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OXALATE OXIDASE AND NON-ENZYMATIC COMPOUNDS OF THE ANTIOXIDATIVE SYSTEM IN YOUNG SERBIAN SPRUCE PLANTS EXPOSED TO CADMIUM STRESS

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Abstract — We studied changes in the concentrations of ascorbate and glutathione, composition of soluble phenolics, and activity of oxalate oxidase in 75-day-old Serbian spruce plants after exposure to 5 μM and 50 μM cadmium for 6-48 h. The presence of OxOx activity in a conifer species is here demonstrated for the first time. Both Cd concentrations induced a decrease of OxOx activity in treated plants in comparison with the control at all sampling dates. The concentrations of reduced glutathione, its oxidized form, and reduced ascorbate in the plants decreased during 48-h treatment with cadmium. Among simple phenolics, only catechin increased significantly during Cd treatment.

Key words: *Picea omorika* (Panč.) Purkyně, cadmium, oxalate oxidase, hydrogen peroxide, glutathione, ascorbate

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INTRODUCTION

Increased contents of heavy metals, especially non-essential metals like Cd, in soils affect physiological processes in plants. Cadmium, a highly toxic environmental pollutant with high toxicity to animals and plants, has no known function in any live cells. It is released into the environment by traffic, metal-working industries, mining, as a by-product of mineral fertilizers, and from other sources (Nriagu and Pacyna, 1988). One of the consequences of the presence of toxic metals in plants is the formation of free radical species, which cause severe damage to different cell components (De Vos et al., 1992; Hendry and Crawford, 1994). Cd treatment affects the activities of antioxidative systems (AOS) (Radotić et al., 2000) and especially GSH in different organisms (Schützendübel et al., 2001; Schützendübel and Polle, 2002).

Antioxidative systems, consisting of several non-enzymatic and enzymatic mechanisms, are activated in the cell as a response to different types of damaging conditions (Rabe and Creeb, 1979; Hendry and Crawford, 1994; Hippeli and Elstner, 1996), particularly the presence of heavy metals (De Vos et al., 1992; Gallego et al., 1996; Sanita di Toppi and Gabrielli, 1999).

Glutathione and ascorbate, the most important non-enzymatic components of AOS, in plants play a major part in their resistance mechanisms to external stress (Noctor and Foyer, 1998) by regulating redox equilibrium in the cell. Glutathione is involved to a considerable extent in detoxification of heavy metals and xenobiotics and also has a role in gene activation and protection against oxidative stress (Noctor and Foyer, 1998; Schützendübel et al., 2001). As an antioxidant, glutathione - together with ascorbate and antioxidative enzymes - controls the cellular concentrations of hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\cdot -}$) (Noctor and Foyer, 1998).

Phenolic compounds also take part in defense mechanisms in plants (Brignolas et al., 1995; Yee-Meiler, 1974) and are considered to be a bioindicator of environmental pollution. Breakdown of the antioxidative systems in plants subjected to metal stress has been taken as an indicator of serious plant damage caused by a given concentration of the metal (Van Grosveld and Clijsters,

1994). There was direct influence of Cd on the phenolic composition of Scotch pine measured *in situ* (Schützendübel et al., 2001).

Oxalate oxidase (E.C. 1.2.3.4.) is an enzyme not directly included in AOS, but very important because it releases H_2O_2 and CO_2 from oxalic acid. It is one of the extracellular enzymes that have been suggested as candidates for the production of extracellular H_2O_2 (Dumas et al., 1993; Lane et al., 1993). Accumulation of H_2O_2, which acts as a signalling molecule, is one of the direct consequences of Cd stress in plants (Schützendübel et al., 2001; Schützendübel and Polle, 2002). Also, H_2O_2 – together with phenolics - is used in plants as fuel for the extracellular peroxidase reaction pathway (Gaspar et al., 2002).

