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A multiple-level study of metal tolerance in *Salix fragilis* and *Salix aurita* clones

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ABSTRACT

The response of two willow clones (*Salix fragilis* (Sf) and *Salix aurita* (Sa)) to the presence of metals (Zn, Cu, Cd, Ni) was studied. Rooted cuttings were planted in control and contaminated soil. After 100 days, different parameters (biomass, chlorophyll fluorescence (F_v/F_m), pigment and sugar concentrations, electrolyte leakage and proteome-level changes) were analyzed. The growth of Sa was not influenced by metals whereas Sf produced significantly less biomass when exposed to the pollutants. Furthermore, although Sa did not show a growth reduction in the presence of metals, the overall view of the physiological results among others the changes in the accumulation of sugars and pigments indicated that metals had a more severe impact on this clone. The response at the proteome level confirmed these observations. The growth reduction and the proteomic changes in Sf indicate that this clone adjusts its metabolism to maintain cellular homeostasis. Sa on the contrary maintains growth but the physiological and proteomics data suggests that this can only be done at the cost of cellular deregulation. Therefore high biomass is not linked with a good tolerance strategy. In a long-term study the survival of Sa might be compromised making it a poorer candidate for phytoremediation efforts.

Biological significance

In the last centuries human activity has resulted in the dispersal of heavy metals with potential phytotoxic effects over large areas. The increased knowledge of the responses of *Salix*-species, a group of trees with potential as biomass producer but also as phytoremediation agent, when growing on metal-polluted substrate provided by this study has the potential to help in the improved selection of clones with more or less potential for these aims. Contrary to most studies the trees in the current study were exposed to a mixture of metals, thereby facing a closer resemblance to the situation on soils polluted by human activity. Whereas many papers focused on the two main phenotypic characteristics (biomass and accumulation), fewer papers studied

Abbreviations: ROS, reactive oxygen species; SOD, superoxide dismutase; POD, peroxidase; CAT, catalase; 2-DiGE, of two-dimensional electrophoresis coupled with multiplexing fluorescent probes; ICP-MS, inductively coupled plasma mass spectrometry; F_o, initial fluorescence; PSII, photosystem II; F_m, maximal fluorescence; $(F_m - F_o)/F_m = F_v/F_m$, efficiency of photosystem II.

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proteomic and physiological parameters which allow to have a global view of the tolerance of probable willow candidates for phytoremediation purposes. Our data demonstrates that higher biomass production in presence of metals is not necessarily linked with higher tolerance whereas growth reduction might indicate longer long-term tolerance. In the long term and in the purpose of a future use in phytoremediation, the survival of this high producer clone could be compromised.

1. Introduction

When metals are in excess in soil, they become toxic as they can disturb plant physiology and thus affect plant development [1,2]. Indeed, metals can interact directly with proteins and lipids of membranes causing membrane damage and thus the loss of cellular electrolytes [2–6]. Moreover, a metal in excess is able to replace essential metals in pigments and enzymes, which can alter their function. The pigment synthesis can be disturbed and their concentrations reduced in the presence of metals, leading to a lower photosynthetic efficiency [6,7]. As has been observed for other abiotic stresses, carbohydrate metabolism can be affected by metal exposure. Several carbohydrates can accumulate, suggesting a role in osmotic adjustments and/or protection of cell constituents [6–9]. Consequently, the disruption of the physiological and biochemical processes in cells reduces plant growth or can even lead to plant death [5,6].

Indirect metal toxicity also exists and lies mainly in the increased generation of reactive oxygen species (ROS) ($O_2^{\cdot-}$, H_2O_2; $HO^{\cdot}$), inducing oxidative stress. Generally, ROS in cells are maintained below toxic levels due to the activity of antioxidative defense mechanisms, which are enzymatic (superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT)) or non-enzymatic (glutathione, carotenoids and ascorbate) [10]. Nevertheless, metals can disrupt the activity of these antioxidative enzymes containing free cysteine residues as they can bind thiol groups which leads to further disturbance of the oxidative balance [5,11]. Because the effects of metals on plants are diverse, several physiological parameters (photosystem efficiency, sugar and pigment concentrations, and electrolyte leakage) are used to evaluate metal-induced cellular damage in this study. For instance, the chlorophyll fluorescence ratio F_v/F_m was used. This ratio estimates the photochemical efficiency of the photosystem II (PSII) as this ratio generally decreases when the photosynthetic apparatus functioning is impaired [12].

In parallel, a proteomic approach can contribute to a better understanding of the impact of metals. While gel-free techniques have become the standard approach for studying the proteome of well-characterized organisms, plant proteomics still largely depends on the use of two-dimensional gel electrophoresis as a quantification tool for proteins. The introduction of fluorescent labels in proteins prior to gel-based separation, as is done in 2D-DiGE, has alleviated some of the limits of this technique. Therefore it is the latter technique that is currently increasingly used to study the influence of biotic and abiotic constraints on the physiology of poorly characterized plant species [4,9,13–16].

Phytoremediation, the use of plants to clean up polluted soils, depends on the potential of plants to tolerate the conditions found on these sites. Furthermore the suitability of plants for phytoextraction, the extraction of polluting metals from soils, depends on the potential of plants to extract metals from the soil and to accumulate these metals in harvestable plant parts. Because they are fast growing and able to tolerate diverse environmental conditions, the ability of *Salix* trees to concentrate metals has been investigated to select good candidates for phytoremediation [17]. However, most of these studies were screenings of different Salix clones and selection of the clones that performed best, therefore relatively few studies added molecular data on the mechanisms of extraction and accumulation of these *Salix* clones.

Based on a previous study [18], two willow clones (*Salix fragilis* and *Salix aurita*) were selected. The general conclusion from this previous study was that clones that produce a high biomass generally have relatively low metals concentrations (in g/g dry weight) in their aerial parts. One notable exception was observed on this general observation, the *S. aurita* clone used in the current study produced a high biomass and accumulated high concentrations of metal. Therefore this clone was selected for further study. The *S. fragilis* clone used in the current study produced the same biomass but, in agreement with the general finding, did not accumulate high concentrations of metal. The detailed study of these clones, equal biomass production but a large difference in concentration of accumulated metals, could therefore shed some light on the differences in the used coping strategy. The aim of the present study was to provide insight in these differences by using not only morphology but also physiological parameters and proteomic analysis on these two willow clones growing with an excess of Cd, Zn, Cu and Ni to explain their metal tolerance/accumulation abilities.

2. Material and methods

2.1. Plant material and growth conditions

A *S. fragilis* clone (Sf) and a *S. aurita* clone (Sa) were chosen from a previous screening [18]. These clones showed a similar biomass production but accumulated different metal concentrations in their annual twigs. Rooted cuttings of each clone were placed outdoors in 10 L containers filled up with a contaminated or non-contaminated (control) substrate. These containers had an open bottom in order to avoid waterlogging. The substrate used was composed of soil sampled from farm land, sand (0–2 mm granule size) and peat (V:V, 2:1:1). To contaminate the substrate, a mixture of metals (Cd, Cu, Zn and Ni as chloride salts) was added. The control and contaminated soils were matured outdoors for three years.

The total concentration of Ca, Mg, Na, K, Fe, Mn and metal trace elements (Al, Cd, Cu, Ni, Mo, Zn) (after *aqua regia* digestion) of the soils was determined using inductively coupled plasma