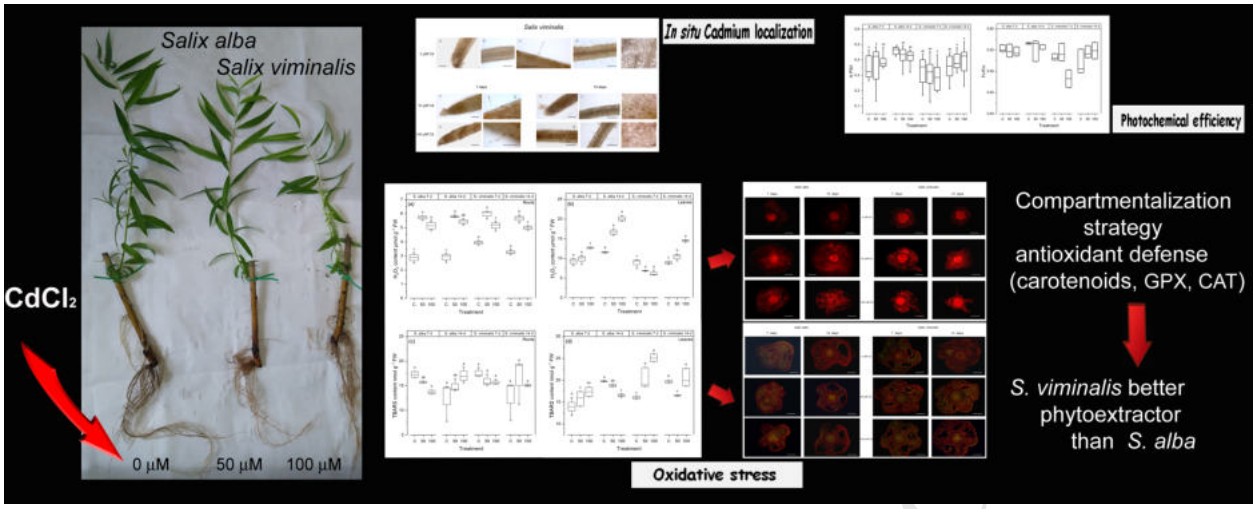

Modulation of the defence responses against Cd in willow species through a multifaceted analysis

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Abstract

Willow, due to the extensive root system, high transpiration rates and ability to accumulate large amounts of cadmium, is considered particularly useful for green remediation practices. In this study two different willow species, *Salix viminalis* and *Salix alba*, were used to assess possible differences in their ability of cadmium accumulation and to analyse in detail the physiology of their response to treatments with this metal using a multidisciplinary approach. Plants were grown in hydroponics and treated with 0, 50 and 100 μM Cd^{2+} ($CdCl_2$) for 7 and 14 days. Cadmium content, oxidative stress, both evaluated by biochemical and histochemical techniques, antioxidant response, leaf stomatal conductance and photosynthetic efficiency were measured in control and treated roots and/or leaves. The two willow species removed cadmium with a high efficiency from the growth solution; however, the highest contents of Cd recorded in plants grown in the presence of the lower Cd concentrations suggest a limited capacity of metal accumulation. No photochemical limitation characterised treated plants, probably due to the ability to store large amounts of Cd in the root compartment, with reduction of damage to the photosynthetic machinery. *S. viminalis*, able to uptake cadmium also in the root apical region, seemed to be a more efficient accumulator than *S. alba* and, thanks to a relatively higher antioxidant response, did not show a higher level of oxidative stress. On the basis of the above, the two plant species, in particular *S. viminalis*, are confirmed as useful for cadmium phytostabilisation/phytoextraction.

Keywords: Antioxidant response; Cadmium; Histochemistry; Oxidative stress; Photosynthetic efficiency; Willow

1. Introduction

Cadmium is one of the most hazardous soil and water contaminants, spread in the environment owing to natural and human activities. If natural sources such as weathering of parent rocks (Khan et al., 2017) and volcanic eruptions account for about 10% of the total release, nearly 90% of soil cadmium derives from anthropogenic sources (mining and smelting of zinc-bearing ores, fossil fuel combustion, waste incineration, sewage sludge, irrigation waters, and fertilizers derived from phosphate rock; Alloway and Steinnes, 1999). It is toxic to most living organisms and due to its high water solubility, mobility and stability is considered one the most dangerous pollutants (Hassan and Aarts, 2011). Its toxicity, combined with its high and continuously increasing

environmental contamination, has raised a great interest for the study of the fate of cadmium in the environment. Cadmium is not an essential element for plants but can be easily taken up by plant roots and in this way it enters the food chain (Wahid et al., 2009). In plants this heavy metal can induce disturbance to growth, photosynthesis (Yu et al., 2013) and water balance (Aghaz et al., 2013). Cadmium may lower photosynthetic efficiency (Dias et al., 2013), probably also through the enhanced degradation of chlorophyll or the inhibition of its biosynthesis (Somashekaraiah et al., 1992). The overall inhibition of this process can ultimately result in a strong reduction of plant growth. The effects of cadmium on water balance are associated with a reduction of root growth and water uptake, and a partial stomatal closure (Barceló and Poschenrieder, 1990; Prasad, 1995), with a consequent further inhibition of the rate of photosynthesis. Even if it is a non-redox reactive metal, it can cause oxidative stress, mostly through indirect mechanisms (Wang et al., 2008), such as the substitution of redox-active metals in proteins (Cuypers et al., 2011), and induction of NADPH oxidase activity, in this way causing the production of reactive oxygen species (ROS, Gallego et al., 2012), among which hydrogen peroxide plays a central role. At low concentrations ROS can exert a positive action, functioning as signalling molecules, able to elicit a protective response. At higher concentrations, however, these reactive molecules can induce oxidative injury, with damage to cellular structures and macromolecules (Benavides et al., 2005). To counteract the negative effects deriving from ROS overproduction, plants have developed an antioxidant machinery based on both enzymatic and non-enzymatic systems. Peroxidases, ascorbate peroxidase, glutathione peroxidase, catalase, among others, can cooperate with low molecular weight antioxidants such as ascorbate and glutathione, to scavenge these reactive, potentially harmful molecules (Lenher et al., 2006).

Despite its toxicity to most organisms, there are plant species that are able to accumulate cadmium to high levels and, due to this characteristic, can be used to remediate cadmium-contaminated matrices. After an initial focus on herbaceous plants able to hyperaccumulate metals, but characterised by a low biomass production, the focus has shifted to fast-growing woody plants. Among them, particular attention was given to the genus *Salix* (about 450 species), tolerant to several soil conditions (Kuzovkina et al., 2004). Willow has an extensive root system, high transpiration rates, and can accumulate large amounts of cadmium in its tissues. In addition, due to the extensive fibrous root system, willow is particularly useful for remediation of deep soil contamination (Kuzovkina and Quigley, 2005). In our study two different species, *S. viminalis and S. alba*, were used. The aim of the present work was to assess if there were differences between the two selected species in the ability of cadmium accumulation and in the tolerance to this heavy metal. The accumulation of Cd in plant tissues is significantly higher when plants are grown in

hydroponic conditions in comparison with growth in soil (Eller and Brix, 2015; Kayser et al. 2000; Schwartz et al. 2003). Our main interest was however in plant physiological response to this metal and so the hydroponic system was used for this research as it allows to better control the experimental conditions, though giving only a preliminary indication of the potential of the plant in the phytoremediation (Dos Santos Utmazian et al., 2007; Lunácková et al., 2003; Zacchini et al., 2009).

The ability of different species of willow to accumulate cadmium has already been studied, to our knowledge, however, the modulation of plant physiological response to this metal has not been investigated through a multifaceted approach yet.

On the basis of the key role of oxidative stress in metal toxicity, oxidative damage and antioxidant response were determined to highlight putative differences in defence strategies. The biochemical approach was associated with histochemical analyses to evidence possible differences not detectable by quantitative methods. The degree of tolerance to cadmium was estimated also through the analysis of photosynthetic efficiency. Moreover, leaf gas exchanges were evaluated by measuring stomatal conductance: this provided information on the rate of photosynthesis and, along with the determination of the relative water content of leaves and roots, on the water balance of the plants. The results obtained may allow to answer our main questions, i.e. which of the two species can be used more successfully in cadmium remediation and which physiological traits might have a pivotal role in their ability of cadmium tolerance and accumulation.

