The final publication is available at De Gruyter via http://dx.doi.org/10.1515/znc-2016-0092

Running title: **Hardening under chronic cadmium stress in poplar**

Full title: **Stress hardening under long-term cadmium treatment depends on the activation of antioxidative defence and the iron acquisition of chloroplasts in *Populus***

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Abstract

Cadmium, a highly toxic heavy metal affects growth and metabolic pathways in plants, including photosynthesis. Though Cd is a transition metal with no redox capacity, it generates reactive oxygen species (ROS) indirectly and causes oxidative stress. Nevertheless, the mechanisms involved in long-term Cd tolerance of plants are not well known. Hydroponically cultured poplar (*Populus jacquemontiana* var. *glauca* cv. 'Kopeczkii') plants were treated with Cd for 4 weeks. Following a functional decline, the plants performed acclimation to the Cd induced oxidative stress evidenced by the decreased leaf malondialdehyde content and the recovery of most photosynthetic parameters. Activation of peroxidases played a key role in the elimination of hydrogen-peroxide, and thus the recovery of photosynthesis, while the role of superoxide dismutase isoforms was less important. Re-distribution of the iron content in leaf mesophyll cells resulted in an increase of the Fe content of chloroplasts, thus contributed to the biosynthesis of the photosynthetic apparatus and some antioxidative enzymes. The delayed increase in photosynthetic activity compared to the decline in the level of lipid peroxidation indicates the energy requirement of acclimation mechanisms during long-term Cd treatment.

Abbreviations: APX – ascorbate peroxidase; BPDS – bathophenantroline disulphonate; CAT – catalase; Chl – chlorophyll; DEEPS – de-epoxidation state of xanthophyll cycle pigments; $\Phi_{f,D}$ – fluorescence/thermal dissipation of the absorbed energy; Φ_{NF} – the thermal dissipation by inactive PSII centres; Φ_{NPQ} – ΔpH dependent, xanthophyll-cycle coupled non-photochemical quenching; Φ_{PSII} – the photochemical efficiency of functional PSII centres; MDA – malondialdehyde; NPQ – non-photochemical quenching; POD – peroxidase; PS –

photosystem; ROS – reactive oxygen species; SOD – superoxide dismutase; VAZ – violaxanthin-antheraxanthin-zeaxanthin.

Keywords: cadmium; oxidative stress; photosynthesis; *Populus*; stress hardening

Introduction

Many cultivated plant species have to cope with increased soil cadmium concentration due to anthropogenic activities, such as deposition of industrial wastes (galvanic batteries, dyes), usage of chemical fertilizers in the intensive agricultural production and zinc mining, where Cd is a by-product. Cd contamination has increasing importance because of its toxicity to plants as well as the high risk to human health [1]. In plants, Cd causes various damages (for review, see: [2]). For instance, it disturbs the metal homeostasis by causing, among others, iron deficiency in the shoot [3,4]. As a result of the induced Fe deficiency, the biosynthesis of chlorophylls (Chl), the formation of Chl-protein complexes and the development of thylakoid membranes are highly disturbed [5,6]. Particularly, Cd was shown to selectively inhibit the biogenesis and the activity of photosystem II (PSII) reaction centres [7], and retard CO_2 fixation [8]. Once photosynthesis becomes inactivated the generation and accumulation of reactive oxygen species (ROS) is elevated. Cd causes oxidative damages in the shoot [9]. In the chloroplasts, one of the most important targets of ROS is the D1 protein in PSII. Under mild stress conditions, damaged D1 proteins are replaced with new ones resulting in operating PSII complexes. Under constant level of stress, however, the capacity to repair is not high enough to replace all the inactivated centres [10]. In addition to the non-photochemical quenching (NPQ) processes in the antennae, these inactivated reaction centres are strong quenchers of the excited state of the antennae that may protect the neighbouring active PSII complexes [11,12].

In aerobic cells, ROS are common by-products of biochemical processes but a well-equipped antioxidative system is also evolved to maintain the redox equilibrium. The elimination of ROS in mesophyll cells has several pathways, such as the water-water cycle in the chloroplasts operated by superoxide dismutase (SOD) and ascorbate peroxidase (APX)

isoforms [13]. Moreover, catalases [CAT] and glutathione peroxidases located in the peroxisomes [14] also contribute to the protection against oxidative stress. In parallel to the occurrence of damages, organisms may change their defence mechanisms influencing the amount and activity of players leading to the restoration of vital functions. Cd, which is able to provoke the disruption of redox equilibrium, has strong effects on the antioxidative defence [14,15]. Moderate Cd stress increased the amount of defence metabolites, and influenced the activation of several antioxidant enzymes in *Phaseolus vulgaris* [15]. While the activity of glutathione reductase [GR], peroxidases [POD], CAT and APX increased under Cd stress, PODs, which need iron for their hem cofactors, showed reduced activity [16].

Poplar species are good candidate for Cd phytoremediation [17]. Since Cd phytoremediation requires plants that perform long-term resistance against Cd stress, the understanding of the defence mechanisms that helps the plant to overcome the acute stress symptoms is essential. Nevertheless, the connection between the alleviation of Cd-induced photosynthesis inhibition and the activity of protective mechanisms is poorly known in poplars. Therefore, we studied here the changes in the excitation energy quenching mechanisms and in the activity of antioxidant enzymes in detail under long-term, moderate Cd stress.

Materials and methods

Plant material

Experiments were performed on micropropagated poplar (*Populus jacquemontiana* var. *glauca* (Haines) Kimura cv. 'Kopeczkii') plants, grown in climatic chamber [14/10 hours light (100 µmol photons m^{-2} s^{-1})/dark periods, 24/22 °C and 70/75% relative humidity] in hydroponics of quarter-strength Hoagland solution [1.25 mM $Ca(NO_3)_2$, 1.25 mM KNO_3, 0.5

mM $MgSO_4$, 0.25 mM KH_2PO_4, 0.08 μM $CuSO_4$, 4.6 μM $MnCl_2$, 0.19 μM $ZnSO_4$, 0.12 μM Na_2MoO_4, 11.56 μM H_3BO_3 and 10 μM $Fe^{(III)}$-citrate as iron source]. Control plants (Ctrl) and plants treated with 10 μM $Cd(NO_3)_2$ from their four-leaf-stage (Cad plants) were investigated during a four-week period . Data were collected on the 6^{th} leaves, emerged during the time of treatment.

Determination of element concentrations

Dried (one week at 60 °C) leaves were digested by HNO_3 for 30 min at 60 °C, and then in H_2O_2 for 90 min at 120 °C. After filtration by MN 640W paper ion contents were measured by ICP-MS (Inductively Connected Plasma Mass Spectrometer, Thermo-Fisher, USA).

