African Journal of Biotechnology Vol. 11(52), pp. 11377-11385, 28 June, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.4114
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Effect of cadmium stress on antioxidative enzymes during the germination of Serbian spruce [*Picea omorika* (Panč.) *Purkyně*]

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Accepted 10 April, 2012

When considering the effect of heavy metals on trees generally and on forest ecosystems especially, importance is given to their influence on seed germination in metal polluted soil. There is insufficient data on this subject, especially when conifers are concerned. In this work, the influence of high cadmium concentrations on percentage germination, specific activities and isoenzyme patterns of catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) during the germination of Serbian spruce [*Picea omorika* (Panč.) *Purkyně*] was studied. Cadmium chloride concentrations of up to 0.1 mM did not cause an inhibition of germination, while 1 mM concentration inhibited germination and the activities of catalase, superoxide dismutase and peroxidase. The isoenzyme profile of catalase and superoxide dismutase did not change at high cadmium concentrations, while peroxidase expression of basic peroxidase (B5) with pI value of 9.1 increased. This isoform of POD can play an important role in the early development of Serbian spruce and its defense mechanism against heavy metals.

Key words: Seed germination, catalase, peroxidase, superoxide dismutase, heavy metals.

INTRODUCTION

Heavy metals are one of the most important environmental pollutants today, with cadmium as the most toxic one whose toxic effect can cause oxidative stress (Chaoui et al., 1997; Agrawal and Mishra., 2009). Defense system against oxidative stress in plants consists of various antioxidative enzymes such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) as well as several non-enzymatic compounds. They eliminate reactive oxygen species (ROS) caused by heavy metals (Blokhina et al., 2003; Hendry and Crawford, 1994). CAT and SOD are the most effective antioxidative enzymes (Scandalios, 1993). On the other hand, POD also plays a role in important physiological processes like growth control by lignification, cross-linking of pectins and structural proteins in cell wall, and catabolism of auxins (Gaspar et al., 1991). Despite the importance of POD in plant development, their exact relationship to developmental events is often obscured by their extensive polymorphism in a single plant species (Van Huystee, 1987). Therefore, it is very important to select POD associated with plant development for purification and further studies (Jackson and Ricardo, 1998). It is known that cadmium can cause a significant increase in oxidative stress parameters such as NAD(P)H oxidase activity (Smiri et al.., 2010) as well as a change in POD activity (Radotić et al., 2000) and oxalate oxidase activity (Dučić et al., 2008). An increase in the activities of SOD, POD and CAT in young seedlings of *Paulownia.*

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Abbreviations: EDTA, Ethylenediaminotetraacetic acid; **DTT,** dithiothreitol; **PVP,** polyvinyl pyrrolidone *fortunei* has been detected after exposure to lead and cadmium, while a decrease in the activity of antioxidative enzymes was dectected when the plants were exposured to zinc and copper (Whang et al., 2010).

In published literature, there have not been numerous data on the effect of heavy metals on conifer trees. The rising importance of this issue is not only related to the increasing pollution of the environment by heavy metals and a related increasing endangerment of forest ecosystems, but also to an increasing importance biotechnological applications in tree breeding. Cadmium (Cd) is especially the most toxic species of heavy metals, since it is not a natural constituent of the environment and living organisms; its presence in ecosystems is entirely antropogenic in origin (Godt et al., 2006). Thus, Cd exerts more toxic effects on plants than other heavy metals. When considering the effect of heavy metals on trees and more particularly on forest ecosystems especially, importance is given to their influence on seed germination in metal polluted soil. There is a lack of data on this subject, especially with respect to conifers.

Serbian spruce [*Picea omorika* (Panč.) *Purkyně*] is a Balkan endemic coniferous species and tertiary relict of the European flora. It is more tolerant of certain environmental factors such as temperature and drought than other conifers (Gilman and Watson, 1994; Král, 2002). This species of trees grow in a large edaphic and altitude range (300 to 1700 m), mostly occupying disturbed and relatively open habitats such as cliffs, forest clearings and vegetation gaps (Čolić, 1957, 1966). On the other hand, cultivated *P. omorika* is used as a decorative species (Jovanović, 1970) as well as for the construction of shelterbelts, due to its wind resistance (Gilman and Watson, 1994). All these facts make this species interesting for investigation.

This work is the first study on the activities and isoenzyme patterns of the antioxidative enzymes CAT, POD and SOD during the germination of Serbian spruce seeds at various Cd concentrations. The aim of this study was to examine the expression of particular parts of antioxidative systems during the early stages of germination of Serbian spruce and to investigate the influence of Cd on isoenzyme pattern. Generally, the results may contribute to the understanding of the response of conifers'seeds/seedlings to heavy metal stress during germination. An important aspect of this study is to identify which isoforms of the studied antioxidative enzymes change during Cd stress and whether certain isoforms may be involved in seed/ seedling protection against Cd. The results of this study may contribute significantly to the understanding of the mechanism of Cd stress effect on the antioxidative enzymes in conifers, considering the fact that this subject has not been sufficiently studied. Furthermore, if Serbian spruce shows ability to germinate at high Cd concentrations, it may be a crucial prerequisite for the use of this species for new forests plantation on Cd polluted soils. This fact is also important when cultivating Serbian spruce as a decorative species and when using it for the construction of shelterbelts.