2. Materials and methods

2.1. Plant growth

The material was provided by the National Institute for Research in Rural Engineering Water and Forestry (INRGREF) of Tunisia. *Salix alba* L. and *Salix viminalis* L. were used as they represent the main *Salix* species in the southern Mediterranean area. Cuttings of the two willow species were collected in the forest nursery of Jendouba (36.5831N, 9.0451E, Tunisia) from the region of Aïn Draham. About 100 mother plants for each species (20-50 m distant each other) were used to generate the collection of stock plants in order to ensure their genetic diversity. From these plants woody cuttings (diameter, 1 ± 0.04 cm, length, 15 ± 1.4 cm) were removed during the winter dormancy period. To assure a comparable hydraulic architecture, the cuttings, used to obtain the seedlings object of study, were taken at the same level of branching and at the same height.

The experiments were performed in a naturally illuminated greenhouse (April - June 2017) at the Department of Biology, University of Pisa (Italy). The temperature was between 23 and 28°C. The

stems cuttings of the two species of *Salix* were grown in plastic pots of 50 cm diameter and 60 cm depth in aerated deionized water. Four cuttings for pot and 3 pots for thesis were used. After 60 days, when leaves were fully developed, water was substituted by ¼ x Hoagland solution (Sigma) and supplemented with 0, 50 and 100 μM Cd^{2+} ($CdCl_2$) for 7 and 14 days. The nutrient solutions were replaced entirely twice a week. After treatment, roots and leaves were collected, measured, washed and used for histochemical analyses or fixed in liquid nitrogen and stored at -80 °C until use for biochemical determinations.

2.2. Flame atomic absorption spectrometry analysis (FAAS)

Cadmium content in roots and leaves was determined according to Ciobanu et al. (2013) with minor modifications. In particular, the samples, after washing with deionized water for 5 min, were reduced to dry ash in muffle furnace at 525°C for 3 hours. Dried plant tissues were ground in porcelain mortar and the powder was digested in 65% HNO_3 and 1N HCl (1:1 v/v) heated at 145°C till white fumes started appearing. The digested samples were made up to 25 ml with deionized water and filtered through filter paper. Heavy metal concentration was measured in a flame atomic absorption spectrometer (Thermo Scientific, ICE 3000 series).

2.3. Removal efficiency, bioconcentration factor and translocation factor

The removal percentage of cadmium by willow plants was determined according to Tanhan et al. (2007) as follows:

$$Removal\ efficiency = \frac{initial\ metal\ concentration\ (mg\ L^{-1}) - final\ metal\ concentration\ (mg\ L^{-1})}{initial\ metal\ concentration (mg\ L^{-1})} x100$$

The bioconcentration factor (BCF) was calculated according to Rahmani and Sternberg (1999) as follows:

$$BCF = \frac{metal\ concentration\ in\ plant\ \ dried\ biomass\ (mg\ kg^{-1})}{initial\ metal\ concentration\ in\ the\ external\ solution\ (mg\ L^{-1})}$$

The translocation factor (TF) was determined according to Luo et al. (2005) as follows:

$$TF = \frac{metal\ concentration\ in\ leaves\ (mg\ kg^{-1})}{metal\ concentration\ in\ roots\ (mg\ kg^{-1})}$$

2.4. Histochemical localization of cadmium

Dithizone (diphenylthiocarbazone) method was used for histochemical detection of cadmium in roots and leaves according to Balestri et al. (2014b). Cadmium localization was evidenced by

reddish colour precipitates produced after reaction of dithizone with this heavy metal. Whole roots and leaf epidermal strips were stained for 1.5 h with a dithizone solution (30 mg dissolved in 60 ml acetone and 20 ml distilled water), rinsed in water and immediately analysed using bright field microscopy.

2.5. Relative water content

Root and leaf relative water contents, RWC, were determined as in Balestri et al. (2014a) with minor modifications and calculated with the formula:

$$RWC = \frac{FW - DW}{TW - DW} x100$$

FW = Fresh weight, *DW* = Dry weight, *TW* = Turgid weight.

After recording fresh weight, roots or leaves were immersed in deionized water over night, blotted dry and then weighed to get the turgid weight. Roots and leaves were then dried in an oven at 60° C to constant weight and weighed again to obtain the dry weight.

2.6. Leaf stomatal conductance

Stomatal conductance (mmol H_2O m^{-2} s^{-1}) was measured at 7 and 14 days by a SC1 steady-state leaf porometer (Decagon Devices, Inc., Pullman, WA, USA). One record per individual was taken on sun-exposed, fully expanded leaves from each replicate, between 12 AM and 1 PM. The value of each thesis was the average of 12 measurements ± SE.

2.7. Pigment content and photosynthetic efficiency

Chlorophylls (a, b and total) and carotenoids were extracted and determined as in Spanò and Bottega (2016), according to Hassanzadeh et al. (2009) and to Lichtenthaler (1987), respectively. Pigment contents were expressed as mg g^{-1} FW. Photosynthetic efficiency was determined as in Pippucci et al. (2015) by analysing chlorophyll fluorescence by a portable fluorometer (MINI-PAM Walz, Effeltrich, Germany), at 7 and 14 days. Two records *per* individual were taken on sun-exposed leaves, thus acquiring the operating Photosystem II (PSII) quantum yield (ΦPSII). Further two leaves were shaded with dedicated clips for 30 min, then measured to evaluate the maximum PSII quantum yield (Fv/Fm) (Genty et al., 1989). All measurements were performed between 12 and 1 PM. The value of each thesis was the average of 24 measurements ± SE.

2.8. Extraction and determination of hydrogen peroxide and thiobarbituric acid reactive substances (TBARS)

H_2O_2 content of leaves and roots was determined according to Jana and Choudhuri (1982) using titanium chloride in H_2SO_4 for peroxide detection. The supernatant was read at 410 nm and the amount of H_2O_2 in the extracts was calculated from a standard curve and expressed as μmol g^{-1}FW. Lipid peroxidation in leaves was estimated by determining the amount of TBARS according to Wang et al. (2013) with minor modifications as in Spanò et al. (2017). The concentration of TBARS, expressed as nmol g^{-1}FW, was measured as specific absorbance at 532 nm by subtracting the non-specific absorbance at 600 nm and calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.9. Histochemical detection of oxidative stress in root system

Six roots of comparable growth level, randomly selected from control and treated plants, were excised and sectioned with hand microtome right above the differentiation zone. At least 10 cross sections for each root were immediately processed with fluorescent probes specific for *in situ* localization of hydrogen peroxide and oxidative damage in cells and membranes. Amplex UltraRed Reagent (Life Technologies, USA) was applied for *in situ* detection of H_2O_2 following the protocol reported in Ruffini Castiglione et al. (2016). After staining, slices were mounted in glycerol and observed with fluorescence microscope (568ex/681em nm). BODIPY 581/591 C11 was used as free radical sensor to visualize lipid peroxidation levels as a change of the fluorescence emission peak from red to green. The slices were incubated and stained following a previous protocol (Ruffini Castiglione et al., 2016). Microscope evaluation was performed acquiring simultaneously the green (485ex/510em nm) and the red fluorescence (581ex/591em nm) signals and merging the two images (Kováčik et al., 2014). Fluorescence microscope analysis was carried out with a Leica DMLB, equipped with appropriate set of excitation/emission filters and with a Leica DC300 ccd camera.

2.10. Enzyme extraction and assays

The extraction of enzymes from roots and leaves was made according to Spanò et al. (2013). Ascorbate peroxidase (APX, EC 1.11.1.11), glutathione peroxidase (GPX, EC 1.11.1.9), catalase (CAT, EC 1.11.1.6) and guaiacol peroxidase (POX, EC 1.11.1.7) activities were measured according to Nakano and Asada (1981), Navari-Izzo et al. (1997), Aebi (1984) and Arezki et al. (2001), respectively, and expressed as U mg^{-1}protein. Protein content was determined according to Bradford (1976), using bovine serum albumin as standard.