Serbian spruce (*Picea omorika* (Panč.) Purkinyě) is a Balkan endemic coniferous species and Tertiary relict of the European flora. Under natural conditions, it is restricted to small communities within disturbed and relatively open habitats such as forest clearings and vegetation gaps (Čolić, 1957, 1966). There are only a few studies on Serbian spruce (Šiljak-Jakovljev et al., 2002; Tucić and Stojković, 2001) and its antioxidative systems (Bogdanović et al., 2005, 2006, 2007).

The antioxidative system (AOS) in Serbian spruce is interesting because it is more tolerant to air pollution and drought compared to other conifers (Gilman and Watson, 1994; Král, 2002) and because trees of this species grow in a wide edaphic and altitudinal range (300-1600 m). Besides, there is no evidence indicating that Cd induces the same defense pathways in plants. We studied some of the physiological defense reactions, e.g., changes in the concentrations of ascorbate and glutathione, composition of phenolics, and activity of oxalate oxidase, occurring in roots and needles of 75-day-old Serbian spruce plants after Cd exposure for 6 - 48 h. The plants were exposed to 5 or 50 µM Cd in hydroponics. The aim of this study was to analyze the part of the AOS that is the main defense line in young Serbian spruce plants during the first days of exposure to the cadmium stress. More generally, this study may deepen our knowledge about the metal pollution resistance of young Serbian spruce trees.

MATERIALS AND METHODS

Plant growth

Prior to planting, seeds of Serbian spruce were spread over filter paper soaked with $1{\cdot}10^{-4}$ % fungicide Benomyl and kept in the dark (4°C, 12 days) in order to synchronize germination. Afterwards, seeds were surface-sterilized for 1 h in 30% (w/v) H_2O_2. After 3 days at 21°C in darkness, the seeds were germinated on sterile 1% (w/v) water-agar, pH 4.5, in a day/night regime of 16 h/8 h. The plants were grown in day/night conditions of 23°C/21°C air temperature and 16 h/8 h daylength under white light of 200 µmol m^{-2} s^{-1} photosynthetic photon flux (Osram L18 W/21 lamps, Munich). After 3 weeks, the plants were transferred to aerated nutrient solutions containing the following nutrient elements: 300 µm NH_4NO_3, 100 µm Na_2SO_4, 200 µm K_2SO_4, 60 µm $MgSO_4$, 130 µm $CaSO_4$, 30 µm KH_2PO_4, 10 µm $MnSO_4$, and 92 µm $FeCl_3$; and 5 mL of a stock solution of micronutrients: 0.1545 g L^{-1} H_3BO_3, 0.012 g L^{-1} $NaMoO_4$, 0.0144 g L^{-1} $ZnSO_4$, and 0.0125 g L^{-1} $CuSO_4$ per liter of nutrient solution. The pH was adjusted to 4.0. The solution was changed every 3 days. After 2 weeks of acclimation, the plants were treated with 5 or 50 µm $CdSO_4$ for 6, 12, 24, and 48 h and sampled at regular intervals for analyses. Roots and needles of plants were used as experimental material. For every measurement, a minimum of three plants were harvested.

Oxalate oxidase activity

Frozen roots (100 mg) were powdered in liquid nitrogen and mixed with 1.1 mL of 0.25 M sucrose in 0.05 M Tris-HCl, pH 7.2 (1:11 (w/v) ratio). The extract was squeezed through 0.5 mm nylon mesh and the filtrate centrifuged at 4000 g for 10 min at 4°C. The pellet was washed twice with an extraction medium containing 1% (w/v) Triton X-100 and three times with the same medium without Triton X-100. After each wash, the pellet was collected by centrifugation at 4000g for 10 min. The final pellet was considered to be the purified cell wall fraction and used for the experiments. Oxalate oxidase was