MATERIALS AND METHODS

Plant material

The seeds of Serbian spruce were obtained from 15-year old *P. omorika* trees grown in a generative seed orchard in Godovik (43° 51' N, 20° 02' E, 400 m), Serbia. The generative seed orchard of *P. omorika* was planted on the basis of previous studies on the collective and individual variability of continuated and discontinuated features of the half-sib *P. omorika* lines (Šijačić-Nikolić, 2001). In the experiments we used the line, "borealis", with broad tree crown and branching similar to the branching in spruce. The seeds were collected in 2002 and stored in a dark place at +5°C.

All the experiments were performed in the year 2004. 50 dry seeds of *P. omorika* were placed on filter paper in 10 cm diameter Petri dishes containing 5 mL of distilled water with cadmium chloride (0, 1, 10 100 and 1000 µM). The seeds were pretreated with 10 days of darkness at +5°C. During the succeeding seven days, at a 12 h photoperiod at 25°C, germination percentage was determined on the 4^{th}, 5^{th}, 6^{th} and 7^{th} day in batches of 50 seeds per sample (one Petri dish), using the protrusion of the radicle by more than one milimeter as criterion. Whole germinated seeds/seedlings separated from non-germinated seeds, which were obtained from one Petri dish, were used for fresh weight (FW) determination.

Enzyme extraction

Whole seedlings separated from non-germinated seeds (between 30 and 40), which were obtained from one Petri dish and were powdered in liquid nitrogen. Frozen powder (between 0.10 and 0.15 g of fresh weight) was added to 1.5 mL of extraction buffer containing 100 mM Tris with a pH of 7.5, 1 mM of ethylenediaminotetraacetic acid (EDTA), 0.5% Triton X-100, 1 mM dithiothreitol (DTT), and 2% polyvinyl pyrrolidone (PVP). The suspension was incubated at 4°C for 1 h and after that, it was centrifuged for 10 min at 10000G and 4°C. The supernatant (1.5 mL) was used for POD, CAT and SOD activities. As no germination and change in enzyme activity were detected in Serbian spruce seeds till the fourth day at a 12 h photoperiod at 25°C, specific enzyme activities of CAT, SOD and POD were examined starting from the 4^{th} day, which we considered as the 1^{st} day after germination.

Enzyme assays

SOD activity was determined spectrophotometrically at 550 nm in 50 mM sodium phosphate buffer at a pH of 7.8 with 1 mM of EDTA and 0.02 mM sodium azide by measuring the percentage of the SOD-induced inhibition of cytochrome C reduction using xantine/xanthine oxidase system as the source of O_2^-, as described by McCord and Fridovich (1968). One unit of SOD activity was defined as the amount of enzyme that causes 50% inhibition of cytochrome C reduction.

CAT activity was determined spectrophotometrically at 240 nm as described by Aebi (1984) by measuring decrease in absorbance of H_2O_2 from 0.850 to 0.750 nm in 3 ml of 100 mM sodium phosphate buffer at a pH of 7.5 and a temperature of 25°C. The extincion coefficient for H_2O_2 was 4.32 cm^2/µmol.

POD activity was determined spectrophotometrically as described by Lagrimini (1991) with guiacol as the substrate in a total volume of 3 ml. The assay mixture contained 50 mM sodium

acetate buffer at a pH of 5.5, 92 mM guiacol, 18 mM H_2O_2 and at a temperature of 25°C. The reaction was monitored at 470 nm.
The reaction rate was calculated to obtain the coefficient of absorbance for tetraguiacol, which was 25.5 cm^2/μmol. One unit of CAT and POD activity was defined as the amount of enzyme that converts one micromol of substrate to end product in one minute, while enzyme activities were defined by the samples' fresh weight.

Isoenzyme patterns in gels

Polyacrylamide gel electrophoresis was carried out on Mini Hoefer sedimentation equilibrium (SE) electrophoresis system under non-denaturing/denaturing conditions in gels containing 8% polyacrylamide with a 4% stacking gel. A constant current of 25 mA was applied to each gel. Electrophoresis buffers and gels were prepared as described by Laemli (1970) except that sodium dodecyl sulphate (SDS) was excluded. Equal volumes of all samples were loaded on the gels.