2.11. Statistical analysis

Statistical analysis was performed using the Statistica package (StatSoft) version 6.0. All the data were the mean of at least three replicates from three independent experiments. Effects of the three factors of experimental design (species: *S. alba* and *S. viminalis*; metal concentration: 0, 50 and 100 μM Cd; time: seven and fourteen days), and their interaction were analysed using multifactor ANOVA. The differences among means were compared with a post-hoc analysis using Tukey test (Tukey Honestly Significant Difference) at $p < 0.05$. For translocation factor (TF) data were analysed after arcsin transformation.

3. Results and Discussion

3.1. Cadmium content

Cadmium content assessed by FAAS (Table 1) depended on species ($F=4608.98$, $p<0.01$), metal concentration ($F=849.16$, $p<0.01$) and time ($F=2802.6$, $p<0.01$) of treatment and interaction among the three factors ($F=232.54$, $p<0.01$). During treatment there was an increase in Cd content in both species, but *S. viminalis* accumulated significantly higher levels of this heavy metal in comparison with *S. alba.* The difference between the two species is not surprising as a difference in the ability of cadmium accumulation among different willow species is often reported in literature (Borišev et al., 2009; Harada et al., 2010) and is in accordance with previous data obtained under low cadmium concentration treatments (Vassilev et al., 2005). With the exception of roots of *S. alba* after 7 days, Cd contents were significantly higher in plants grown under the lower cadmium concentration, suggesting that, in our experimental conditions, there is a limited capacity for the accumulation of cadmium in these willow species. Indeed, the highest content of this heavy metal (about 5076 ppm) was recorded in roots of *S. viminalis* after 14 days of treatment under 50 μM $CdCl_2$. There are no consistent data on the subject in literature since both increase and decrease in Cd accumulation have been recorded according to its increasing concentration in growth medium (Vassilev et al., 2005; Liu et al., 2011; Cosio et al., 2006).

In situ localization by dithizone staining (Fig. 1, 2) revealed Cd as the formation of reddish/brown precipitates within root hairs and cortical cells in both species (Fig. 1e, g, h, j, k, l; Fig. 2g, i, l, m, n). In *S. viminalis* also root apex was positive to the histochemical probe (Fig. 2f, h, k), indicating that this species is able to take up the metal outside the root hair region. This ability, recorded also for other species and cations (Pineros et., al 1998), could in part explain the higher capacity of Cd extraction of this species.

The content of Cd was significantly lower in leaves than in roots. In particular leaf concentration was well below 100 ppm, the threshold value to define a plant as a Cd hyperaccumulator in a natural environment (Brown et al., 1994). There were significant effects for species ($F= 400.3$, $p<0.01$), metal concentration ($F= 101.85$, $p<0.01$), time of treatment ($F= 1432.9$, $p<0.01$) and interaction among the three factors ($F=28.29$, $p<0.01$). While cadmium content progressively increased in *S. viminalis* leaves with time and Cd concentration, in *S. alba* the highest contents of this metal were recorded in plants treated with 50 µM $CdCl_2$ (Table 1). The maximum value of cadmium content in leaves was recorded in *S. viminalis* after 14 days of treatment with 100 µM $CdCl_2$ (54.94 ppm). This data was also confirmed by *in situ* detection on leaf epidermal strips, in which stain precipitates were more abundant, with a localization mainly involving subsidiary cells surrounding guard cells as well as the other epidermal cells (Fig. 2o).

Bioconcentration factor values in roots of both species were always higher under 50 µM $CdCl_2$ than 100 µM $CdCl_2$ reaching the highest value in roots of *S. viminalis* after 14 days of treatment (903.22, Table 1). In *S. alba* roots the BCF values were generally significantly lower than those of the other species and the highest value was detected in 50 µM $CdCl_2$ after 14 days (358.65). Differences were significant in function of species ($F= 5109.79$, $p<0.01$), metal concentration ($F= 8434.69$, $p<0.01$), time of treatment ($F= 3388.01$, $p<0.01$) and interaction among the three factors ($F=550.91$, $p<0.01$). In leaves there was a decrease in the BCF with increasing cadmium concentration with the exception of *S. viminalis* after 7 days of treatments where the BCF of plants grown under the two concentrations were comparable (Table 1). There were significant effects for species ($F= 152.5$, $p<0.01$), metal concentration ($F= 1444.62$, $p<0.01$), time of treatment ($F= 1556.05$, $p<0.01$) and interaction among the three factors ($F=32.90$, $p<0.01$). Bioconcentration factor is an index of the capacity of the plant to accumulate cadmium in function of the concentration of this metal in the growth medium and of the efficiency of metal sequestration: the high values recorded (Bakar et al., 2013) in this experiment confirm that Salicaceae plants have a considerable potential to remove cadmium from a contaminated medium (Zacchini et al., 2009). Between the two species *S. viminalis* seems to be a more efficient bioaccumulator, but BCF of *S. alba* was however higher than those reported in literature for this species of willow (Zacchini et al., 2009).

Translocation factor was always < 1 for both species in all treatments with a decrease at the higher cadmium concentration in *S. alba* (Table 1). This was in agreement with Yao et al. (2018) but in contrast with data reported in literature for other *Salix* species (Zacchini et al., 2009). Differences were significant in function of species ($F= 56.69$, $p<0.01$), metal concentration ($F= 34.09$, $p<0.01$), time of treatment ($F= 47.47$, $p<0.01$) and interaction among the three factors ($F=14.73$, $p<0.01$). Relatively low TF values (Dai et al., 2013), together with the leaf cadmium content below 100 ppm,

clearly indicate our species as non-hyperaccumulating plants. The ability to accumulate cadmium mostly in roots is already reported in literature for willow (Luković et al., 2012) and is consistent with the low Cd mobility in the two species studied that in our experimental conditions seem to adopt a compartmentalization strategy aimed to fix most of the Cd in their underground parts as a defence mechanism against the harmful effects of Cd on photosynthesis (Bonanno and Vymazal, 2017). Nevertheless, the high removal efficiency (data not shown), ranging from about 97% to 99%, observed in both species indicates their good ability to extract cadmium from the growth medium.

3.2. Stomatal conductance

Cadmium appeared to affect stomatal conductance depending on species ($F= 23.17$, $p<0.01$), metal concentration ($F= 12.17$, $p<0.01$) and time of treatment ($F= 42.84$, $p<0.01$). Significant were also the interactions between species and metal concentration ($F= 8.01$, $p<0.01$) and metal concentration and time ($F= 5.32$, $p<0.01$). In *S. alba* after 7 and 14 days stomatal conductance was significantly lower in plants treated with 50 µM $CdCl_2$ than in the control, while the highest concentration (100 µM) induced a decrease in stomatal conductance only after 14 days, although the effect was not statistically significant (Fig. 3). In *S. viminalis* no significant difference was detected between control and treated plants (Fig. 3); this species seemed to tolerate greater amounts of Cd in the leaves without consequences on stomatal conductance, in comparison with *S. alba*. This could lead to conclude that *S. viminalis* might perform better than *S. alba* regarding this physiological trait, but it must be taken into account that, overall, the values of stomatal conductance in the treated plants were similar for the two species. At concentrations lower than those applied in the present work, Cd did not show negative effects on stomatal conductance in one clone of *S. alba* (LUC-31) and one of *S. viminalis* (STOTT) (Vassilev et al., 2005). Conversely, when plants of *S. alba* clone SS5 were treated with 50 µM $CdSO_4$, stomatal conductance displayed a significant decline (Pietrini et al., 2010).

3.3. Relative water content, pigment concentration and chlorophyll fluorescence

In roots, relative water content changed according to metal concentration ($F= 9.22$, $p<0.01$) and time of treatment ($F= 10.59$, $p<0.01$) with no significant effect of species ($F= 0.44$, $p>0.1$). In *S. alba* roots, cadmium treatment never induced significant differences of RWC in comparison with controls (Table 2). In *S. viminalis* a significant increase in comparison with control was detected under the higher cadmium concentration in the shorter period while a significant decrease in RWC was recorded in roots from plants under 50 µM $CdCl_2$ after 14 days (Table 2). In leaves, RWC changed according to species ($F= 6.64$, $p<0.05$), metal concentration ($F= 34.74$, $p<0.01$) and time of

treatment (F= 19.77, p<0.01). With the exception of *S. alba* after 14 days, relative water content was significantly lower in leaves from plants under 100 µM $CdCl_2$ than in control leaves (Table 3) but only in *S. viminalis* a progressive Cd-concentration dependent decrease, not always significant, was detected (Table 3). On the whole there were not dramatic differences in RWC upon cadmium treatment, which demonstrated the ability of willow to maintain a good water balance even under metal treatment.

