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Effect of short-term cadmium stress on *Populus nigra* L. detached leaves

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ABSTRACT

Pollution by toxic metals, accumulating into soils as result of human activities, is a worldwide major concern in industrial countries. Plants exhibit different degrees of tolerance to heavy metals, as a consequence of their ability to exclude or accumulate them in particular tissues, organs or sub-cellular compartments. Molecular information about cellular processes affected by heavy metals is still largely incomplete. As a fast-growing, highly tolerant perennial plant species, poplar has become a model for environmental stress response investigations. To study the short-term effects of cadmium accumulation in leaves, we analyzed photosystem II (PSII) quantum yield, hydrogen peroxide (H_2O_2) generation, hormone levels variation, as well as proteome profile alteration of 50 μM $CdSO_4$ vacuum-infiltrated poplar (*Populus nigra* L.) detached leaves. Cadmium management brought about an early and sustained production of hydrogen peroxide, an increase of abscisic acid, ethylene and gibberellins content, as well as a decrease in cytokinins and auxin levels, whereas photosynthetic electron transport was unaffected. Proteomic analysis revealed that twenty-one proteins were differentially induced in cadmium-treated leaves. Identification of fifteen polypeptides allowed to ascertain that most of them were involved in stress response while the remaining ones were involved in photosynthetic carbon metabolism and energy production.

Introduction

Nowadays, contamination of soils by toxic metals and metalloids is a major concern all over the world. In soils, levels of toxicity from metals such as aluminium (Al), cadmium (Cd), cesium (Cs), copper (Cu), chromium (Cr), lead (Pb), manganese (Mn), mercury (Hg), nickel (Ni), zinc (Zn) and iron (Fe), or from metalloids such as arsenic (As) and boron (B), are rapidly increasing as a result of human activities or environmental causes (Ahsan et al., 2009).

Soils perform a number of key environmental features that profoundly impact not only biological but also economic, social and cultural functions. Hence, alteration of soil composition, due to introduction of toxic contaminants, can drastically change the equilibrium of ecosystems.

Among different plant species, a wide range of plasticity in heavy metal tolerance has been observed (Salt et al., 1998). Hyperaccumulating plants can naturally absorb high levels of metal ions because they have evolved multiple mechanisms of resistance and different strategies to cope with heavy metal stress, including detoxification and exclusion (Hall, 2002). In particular, detoxification involves the increased synthesis of metal-binding proteins such as metallothioneins and phytochelatins, as well as the increased production of organic acids or aminoacids (Cobbett, 2000).

Cadmium (Cd) is a highly toxic trace element which enters the environment mainly from industrial processes and phosphate fertilization (Liu et al., 2008). Cd is toxic to many plant species at very low concentrations. Indeed, plants cannot prevent Cd uptake since it belongs to the group of metals whose ions are rapidly taken up by plant roots (Schützendübel and Polle, 2002) via metal transporters,

Abbreviations: ACCox, 1-aminocyclopropane-1-carboxylate oxidase; DAB, 3,3′-diaminobenzidine; IAA, auxin; ABA, abscisic acid; CK, cytokinin; GA_3, gibberellin A_3; GA_4, gibberellins A_4; H_2O_2, hydrogen peroxide; KN, kinetin; PSII, photosystem II.

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and once taken up it is loaded into the xylem for transport to leaves (Verbruggenl et al., 2009).

Different studies demonstrated that Cd affects plant metabolism and nutrient uptake (Hall, 2002). Furthermore, evidences have been produced showing that leaves represent the main target of Cd toxicity, where it affects the photosynthetic process, disturbing the chlorophyll metabolism, altering chloroplasts structure, as well as impairing photosystems and carbon metabolism enzymes activities (Krantev et al., 2008). Cd effects have also been related to alteration of the plant antioxidant system (Romero-Puertas et al., 2002) and to increased lipid peroxidation, which in turn may lead to impairment of membrane functionality or of associated enzymes, such as H^+-ATPase (Popova et al., 2009).

The genus *Populus* is easily propagated, highly tolerant and characterized by a deep root system perennial, which is therefore acknowledged as an economically very valuable non-food biomass source. Due to these favourable features, it has been proposed as a model tree species and is now widely investigated to understand how tree plant species respond to environmental stress (Trupiano et al., 2013, 2014; Romeo et al., 2014). Recently, due to its fast growing features, interest has been focused on *Populus nigra* L. (Stobrawa and Lorenc-Plucińska, 2008), prompting investigations to identify tolerant genotypes (Zacchini et al., 2011). However, despite the increasing body of interest on poplar tolerant genotypes, information about molecular mechanisms involved in Cd response in terms of tolerance or toxicity is still very scarce. This lack of knowledge may be partially due to the difficulty of investigations on heavy metal accumulation and response in tree species, usually very time-consuming and labour-intensive.