Isoelectric focusing was performed horizontally in the LKB 2117 Multiphor II system containing 4% 3.5 to 10.0 ampholites using 1 mm thick polyacrylamide gels (5% T and 3% C). The gels were solidified with 50 µL of 10% ammonium persulfate and 7 µL of TEMED per 15 ml of gel solution. They were run at 4°C with a constant power of 0.5 W/cm width of gel and a voltage limit of 2000 V for 2 h.
CAT was stained on gel by incubating it in the dark for 20 min in 10 mM H_2O_2 disolved in sodium acetate buffer with a pH of 5.5, followed by an incubation in a mixture of 1% $K_3Fe(CN)_6$ and $FeCl_3$ for 15 min (Woodbury et al., 1971).

SOD isoenzymes were detected on the gels by the method of Beuchamp and Fridovich (1971). Briefly, the gels were incubated for 20 min in the dark in 20 mL 100 mM sodium phosphate buffer of pH 7.8 with 4 mg of nitrobluetetrazolium, 0.6 mg of riboflavine, 2 µL of TEMED, and 40 µL of 0.25 M NaEDTA. After that, the gels were briefly rinsed with distilled water and illuminated for 15 min. POD was stained on gel with 9.2 mM guiacol and 5 mM H_2O_2 in sodium acetate buffer of pH 5.5 for 10 min at a temperature of 25°C. The relative activities of POD isoforms on the gels were determined from scanned images of electrophoretic gels using freeware software GelAnalyzer2010a developed by Dr. Istvan Lazar (http://www.gelanalyzer.com/). They were expressed as percentage of total POD activity for every considered sample.

Statistical analysis

Statistical analysis was performed by using Microsoft Excel 2010, Origin 8.0 and Statistica 7.0. The data represents means calculated for tetraplicates, while the bars on the graphs represent 95% confidence interval (95% CI). Significant diferences between the germination treated and untreated were determined by using unpaired t-test ($p<0.05$).

RESULTS

Percentage of germination

Percentage of germination was determined for Serbian spruce in seven days at a 12 h photoperiod at 25°C in the presence of various cadmium chloride concentrations.
Radicle protrusion occurred on the second day but germination percentage increased significantly on the fourth day as shown in Figure 1.

Germination percentage was not influenced by Cd concentration of about 1 mM. In the presence of 1 mM Cd, germination was significantly more inhibited (18%) compared to the control (74%).

In the experiments, we measured the specific enzyme activities of CAT, SOD, and POD per fresh weight of seedlings from the 1th to the 4th day after germination in the absence and presence of 1 mM cadmium chloride.

Enzyme activities and isoenzyme patterns

Catalase

During the germination of control seeds, CAT activity did not change significantly and was very similar to the CAT activity in seeds before germination. Specific CAT activity was higher in untreated seedlings than in the ones treated with 1 mM cadmium chloride (Figure 2). Thus, CAT activity dropped from 3511 to 1222 U/g ($p=0.002$) on the 4th day after germination. There was no CAT activity in non-germinated seeds on the 4th day after germination.

CAT isoenzyme pattern did not change in all samples observed; only two close bands of CAT activity were found on native poly-acrylamide gels (Figure 3).

Superoxide dismutase

In the control group, SOD activity did not change significantly in seedlings. There was a decrease in SOD activity in the seeding treated with 1 mM Cd on all sampling dates (Figure 4). The specific activitity of SOD on the 4th day after germination was 279 U/g in the control group; however, in the presence of Cd, even if the activity increased to 62.7 U/g on the 4th day, the increase was still significantly lower ($p=0.002$) than in untreated seedlings.

After isoelectrofocusing, five different bands were found in all the samples (Figure 5). The isoenzyme pattern of SOD did not change after germination at high cadmium concentrations.

Peroxidase

In contrast to CAT and SOD activities, POD activity showed significant change both in treated and untreated seedlings. As shown in Figure 6, the specific activity of POD increased con-tinuously and was highest on the 4th day after germination. The specific activity of POD per fresh weight of seedlings treated with cadmium chloride decreased with respect to the untreated ones in the first days after germination, but due to a higher rate of activity increase on the 4th day, specific activity was 31.0 U/g, which was not significantly different ($p=0.761$) from the activity (28.3 U/g) in untreated seedlings (Figure 6).