Total chlorophyll and carotenoid contents changed according to species (F= 4.68, p<0.05 and F= 14.88, p<0.01 respectively) and to the concentration of cadmium in the growth medium (F= 31.89, p<0.01 and F= 8.07, p<0.01 respectively). For both parameters significant was also the interaction among the three factors (F= 3.74, p<0.05 and F= 4.15, p<0.01 respectively). In most cases, total chlorophyll and carotenoid contents progressively decreased, with increasing Cd concentration, in treated plants of both species (Table 3). This is in accordance with data in literature (Vassilev et al., 2005; Gowthami and Vasantha, 2015) showing a decrease in the content of these pigments in cadmium treated plants. There were not significant differences in Chla/Chlb ratio among different species (data not shown). The carotenoids/Total Chl ratio changed depending on species (F= 4.94, p<0.05) and time of treatment (F= 7.45, p<0.05) with an increase in treated plants of both species after 14 days of treatment (Table 3). The increase from 7 to 14 days was however significant only in *S. viminalis*, in which this ratio was 1.4 (50 µM $CdCl_2$) and 1.7 (100 µM $CdCl_2$) that of the control leaves (Table 3). In the context of a general decrease in photosynthetic pigments, therefore, there was a preferential investment in carotenoids under stress condition, probably to enhance protection of photosystems (see Young and Frank, 1996 and references therein). Photosynthetic efficiency assessed measuring ΦPSII and Fv/Fm changed according to species (F= 20.57, p<0.01; F= 18.83, p<0.01) and time of treatment (F= 32.27, p<0.01; F= 5.43, p<0.05). Significant was also the interaction among the three factors (F= 3.79, p<0.05; F= 4.74, p<0.05) for ΦPSII and Fv/Fm respectively. Control and treated plants did not show any significant difference when compared for these two parameters (Fig 4a and 4b). The sole exception was a slight decrease of Fv/Fm of *S. viminalis* treated with 100 µM $CdCl_2$ after 7 days, but this drop could not be attributed to a weakening of the photoprotecting processes belonging to non-photochemical quenching (NPQ), since the latter parameter did not change according to either ΦPSII or Fv/Fm (data not shown). Nevertheless, the effect of Cd on Fv/Fm of *S. viminalis* was transient and after 14 days of treatment these plants had fully recovered. Pietrini et al. (2010) treated *S. alba* plants, clone SS5, with 50 µM $CdSO_4$ and found that ΦPSII was significantly lower than in the control, whereas Fv/Fm did not change. Stable values of Fv/Fm under Cd treatments could be a clue of the stability of the structure of thylakoids (Pajević et al., 2009). In a cadmium-induced decline of net photosynthesis,

conceivable on the basis of literature data on *S. alba* and *S. viminalis* (Vassilev et al., 2005), photochemical limitations could be therefore reasonably ruled out as possible causes.

3.4. Oxidative stress and antioxidant response

Treated root cuttings had higher contents of hydrogen peroxide in comparison with control plants, as shown by both biochemical and histochemical data (Fig 5 and Fig 6). The content of this signalling molecule changed in a species- ($F= 8.69$, $p<0.01$), metal concentration- ($F= 630.56$, $p<0.01$) and time-($F= 4.54$, $p<0.05$) dependent manner, without significance for the interaction among the three factors ($F= 0.59$, $p=0.56$). In both species treatments with the lower cadmium concentration generally induced the occurrence of higher H_2O_2 content in accordance with the higher cadmium contents recorded in roots grown in these conditions (Fig 5). In addition, *in situ* H_2O_2 localization showed a different and peculiar staining pattern. While in *S. viminalis* (Fig 6) the red fluorescence was localized in the vascular cylinder and in a limited area of the cortex, in *S. alba* (Fig 6) the H_2O_2 signal was also relieved in the periphery of the cortical cylinder, including root epidermis. It is worth noting that, in *S. viminalis* under 100 µM Cd, the staining pattern also involved the phloem arcs, probably as systemic stress response, as previously demonstrated following both biotic (Musetti et al., 2005) and abiotic (Ruffini Castiglione et al., 2016; Giorgetti et al., 2019) stress. In leaves, just as in roots, there was a significant effect on the content of hydrogen peroxide of the three factors (species: $F= 548.17$, $p<0.01$; metal concentration: $F= 151.13$, $p<0.01$; time: $F= 726.35$, $p<0.01$) also in interaction ($F= 13.33$, $p<0.01$). Cadmium treatment always induced a progressive increase of H_2O_2 content with the exception of 7 days-treated *S. viminalis* plants where a decrease in the concentration of this molecule was recorded in treated leaves (Fig 5). In accordance with data in literature (Balestri et al., 2014a), cadmium, despite being a non-redox reactive metal, can therefore induce the production of ROS, (Gallego et al. 2012). These increases in hydrogen peroxide content however were not always associated with increases in TBARS content (Fig 5), indicative of membrane damage. In fact, cadmium treatment induced in roots a non-significant increase in lipid peroxidation in comparison with controls only after 14 days of exposition in both species (Fig 5). In leaves, showing changes in TBARS content depending on species ($F= 43.21$, $p<0.01$) and metal concentration ($F= 15.11$, $p<0.01$) also in interaction ($F= 16.73$, $p<0.01$), a progressive increase in the concentration of this parameter with increasing cadmium content was recorded only in the short-term treatment (Fig 5). In literature both increase and decrease of lipid peroxidation with increasing cadmium concentration (Hassan et al., 2005; Skórzyńska-Polit and Krupa, 2006) have been reported. Our species were able to maintain low levels of lipid peroxidation, even lower than the control (roots in the short term, and leaves in the

long term), showing a good ability to limit oxidative damage. Further information may derive from the analysis of the histochemical data obtained on the root system (Fig 7), demonstrating that cadmium can induce a specific pattern of lipid peroxidation that involved the central cylinder, while the signal in the cortical cylinder, present in all samples, was probably related to the lysigenous aerenchyma formation in willow species (Kawase and Whitmoyer, 1980).

To counteract negative effects of ROS plants are able to activate a complex antioxidant machinery in which enzymes play a significant role. Both inhibition and stimulation of antioxidant enzymes by cadmium have been reported (Balestri et al., 2014a; Martins et al., 2011; Sandalio et al., 2001; Sanità di Toppi and Gabrielli, 1999). Glutathione peroxidase scavenges hydrogen peroxide using glutathione and other substrates, including lipid hydroperoxides, as reducing agent (Herbette et al., 2002). Changes in the activity of this enzyme in roots depended on species ($F= 76.00$, $p<0.01$), metal concentration ($F= 120.52$, $p<0.01$) and time ($F= 94.47$, $p<0.01$). Significant were also interactions among the three factors ($F= 10.87$, $p<0.01$). GPX activity generally reached the maximum value in 100 µM $CdCl_2$ treated roots (Table 2). Plants under 50 µM $CdCl_2$ had lower GPX activity than control plants only in *S. alba* (Table 2). Only in *S. alba* plants treated for 14 days the maximum activity characterised control roots (Table 2). In leaves of *S. viminalis* after 7 days of treatment with the higher Cd concentration, GPX activity was significantly higher than the other two treatments (Table 3). In leaves of *S. alba* 14 days of Cd treatment induced an increase in enzymatic activity (Table 3). The activity of this enzyme in leaves changed according to species ($F= 47.31$, $p<0.01$), metal concentration ($F= 28.31$, $p<0.01$) and time ($F= 997.40$, $p<0.01$) also in interaction ($F= 17.08$, $p<0.01$).