detected spectrophotometrically as described by Pundir (1991). Containing 0.9 mL of 40 mM Na succinate (pH 3.2) and 0.1 ml of the sample with cell wall, the assay mixture (1 ml) was incubated for 10 min at 37°C in the dark. The reaction was started by adding oxalic acid to a final concentration of 10 mM. After 5 min, 0.5 ml of color reagent for H_2O_2 measurement was added to stop the reaction, and color was allowed to develop for 30 min at room temperature. The color reagent consisted of 100 mg of solid phenol, 50 mg of 4-aminophenazine, and 5 U · ml^{-1} of horseradish peroxidase in 0.4 M Na-phosphate buffer (pH 7). Absorbance of the solution was measured at 520 nm and corrected for absorbance obtained when oxalic acid was omitted from the assay mixture. Hydrogen peroxide generated during the reaction was determined by interpolation from a standard curve in the range of from 0.01 to 0.25 µmol H_2O_2 in 50 mM succinate buffer. Enzyme activity expressed as the amount of H_2O_2 produced per min and per mg fresh weight.

Extraction and Analysis of Antioxidants

For the extraction of water-soluble antioxidants, 100 mg of frozen plant tissue (roots and needles) were powdered in liquid nitrogen, mixed with 1 mL of 2% (w/v) meta-phosphoric acid containing 1 mM EDTA and 1 mg of polyvinylpolypyrrolidone per mg of sample, and centrifuged (20 min, 4°C, 30,000g). The supernatant was used for analyses of antioxidants. Ascorbate was determined at 268 nm after separation by capillary electrophoresis as described by Davey et al. (1996). The system consisted of a high-performance capillary zone electrophoresis (model P/ACE 5500 HPCE) system (Beckman Instruments, Fullerton, CA) fitted with a UV-VIS diode array detector (P/ACE 5500 DAD, Beckman) and equipped with GOLD software (Beckman) for peak analysis. Injections were made for 5 s under hydrostatic (N_2) pressure (0.5 pounds per square inch) into a 57-cm fused silica capillary at a constant temperature of 25°C and constant voltage of 25 kV. Separations were run for 10 min with 100 mM borate buffer, pH 9, as a carrier electrolyte. The capillary was subsequently conditioned for the next run with 0.1 M NaOH, water, and the carrier electrolyte. For determination of total ascorbate, DHA was reduced by dithiothreitol at pH 8.3 to 8.5 for 60 min at room temperature (Anderson et al., 1992). This was achieved by mixing 100 µL of the sample with 150 µL of 60 mM dithiothreitol in 1 M 2-[*N*-cyclohexylamino] ethansulfonic acid. The mixture was analyzed as above and DHA was calculated by subtracting ascorbate from total ascorbate.

Glutathione was determined after reduction, derivatization with monobromobimanes, HPLC separation (Beckman System Gold, Munich, Germany) on a C-18 column, and detection with a fluorescence detector (RF-550, Shimadzu, Duisburg, Germany) according to the method of Schupp and Rennenberg (1988). GSSG was determined in the same manner after removal of glutathione by alkylation with *N*-ethylmaleimide (Gorin et al., 1966). GSH was calculated as the difference of glutathione and GSSG. The redox state was defined as the GSSG content in percent of glutathione content.

Determination of soluble phenolics was performed by HPLC after isolation from 200 mg of frozen needles grounded in liquid nitrogen and transfer to 5 mL of 50% (v/v) methanol and water. After shaking and incubation for 60 min at 40°C on an ultrashaker, samples were centrifuged 10 min at 2500g at 4°C. The supernatant was taken, while the pellet was washed with 2 mL of 50% methanol. After incubation of 10 min at room temperature and centrifugation, this supernatant was then combined with the first one. Reverse phase HPLC analysis was carried out on the 1100 Hewlett Packard (Palo Alto, CA, USA) system with diode-array detector adjusted to 210, 280, and 330 nm and reference signal at 600 nm. Separations were performed on a Waters Symmetry C-18 RP column (125x4 mm) with 5-µm particle size and a corresponding precolumn (Waters, Milford, MA, USA). Mobile phases were 0.1% phosphoric acid (mobile phase A) and acetonitrile (mobile phase B). Acetonitrile (J. T. Baker, USA), methanol (Carbo Reagenti, Milan), and p.a. grade phosphoric acid were used.