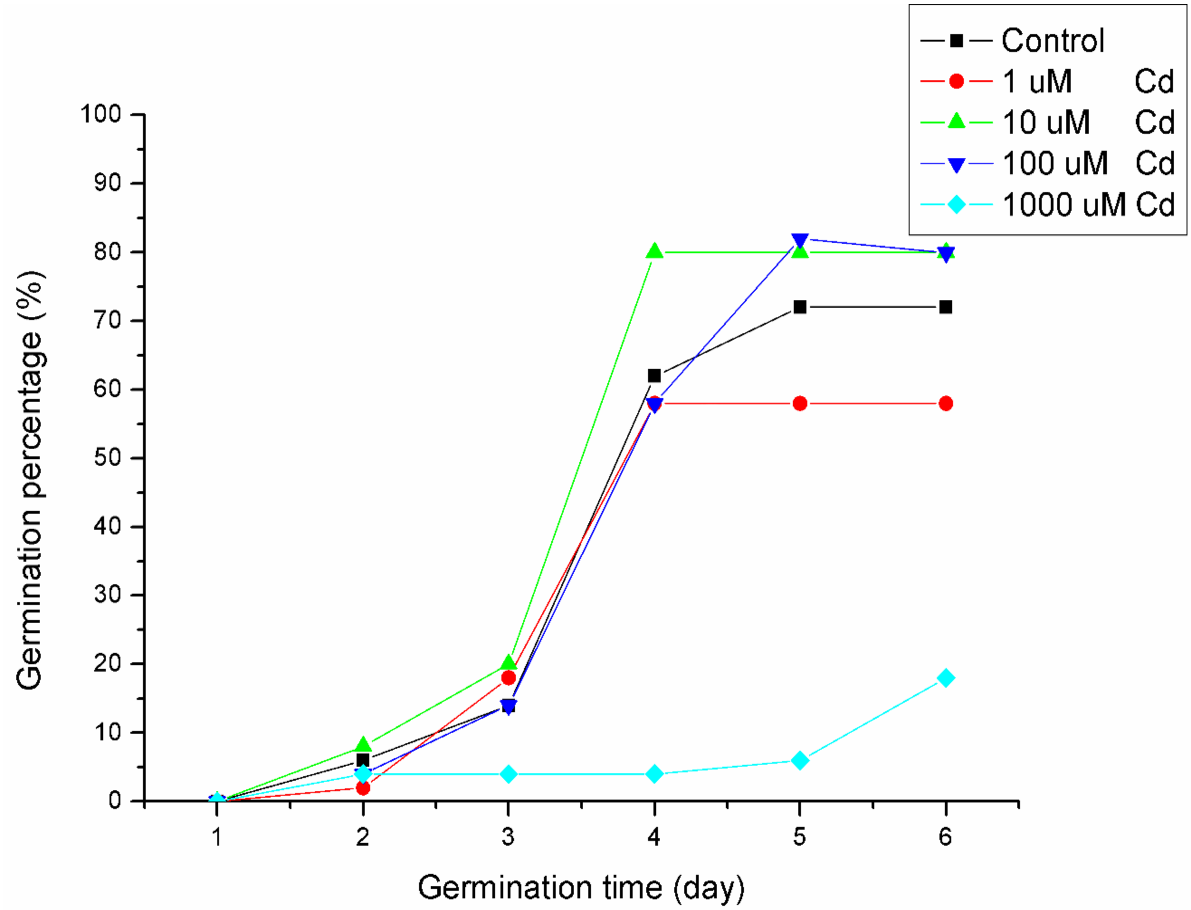

Figure 1. Seed germination of Serbian spruce at different cadmium concentrations.

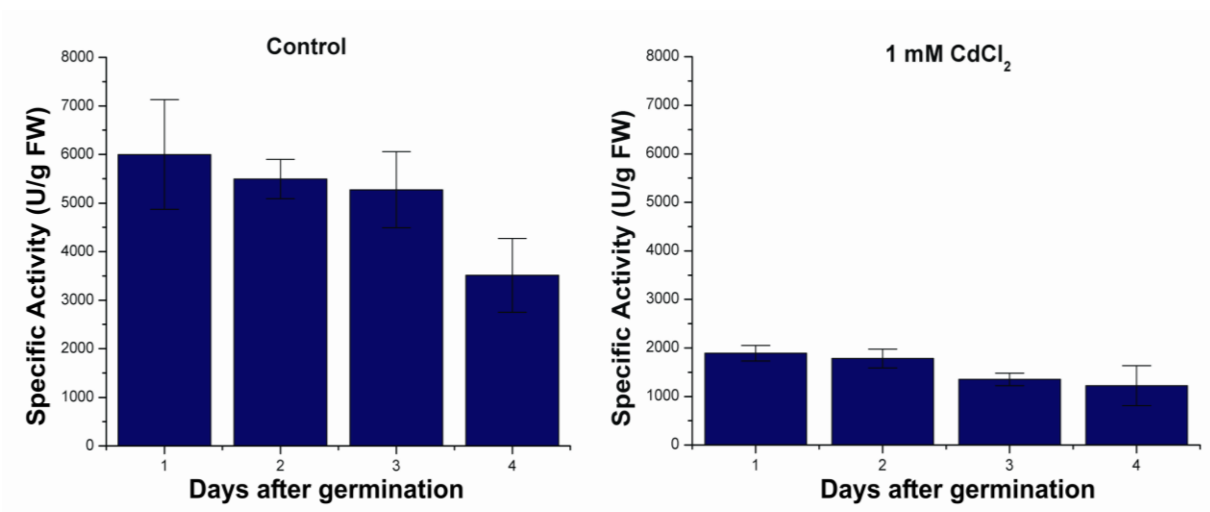

Figure 2. Specific activity of catalase per fresh weight of seedlings of Serbian spruce in the absence and presence of 1 mM cadmium chloride**,** respectively. Error bars show 95% confidence interval (95% CI).