Ascorbate peroxidase, like GPX, can scavenge H_2O_2 using ascorbate as reducing agent. In *S. alba* APX activity was always lower in cadmium treated roots than in control ones (Table 2) while in *S. viminalis* there were not significant differences among the different treatments (Table 2). Changes in the activity of this enzyme depended from metal concentration ($F= 11.28$, $p<0.01$) also in interaction with species ($F= 7.96$, $p<0.01$) and time ($F= 11.13$, $p<0.01$). In leaves this parameter was dependent on the species ($F= 21.20$, $p<0.01$), metal concentration ($F= 7.27$, $p<0.01$) and time of treatment ($F= 67.63$, $p<0.01$) also in interaction ($F=4.28$, $p<0.05$). There were not significant differences in APX activity in treated plants in comparison with respective controls (Table 3). As a consequence, in our conditions, the cadmium-decrease generally recorded in the activity of this enzyme (Vestena et al., 2011) was not observed. Peroxidases (POX) scavenge H_2O_2 in chloroplast and cytosol of plant cells (Gill et al., 2011; Gill and Tuteja, 2010) and catalyse H_2O_2 dependent oxidation of substrate. In accordance with literature (Vassilev et al., 2005) different trends of peroxidase activity were recorded depending on the organ and species. In roots significant were the

effects of species (F= 59.63, p<0.01), metal concentration (F= 6.28, p<0.01), time (F= 205.83, p<0.01) of treatment and interaction among the three factors (F= 6.36, p<0.01). In *S. alba* roots POX activity decreased in plants supplemented with the heavy metal with lower values recorded in 50 µM $CdCl_2$ treated plants (Table 2). In *S. viminalis* on the contrary, the activity of this peroxidase increased significantly in particular in roots under 50 µM $CdCl_2$ treatment (Table 2). In leaves of both species POX activity generally decreased in cadmium-treated plants (Table 3), in accordance with previous data recording a down regulation of this enzyme in leaves of *Vigna radiata* under treatment with comparable Cd concentrations (Gowthami and Vasantha, 2015). Changes in the activity of this enzyme depended on species (F= 53.09, p<0.01), metal concentration (F= 575.58, p<0.01) and time (F= 112.89, p<0.01), also in interaction (F= 423.84, p<0.01). Catalase (CAT) is able to scavenge hydrogen peroxide without using a reductant as it catalyses a dismutation reaction (Mhamdi et al, 2010). There were not significant differences in the activity of this H_2O_2 scavenging enzyme among different roots in *S. alba* and in *S. viminalis* both after 7 and 14 days of treatment (Table 2). In leaves there were not significant differences among treated plants and respective controls (Table 3). In *S. viminalis* the low activity recorded in leaves under 50 µM $CdCl_2$ after 7 days of treatment was recovered in the longer period (Table 3).

Overall, the extent and the direction of the enzymatic antioxidant response to cadmium treatment was different depending generally on species, metal concentration and time of exposure. In addition, the trend was different in the various enzymes studied with an enzymatic antioxidant response mainly relying on CAT and GPX whose activity was generally higher, even if not always significantly, in treated plants.

3.5. Conclusions

Though being able to accumulate cadmium with a high efficiency of removal from the substrate, our two willow species on the base of the relatively low TF and leaf cadmium content (below 100 ppm) cannot be considered hyperaccumulating plants. Consistently, the higher contents of Cd recorded in plants grown in presence of the lower cadmium concentration could indicate that under the present experimental conditions there is a limited capacity for the accumulation of this metal. Both species seem to adopt a compartmentalization strategy, fixing most of the Cd in roots to protect the plant against the harmful effects of cadmium on photosynthesis. Thanks to this strategy and to the protective action of carotenoids, a good stability of the structure of thylakoids without stomatal and photochemical limitations characterised our treated plants. *Salix viminalis*, able to uptake cadmium also in the apical region of the roots, seems to be a better accumulator than *Salix alba*; the former species, despite the higher contents of cadmium both in roots and in leaves, did not

show a higher level of oxidative stress, thanks to a relatively higher antioxidant response. On the base of the above, considering the high BCF associated with the low TF, our two species, in particular *S. viminalis*, could be useful for wastewater decontamination, in which the whole plant can be removed. A role in soil phytostabilisation, when a reduction in metal mobility and bioavailability is requested, might be also suggested. Bearing in mind differences between soil and hydroponic conditions, these statements however needs confirmation from experiments made using soil as growth medium.

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Legends of figures

Fig.1. *In situ* cadmium localization in roots and leaves of *Salix alba* after treatment with dithizone. a-d, 0 µM Cd (a, root apex and elongation region; b, c, root maturation region; d leaf epidermal strips). e-i, 50 µM Cd; j-n, 100 µM Cd: Cd precipitates were detectable within root hairs (e, h, j, k), as well as in cortical cells (g, h, j, k, l), but never in the root apical and elongation region (f, m); leaf epidermal strips showed areas of Cd precipitates (i, n). Bars indicate 100 µm.

Fig.2. *In situ* cadmium localization in roots and leaves of *S. viminalis* after treatment with dithizone. a-e, 0 µM Cd (a, root apex; b, elongation region; c, d, root maturation region; e leaf epidermal strips). f-j, 50 µM Cd; k-o, 100 µM Cd: large Cd precipitates were detectable in both root apex and elongation region (f, h, k, m) as well as in the root maturation region (g, i, l, n); leaf epidermal strips showed areas of Cd precipitates (j, o). Bars indicate 100 µm.

Fig.3. Stomatal conductance recorded on leaves of *S. alba* (right) and *S. viminalis* (left) plants treated with 0, 50 and 100 µM Cd for 7 and 14 days. The values reported are the results of 12 measures per treatment. In the box plots, the lower boundary of the box indicates the 25th percentile, the line within the box marks the median and the upper boundary of the box indicates the 75th percentile. Whiskers above and below the box indicate the 10th and 90th percentiles. Filled points above or below the whiskers indicate outliers outside the 10th and 90th percentiles. Different letters denote significant differences among species and different treatments ($p<0.05$)

Fig.4. Operating (ΦPSII, left) and maximum potential (Fv/Fm, right) efficiency of photosynthesis of *S. alba* (right of each panel) and *S. viminalis* (left of each panel) plants. The values reported are the results of 24 measures per treatment. Different letters denote significant differences among species and different treatments ($p<0.05$). For details on the symbols, see Fig.3 caption

Fig.5. Hydrogen peroxide (above) and thiobarbituric acid reactive substances (TBARS, below) in roots and leaves of *Salix alba* and *S. viminalis* plants treated with 0, 50 and 100 µM Cd for 7 and 14 days. Values are the results of 9 measures per treatment. among species and different treatments ($p<0.05$). For details on the symbols, see Fig.3 caption

Fig. 6. Cross hand sections of roots of *Salix alba* and *S. viminalis* plants treated with 0, 50 and 100 µM Cd for 7 and 14 days stained for *in situ* detection of H_2O_2 by Amplex UltraRed reagent. Bars indicate 50 µm.

Fig. 7. Cross hand sections of roots of *Salix alba* and *S. viminalis* plants treated with 0, 50 and 100 µM Cd for 7 and 14 days stained for *in situ* detection of lipid peroxidation by BODIPY 581/591 C11. Bars indicate 50 µm.

Table 1. Cadmium content, bioconcentration factor (BCF) and translocation factor (TF) in roots and leaves of *Salix alba* and *S. viminalis* plants treated with 0, 50 and 100 µM Cd for 7 and 14 days. Values are means of triplicate ± SE. Different letters denote significant differences within each row at $p<0.05$