For measurement of chloroplast iron content, intact chloroplasts were isolated, and iron content was measured photometrically as $[Fe(BPDS)_3]^{4-}$ complex at 535 nm by UV-VIS spectrophotometer (Shimadzu, Japan) as described in Sárvári *et al*. [18]. An absorption coefficient of 22.14 mM^{-1} cm^{-1} for the $Fe^{(II)}$-BPDS complex [19] was used for calculations. Chloroplast density was determined by counting in a Nikon Optiphot-2 microscope equipped with Nikon *D*70 DSLR camera.

Pigment contents

Chlorophyll contents were determined in 80% (v/v) acetone extracts by a UV-VIS spectrophotometer (Shimadzu, Japan) using the absorption coefficients of Porra *et al*. [20].

For the determination of xanthophyll cycle components, leaf discs adapted to 100 μmol photons m^{-2} s^{-1} or kept in dark for 30 minutes were used. Carotenoid components were separated by HPLC method and quantified using zeaxanthin standard [20,21]. The de-epoxidation state of xanthophyll cycle pigments (DEEPS) was calculated as (DEEPs=Z+0.5A)/(V+A+Z) and diurnal change as $\Delta DEEPS=DEEPS_{light}-DEEPS_{dark}$.

Chlorophyll *a* fluorescence induction

Fluorescence induction measurements were performed using a PAM 101-102-103 Chlorophyll *a* Fluorometer (Walz, Effeltrich, Germany) as in Solti *et al*. [22]. Maximal efficiency of PSII centres were determined as $F_v/F_m=(F_m-F_0)/F_m$. For excitation energy allocation experiments, the maximal quantum efficiencies of the corresponding Ctrl leaves were used as reference value (F_{vM}/F_{mM}). Parameters were determined according to Hendrickson *et al*. [23] modified in the application by Solti *et al*. [24]:

$$\Phi_{PSII} = (1-\frac{F_t}{F_m'})*(\frac{F_v/F_m}{F_{vM}/F_{mM}}); \qquad \Phi_{NPQ} = (\frac{F_t}{F_m'}-\frac{F_t}{F_m})*(\frac{F_v/F_m}{F_{vM}/F_{mM}});$$

$$\Phi_{f,D} = (\frac{F_t}{F_m})*(\frac{F_v/F_m}{F_{vM}/F_{mM}}); \qquad \Phi_{NF} = 1-(\frac{F_v/F_m}{F_{vM}/F_{mM}});$$

where $\Sigma E_{exc}=\Phi_{PSII}+\Phi_{NF}+\Phi_{NPQ}+\Phi_{f,D}=1$.

Oxidative damages and antioxidative defence

Lipid peroxidation was estimated by measuring the malondialdehyde (MDA) content according to Heath and Packer [25]. 100 mg leaf samples were homogenized in 1 ml 0.1% (v/v) trichloroacetic acid and centrifuged at 15,000 ×*g* for 15 min. Equal volume of 20% (v/v) trichloroacetic acid and 0.5% (w/v) thiobarbiturate was added to the supernatant and incubated at 90 °C for 30 min. MDA content was determined by measuring the absorption at 532 nm (ε = 155 mM^{-1} cm^{-1}) by UV-VIS spectrophotometer (Shimadzu, Japan).

The activity of ascorbate peroxidase (APX, L-ascorbate: H_2O_2 oxidoreductase, EC 1.11.1.11) was measured according to Nakano and Asada [26]. 100 mg leaf samples were homogenized in 1 ml isolating buffer (50 mM phosphate buffer [pH 7.0], 1.0 mM EDTA, 0.1% (w/v) Triton X-100, 5 mM ascorbic acid, 2 mM PVP) at 0 °C. Reaction mixture contained 50 mM phosphate buffer [pH 7.0], 0.1 mM H_2O_2, 0.5 mM ascorbic acid, 0.1 mM EDTA and 100 µl

crude enzyme extract. Reaction was started by adding H_2O_2. Enzyme activity was measured by following the absorption decrease of ascorbate at 290 nm (ε = 2.8 mM^{-1} cm^{-1}) at 25 °C by UV-VIS spectrophotometer (Shimadzu, Japan).

The activity of superoxide dismutase (SOD, EC 1.15.1.1) was measured according Giannopolitis and Ries [27] with some modifications. 100 mg leaf samples were homogenized on ice in 1 ml isolating buffer (50 mM phosphate buffer [pH 7.0], 1.0 mM EDTA, 0.1% (w/v) Triton X-100, 2 mM PVP) and centrifuged at 15,000 ×*g* for 15 min, and the supernatants were collected as crude extract. Samples were solubilised mildly (5 mM Tris-HCl, pH 6.8, 0.01% SDS, 10% glycerol and 0.001% bromophenol blue). SOD isoforms were separated by native PAGE using 10–18% gradient acrylamide gels by Laemmli [28] containing only 0.01% SDS. Gels were negatively stained for SOD activity in 50 mM phosphate buffer [pH 7.8], 0.1 mM EDTA, 13 mM methionine, 60 μM riboflavin, 2.25 mM Nitro Blue Tetrazolium. Activity stained gels were scanned using an Epson Perfection V750 PRO gel scanner and evaluated by densitometry using the Phoretix software (Phoretix International, Newcastle upon Tyne, UK).

To extract and separate peroxidases (POD; EC 1.11.1.7), the same procedure was done as described for the SOD isoforms. POD isoforms were stained by incubating the native gels in 50 mM acetate buffer (pH 4.5), containing 2 mM benzidine (dissolved in DMSO). The reaction was initiated by adding 3 mM H_2O_2 and allowed to continue for 30 min [29]. Bands were evaluated by densitometry using the Phoretix software as above.

To measure protein content of samples, solubilised proteins (in 5 mM Tris-HCl, pH 6.8% SDS, 2% dithiothreitol, 10% glycerol and 0.001% bromophenol blue) were run on 10–18% gradient acrylamide gels containing 0.1% SDS [28]. Protein content was calculated by comparing the density of protein samples to that of protein standards of known protein content in Coomassie-stained gels.

Statistical analysis

Enzymatic activities, pigment and element contents were measured as 2 replicates per 3–4 independent experiments. Fluorescence induction measurements were performed on the same 4–5 plants during the experimental period in 3 independent experiments. Unpaired Student's t-tests and one-way ANOVAs with Tukey-Kramer *post-hoc* tests were performed on data. The term 'significantly different' means, that the similarity of samples is $p<0.05$.