POD isoenzyme pattern of untreated seedlings did not change and on the 3rd day after germination when POD activity increased signifi-cantly and all basic POD isoforms were visible on the gel; the most dominant

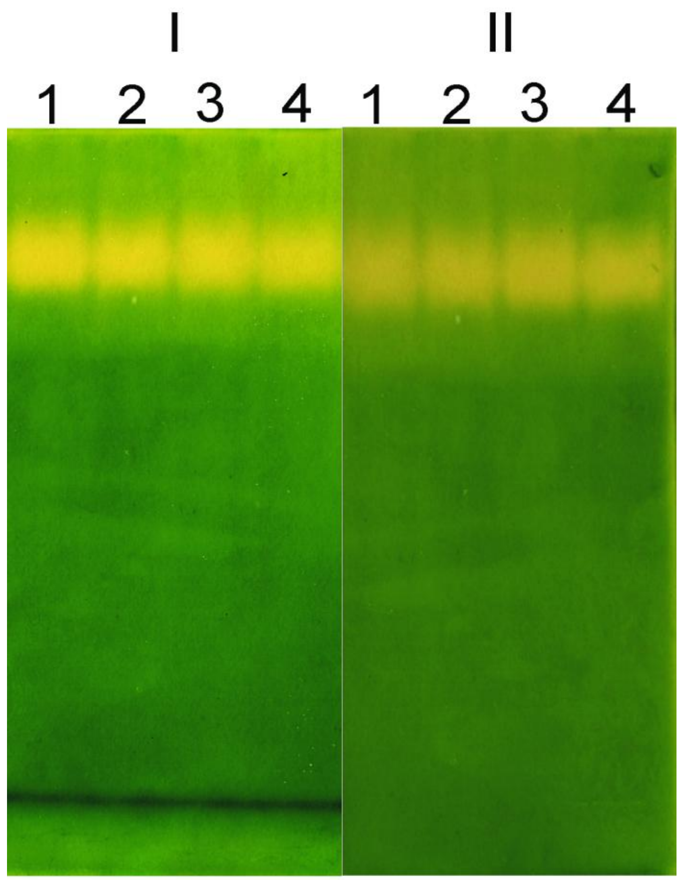

Figure 3. Isoenzyme pattern of catalase on polyacrylamide gels using native electrophoresis. 1 **to** 4 denote**s** 1^{st} to 4^{th} day after germination. I, Control without cadmium chloride; II, 1 mM cadmium chloride.

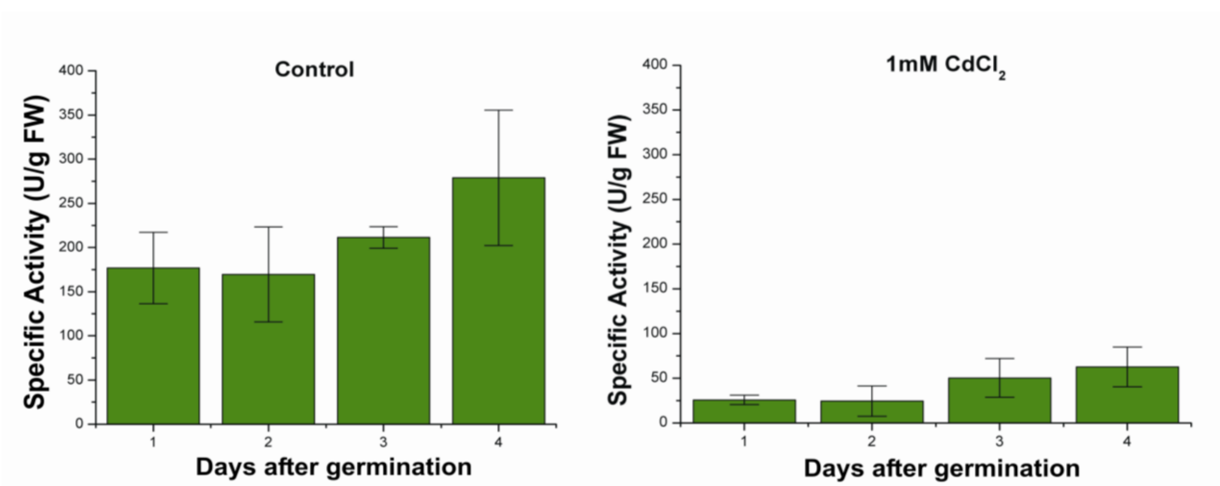

Figure 4. Specific activity of superoxide dismutase per fresh weight **of** seedlings of Serbian spruce **in** the absence and presence of 1 mM cadmium chloride**,** respectively. Error bars show 95% confidence interval (95% CI).

basic form was B2 (50.1% of total POD), followed by minor B5 form (20.3% of total POD). The seedlings treated with cadmium chloride showed a change in isoenzyme profile. The activity of B2 (20.0% of total