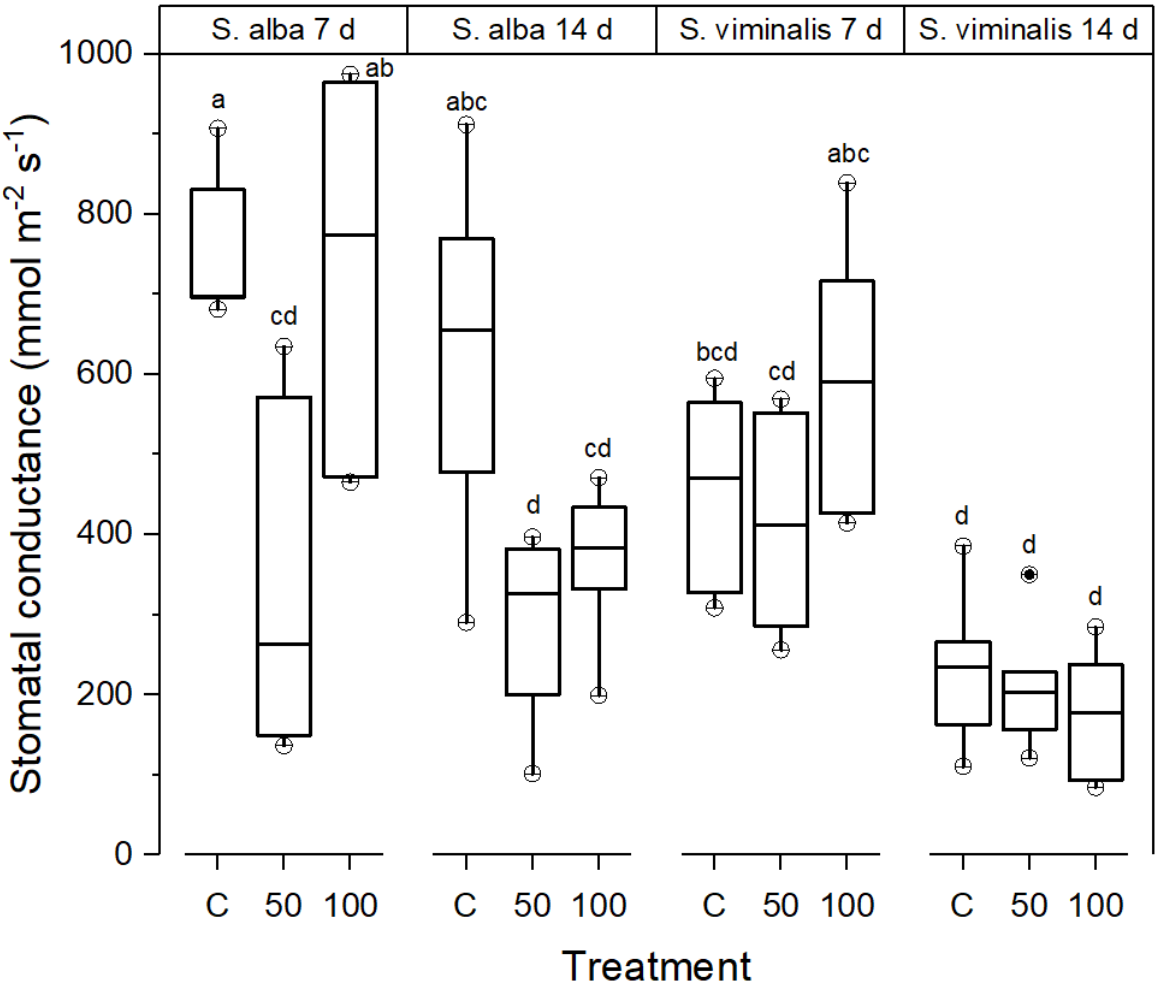
	Salix alba						*Salix viminalis*					
	7 days			14 days			7 days			14 days		
	0 µM Cd	50 µM Cd	100 µM Cd	0 µM Cd	50 µM Cd	100 µM Cd	0 µM Cd	50 µM Cd	100 µM Cd	0 µM Cd	50 µM Cd	100 µM Cd
Cd content in roots (mg Kg^{-1} DW)	BDL	1229.04±41.71 f	1296.34±57.38 f	BDL	2015.62±21.23 d	1631.56±15.51 e	BDL	2291.36±15.00 c	2039.84±9.04 d	BDL	5076.10±30.56 a	2977.77±24.13 b
Cd content in leaves (mg Kg^{-1} DW)	BDL	23.84±0.65 d	11.57±0.10 e	BDL	33.35±0.80 c	7.34±0.03 f	BDL	5.91±0.02d f	13.87±0.20 e	BDL	48.26±1.05 b	54.94±1.75 a
BCF in roots		218.69±9.01 e	115.33±5.10 h		358.65±3.79 c	145.16±1.38 g		407.71±2.67 b	181.48±0.80 f		903.22±5.44 a	264.92±2.15 d
BCF in leaves		4.24±0.14 d	1.03±0.01 ef		5.93± 0.14 b	0.65± 0.00 f		1.05±0.00 ef	1.23±0.02 e		8.59±0.19 a	4.89±0.16 c
TF		0.019±0.00 a	0.009±0.00 c		0.017±0.00 b	0.004±0.00 de		0.003±0.00 e	0.007±0.00 cd		0.009±0.00 c	0.018±0.00 ab

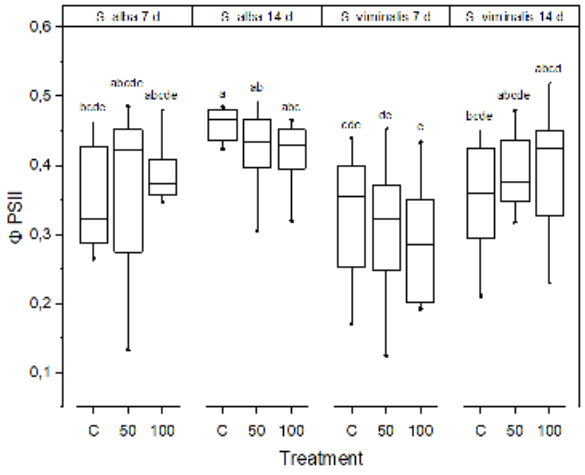
Table 2 Relative water content (RWC) and glutathione peroxidase (GPX), ascorbate peroxidase (APX), guaiacol peroxidase (POX), catalase (CAT) activities in roots of *Salix alba* and *Salix viminalis* plants treated with 0, 50 and 100 µM Cd for 7 and 14 days. Values are means of triplicate ± SE. Different letters denote significant differences within each row at $p<0.05$

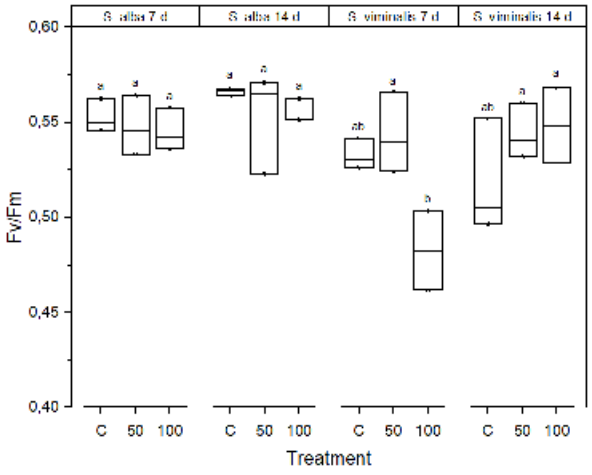
	Salix alba						*Salix viminalis*					
	7 days			14 days			7 days			14 days		
	0 µM Cd	50 µM Cd	100 µM Cd	0 µM Cd	50 µM Cd	100 µM Cd	0 µM Cd	50 µM Cd	100 µM Cd	0 µM Cd	50 µM Cd	100 µM Cd
RWC (%)	88.36±0.00 abcd	73.40±0.78 d	91.48±1.59 abc	84.18±0.01 abcd	99.70±0.30 a	90.78±6.41 abc	75.92±0.17 cd	82.28±4.81 bcd	93.04±5.66 ab	98.03±1.29 ab	74.48±3.70 cd	96.19±5.23 ab
GPX (U mg^{-1} protein)	1.97±0.06 e	1.70±0.03 e	3.25±0.05 b	2.72±0.03 bcd	1.69±0.02 e	1.74±0.01 e	2.83±0.40 bc	2.14±0.02 cde	5.71±0.04 a	1.69±0.02 e	2.08±0.15 de	2.90±0.17 b
APX (U mg^{-1} protein)	0.16±0.02 a	0.08±0.02 abcd	0.05±0.01 d	0.13±0.00 abc	0.02±0.01 d	0.07±0.01 bcd	0.08±0.01 abcd	0.05±0.01 d	0.07±0.02 bcd	0.11±0.05 abcd	0.08±0.02 abcd	0.16±0.02 a
POX (U mg^{-1} protein)	1.36±0.06 a	0.76±0.03 fg	1.07±0.04 bcd	0.93±0.02 cde	0.68±0.01 g	0.74±0.02 fg	1.08±0.02 bcd	1.34±0.02 a	1.23±0.01 ab	0.82±0.02 efg	1.08±0.08 bc	0.90±0.01 def
CAT (U mg^{-1} protein)	3.09±1.00 a	2.59±0.92 a	2.90±1.11 a	3.60±0.42 a	2.55±0.41 a	2.89±0.35 a	1.94±0.19 a	3.09±0.21 a	2.08±0.16 a	3.13±0.50 a	2.78±0.25 a	3.16±0.36 a