Results

Leaf development

Investigations were performed on 6th leaves developed during the treatment period. The rate of leaf growth both in weight and area began to decline in leaves of Cad plants from the beginning of the treatment. Cad and Ctrl 6th leaves reached their maximal size to the 9–10th and 10–11th day of treatment, respectively, thus no further growth was found (the maximal size of Cad leaves were 58.1±2.3 cm^2 and 518.6±20.6 mg fresh weight and those of Ctrl leaves were 129.1±6.1 cm^2 and 785.7±168.26 mg fresh weight, respectively). In Ctrl leaves, the dry weight growth had a similar run to that of the fresh weight (reaching 124.0±6.9 mg at the 11th day). However, in Cad leaves, increase in the leaf dry weight did not stop together with leaf expansion but continued in the second and third week of treatment, and increased from 52.0±1.7 mg (7th day of treatment) to 88.9±1.2 mg (21st day of treatment).

Leaf metal content

The concentration of Cd reached 268.0±45.9 µg g^{-1} dry weight (DW) in Cad leaves by the 7th day and 373.0±43.8 µg g^{-1} DW by the 21st days of treatment (Fig. 1A), whereas Ctrl leaves

contained less than 3 µg Cd g^{-1} DW by the end of the investigated period. Concerning the essential transition metals, the Fe concentration of leaves was strongly affected by Cd treatment (Fig. 1A): it decreased to 45.0±3.3% of the Ctrl at the 9th day. It did not change significantly during the further treatment period. The concentration of Mn in the Cad samples was hardly affected, i.e. it decreased a little but did not differ significantly from that of the Ctrl during the experimental period. The concentration of Zn increased under Cd treatment (to 175.3±17.5% of Ctrl by the end of the third week).

Iron content of chloroplasts

As a result of acute Cd stress, the Fe accumulation of chloroplasts was strongly retarded (to around 60%) compared to those of the Ctrl, the Fe contents of Cad and Ctrl leaves were 0.41±0.04 and 0.67±0.04 femtomol (fmol) Fe chloroplast^{-1}, respectively. Nevertheless, Fe content of chloroplasts isolated from Cad leaves started to increase from the end of the first week of treatment (Fig. 1B). It is important to mention that there was a Fe accumulation in Ctrl chloroplasts, but the increase in Fe content was more intensive in the Cad chloroplasts. Thus, by the end of the 4th week of treatment, the Fe content in the Cad chloroplasts reached around 80% of the Fe content of the Ctrl chloroplasts (1.23±0.05 fmol Fe chloroplast^{-1}).

Photosynthetic pigment content

In Ctrl leaves, the Chl concentration stagnated or slightly increased from the middle of the second week of treatment (Fig. 2A) as a result of their transformation into shade leaves. At the same time, the Chl concentration of Cad leaves was strongly reduced in the first two weeks, but an increasing trend was observed in the third week. Nevertheless, their Chl concentration did not reach the level of not-shaded Ctrl leaves (their difference was significant at the end of the fourth week of treatment).

Cd treatment also affected the Chl *a*/*b* ratio (Fig. 2B). While the Chl *a*/*b* ratio increased in Ctrl leaves in the first week, and some decreasing trend was found from the second week, the initially increasing trend stopped soon in Cad leaves, and turned into decrease during the first and second weeks. However, the relative amount of Chl *a* increased in Cad leaves during the third-fourth week period resulting in an elevated Chl *a*/*b* ratio which did not differ from that of the fully developed (7 day-old) Ctrl (Fig. 2B).

Concerning the carotenoids, the relative amount of *β*-carotene showed a decreasing trend in Ctrl plants under the time of treatment, whereas an increase occurred from the third week in Cad plants (Fig. 2C). Cd treatment had little effect on the relative amount of xanthophylls during the treatment period (Table 1), lutein and VAZ decreased in both Ctrl and Cad plants, but the decrease was less pronounced in Cad plants. ΔDEEPS, indicating the light-inducible de-epoxidation of violaxanthin, increased in Cad plants from the second week to the end of the investigated period (Table 1), thus it became significantly higher than the Ctrl value by the end of third week.

Photochemical efficiency and excitation energy allocation

During one week Cd treatment, the maximal quantum efficiency of PSII reaction centres (F_v/F_m) decreased continuously up to the middle of the second week but it turned into an increase thereafter until it reached the level of Ctrl plants. The F_m level was more affected (from 0.661±0.081 at the 9th day to 0.885±0.105 at the 21st day), the values of F_0 was less variable (from 0.316±0.057 at the 9th day to 0.220±0.026 at the 21st day). Ctrl plants did not show significant changes during the experimental time, F_0 and F_m values were stable (F_0: 0.190±0.015 and F_m: 0.920±0,096).

Concerning the excitation energy allocation, no significant changes were found in Ctrl plants except the decrease of Φ_{NPQ} in the third week of treatment. In Cad plants, the changes in the

Φ_{PSII} were similar to that of F_v/F_m (Fig. 3A). Φ_{NF} changed in opposite compared to Φ_{PSII} in Cad plants, while did not differ significantly from zero in Ctrls (Fig. 3C). Φ_{NPQ} showed an increasing trend from the second half of the first week, and exceeded the level of Ctrl from the second week (Fig. 3B). The fluorescence and constitutive heat dissipation ($\Phi_{f,D}$) rose in the initial phase of Cd treatment and only showed a trend of decrease during the fourth week of treatment (Fig. 3D).

Oxidative stress and antioxidative defence

The concentration of MDA strongly increased in Cad plants compared to the Ctrls. It reached 241.0±19.8% of the well-developed Ctrl up to the end of the second week of treatment (Fig. 4A). However, it decreased in the third and fourth week, the end of which the difference became not significant.

Cd treatment decreased the APX activity in the acute phase of the stress but the activity started to increase from the first week. Thus, the APX activity became significantly higher in Cad leaves compared to the unchanged activity of Ctrl leaves in the third and fourth week (Fig. 4B).

Cd treatment decreased the activity of SOD in general (Table 2). Nevertheless, it showed two-week periodical changes in time: there was no significant difference between Ctrl and Cad plants at the 14^{th} and 29^{th} day of treatment, but, SOD activity was significantly lower in the remaining experimental time. In native gels, four SOD isoforms were separated (Fig. 5). The activities of isoforms I III, and IV were the most sensitive to Cd treatment (Fig. 6A–C), while any significant differences from the Ctrl was not detected in the activity of the isoform II (not shown). Periodical changes were found only in the activity of the isoform III: the trend of changes was the same in Cad and Ctrl leaves, but there was an about five-day delay of appearance of activity maxima in Ctrl compared to Cad leaves.

