0.39 ± 0.02 ab	0.166	0.945	0.048
Antheraxanthin	0.19 ± 0.02 b	0.28 ± 0.02 a	0.25 ± 0.01 ab	0.22 ± 0.01 bc	0.21 ± 0.02 bc	0.22 ± 0.02 bc	0.136	0.124	0.037
Zeaxanthin	0.02 ± 0.004	0.02 ± 0.002	0.02 ± 0.001	0.01 ± 0.002	0.02 ± 0.002	0.02 ± 0.002	0.079	0.429	0.141
VAZ	0.53 ± 0.04 c	0.69 ± 0.05 a	0.63 ± 0.01 abc	0.66 ± 0.05 ab	0.58 ± 0.03 bc	0.63 ± 0.02 abc	0.874	0.459	0.012
Xanthophylls	4.92 ± 0.15	5.29 ± 0.32	4.98 ± 0.13	5.15 ± 0.29	4.73 ± 0.14	4.88 ± 0.12	0.422	0.864	0.181
DEPS index	22.23 ± 1.13	22.68 ± 1.08	22.76 ± 0.67	19.32 ± 0.95	20.49 ± 1.24	20.28 ± 1.38	0.008	0.716	0.947
β-carotene	2.17 ± 0.0.7	2.39 ± 0.14	2.18 ± 0.07	2.25 ± 0.11	2.02 ± 0.07	2.01 ± 0.15	0.092	0.478	0.130
Carotenoids	7.08 ± 0.21	7.68 ± 0.46	7.15 ± 0.17	7.40 ± 0.41	6.76 ± 0.19	6.89 ± 0.23	0.243	0.718	0.132

Abbreviations: V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; DEPS, de-epoxidation index, calculated as (Z + 1/2A)/(V + A + Z) × 100; ×, clone and Cd treatments interaction.

(Hart et al., 2006; DalCorso et al., 2008; Pietrini et al., 2010; Wu et al., 2010). In I-214 and Eridano, the highest Cd concentrations were effectively detected in the roots, at more elevated levels under H Cd than L Cd (Table 3), while the most pronounced Cd contents were revealed in the leaves of both clones under H Cd treatment (Fig. 1 A–C).

When the plants were exposed to H Cd treatment, 67% and 50% of the total Cd content was concentrated in leaves of I-214 and Eridano, respectively, while only 27% and 24% in the roots and 23% and 26% in the stems under the same conditions. These results on the Cd removal capacity by different plant organs suggest an active translocation of Cd from the roots to the leaves throughout the stems in both clones. This hypothesis is also supported by the relative high values (2.5 in I-214 and 3.5 in Eridano) of the translocation factor (Wu et al., 2010) in the same condition (Fig. 1E). When the two clones were exposed to L Cd treatment, the metal removal capability by I-214 was still higher in the leaves (47%) than in the roots (35%), while in Eridano the Cd accumulation was of the same extent in the roots (44%) and in the leaves (40%). Also in this condition the translocation factor was higher than 1 (1.5–2.3), meeting the definition criteria of accumulator plants (Wu et al., 2010). This index, together with the bioconcentration factor, gives a good evaluation of the metal distribution between the above- and belowground biomass by the plant (Baker, 1981; Pietrini et al., 2010) and, consequently, of the phytoextraction capacity. The more the BCF is near to 1, the more elevated is the plant ability of uptaking and translocating the metal to the shoots. In our experiment the highest BCF values were registered in the roots of both clones, being

Table 6
Anion and cation concentrations in the leaves of the two hybrid poplar clones I-214 and Eridano grown in pots filled with soil treated with Cd ($CdCl_2$): Cd 0, control; L Cd, 50 μM Cd; H Cd, 150 μM Cd. Each value represents the mean of four ($n = 4$) biological replicates ± SE. Analysis of data is as in Table 4.