RESULTS AND DISCUSSION

Metals can induce oxidative stress directly or indirectly, by interaction with biochemical redox

processes (Van Assche and Clijsters, 1990). One of the consequences of the presence of toxic metals in cells is the formation of free radicals and activated oxygen species (AOS), which cause severe damage to different cell components.

Figure 1 shows 75-day-old Serbian spruce plants, untreated and treated with 5 μM and 50 μM cadmium. Exogenous symptoms of metal stress are not pronounced on the treated plants.

Oxalate oxidase (OxOx) activity has been detected in a few plant species, for example wheat (Lane et al., 1986; Hamel et al., 1998), sorghum (Pundir, 1991), barley (Zhang et al., 1995; Zhou et al., 1998;), and maize (Vuletić and Hadži-Tašković, 2000). The presence of OxOx activity in a conifer species is here demonstrated for the first time. After exposure of plants to both 5 μM and 50 μM cadmium, a decrease in OxOx activity was observed in comparison with the control at all sampling dates (Fig. 2). There was a slight increase of OxOx acivity in treated plants with treatment duration. It seems that the decrease of enzyme activity was not Cd concentration-dependent, especially in short-time treatment. OxOx has been considered to be a candidate for production of extracellular H_2O_2 (Zhang et al., 1995; Lane et al., 1993; Dumas et al., 1993), which is involved in the signal transduction events associated with defense responses to various kinds of stress (Levine et al., 1994). In this way, oxalate oxidase may play a role in regulation of the hypersensitive response (Zhou et al., 1998). Additionally, increased transcription of a gene for germin-like oxalate oxidase in wheat and barley leaves following pathogen attack (Zhou et al., 1998; Hurkman and Tanaka, 1996) and modulation of germin gene expression in wheat seedlings by heavy metal ions (Berna and Bernier, 1999) suggested the involvement of this enzyme in the plant response to stress. On the other hand, it is known that metal toxicity depends on soil pH, organic matter, and the phosphate status of the soil (Das et al., 1997). Under conditions of low pH, cadmium appears to be absorbed passively (Cutler and Rains, 1974) and translocated freely (Hemphill, 1972). In the cell, it can interfere with metabolism through competition for uptake, inactivation of enzymes, and displacement of elements from functional sites (Das et al., 1997). Our results show a toxic effect of Cd on OxOx, which implies that this enzyme produces a lower amount of H_2O_2 in comparison with the control. This may affect further reactions involved in the plant response to Cd stress. An increase of OxOx content and activity was found in plants exposed to different treatments (Zhang et al., 1995; Delisle et al., 2001). Our results show that Cd treatment caused a decrease of OxOx activity in young Serbian spruce plants. The slight temporary increase of OxOx acitivity after initial decline in treated plants may be due to partial recovery of the enzyme.

Decrease in concentration of both reduced and oxidized glutathione was observed in plants treated with cadmium (Fig. 3), the highest difference in relation to untreated plants being observed after 24- and 48-h treatment. There was no significant considerable difference of glutathione concentration between plants treated with 5 μM and 50 μM Cd.

Decrease of total ascorbate and increase of ascorbic acid (AA) concentration with the passage of in the control plants (Fig. 4) are due to changes of this compound during growth and development of young plants. In plants treated for 6 h, there was no difference of ascorbic acid concentration in comparison with the control plants, while total ascorbate was increased, which indicates that the dehydroascorbate (DHA) concentration increased. In further treatments, there was a considerable decrease of reduced ascorbate in treated plants in comparison with untreated ones, especially after 12 and 48 hours. However, a decrease of AA concentration was observed in relation to the control, being independent of cadmium concentration in the case of plants treated for 12 and 24 h. A decrease of AA concentration in the case of higher Cd concentration was observed in plants treated for 48 h. These results show that both the glutathione and the ascorbate defense systems were damaged after short-term exposure to cadmium, and the decrease was proportional to the duration of treatment.