In contrast to SOD activity, the activity of POD was elevated by Cad treatment in general (Table 2). The POD activity increased nearly continuously in Cad leaves during the experimental time. On native gels, 12 POD isoforms were identified (Fig. 5), three of them (I, II and VII) were not present in the first two weeks of treatment, and the amount of all isoforms increased during the hardening period (from the second week). Cd treatment affected the activity of the isoforms differently. Four major groups can be distinguished. In the first group (isoforms I, III and IV), the activity did not differ significantly in Cad and Ctrl leaves (not shown). In the second group (isoforms VIII, X, XII and partially V), the activity was higher in Cad leaves in the first two weeks of treatment, but did not differ significantly from that of the Ctrl leaves in the last two weeks (Fig. 7A). In the third group (isoforms II, VI and VII), the activity did not differ significantly from the Ctrl in the first two weeks but was significantly lower in the last two weeks (Fig. 7B). In the fourth group (isoforms IX and XI), the activity was significantly higher in Cad leaves during the whole experimental time (Fig. 7C).

Discussion

Poplar plants were able to survive long-term exposure to Cd in the nutrient solution. Since Cd induces acute damages, the long-term survival requires inducible defence mechanisms against the direct and indirect effects of Cd.

Metal re-distribution and hardening

Cd is known to cause many of its effects indirectly, through influencing metal distribution [3,28]. Cd treatment strongly reduced the concentration of Fe, slightly decreased that of the Mn, but increased the amount of Zn in leaves of poplar. Since severe Mn deficiency produces

similar effects to Cd [30], it can contribute to the Cd caused inhibition of photosynthesis, particularly the PSII activity. In turn, increased Zn content of Cad leaves may be connected to the Cd induced transcription of root Zn transporter ZIP9 [31].

From the point of view of both damage and hardening the most important is, indeed, the Cd induced Fe deficiency in leaves [3,4] and also in the chloroplasts [18]. In fact, the translocation of Fe from roots to the shoots was negligible here, during the whole experimental period. It may be connected with the fact that Cd is known to down-regulate the expression of the root xylem parenchyma citrate transporter FRD3, which plays a key role in the citrate efflux in the xylem and thus in the root-to-shoot Fe translocation [32]. Since the Fe translocation did not restore under the longer term Cd stress, the later recovery in some Fe-requiring physiological parameters indicates, indeed, the re-distribution of Fe in the mesophyll cells that also involves the allocation of Fe into the chloroplasts. The Fe acquisition of chloroplasts can also be monitored by the slow increase in the Chl content [33], and the recovery of photochemical activity.

Changes in the excitation energy allocation during hardening

Acute Cd stress inhibited Chl accumulation, decreased Chl *a/b* ratio due to its effects on the developmental stability and activity of reaction centres but did not significantly changed the carotenoid/Chl ratio. Together with the developmental disturbances in thylakoids, the F_v/F_m, i.e. the maximal quantum efficiency of PSII centres, also dropped down due to the alterations in both F_0 and F_m. Since Cd inhibits the electron transport in PSII reaction centres [7] the excitation energy transfer to molecular oxygen can increase leading to damages in the D1 protein. The increase of $\Phi_{f,D}$ together with the elevated level of F_0 indicated the loosing connection between the PSII reaction centres and the antennae [34]. Nevertheless, F_m was more affected in this period indicating an inhibition-based non-photochemical quenching of

the reaction centres [23]. The strongly increased Φ_{NF} together with the decreased F_m and Φ_{PSII} means that the quenching of the inactive reaction centres become one of the most important excitation energy quenching mechanisms in the acute period of Cd stress. The diminished function of PSII, the decreased synthesis of PSI centres together with the developing oxidative stress shown by the increasing MDA-content (Fig. 4A) can be a self-enhancing driving force in the formation of quenching centres.

In the hardening period, the opposite trend of decrease in PSII inactivation and increase in PSII quantum efficiencies indicates the recovery of photosynthetic activity. Whereas the concentration of Fe did not grow significantly in the leaves, the chloroplast-directed re-distribution of Fe may have had special importance in the hardening. Any increase in the chloroplast Fe content results in an increase of strongly Fe-dependent processes such as biosynthesis of Chls and formation of Fe-containing PSI complexes [22]. In addition to the increase in Φ_{PSII}, the observed increase in the *β*-carotene/Chl and Chl *a/b* ratios indicates, in fact, a *de novo* accumulation of reaction centres. The elevated concentration of zeaxanthin under light conditions contributed to the increase of Φ_{NPQ}. Nevertheless, it has to be noticed that the ΔpH-dependent quenching was low during the whole experiment even in Ctrl samples because of the applied low light intensity (Cd treated plants are light sensitive, see: [35]).

Antioxidative defence

Cd was shown to accumulate in the cytoplasm and in the cell walls of *Populus* × *canescens* leaves [36]. Since Cd cannot take part in Fenton reactions, it generates an oxidative burst indirectly, by interacting with the signalling pathway of calmodulin/Ca^{2+}-dependent protein kinases [37]. Peroxidases are also important in scavenging H_2O_2, especially in the cell wall. In fact, the relatively fast induction of some peroxidase isoforms in the high mobility region upon the start of Cd stress indicates that they may be in the first line of protection against

ROS. Marmiroli *et al*. [38] showed that Peroxidase 5 is among the first proteins that are induced by Cd treatment in *Populus* spp. Since all class III peroxidases contain hem prosthetic group, the limited availability of Fe may have also contributed to the alterations in their activity. Acidic peroxidases, which have partial role in lignin synthesis [16] did not show inactivation. Moreover, the high mobility, and potentially acidic isoforms showed increased activity under Cd stress. In fact, they may also have a role in increase of leaf dry weight and also in the accumulation of phenolic compounds [34,35].

During long-term Cd treatment, poplar plants could overcome on oxidative damages. Thus, following the acute phase of the Cd stress, lipid peroxidation decreased, while the trend of decrease in the MDA content was similar to that of Φ_{NF} indicating the involvement of ROS both in development of membrane damages and in PSII inactivation. According to Giacomelli *et al*. [39], APX and SOD isoforms have importance to eliminate ROS in chloroplasts. The rise in the activity of APX was observed in parallel to the increase of Φ_{PSII} and to the decline of Φ_{NF}. The separated SOD isoforms can be identified as I: MnSOD, II: Cu/ZnSOD, III: FeSOD and IV: Cu/ZnSOD [40,41]. Since, in contrast to the MnSOD, that is present in peroxisomes, mitochondria and cell wall [42], the activity of chloroplast Cu/ZnSOD isoforms (II and IV) did not increase during the experiment, their role in Cd stress alleviation may be less important in poplar. Periodic changes in the activity of the isoform III, identified as FeSOD, might be a result of re-distribution of intracellular Fe content. FeSOD is only present in the chloroplasts [42] thus any changes in its activity are strongly connected to the Fe content of chloroplasts. In Cad leaves, maximum activity of FeSOD appeared when APX activity reached the level of Ctrl leaves and Φ_{NF} started to decline, indicating that the FeSOD isoform has an importance in hardening. Thus, elimination of ROS *via* water-water cycle in chloroplasts [13] could have induced a self-enhanced process: the decreased amount of ROS

allowed an elevated ratio of non-inhibited PSII reaction centres and increasing photosynthetic activity probably facilitated the production of reducing capacity for ROS elimination.