Clone	I-214			Eridano			ANOVA (P-value)		
Cd treatment	Control	L Cd	H Cd	Control	L Cd	H Cd	Clone	Cd	×
Parameter									
NO_3 (μg g^{-1} DM)	25.47 ± 2.33	26.30 ± 3.81	17.53 ± 2.17	9.07 ± 2.08	10.75 ± 1.35	8.10 ± 0.01	≤0.001	0.084	0.308
PO_4 (μg g^{-1} DM)	479.00 ± 21.00	473.50 ± 39.50	444.00 ± 10.00	244.00 ± 13.00	331.50 ± 25.11	234.67 ± 23.33	≤0.001	0.086	0.227
SO_4 (%)	0.34 ± 0.02 a	0.28 ± 0.02 ab	0.27 ± 0.01 bc	0.20 ± 0.01 cd	0.21 ± 0.01 bcd	0.19 ± 0.004 d	≤0.001	0.016	0.044
Cl (μg g^{-1} DM)	592.00 ± 49.00	551.33 ± 26.03	372.67 ± 20.46	486.67 ± 63.36	375.50 ± 91.86	500.33 ± 66.67	0.308	0.235	0.054
K (%)	2.58 ± 0.14	2.45 ± 0.09	2.38 ± 0.03	2.07 ± 0.13	1.78 ± 0.01	1.76 ± 0.06	≤0.001	0.035	0.706
Ca (%)	0.60 ± 0.02	0.46 ± 0.06	0.39 ± 0.02	0.82 ± 0.03	0.59 ± 0.04	0.54 ± 0.07	≤0.001	≤0.001	0.652
Mg (%)	0.42 ± 0.01 a	0.38 ± 0.03 ab	0.28 ± 0.02 b	0.39 ± 0.04 a	0.34 ± 0.02 ab	0.36 ± 0.002 ab	0.724	0.005	0.036
Fe (μg g^{-1} DM)	197.84 ± 31.39	220.98 ± 31.22	274.56 ± 45.11	278.16 ± 68.69	180.44 ± 12.94	270.97 ± 43.70	0.734	0.274	0.375
Zn (μg g^{-1} DM)	167.66 ± 4.56	143.86 ± 33.72	136.68 ± 0.13	115.16 ± 3.36	99.53 ± 7.50	96.76 ± 6.07	0.002	0.238	0.909