Table 1 shows phenolic acids detected in young Serbian spruce plants. It is evident that the concen-

Fig. 1. Seventy-five-day-old Serbian spruce plants grown on a hydroponic solution. From left to right: untreated plants and plants treated with 5 μM and 50 μM CdSO4.

tration of catechin is considerably higher than that of other phenolic species. Catechin was also found in significantly higher concentration in relation to other soluble phenolic compounds in the needles of older Serbian spruce trees (Bogdanović et al., 2006). In young plants, only catechin content increased considerably during Cd treatment (Table 1). There was a slight increase in coniferyl alcohol and coumaric acid content. Schützendübel and co-workers (2001) found that Cd caused accumulation of soluble phenolics in the cytosol, and this reaction was much faster than lignification. Together with ascorbate, phenolics may contribute to H_2O_2 destruction in the so-called phenol-coupled ascorbate peroxidase reaction (Polle et al., 1997), thereby protecting the plants from oxidative stress. It is unknown whether phenolics are directly involved in protection against Cd, but they were found to protect cultured tobacco cells from aluminium toxicity (Yamamoto et al., 1998).

The fact that cadmium treatment of young *P. omorika* plants for 48 hours induced a decrease in concentration of the antioxidant compounds ascorbate and glutathione and a parallel decline of OxOx activity demonstrates a general toxic effect of this metal on the antioxidative capacity of these young plants. Moreover, since Cd reduced the concentration of both ascorbate and glutathione, a disturbance

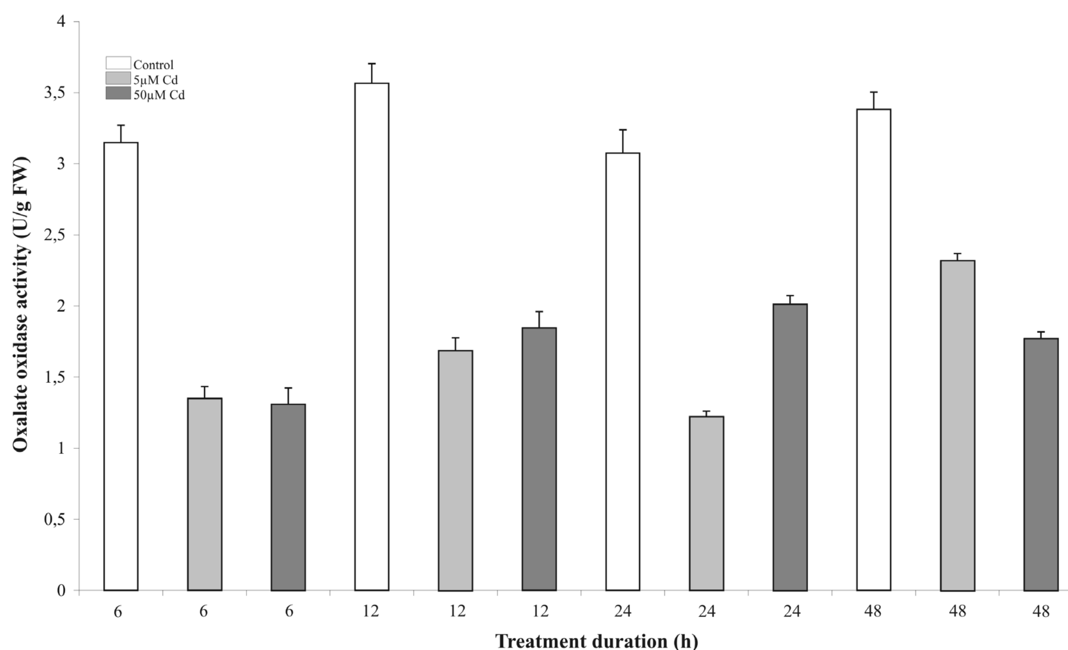

Fig. 2. Oxalate oxidase activity in 75-day-old Serbian spruce plants treated with 5 and 50 μM CdSO4 over 48 hours.