Conclusion

In poplar, the long-term Cd treatment induces hardening that lead to an alleviation of the acute damages evidenced by the decrease in leaf MDA content and recovery of PSII activity. Activation of antioxidative enzymes, especially POD and APX seems to play a role in the elimination of ROS. The re-distribution of leaf Fe content and the allocation of Fe to the chloroplasts in particular, promote the recovery of photosynthetic structures and activity, which, in fact, also contributes to the reductant requirement of the antioxidative defence.

Acknowledgments

We would like to thank Zsuzsanna Ostorics for her technical assistance. This work was supported by the National Office for Research, Development, and Innovation, Hungary (NKFIH, PD-112047). Á. Solti was also supported by the Bolyai János Research Scholarship of the Hungarian Academy of Sciences (BO/00207/15/4).

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Table 1: Xanthophyll contents (mmol mol^{-1} Chl $a+b$) of Ctrl and Cad leaves in the acute (7^{th} day – 7d) and in the hardening phase (21^{st} day – 21d) of the stress. ΔDEEPS – light minus dark de-epoxidation index, i.e. light-induced de-epoxidation. One-way ANOVAs with Tukey-Kramer *post-hoc* tests were performed on data ($p<0.05$).

		Ctrl	**Cad**
lutein	7d	142.3±*32.6*^{a}	159.3±*21.5*^{b}
	21d	111.8±*16.6*^{ab}	139.6±*10.2*^{ab}
VAZ	7d	32.8±*10.5*^{a}	41.9±*10.3*^{a}
	21d	27.4±*4.5*^{a}	31.6±*5.4*^{a}
ΔDEEPS	7d	0.283±*0.044*^{ab}	0.359±*0.050*^{b}
	21d	0.183±*0.106*^{a}	0.744±*0.159*^{c}

Table 2. SOD and POD activities in Ctrl and Cad leaves. Values are given in percentage of the activity of full-grown Ctrl leaves (SOD: 6565.66±594.03 pixel density μg^{-1} protein; POD: 39837±9337 pixel density μg^{-1} protein). One-way ANOVAs with Tukey-Kramer *post-hoc* tests were performed on data ($p<0.05$).

	SOD		POD	
	Ctrl	**Cad**	**Ctrl**	**Cad**
7d	94.6±*18.8*^{b}	36.3±*25.0*^{a}	110.6±*14.8*^{b}	58.7±*22.7*^{a}
14d	121.6±*14.9*^{bc}	98.5±*8.2*^{b}	89.4±*14.8*^{a}	258.5±*69.2*^{c}
21d	96.4±*17.6*^{b}	64.8±*14.7*^{ab}	477.2±*109.9*^{d}	365.2±*56.7*^{c}
29d	126.9±*21.8*^{bc}	111.4±*2.1*^{c}	307.0±*79.0*^{c}	593.5±*87.0*^{e}

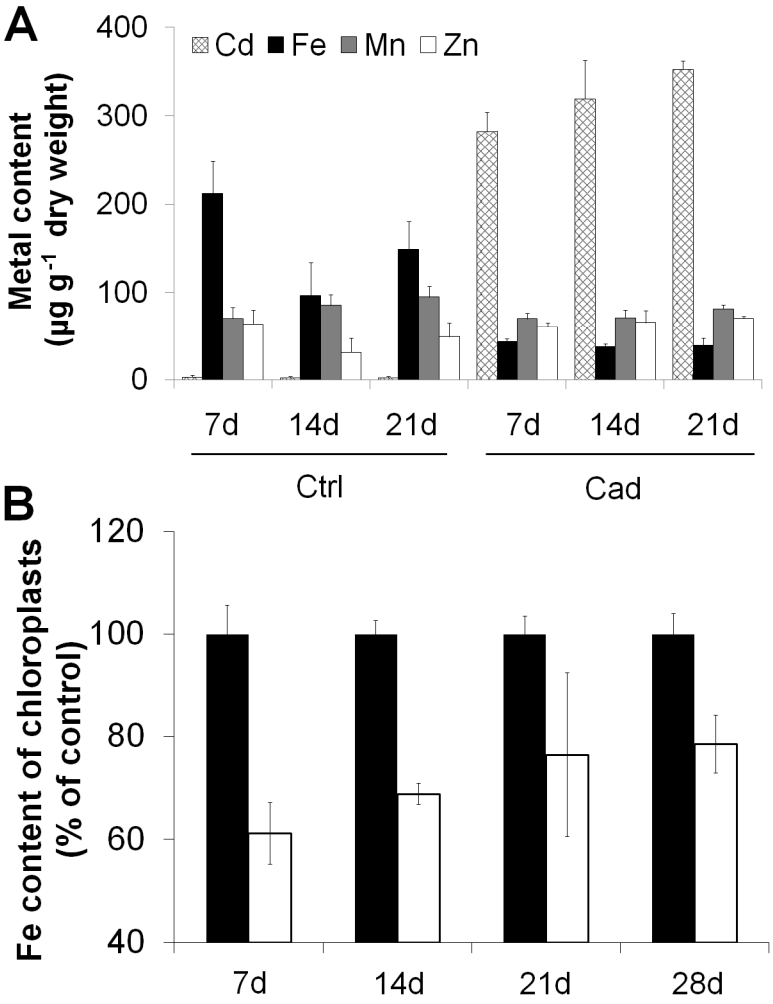

Figure 1. (A) Cd, Fe, Mn and Zn content (striped, black, grey and open columns, respectively) in Ctrl and Cad leaves in the acute phase (9th day), inflexion point (16th day) and hardening phase (20th day) of the Cd stress. (B) Fe content of the Ctrl (filled marks) and Cad chloroplast (open marks) in the acute and hardening phase of Cd stress. Cad values are expressed in the percentage of the corresponding Ctrl (0.67±0.04; 0.77±0.02; 1.13±0.04 and 1.23±0.05 fmol Fe chloroplast^{-1} at the 7th, 13th, 21st and 27th day of treatment, respectively).