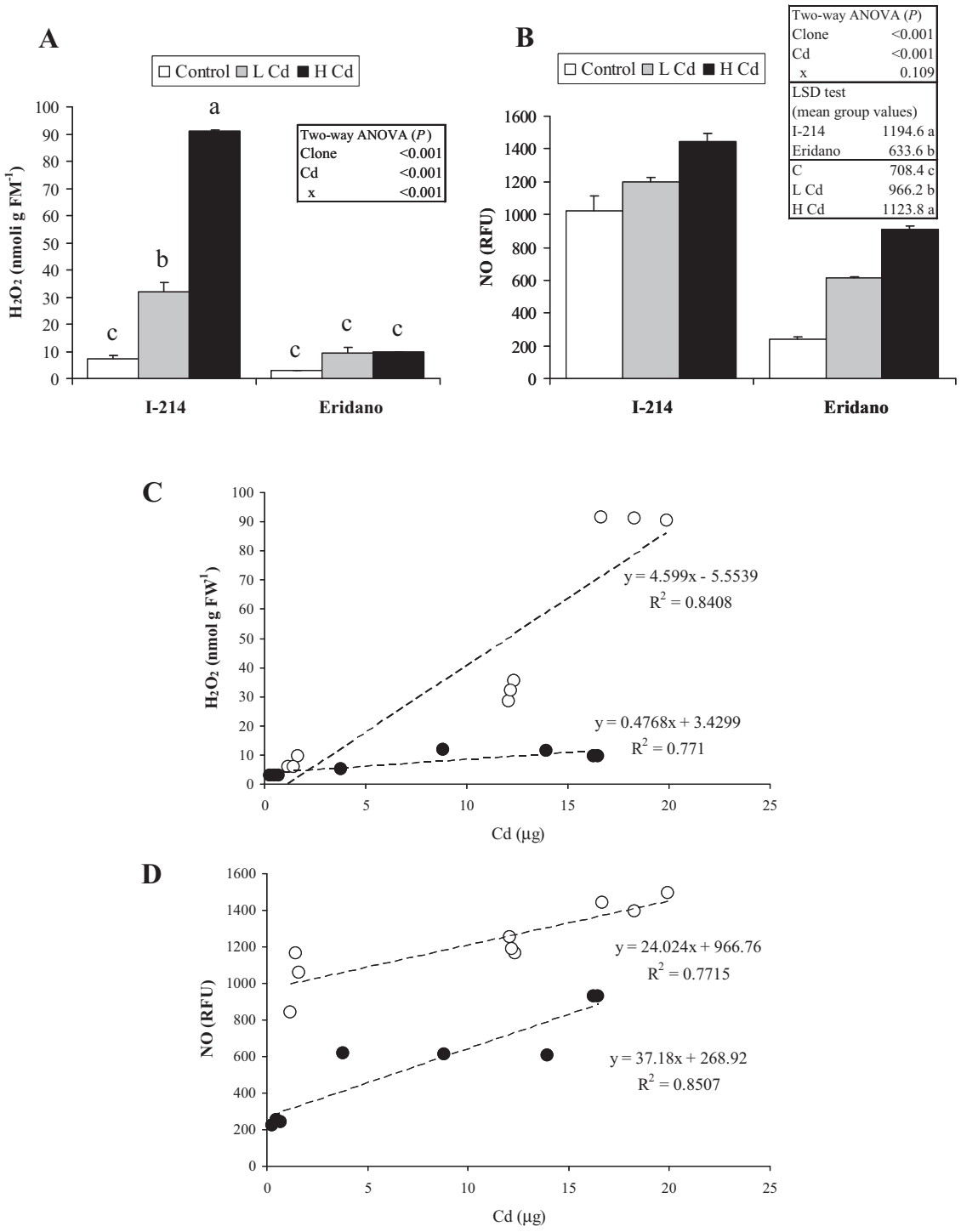

Fig. 3. Hydrogen peroxide (H_2O_2) and nitric oxide (NO) quantification ((A) and (B), respectively) by the mean of the fluorescence probes in the leaves of the two hybrid poplar clones I-214 and Eridano grown in pots filled with soil treated with Cd ($CdCl_2$): Cd 0, control; L Cd, 50 mM Cd; H Cd, 150 μM Cd. RFU, relative fluorescence units. Each value represents the mean of four ($n = 4$) biological replicates ± SE. Analysis of data is as in Fig. 1. (C) and (D): correlation between H_2O_2 and NO concentration, respectively, and Cd content (μg) in the leaves of I-214 (open circle) and Eridano (filled circle). R^2 values and equations are shown.

1 at L Cd and 0.4–0.5 at H Cd treatment (Table 3). These values and their dependence to Cd content in the soil define the two clones as indicator and non-hyperaccumulator plants (Baker, 1981). Either in I-214 or in Eridano the root apparatus was particularly active in the uptake and, eventually, in the re-translocation of excess Cd, with the highest efficiency at the lowest Cd level tested. Indeed, it is undoubted that Cd, as the other metal ions, enters first the roots and consequently they experience Cd toxicity first. In this context, non-hyperaccumulator plants (the most) have developed two basic metal tolerance mechanisms: the regulation of metal homeostasis and the metal retaining in the root (Clemens, 2001).

Impairments in photosynthesis and pigment concentrations

Inhibition of photosynthesis, stomatal opening and disruption of water balance are well-known damages of Cd on plant physiology (Sanità di Toppi and Gabbrielli, 1999; Benavides et al.,