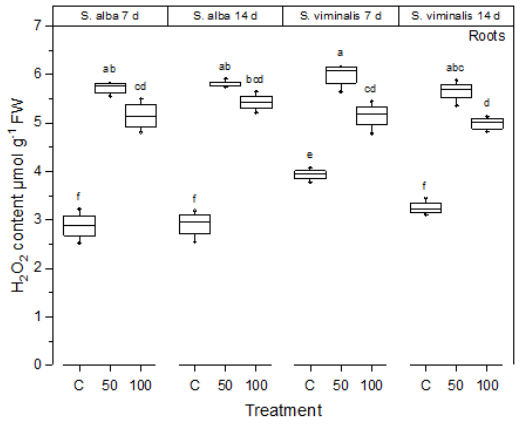
Table 3 Relative water content (RWC), total chlorophylls (total Chl), carotenoids, carotenoids/total chlorophyll ratio (Car/tot Chl) and glutathione peroxidase (GPX), ascorbate peroxidase (APX), guaiacol peroxidase (POX), catalase (CAT) activities in leaves of *Salix alba* and *Salix viminalis* plants treated with 0, 50 and 100 µM Cd for 7 and 14 days. Values are means of triplicate ± SE. Different letters denote significant differences within each row at $p<0.05$

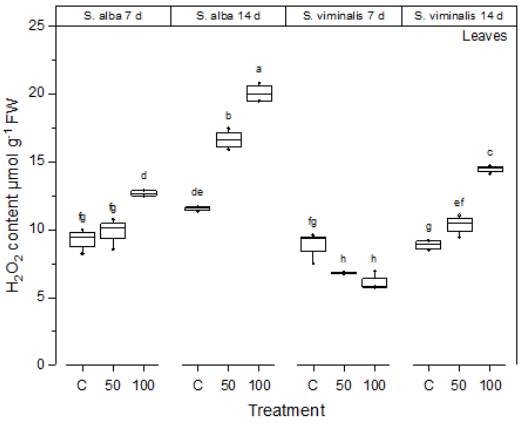
	Salix alba						*Salix viminalis*					
	7 days			14 days			7 days			14 days		
	0 µM Cd	50 µM Cd	100 µM Cd	0 µM Cd	50 µM Cd	100 µM Cd	0 µM Cd	50 µM Cd	100 µM Cd	0 µM Cd	50 µM Cd	100 µM Cd
RWC (%)	100.00±0.50 a	93.23±5.76 abc	88.17±1.06 c	95.26±0.92 abc	97.40±0.23 ab	95.60±0.68 abc	100.00±1.00 a	87.35±0.52 cd	78.35±0.27 d	100.00±0.48 a	97.31±0.69 ab	90.07±4.20 bc
Total Chl (mg g^{-1} FW)	1.86±0.13 a	0.63±0.24 de	1.32±0.11 c	1.79±0.14 ab	1.10±0.16 c	0.51±0.04 e	1.36±0.14 bc	1.06±0.23 cd	0.63±0.13 de	1.36±0.15 bc	1.09±0.17 c	0.57±0.05 e
Carotenoids (mg g^{-1} FW)	0.22±0.01 a	0.08±0.02 de	0.18±0.04 ab	0.19±0.01 ab	0.16±0.04 abc	0.08±0.00 de	0.09±0.01 cde	0.07±0.00 e	0.04±0.00 e	0.14±0.04 bcd	0.15±0.02 bc	0.10±0.01 cde
Car/tot Chl	0.12±0.01 abc	0.13±0.02 abc	0.14±0.04 ab	0.11±0.01 abc	0.14±0.05 a	0.16±0.00 a	0.07±0.02 bc	0.07±0.01 bc	0.06±0.01 bc	0.10±0.03 abc	0.14±0.00 abc	0.17±0.00 a
GPX (U mg^{-1} protein)	0.78±0.07 d	0.79±0.03 d	0.76±0.04 d	1.45±0.06 bc	1.64±0.04 ab	1.72±0.02 a	0.86±0.04 d	0.73±0.03 d	1.35±0.01 c	1.71±0.01 a	1.73±0.01 a	1.83±0.06 a
APX (U mg^{-1} protein)	0.21±0.01 a	0.17±0.02 abcd	0.18±0.02 abc	0.16±0.01 abc	0.14±0.01 bcd	0.11±0.03 cd	0.19±0.01 ab	0.17±0.00 bcd	0.13±0.01 bcd	0.10±0.01 d	0.08±0.01 d	0.10±0.01 d
POX (U mg^{-1} protein)	1.06±0.04 a	0.08±0.00 e	0.18±0.01 cd	0.18±0.01 cd	0.11±0.00 de	0.12±0.01 de	0.18±0.02 cd	0.11±0.00 de	0.21±0.02 c	0.47±0.04 b	0.16±0.00 cd	0.20±0.01 c
CAT (U mg^{-1} protein)	1.76±0.25 ab	3.13±0.26 ab	3.28±0.50 a	3.29±0.35 a	2.55±0.32 ab	3.14±0.20 ab	2.80±0.38 ab	1.73±0.22 b	3.18±0.18 ab	2.64±0.17 ab	2.82±0.28 ab	2.19±0.19 ab