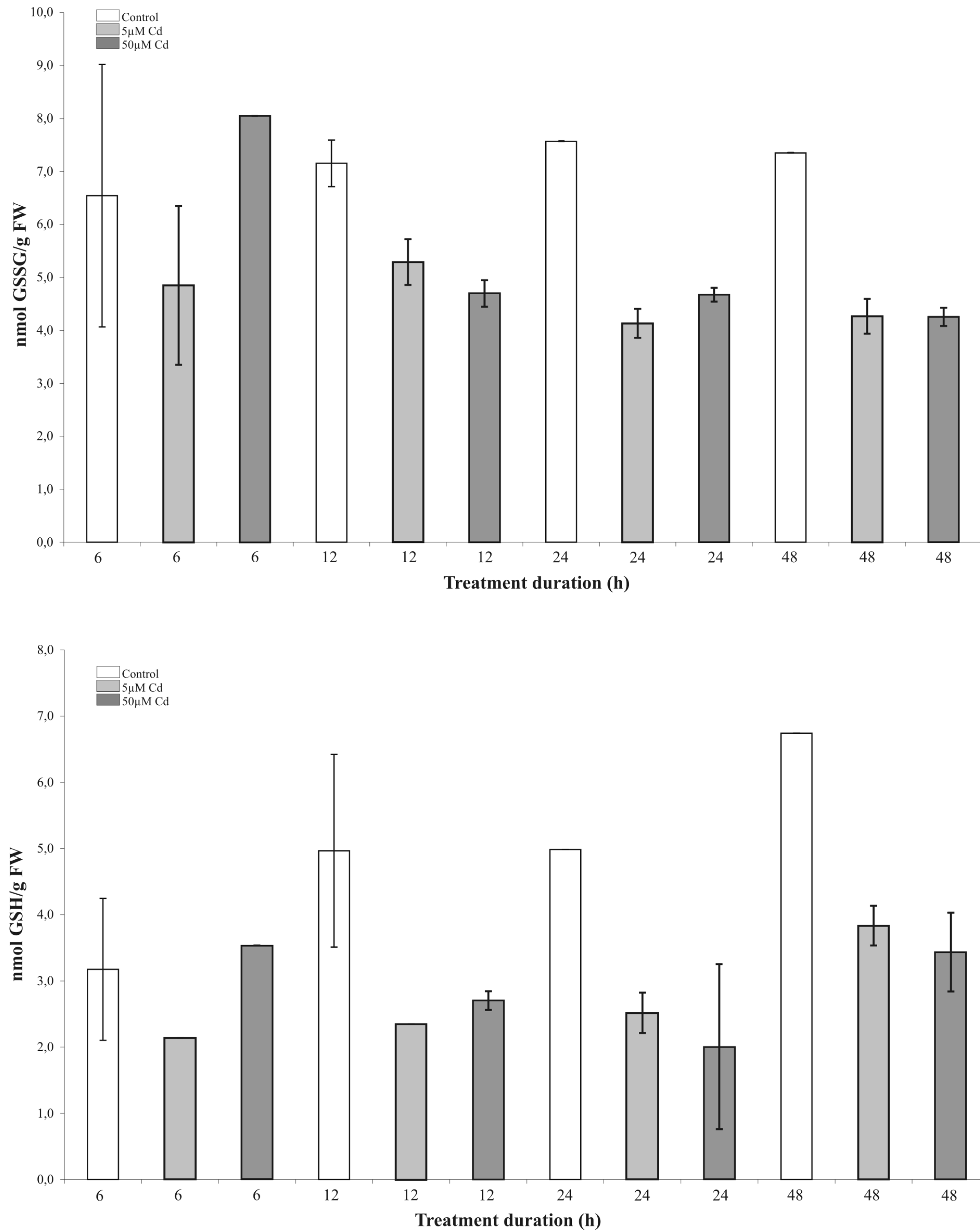

Fig. 3. Oxidized and reduced glutathione in 75-day-old Serbian spruce plants treated with 5 and 50 µM CdSO4 over 48 hours.