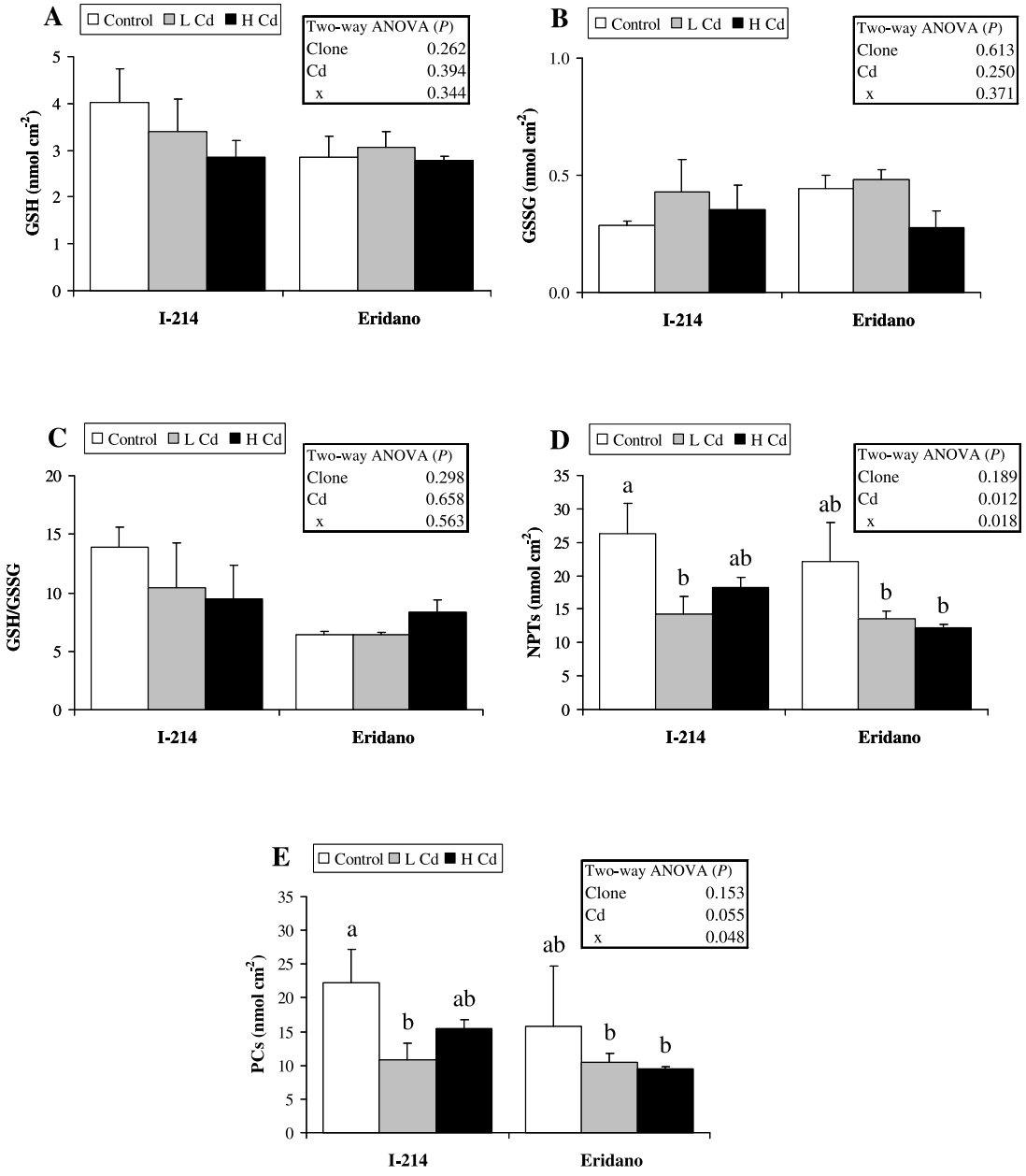

Fig. 4. Glutathione (reduced, GSH, and oxidized, GSSG, forms), GSH to GSSG ratio, total non protein thiols (NPTs), and phytochelatins (PCs) concentration (nmol cm^{-2}) in the leaves (2nd–6th leaf from the apex) of the two hybrid poplar clones I-214 and Eridano grown in pots filled with soil treated with Cd ($CdCl_2$): Cd 0, control; L Cd, 50 μM Cd; H Cd, 150 μM Cd. Each value represents the mean of four ($n = 4$) biological replicates ± SE. Analysis of data is as in Fig. 3.

2005). At biochemical level, the reduction of total chlorophyll and carotenoid contents, with the consequent increase of non-photochemical quenching, oxidative stress occurrence and cellular damages are other effects of Cd stress, mainly due to the impairment of the light harvesting complex II and the photosystems II and I (Mobin and Khan, 2007; Pietrini et al., 2010). In our experiment, the photosynthetic activity and stomatal conductance were negatively affected by Cd treatments, while C_i remained unvaried. The F_v/F_m was higher in I-214 than in Eridano, independently of Cd factor; in control condition, I-214 showed the highest values of ΦPSII and qP and the most pronounced decreases after L and H Cd treatments.