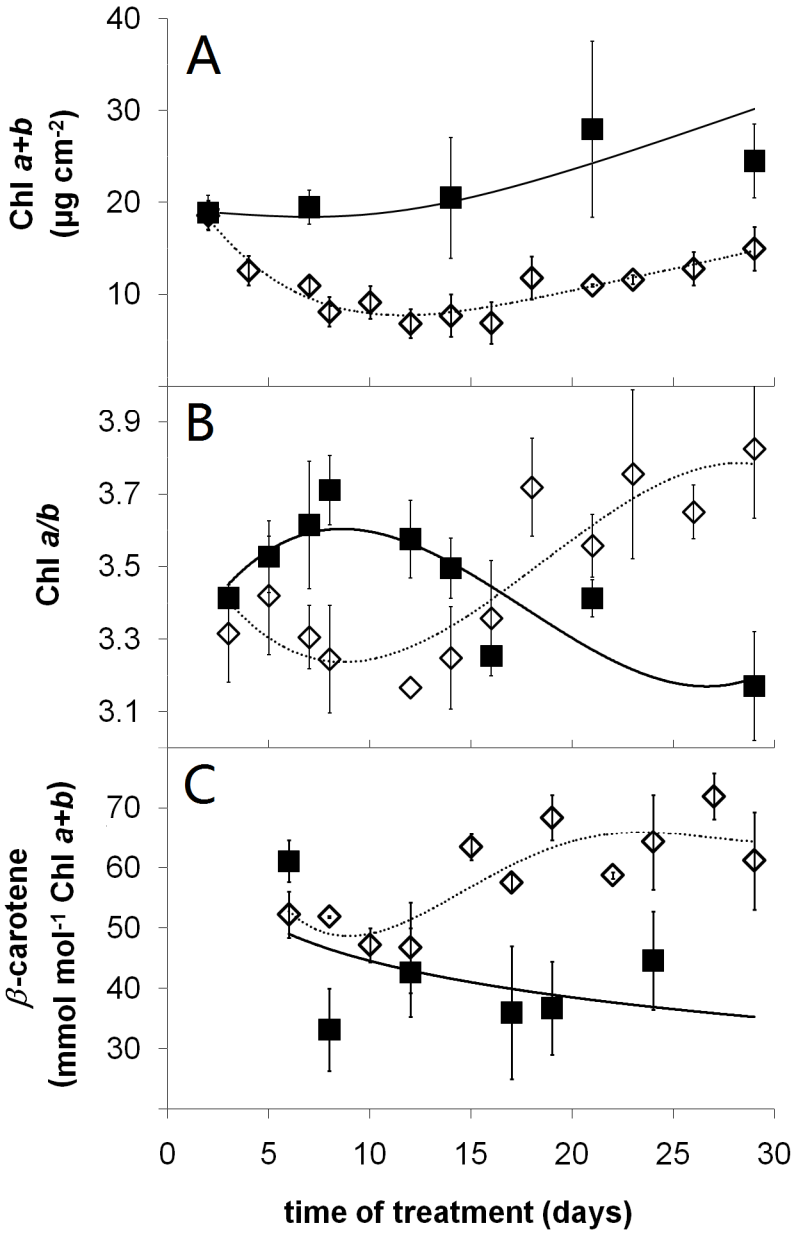

Figure 2. Changes in the Chl *a+b* content (per leaf area) (A), Chl *a/b* ratio (B), and *β*-carotene content (per Chl *a+b* content; C) in Ctrl (filled marks) and the Cad (open marks) leaves during the time of treatment. Error bars represent SD values, n=8.

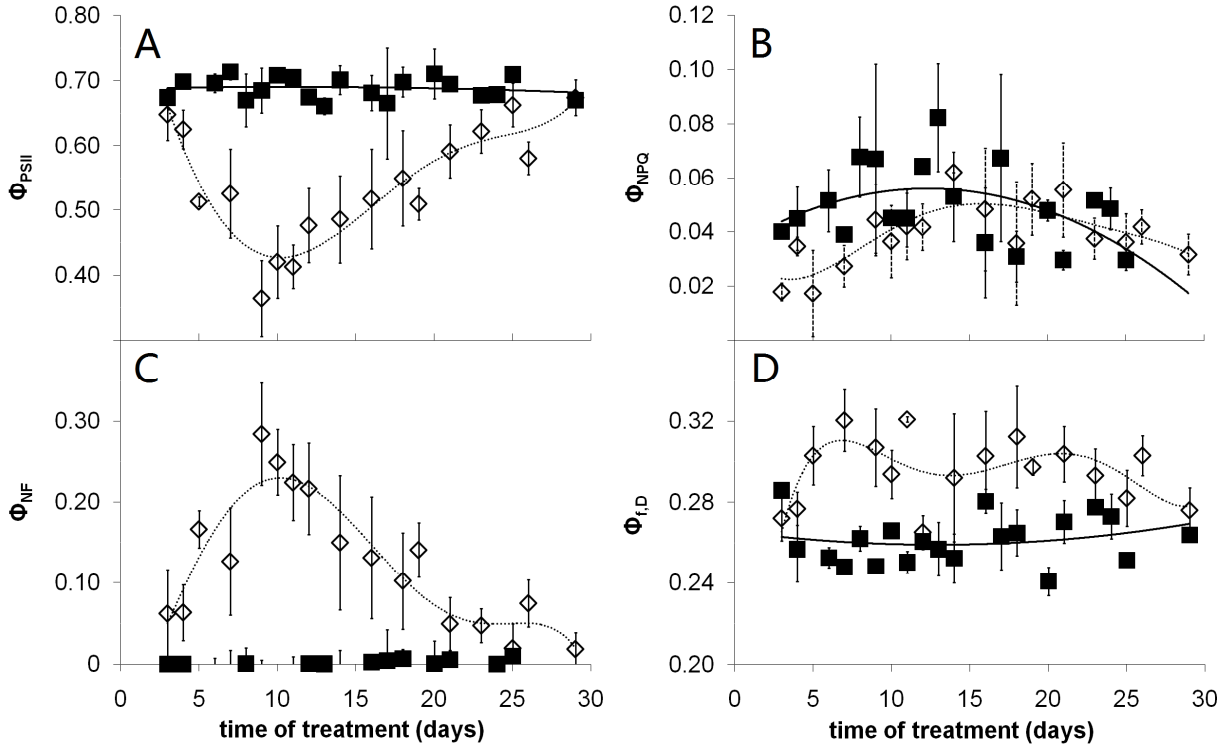

Figure 3. Differences in the excitation energy allocation in Ctrl (filled marks) and the Cad (open marks) leaves during the time of treatment, (A) photochemical quenching (Φ_{PSII}); (B) energization-dependent non-photochemical quenching (Φ_{NPQ}); (C) non-radiative dissipation related to the inactivation of PSII reaction centres (Φ_{NF}) and (D) fluorescence and constant heat dissipation of the photosynthetic apparatus ($\Phi_{f,D}$). Error bars represent SD values, n=5.