of the cell's redox equilibrium will probably occur as a consequence. An increase in phenol concentration was commonly observed after exposure of plants to polluted environments (Karolewski and Giertych 1995; Giertych and Karolewski, 1993). This increase under pollution stress is probably connected with the higher energy requirements of plants in such conditions and consequent increase of respiration (Tomaszewski, 1961). The fact that among soluble phenolics found in

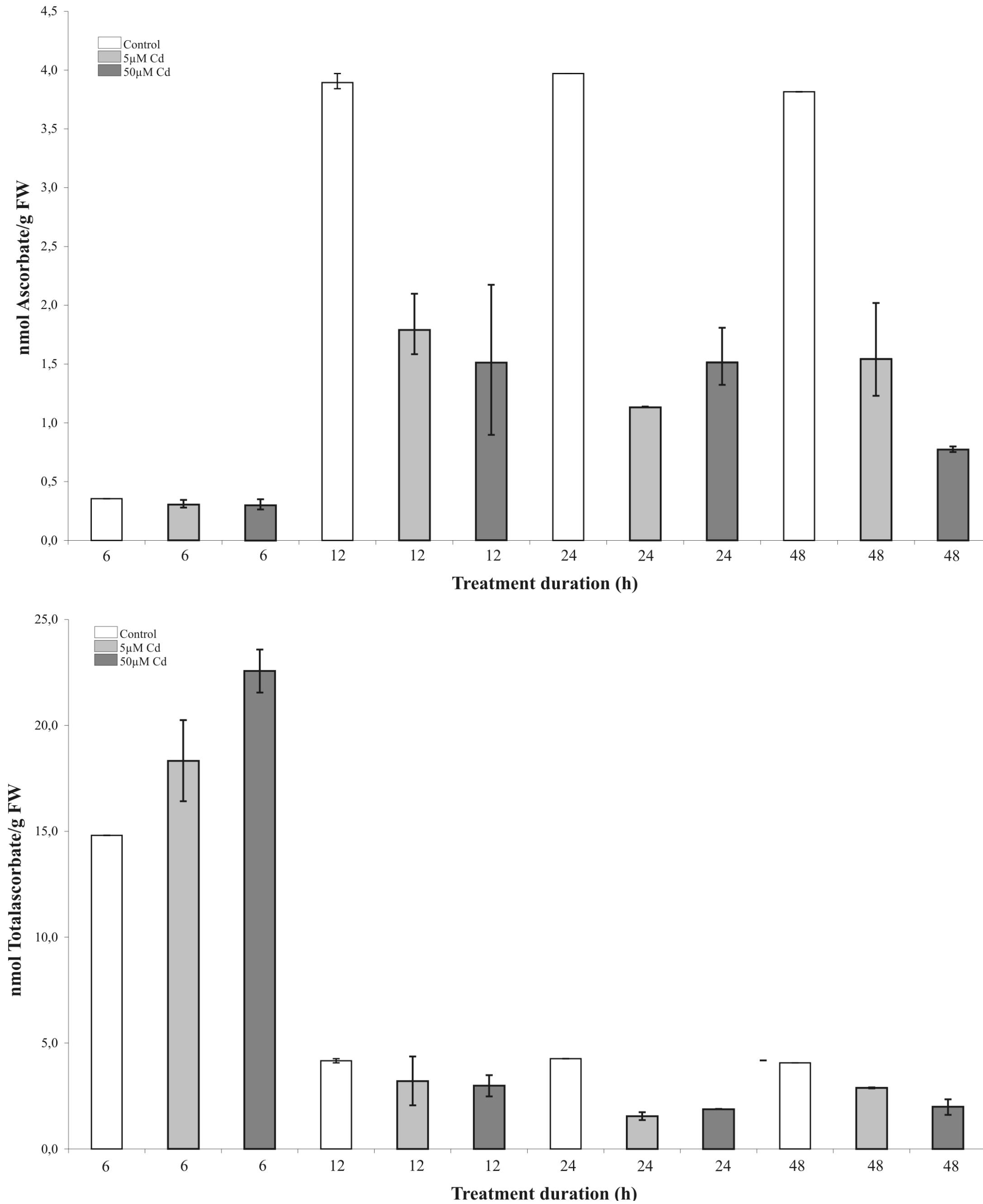

Fig. 4. Ascorbic acid and total ascorbate in 75-day-old Serbian spruce plants treated with 5 and 50 µM CdSO4 over 48 hours.

young Serbian spruce plants only catechin reacted significantly to cadmium stress with an increase in content could mean that the protective mechanism of soluble phenolics is not yet activated in young plants after short-term stress. On the other hand, catechin has been considered to be a possible bio-indicator of early stress in Scotch pine, since among many phenolics, it was only the content of catechin in needles that followed the dynamics of external pollution (Härtlig and Schulz, 1998).

Table 1. Table 1. Individual soluble phenols in the extract of 75-day-old Serbian spruce plants: untreated plants and plants treated with 5 μM and 50 μM Cd for 6 and 48 h as measured by HPLC [μmol /g FW].

Soluble phenol	Control	Treatment with cadmium			
		5μM		50μM	
		6h	48h	6h	48h
Catechin	55.8	67	88.5	71.2	76.7
Chlorogenic acid	4.8	5.3	4	7.6	6.5
Syringic acid	10.1	14.3	7.8	16.5	16.7
Coniferyl alcohol	7.4	10.4	11.9	11.3	14.5
Coumaric acid	1.2	0.9	3.8	1.9	3.4
Isoferulic acid	7	11.4	9.4	5.3	9.5