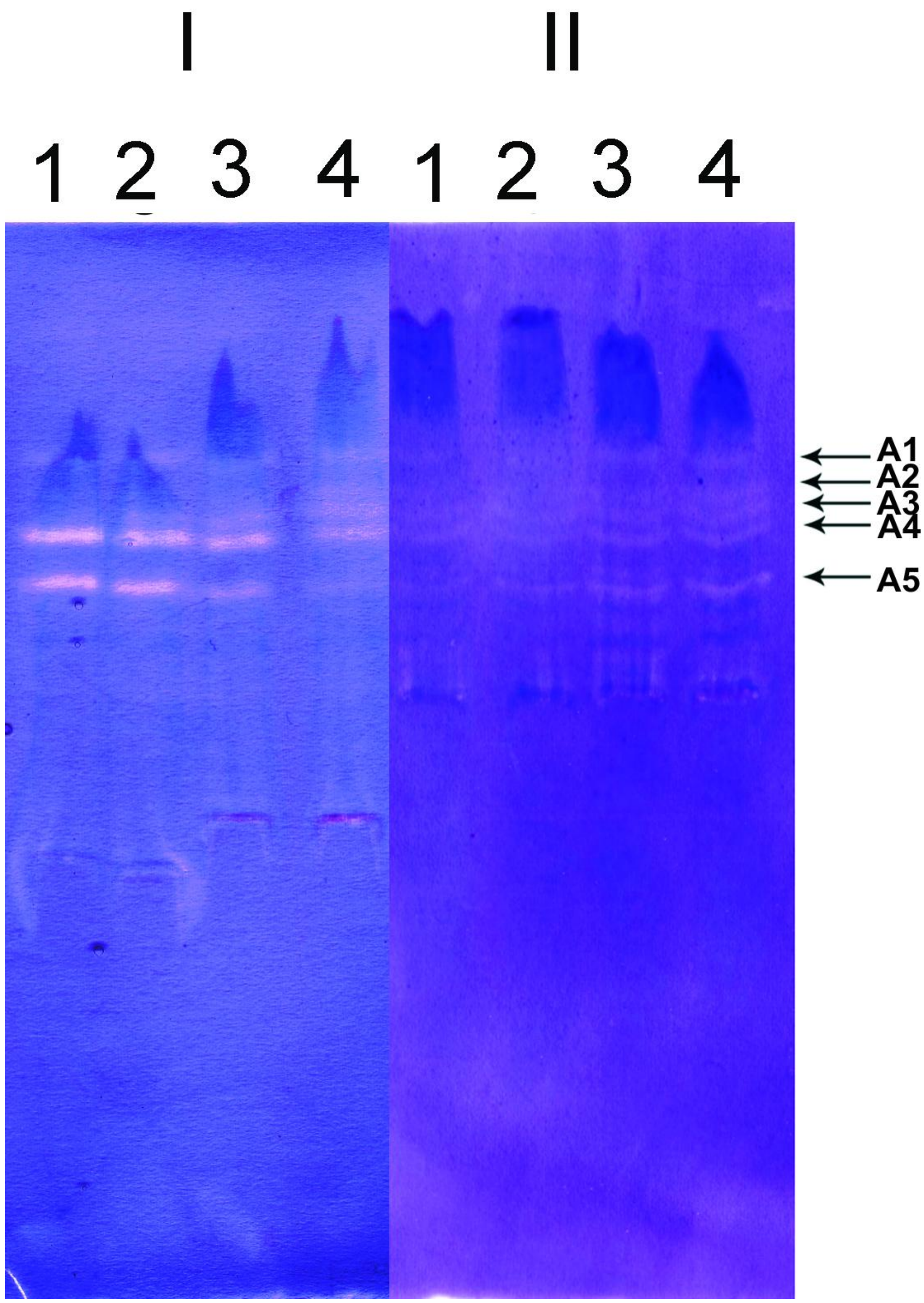

Figure 5. SOD activity in polyacrylamide gels using isoelectrofocusing and native electrophoresis. 1 **to** 4 denote**s** 1st to 4th day after germination. I, Control without cadmium chloride; II, 1 mM cadmium chloride. **SOD,** Superoxide dismutase.

POD) form of POD decreased, while that of B5 (35.5% of total POD) isoform increased as shown in Figure 7. Thus, in the treated seedlings the most abundant POD isoform was B5.

