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The response of *Populus* spp. to cadmium stress: Chemical, morphological and proteomics study

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HIGHLIGHTS

- Poplar clones analyzed showed a marked physiological and proteomic different response to Cd toxicity.
- Cd exposure caused after 14 d more visible signs of toxicity in clones Poli and 58-861 than in A-4A.
- Proteins expression changed in time and according to the different clones.
- The clone A-4A might be useful for Cd phytoremediation.

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ABSTRACT

Poplar (*Populus*) species are seen as candidates for removing heavy metal contamination from polluted soil. A bottom-up multidisciplinary approach was utilized to compare the performances of clones 58-861 and Poli (*Populus nigra*) and A4A, a *Populus nigra* × *Populus deltoides* hybrid to Cd toxicity. Qualitative and quantitative differences in their tolerance to Cd exposure and the uptake, accumulation and translocation of Cd were noted following the hydroponic exposure of rooted cuttings to 20 μM $CdSO_4$ for either 48 h or 14 d. Cadmium was less toxic for the hybrid clone A4A as compared to Poli and 58-861. Cd uptake and root to shoot translocation were determined by AAS, and its compartmentation was analyzed using SEM/EDX. A comparative proteomic approach was utilized to identify changes in proteins expression according to dose and time of exposure. Toxicity to Cd mainly influenced proteins related to general defense, stress response and carbohydrate metabolism.

1. Introduction

Few soil organisms can tolerate cadmium presence at any appreciable concentration, as its toxicity is many folds higher than that of most of the other heavy metals (Vassilev et al., 1998). Even at low concentrations, it can be toxic to a range of microorganisms, plants and animals (Benavides et al., 2005). The primary risk associated with Cd-contaminated soil reflects the amplification in concentration which occurs as it passes along the food chain (Page et al., 1982). It has been identified as a primary agent of liver cancer (Arroyo et al., 2012) and is known to accumulate in the mammalian kidney, liver and reproductive organs.

Cd contamination is actively monitored by the US Environmental Protection Agency (EPA, 2013) (http://www.epa.gov/osw/hazard/wastemin/priority.htm). The most recent Agency for Toxic Substance and Disease Registry (ATSDR, 2013) (http://www.atsdr.cdc.gov/SPL/index.html) priority list of hazardous substances ranks Cd seventh for toxicity. In natural environments, soil concentrations of 0.1–0.5 mg kg^{-1} are common, but concentrations of up to 150 mg kg^{-1} have been recorded at highly contaminated sites, associated with the disposal of batteries and waste products from mining, electroplating, plastics and paint manufacture, alloy preparation (Adriano, 2001; Gallego et al., 2012). Many metallic appliances and components are electroplated with Cd to prevent rusting; the metal is also used to create luminescent dials, to cure rubber, and as an ingredient of some photographic materials and fungicides (Adriano, 2001). Cd enters the soil through the incorporation of sludges, composts or fertilizers; it is also abraded from rubber tyres from whence it finds its way into the sewage system.

Phytoremediation has been proposed as a useful means of removing many types of toxics, both organic and inorganic (Salt et al., 1995a). It is cheaper than chemical removal and has a favorable public profile (McGrath et al., 2001). The idea behind it is to exploit the natural capacity of certain plant species in conjunction with rhizosphere microbial flora to degrade and/or sequester specific pollutants.

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The genus *Populus* as a whole offers much variation with respect to morphology, anatomy, physiology, phenology and response to biotic and abiotic stress. Individual trees are dioecious, wind-pollinated, and are capable of rapidly invading disturbed sites such as riverine floodplains (Braatne et al., 1996). The ease with which most species can be vegetatively propagated is particularly attractive, as is their rapid growth rate. Juvenile trees grow fast enough to allow short term responses to biotic (for example, disease) and abiotic (for example, drought, elevated CO_2, ozone) stresses to be monitored. The large physical size of poplars, coupled with a good understanding of assimilate and how signals move within the tree (Davis et al., 1991) gives it a significant edge over standard small-stature plant models such as *Arabidopsis thaliana* (L.) Heynh. The various species are organized in sections based on shared morphological and reproductive characters, and frequently members of a single section are able to cross-hybridize with one another (Cervera et al., 2005). *Populus trichocarpa* Torr. & A. Gray, the first tree species for which a full genome sequence has been acquired (Brunner et al., 2004), was chosen because its genome size, although four times larger than that of *A. thaliana*, is still 40 folds smaller than that of most conifer genomes. So far poplar species have been used in phytoremediation (Robinson et al., 2000), in the production of energy and biomass through short rotation coppice (Labreque and Teodorescu, 2005).

Proteomic analysis provides a systematic analysis of the protein complement of an organism or of specific cells or organs of an organism (Phizicky et al., 2003). The aims of this work was to gather more knowledge about the capacity of poplars to withstand Cd stress and how Cd tolerance varies among different poplar species and clones from a morphological, chemical and proteomics points of view. Therefore the main thrust of the investigation was to seek to exploit poplar species for phytoremediation in all the best possible ways.