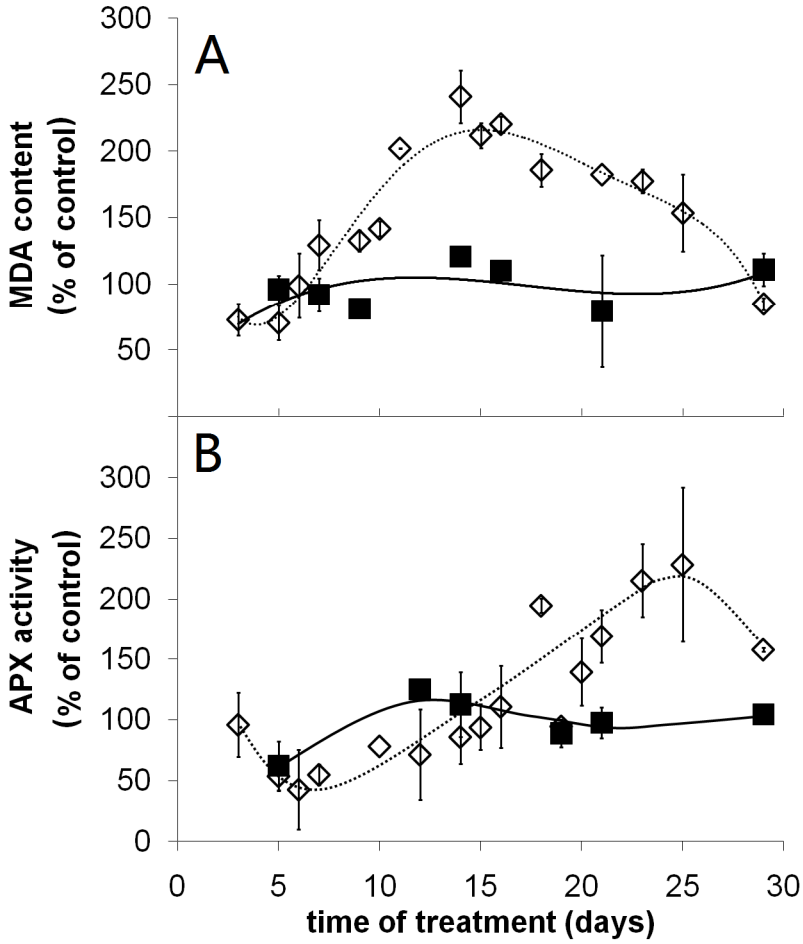

Figure 4. Changes in the malondialdehyde (MDA) content (A) and ascorbate peroxidase (APX) activity (B) in the Ctrl (filled marks) and Cad (open marks) leaves during the time of treatment. Average values of the Ctrls were: 82.6±12.2 nM MDA g^{-1} fresh weight and 9.0±4.1 pmol ascorbate μg^{-1} protein min^{-1}, respectively. Error bars represent SD values, n=8.

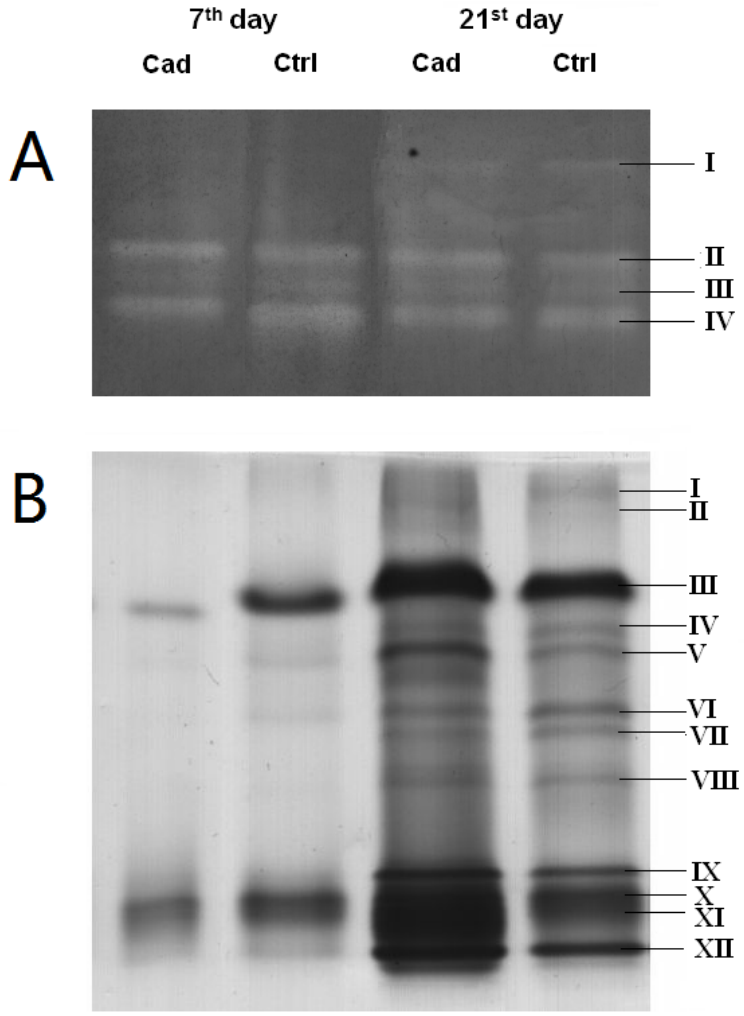

Figure 5. Superoxide dismutase (A; SOD) and peroxidase (B; POD) isoforms separated on native gels in the Ctrl and Cad leaves. Numbers indicate the separated isoforms for both SOD and POD enzymes.