The photosynthetic rate (A) and g_s can either increase or decrease concomitantly, without appreciable variations in C_i, rather responding to processes in the guard cells or in the mesophyll, but independently of C_i (Messinger et al., 2006). Alterations to the stomatal apparatus and internal damage to the mesophyll cells, in general, result in declining A in parallel with declining g_s (von Caemmerer et al., 2004). However, the stomatal closure might have a patchy pattern on the leaf blade, as well as the inhibition of photosynthetic machinery induced by pollutants, with affected areas having low photosynthesis and non-affected areas retaining high photosynthesis rates. Recent works suggest that the response of g_s to C_i is linked to the photosynthetic electron transport and controlled by the balance between electron transport capacity and carbon assimilation. Nevertheless, the mechanisms underlying this response are not fully understood. So far, stress-induced loss of A might be due to both stomatal and non-stomatal limitations, and decline in A accompanied by a decline in g_s might result in slight changes in C_i.

In this experiment the inhibition of photosynthesis and stomata functionality caused by Cd treatment was so strong to impair

carbon (C) assimilation and growth, as shown by the general decrease of leaf, stem and root biomass, but it was not sufficiently severe to alter the photochemical efficiency of PSII (F_v/F_m) and to develop visible symptoms as chlorosis. Elevated F_v/F_m values of I-214 are associated to high ΦPSII and qP, although these parameters showed a slight decrease after L and H Cd treatment (Table 4). The concentration of chlorophylls and carotenoids was not affected by the presence of Cd, but some modifications in the composition of xanthophylls were observed (Table 5). Violaxanthin and anteraxanthin were influenced by Cd and clone factor interactions, with a consequent slight increase of VAZ value in I-214 at the L Cd treatment. This indicated that under the lower Cd concentration I-214 presented a higher level of photoprotection capacity than Eridano, with higher potential capacity to dissipate the excessive absorbed light energy as heat (Fernàndez-Martínez et al., 2014). The DEPS index, which is known to be directly correlated to the de-epoxidation of xanthophylls-cycle, was in any case more elevated in I-214 than in Eridano. These data, together with the relative lower decrease of A_{max} and g_s in I-214 than Eridano, suggest less sensitivity of photosynthetic performance to Cd stress in I-214. The higher photoprotective capacity of I-214 was also associated to greater GSH concentration and antioxidative status (GSH/GSSG), which potentially improved antioxidant defence, as observed in *Populus* spp. by Fernàndez-Martínez et al. (2014).

Cd interference with the uptake of principal nutrients

Excessive Cd in the medium has been shown to interfere with the uptake, transport and utilization of several macro- and micronutrients by plants, although the mechanisms underlying this interference are not completely understood yet. To investigate if exposure to Cd interfered with the nutrient uptake of I-214 and Eridano, major nutrient elements were measured in leaves. In our experiment, the nitrate, phosphate and Zn concentration in leaves was relatively higher in I-214, but it was not affected by Cd treatment (Table 6). Uptake of K, Ca and Mg was influenced by Cd treatment and SO_4 and Mg also by Cd × clone interaction.

The uptake of Cd ions seems to be in competition for the binding sites of the same proteins or transporters with nutrients such as K, Ca, Mg, Fe, Mn, Cu, Zn, Ni (Clemens, 2001; Li et al., 2012). However, among plant macronutrients nitrate, phosphate and sulphate are anions. Hasson et al. (2008) demonstrated that Cd^{2+} exposure significantly reduced nitrogen (N) accumulation in rice roots and shoots irrespective of N forms, nitrate (NO_3^-) or ammonium (NH_4^+). Hernández et al. (1996) attributed the decrease of nitrate absorption and transport from roots to shoots in pea seedlings under Cd stress to the inhibition of the nitrate reductase activity in the shoots. The impairments in plant sulphur assimilation and content caused by Cd stress are mainly attributed to the induction of synthesis of metal binding molecules rich of sulphydryl groups such as glutathione (GSH) and phytochaletins (Nocito et al., 2007).