2. Materials and methods

2.1. Plant material

The three *Populus* spp. clones (*Populus nigra* clones 58-861 and Poli, and *Populus euramericana* [=*P. nigra x P. deltoides* hybrid] clone A4A) used were obtained from the collection maintained by the Institute of Agro-Environment and Forest Biology, National Research Council, Monterotondo Scalo, Rome, Italy. Cuttings of each clone were established in pots of garden soil (VigorPlant Italia S.r.l., Fombio, Lodi). The clones were already documented as showing differential responses to Cd stress (Zacchini et al., 2009). Furthermore each clone had previously been SNP (single nucleotide polymorphism) genotyped with respect to various genes, both associated and not associated with metal homeostasis (Marmiroli et al., 2011b).

2.2. Hydroponic culture and treatments

To promote root growth, fresh cuttings of each clone were held for 3 wk in half-strength Hoagland's nutrient solution (J.T. Baker, Deventer, Holland; composition in Table SM-1 of Supplementary Material (SM)) and under a 14 h photoperiod provided by metal halide lamps (photon flux density 300 $\mu mol\ m^{-2}\ s^{-1}$) with the temperature set to 25 °C during the lit period and 22 °C during the dark; the relative humidity was kept at 60%. The hydroponics solution was topped up daily to compensate for evapotranspirative loss. Five cuttings per clone were then exposed to the same nutrient solution supplemented with either 0 or 20 μM $CdSO_4$ for either 48 h (short term treatment) or 14 d (long term treatment). In the latter case, the nutrient solution was replaced weekly. At the end of the treatment, a 5 g leaf sample and a 2 g root sample was harvested, snap-frozen and stored at −80 °C. The remaining roots were pooled (separately for the control and Cd stressed plants), and rinsed in 0.05 M $CaCl_2$ for 30 min to remove any surface-adhering Cd. During the course of the Cd treatments, the number of leaves, total leaf area and the growth of the main root were all monitored. Differences between control and treated plants were statistically compared using the Student's *t*-test and one way analyses of variance were performed using routines implemented within the statistical software package SPSS Statistics v17.0 (http://www-01.ibm.com/software/analytics/spss/). A "bio-concentration factor" (BCF) was derived, following Zayed et al. (1998), based on the ratio between the Cd concentration in the plant material and that in the nutrient solution. Finally, a "translocation factor" (T_f) was calculated from the ratio of the quantity of Cd present in the aerial part of the plant to that present in the root.

2.3. Elemental analysis by Atomic Absorption Spectroscopy (AAS)

Pooled root, stem and leaf material harvested from plants grown in the presence or absence of Cd was baked for 3 d at 70 °C, and a three replicate 300 mg aliquots of each were digested in 10 mL 15.8 M HNO_3 (69% [v/v]) at 120 °C for 25 min, followed by 15 min at 250 °C (DK20, Velp Scientifica S.r.l., Usmate MI, Italy), after which 7 mL MilliQ water were added. The Cd concentration of the solute was determined using a flame AAS device (AA240FS Fast Sequential AAS, Varian, Palo Alto, California). Calibration curve was constructed by using a standard solution of Cd.

2.4. SEM/EDX

Five biological replicates per organ per treatment (short term treatment only) were used for the quantification and localization of elements. The leaf material was removed from the stem at the nodal intersection, while the stem and root were cut into 1 mm thick horizontal sections and then dried at room temperature in a sterile container. The dry sections were placed on a microscope slide, covered with colloidal graphite to promote electron conductivity, and prepared following the Marmiroli et al. (2004) procedure. Each sample was characterized morphologically and the elements present were quantified and localized by SEM (Jeol model 6400, Osaka, Japan) coupled to an X-ray detector (Oxford Instruments, Oxford, UK), supported by INCA software (http://www.e-tas.com/en/products/inca_software_products.php). The SEM/EDX parameters were: working distance: 14 mm; electron beam energy: 20 keV; dead time in X-ray data acquisition: 15–25%. The number of frames was 1130 (acquisition time of 2 h for each map). The magnification was 100–700 x, according to tissue. Line-scan analysis of elemental content along a transect, within a sample, was performed to acquire information on the relative abundance of elements along that transect.

2.5. Protein extraction, quantification, separation and identification

Crude protein was extracted from a 3.5 g aliquot of powdered, frozen leaf by suspension in 50 mM Tris HCl (pH 7.8), 10 mM $MgSO_4$, 0.1 vol% β mercaptoethanol and 0.1 vol% protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA), followed by a 10 min sonication at 35 kHz (Transsonic T460, Elma, Singen, Germany). The preparation was placed in ice for 40 min, re-sonicated and centrifuged (16000*g*, 5 min, 4 °C). The pellet was discarded and the supernatant re-centrifuged (16000*g*, 30 min, 4 °C). Leaf samples from each treatment were extracted in triplicate. The final supernatants were desalted by passing through a PD-10 column (GE-Healthcare, Uppsala, Sweden). The protein concentration was quantified using a Quick Start Bradford Protein Assay (BioRad,