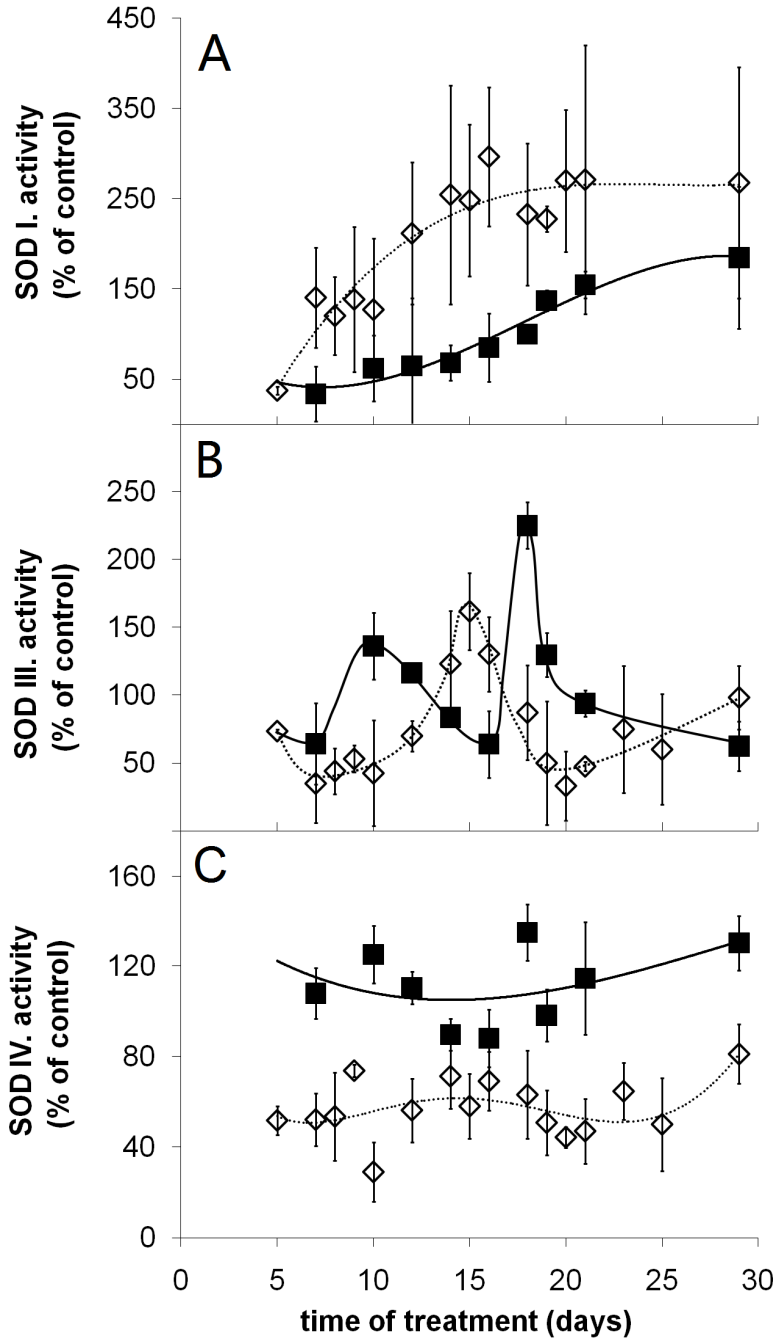

Figure 6. Changes in the activity of selected SOD isoforms representing three different types of changes under long-term Cd treatment; (A) activity increase under the treatment; (B) periodic changes in the activity; and (C) reduced activity compared to the corresponding Ctrls. Values of the normalizations were: (A) 37.1±0.0 pixel density μg^{-1} protein; (B) 136.2±15.4 pixel density μg^{-1} protein; (C) 569.9±212.9 pixel density μg^{-1} protein. Error bars represent SD values, n=8.

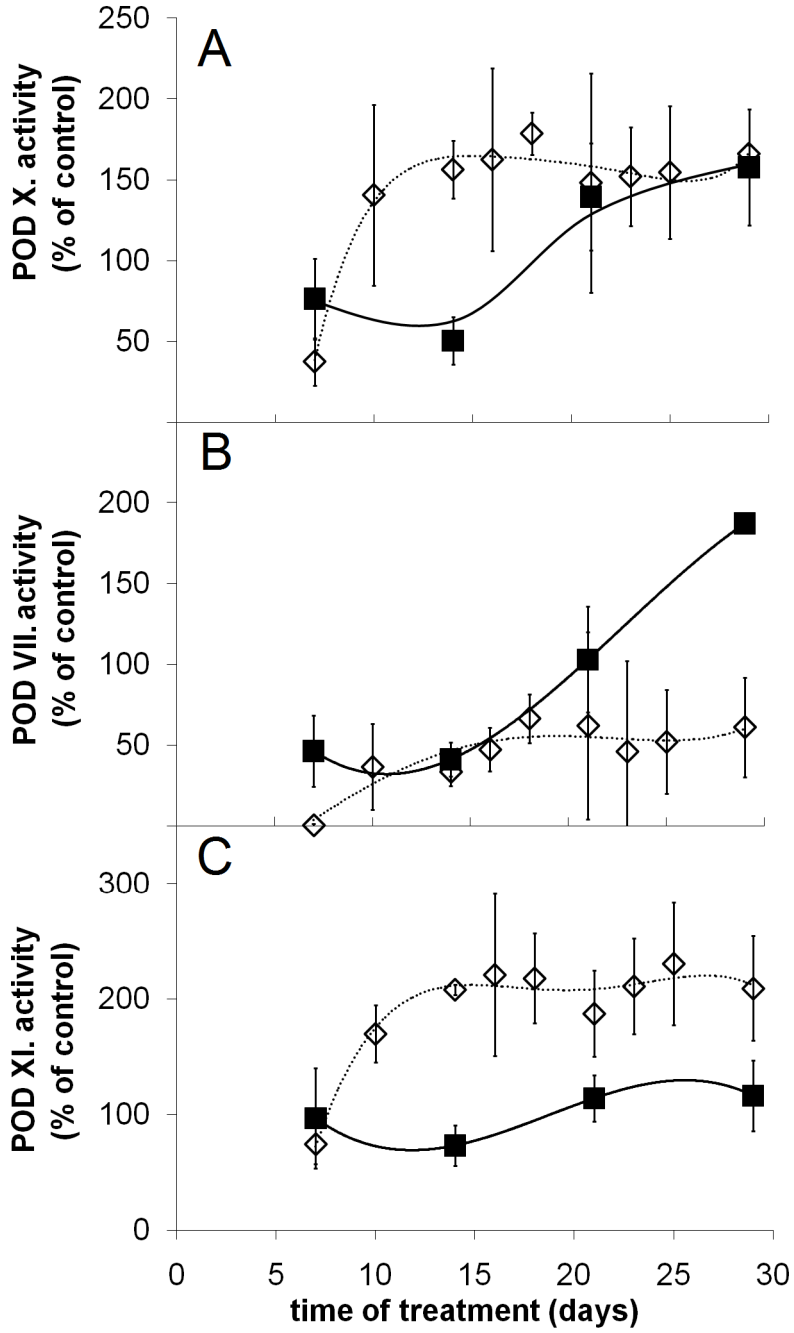

Figure 7. Changes in the activity of selected POD isoforms representing three different types of changes under long-term Cd treatment; (A) activity increase in both Ctrl and Cad leaves; (B) activity decrease and (C) activity increase under Cd treatment. Values of the normalizations were: (A) 1168.1±238.0 pixel density μg^{-1} protein; (B) 4582.0±1634.3 pixel density μg^{-1} protein; (C) 11228.5±3779.3 pixel density μg^{-1} protein. Error bars represent SD values, n=8.