In the present study, *P. nigra* L. detached leaves were vacuum-infiltrated with $CdSO_4$ with the aim of assessing if this method may be an efficient experimental system for analyzing the short-term response of poplar leaf to cadmium. Indeed similar methods have been successful used by other authors to asses the heavy metal tolerance and changes of leaf gene expression in herbaceous plant species (Horling et al., 2002; Cho et al., 2003; Lin and Kao, 2007). With this purpose poplar detached leaves were vacuum-infiltrated with 50 μM $CdSO_4$ and then subjected to the analysis of photosystem II (PSII) quantum yield, which proved to adequately describe the effects on photosynthesis, H_2O_2 generation, hormone levels, as well as proteome profile.

Materials and methods

Cadmium treatment of Populus nigra L. *leaves*

First expanded leaves in a number of ten, excised with the aid of a metal razor blade, were taken from the stem of two years-old plants of poplar *Populus nigra* L. (seed origin, Alasia Franco Vivai, Savigliano (CN), Italy) grown in large pots in a greenhouse under controlled water regime and natural photoperiod and temperature; soil was fertilized with 1 kg m^{-3} of a slow release fertilizer osmocote (12-11-17). Main morphological features of leaves used are reported in Table 1 Supporting information.

The leaf petioles were positioned within polycarbonate magenta boxes containing 30 mL of a 50 μM $CdSO_4$ solution in distilled H_2O. Magenta boxes were then placed within a plastic bell to start cadmium leaves treatment by vacuum infiltration. The infiltration had duration of 15 min, followed by 45 min incubation, for a total of 1 h treatment at room temperature (25 °C) and light intensity of 800 μmol quanta m^{-2} s^{-1}. For the determination of hydrogen peroxide (H_2O_2) release, some leaves were removed from the solution after 15 min or 1 h of cadmium treatment and then subjected to histochemical H_2O_2 determination, as reported below. Control leaves were infiltrated with distilled H_2O, under the same conditions. Three independent experiments were performed. To reduce noise arising from biological variation, in each experiment ten leaves were taken from the stem of five plants of poplar and pooled together in order to have three biological replicates to be used for further analysis.

Measure of Cd concentration

Cd content was measured as reported by Durand et al. (2010) on digested leaves samples including petioles. In detail, dried material was milled to a fine powder (Grinder, IKA, Staufen, Germany) and then mineralized. Mineralization was performed by treating 200 mg of powdered samples with 3 mL of 33% hydrochloric acid and 6 mL of 67% nitric acid in a microwave digestion system (Multiwave ECO Anton Paar, Graz, Austria). The Cd content of digested leaves samples was measured by ICP–MS (Inductively Coupled Plasma Mass Spectrometry; Thermo Finnigan Element 2XR, Thermo Scientific, Waltham, MA, USA). Five biological replicates of control and Cd vacuum-infiltrated leaves were analyzed.

Measure of quantum yields of PSII

The true quantum yield of photosystem II (PSII) in the light (ΦPSII, estimated as $\Delta F/F_m'$, where ΔF is the difference between maximal and steady state fluorescence in the light (Govindjee, 1995) was measured according to Delfine et al. (2001), by means of a PAM 101 modulated fluorimeter (Walz, Effeltrich, Germany). Fluorescence measurements were made at a leaf temperature of 25 °C, after 1 h infiltration of leaves with 50 μM $CdSO_4$ at a light intensity of 800 μmol quanta m^{-2} s^{-1}, in three independent experiments, each using ten different leaves for treatment. Leaves infiltrated with H_2O under the same conditions, were used as control. Thirty measurements of three independent experiments for each sample (control and treaded) were performed.

Histochemical detection of hydrogen peroxide

The *in situ* histochemical determination of H_2O_2 in *P. nigra* leaves was performed by using the 3,3′-diaminobenzidine (DAB) dye, as described by Donnini et al. (2011). Control or cadmium-treated leaves were vacuum-infiltrated with 0.1 mg mL^{-1} of DAB in a buffer solution of Tris–acetate 50 mM, pH 5.0, at room temperature (25 °C) for 5 min. The samples were then incubated in the dark at room temperature for 24 h. Samples were then illuminated until appearance of brown spots typical of the reaction between DAB and H_2O_2. Leaves were finally bleached in boiling ethanol for 10 min to visualize brown spots. Sections of leaf tissues were observed under Zeiss Primo Star optical microscope equipped with AxioCam ERc5s camera (Zeiss, Jena, Germany) and images acquired by using the AxioVision Rel 4.8 software (Zeiss). Experiment was repeated in triplicates.

Protein extraction

Total proteins from 2.5 g of control or Cd-treated poplar leaves were extracted following the TCA/acetone protocol, as described in Ialicicco et al. (2012). Final protein exctracts were vacuum-dried and solubilized in rehydration buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (v/v) Triton X-100, 20 mM Tris–HCl, 1% (w/v) DTT, 0.2% (w/v) ampholine 3–10 and 0.15% (w/v) ampholine 5–7. Protein concentration was determined with Bradford's method (1976) by using the BioRad protein assay (Biorad, Hercules, CA, USA) and bovine serum albumin as standard.