Potassium and calcium are important macronutrients required in plant leaf cell for membrane and stomata functionality. Cd^{2+} interferes with K^+ and Ca^{2+} movements and abscisic acid in the guard cells, inhibiting stomatal opening (Li et al., 2012). In I-214 and Eridano leaves, K and Ca concentrations were independently affected by clone and Cd treatments. Cd exposure caused a severe reduction in Ca and a moderate decrease of K concentrations in leaves of both clones, alterations consistent with the inhibition of stomatal conductance observed in I-214 and Eridano at L and H Cd.

In contrast to many literature results, no significant changes in any of the micronutrients recovered (Cl, Fe, Zn) were found in response to Cd in the leaves of the two poplar clones (Table 6). Anyway, in our previous study (Castagna et al., 2013) Fe and Zn leaf concentrations were not influenced by Cd exposure. Indeed, Cd stress is often accompanied by chlorosis due to Fe and/or phosphorous deficiency, the former causing the inhibition of root Fe(III) reductase (Benavides et al., 2005). On the other hand, Fe can increase in the leaves of Cd-treated plants (Pietrini et al., 2003; Polle et al., 2013) or remain unaltered (Mohamed et al., 2012), depending on Cd dose, plant species, organs and experimental conditions used in various studies.

Cd-induced oxidative stress: H_2O_2 and NO production and interaction, sulphydryl metal-binding molecules

Cadmium was found to induce oxidative stress, as evidenced by the formation of reactive oxygen species (ROS), like superoxide anion and hydrogen peroxide (H_2O_2) (Nocito et al., 2007; Romero-Puertas et al., 2012). Among ROS, H_2O_2 is the most stable and it is capable of rapid diffusion across cell membranes (Pellinen et al., 2002; Foyer and Noctor, 2003). In our experiment Cd increased H_2O_2 concentration in I-214 leaves, more markedly at the H Cd than L Cd treatment. Yet, leaf H_2O_2 accumulation by excess Cd in I-214 was previously observed when the clone was tested, together with Eridano, for tolerance to Cd and O_3 (Castagna et al., 2013), while the opposite effect (H_2O_2 accumulation in Eridano leaves and basal level in I-214) was shown under chronic O_3 exposure (Di Baccio et al., 2008). The induction of oxidative burst by Cd in I-214 leaves was confirmed by the high correlation between H_2O_2 concentration in leaf tissues and their relative Cd content at the different metal treatments (0, L and H Cd, Fig. 3C).

The nitric oxide (NO) as active N species and signal molecule plays crucial functions in the regulation of many plant physiological processes and in the modulation of oxidative stress and cell death (Delledonne et al., 2001; Laspina et al., 2005). In particular, it has been recently found a strict interplay between NO and ROS in the regulation of antioxidant system in plant stress responses (Cui et al., 2011; Groß et al., 2013; Liao et al., 2013). Besides, NO has been indicated as a key modulator in plant resistance response to heavy metals such as Cd (Laspina et al., 2005; Romero-Puertas et al., 2012).

In I-214 leaves, NO concentrations was 2-fold higher than Eridano leaves, irrespective of Cd levels in the medium, and it reached the highest values in I-214 under Cd treatment just when H_2O_2 showed a dramatic increase. A NO rise and high correlation with Cd content was revealed in Eridano leaves, where no significant H_2O_2 enhancement was measured. These results are consistent with the theory that under stress condition NO can interfere with ROS, especially non radical oxygen species such as H_2O_2 (Neill et al., 2002; Groß et al., 2013; Liao et al., 2013). However, the exact molecular mechanisms by which NO interacts with H_2O_2 has not been completely deciphered yet. Many evidences show that endogenous NO is often involved in tolerance mechanisms against oxidative stress (overproduction of ROS), and exogenous NO improves abiotic stress tolerance, including heavy metal stress, often concomitant with a decrease in H_2O_2 and MDA levels (Laspina et al., 2005; Romero-Puertas et al., 2012; Groß et al., 2013). Nevertheless, an increase in NO plant content is not always correlated to ROS reduction, these modifications being dependent on the kind and strength of stress, in addition to the complexity of NO and ROS formation and roles (Delledonne et al., 2001; Neill et al., 2002; Foyer and Noctor, 2003). In several plant metabolic processes and defence mechanisms (Pellinen et al., 2002; Kangasjärvi et al., 2005; Di Baccio et al., 2012, Pető et al., 2013) if the nature of NO/ROS (H_2O_2) interaction is mutualistic or antagonistic is still unclear. What has been shown is that the plant tolerance to stress is regulated by a fine-tuned NO/ROS balance (Cui et al., 2011; Groß et al., 2013; Liao et al., 2013). In our case the apparent similar sensitivity of I-214 and Eridano to Cd stress in terms of visible symptoms, photosynthesis performance and biomass production could lye on the capacity of these poplar clones of containing the oxidative stress thanks to different