DISCUSSION

The inhibition of germination of Serbian spruce was noticed only at high cadmium chloride concentrations (1 mM), while the other plant species are seriously damaged by considerably lower Cd concentrations during germination. This shows a contrast in the way Serbian spruce and other plant species respond to high Cd concentrations. For example, in the case of *Achnatherum inebrians,* the inhibition of germination was noticed at a lower concentration of Cd (0.2 mM) (Zhang et al., 2010). The germination of wheat seeds was inhibited at 0.025

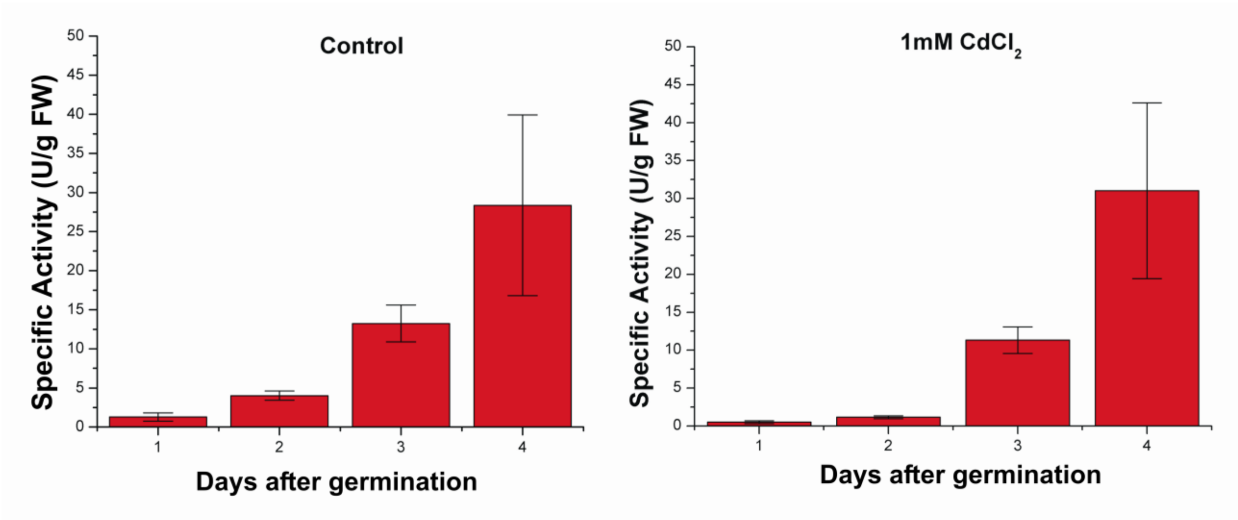

Figure 6. Specific activity of peroxidase per fresh weight of seedlings of Serbian spruce **in the absence** and presence of 1 mM cadmium chloride. Error bars show 95% confidence interval (95% CI).

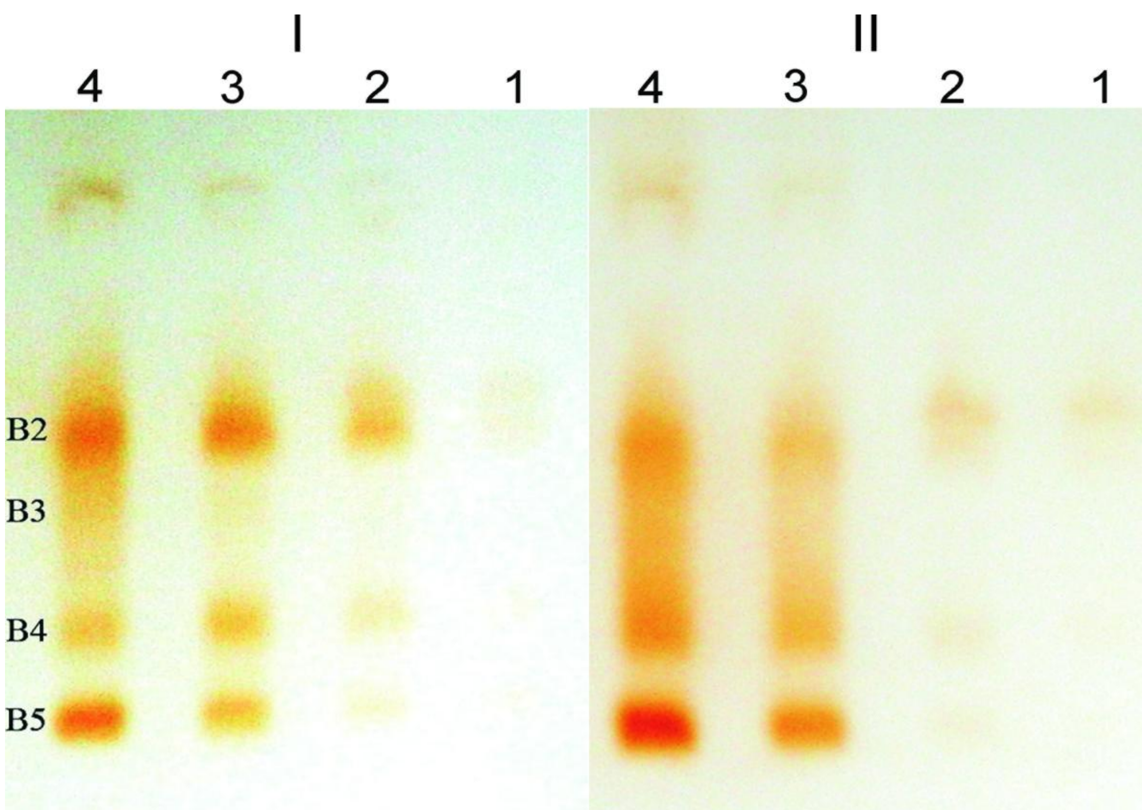

Figure 7. Isoelectrofocusing of POD isozymes from the seedlings of Serbian spruce. 1 **to** 4 denote**s** 1^{st} to 4^{th} day after germination. I, Control without cadmium chloride; II, 1 mM cadmium chloride. POD, Peroxidase.