The obtained results show that antioxidative systems in young Serbian spruce plants are depressed after short-term stress caused by both 5 μM and 50 μM cadmium, and this effect is Cd-dose independent. The given toxic effect is reflected in an early decrease of OxOx activity, as well as a later decrease of ascorbate and glutathione content (24- and 48-h treatment duration). OxOx is thus shown to be a sensitive indicator of stress in young Serbian spruce plants. Taken together, our results suggest that inhibition of oxalate oxidase by Cd may partly contribute to decline of H_2O_2 production. This might be an adjustment of plants to generally increasing H_2O_2 production after Cd- stress (Schützendübel et al., 2002). Our study shows that the measured biochemical parameters indicate injury to young plants at a time when symptoms of damage are not yet visible. Increase in the content of catechin in treated plants shows that this phenol is one of the first indicators of early stress in young Serbian spruce plants.

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ОКСАЛАТ ОКСИДАЗА И НЕ-ЕНЗИМСКА ЈЕДИЊЕЊА АНТИОКСИДАТИВНОГ СИСТЕМА У МЛАДИМ ОМОРИКАМА НАКОН КРАТКОТРАЈНОГ ТРЕТМАНА КАДМИЈУМОМ

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Проучаване су промене у концентрацији аскорбата и глутатиона, саставу простих фенола и активност оксалат оксидазе, у 75 дана старим оморикама, после излагања 5 μM и 50 μM кадмијуму у току 6-48 сати. Овде је први пут показана оксалат оксидазна (OxOx) активност у једној четинарској врсти. Обе концентрације Cd су индуковале смањење OxOx активности у третираним младим биљкама у односу на контролу, за све дужине третмана. Утврђено је смањење концентрације редукованог и оксидованог глутатиона, као и редукованог аскорбата у биљкама у току 48 сати третмана кадмијумом. Међу простим фенолима, само количина катехина је знатно повећана у биљкама у току третмана.