cross-talking and interplay between NO and ROS, finally regulating the antioxidant cell status.

Glutathione (GSH) and phytochelatins (PCs) are well-known plant thiol-containing peptides and natural chelators of metals and Cd (Sanità di Toppi and Gabbrielli, 1999; Clemens, 2001). Thanks to their sequestrating capacity, together with the antioxidant properties of GSH, thiol compounds are often positively related to plant tolerance to heavy metals (Benavides et al., 2005; Nocito et al., 2007). The constitutive concentration of the reduced form of glutathione was higher (1.4-fold) in I-214 than Eridano, as well as its antioxidant capacity, GSH/GSSG (2.2-fold, Fig. 4), findings consistent with previous determinations on the two clones exposed to O_3 stress (Di Baccio et al., 2008). Despite these differences between I-214 and Eridano leaves, the glutathione pool (oxidized and reduced forms) was not significantly affected by Cd treatment. NPTs and PCs were similarly influenced by both clone and Cd factors, with a decrease in I-214 under L Cd (Fig. 4). These results are not surprising, as some cases are described in which the synthesis of glutathione and/or phytochelatins was either not stimulated by Cd stress or not fundamental for tolerance to the toxic metal (Ebbs et al., 2002; Lee et al., 2003). Possible explanations are that, in the investigated Cd stress conditions, the two clones activated other complementary defence systems or perhaps the constitutive levels of PCs, genetically controlled in all plant species, present in both poplar clones, were sufficient to cope with the effects of Cd uptake.

Conclusions

From the overall data obtained in this work, it can be inferred that I-214 (*Populus* × *canadensis*, O_3-tolerant) and Eridano (*Populus deltoides* × *maximowiczii*, O_3-sensitive) poplar clones showed similar sensitivity to the investigated Cd stress conditions, although physiological and biochemical traits revealed different defence strategies. Indeed, the decreases of leaf area, photosynthetic activity and biomass allocation were comparable in both clones, as well as their Cd uptake, translocation and distribution in leaves, stems and roots. However, Eridano maintained in any Cd treatment the number of its constitutively wider leaves, while the number of I-214 leaves (with lower surface area) was reduced by Cd effect. These morphological traits might account for the relatively higher concentration of Cd in I-214 leaves (although corresponding to comparable Cd accumulation in comparison with Eridano), and it might partially explain the remarkable increase of H_2O_2 in this organ, indicating induction of oxidative burst by Cd stress. At biochemical level, I-214 leaves were constitutively richer in GSH, and showed potentially higher antioxidative capacity (GSH/GSSG), photochemical efficiency and quenching of chlorophyll (F_v/F_m, ΦPSII and qP), as well as xanthophylls de-epoxidation degree (DEPS) than Eridano. These biochemical characteristics, together with a high NO concentration and induced production under Cd exposure, made I-214 able to counteract the incipient oxidative stress.

Young plants of I-214 and Eridano exhibited a high capacity of Cd translocation to the leaves: in presence of the highest Cd level, in both clones Cd accumulation increased in the order: leaf > stem ≥ roots. Indeed, I-214 and Eridano developed different tolerance mechanisms at morpho-physiological and biochemical level to reach a similar outcome. Further studies and additional analyses will unravel the molecular basis of these differences. Here we have shown that the two poplar clones I-214 and Eridano are suitable to grow in environments contaminated with the levels of Cd tested, from the earliest stages of development (rooting and first weeks after the full expansion of the leaves).

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