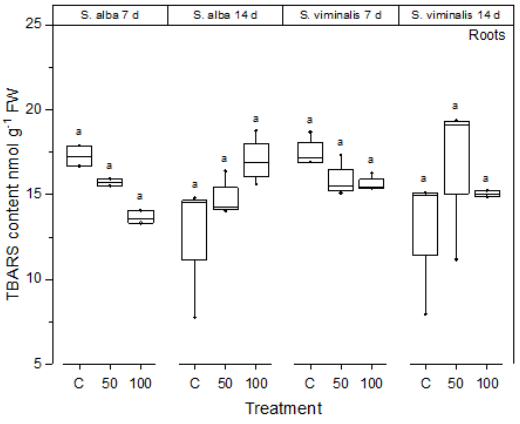


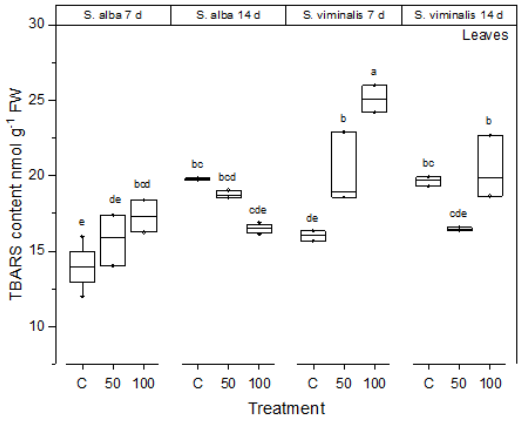


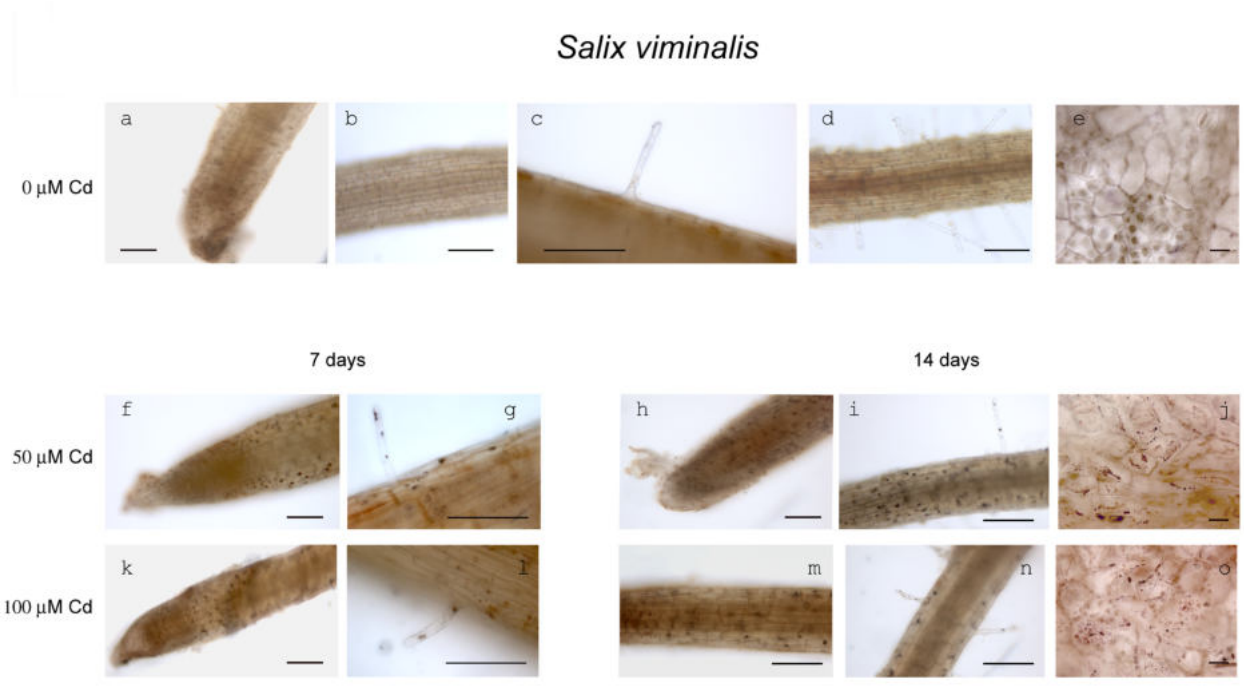


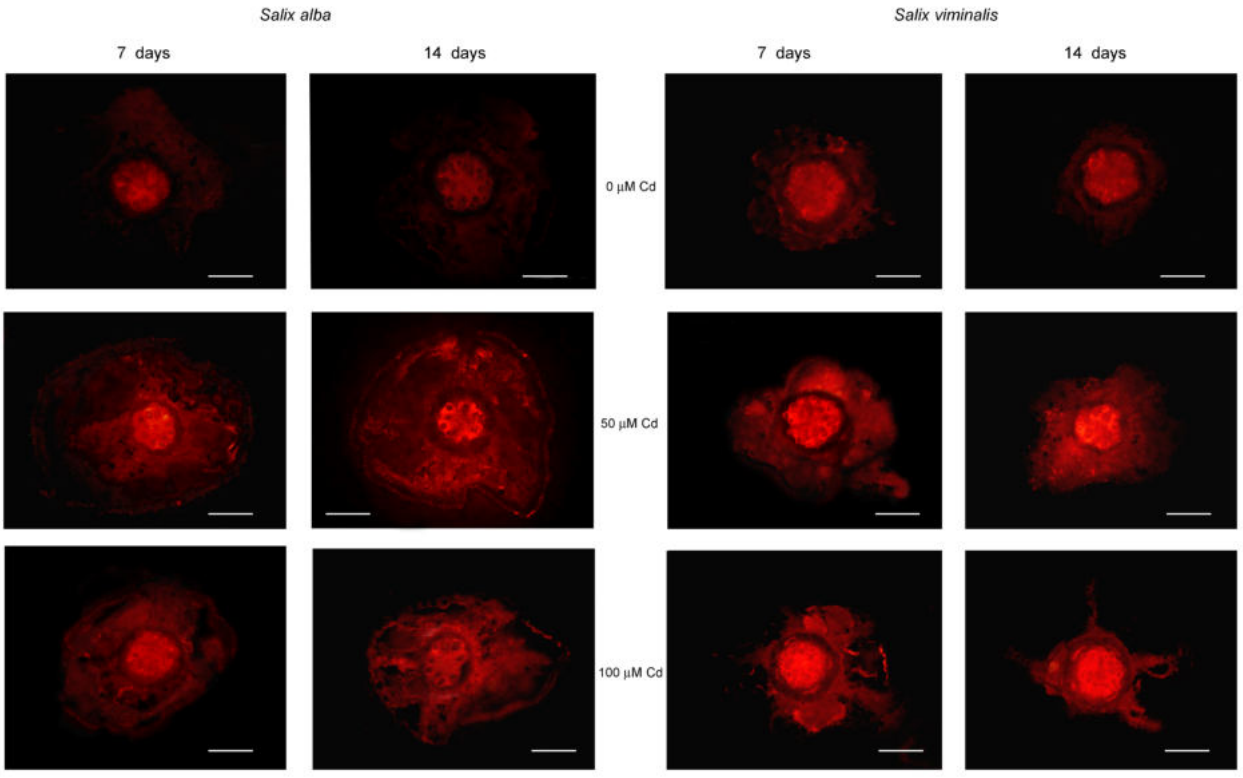


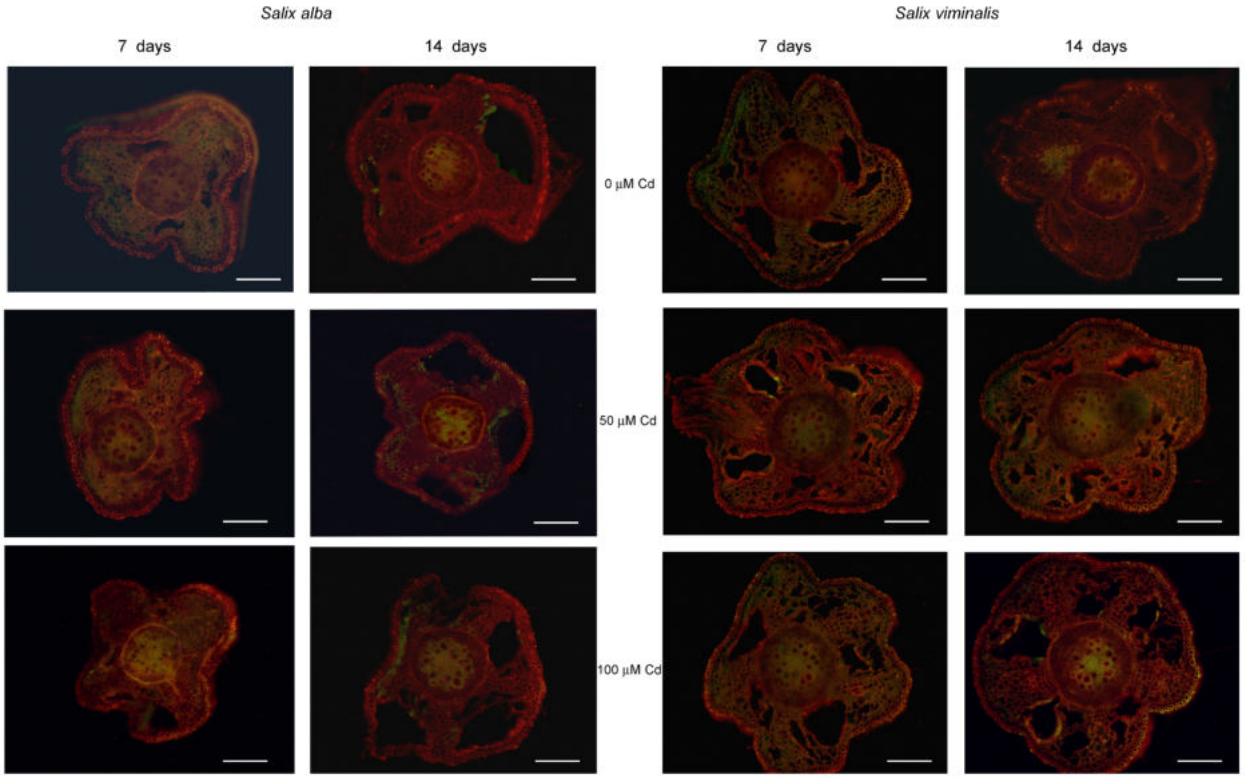












Highlights

- *Salix alba* and *S. viminalis* can efficiently remove cadmium from substrate
- Low translocation factor value indicates a compartmentalization strategy
- Cadmium treated plants had no photochemical limitations
- *S. viminalis*, able to uptake Cd also in root apical region, is a better accumulator
- In *S. viminalis* higher Cd content did not induce a higher level of oxidative stress

Contributions

Mouna Touati, Carmelina Spanò and Zoubeir Béjaoui conceived the original idea, Mouna Touati, Carmelina Spanò and Stefania Bottega performed biochemical analyses and the majority of experiments, Carlo Sorce carried out photochemical efficiency experiments and designed graphical artwork, Monica Ruffini Castiglione performed histochemical analyses. All authors discussed the results and wrote the manuscript