mM Cd (Liu et al., 2007). It was shown that Cd^{2+} stimulated the growth of sorghum plants at a certain lower concentration range, but the growth was inhibited significantly when Cd^{2+} content was more than 25 mg kg^{-1} (Da-lin et al., 2011). This is a considerably lower concentration than that used in our experiments, which inhibited Serbian spruce germination. This strongly suggests that Serbian spruce seeds have much higher tolerance to the stress induced by Cd, when compared with other plant species.

The results obtained from our previous study showed a correlation of CAT activity with germination percentage of Serbian spruce seeds (Prodanović et al., 2007). In the present study, high Cd concentrations induced a simultaneous decrease in percentage of germination and CAT activity, confirming a correlation between these two phenomena. The fact that CAT isoenzyme pattern did not changed probably implies that all isoenzymes are affected by similar concentration levels of Cd. Some previously reported results revealed that Cd induced increase in CAT activity if it acts alone, but together with arsen, it reduced CAT activity (Liu et al., 2007).

The decrease in SOD activity in the presence of Cd was at a high rate during germination in untreated seeds/ seedlings; this may be as a result of increased superoxide anion radical production in the presence of Cd. Higher SOD expression was also observed in the case of corn seedlings (Chaudhary and Sharma, 2009), while wheat seedlings showed an increase followed by a drop in SOD activity in the presence of Cd (Liu et al., 2007). The unchanged isoenzyme profile of SOD (Figure 5) probably indicates that all isoforms had equal importance in the protection of seedlings from free radical species.

In our previous publication, we showed that POD activity in Serbian spruce seeds has a role to play in the period between the 1st and 4th day after germination with B2 being the most abundant isoform (Prodanović et al., 2007). POD activity was also shown to increase during late germination and early seedling growth in some annual herbaceous species like *Chenopodium rubrum* (Dučić et al., 2003) and tomato (Morohashi., 2002), biennual species like *Brassica oleracea* (Belani et al., 2002), and perennial species like *Viola carnuta* (Mitchell and Barrett, 2000). The basic isoforms of POD have distinct associations with the vegetative development of lupin and play important roles in the early stages of lupin growth (Jackson and Ricardo, 1998). At high Cd concentrations, the specific activity of POD on the 1st and 2nd day after germination was lower than the corresponding controls (Figure 6). Due to the high rate of increase in specific activity, on the 4th day after germination the specific activity of POD in the treated seedlings was similar to that in the untreated ones, but the isoenzyme profile changed. The expression of B5 isoform increased, while the expression of B2 decreased in the presence of Cd. This could imply that the basic isoforms of POD play different roles in the early seedling development of spruce and in its defense against stress caused by heavy metals like Cd. We propose that *de novo* synthesis of B5 isoform of POD is involved in the antioxidative protection of seedlings and further studies of this isoform is needed. The results demonstrate that the change in the activity of individual POD isoforms is crucial in Cd induced stress as the activation of particular isoforms is a mechanism involved in protection against metal stress. However, this is not visible on the level of the overall POD activity. These results are fundamental to the understanding of the individual isoforms of enzymes involved in the defense against metal stress and the mechanism of metal induced plant stress and further decay. This may favor the use of biotechnology in the production of a desired species containing the isoforms that are crucial for its protection against a particular metal stress.

The fact that Serbian spruce maintains satisfactory germination potential at high Cd concentrations (1 mM) indicates that this conifer species may be considered as a promising candidate for the planting of new forests in Cd polluted soils. Since cultivated Serbian spruce is an amiable decorative species, and also used for construction of shelterbelts, its high germination potential in Cd contaminated soils is even more significant.

Conclusion

High Cd concentration of 1 mM caused the inhibition of germination and decrease in the specific activities of CAT, SOD, and POD in the first few days after germination. Afterwards the activities of SOD and POD increased more sharply. The expression of the B5 basic POD isoform increased in the presence of Cd, indicating its role in the protecting Serbian spruce from Cd stress. Further studies of this enzyme may lead to a better understanding of its role in protecting seedlings from the toxicity of heavy metals.

ACKNOWLEDGEMENTS

This work was supported by the grant No: ON173017 from the Ministry of Education and Sciences of the Republic of Serbia. Our thanks go to Professor Vasilije Isajev and Professor Mirjana Šijačić for providing the Serbian spruce seeds from the seed orchard in Godovik.

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