The Level of Oxidative Stress in Poplars due to Heavy Metal Pollution in Soil

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Abstract

Poplar trees are commonly used in phytoremediation thanks to its specific characteristics such as large leaf surface, the conductivity of water and minerals throughout the section of the tree (diffuse porous species) and easy vegetative propagation through cuttings. The use of poplars in detoxification of soil and water contaminated with heavy metals has been demonstrated for various species.

The effect of different concentrations of three heavy metals ions, Cu^{2+}, Ni^{3+} and Cd^{2+}, on oxidative stress in three poplar clones from two different species (PE 19/66 and B229), belonging to *Populus deltoides* (Marshall) and to (M1 clone) *Populus euramericana* (Dode-Guinier) were analysed. Biochemical parameters which were applied for identifying the level of oxidative stress in these three clones were the following: soluble protein content, lipid peroxidation, ferric reducing antioxidative power assay and superoxide dismutase activity. Possible antioxidant capacity of these clones in the response to different concentrations of heavy metals ions in substrate was measured in order to find which clone is most appropriate for phytoremediation processes. Results showed variable responses within poplar leaves and roots in response to oxidative stress induced by heavy metals and the most promising clone for phytoremediation of contaminated soils is B229 clone, while M1 and PE 19/66 showed variable antioxidant response.

Key words: heavy metals, poplar, oxidative stress, antioxidative activity, phytoremediation

Introduction

In plants, the appearance of mostly toxic oxygen and nitric oxide molecule species causes disruption of cell membrane components and lipid peroxidation, whereas activated forms of oxygen may damage the molecules of chlorophyll which is manifested by the loss of green colour and reducing the intensity of photosynthesis. Plants may be used for reducing the concentration of heavy metals in the soil and groundwater.

The concentrations of heavy metals in soils depend on the weathering of the bedrock and on atmospheric inputs of metals. Natural sources of heavy metals in the atmosphere are volcanoes and continental dusts. Anthropogenic activities like mining, combustion of fossil fuels, metal based industries, phosphate fertilizers, etc., lead to the emission of heavy metals and their accumulation in the ecosystems. The availability of heavy metals to plants and, thus, their toxicity depends on complex rhizospheric reactions involving not only exchange processes between the soil and plants but also microbial activities (Schützendübel and Polle 2002).

The genus *Populus* is geographically widespread in various climatic areas and its presence can be observed in the severe soil conditions (pioneer species) that characterize heavily contaminated areas (Pulford and Watson 2003). By screening some *Populus nigra* genotypes for cadmium removal from hydroponic medium, Dos Santos Utmazian et al. 2007, showed that only around 1% of the total absorbed cadmium is reported to accumulate in leaves (Pietrini 2008).

There is evidence in the literature on the phytoremediation capacity of the genus *Populus* and, at the same time, an obvious lack of biochemical data regarding the intensity of oxidative stress caused by the accumulation of different heavy metals ions in tree species. Poplar species can accumulate high concentrations of heavy metal ions like cadmium (Cd), nickel (Ni), zinc (Zn), copper (Cu) and lead (Pb) in the aboveground tissues. The accumulation of cadmium ions in poplar is 30 times higher compared to other tree species, which is reflected by the highly contaminated regional reforestation (Migeon et al. 2010). These plants have efficient mechanisms for the transfer of heavy metals in aboveground tissues (Migeon et al. 2010). However, the exact localization of heavy metals, especially zinc and cadmium in plant cells is still unknown in this species. So far, it was discovered that cadmium and zinc are accumulated in high concentrations in the mesophyll and epidermal layer of leaves (Migeon et al. 2010). Current evidence suggests that plants share several common mechanisms of tolerance to metals.

More than 400 different species belonging to taxonomically very different groups are described as hyperaccumulators (Ninkov et al. 2010; Zeremski-Skorić 2010). Hyperaccumulators are officially considered as plants that are able to accumulate more than 0.1% dry weight of elements such as Ni, Co (Cobaltum) or Pb. The limit for Zn is more than 1%, while for Cd it is > 0.01 % of dry matter (Zeremski-Skorić 2010). The phenotypes characterized as hyperaccumulators provide the basic concept for the process of phytoremediation and the use of these plants to clean soil polluted with various organic and inorganic pollutants. Hyper accumulation is usually observed for Ni, Zn, Co and Se (Selenium). So far, tolerance to heavy metals has been mostly studied in the cases of Zn, Cu, Ni, Cd ions and the study of these mechanisms is further complicated by the fact that some of the metals occur in different oxidation states (Sanita di Toppi and Gabbrielli 1999).

Some species can grow at sites with moderate to sizable HM contamination (Watmough and Dickinson 1995, Lepp and Madejon 2007) or can be planted for phytoremediation purposes, taking advantage of their rapid growth and large biomass (Laureysens et al. 2004, 2005, Maxsted et al. 2007). Applications using genetically modified poplars to enhance metal tolerance/accumulation are under development (Peuke and Rennenberg 2005, Merkle 2006). Information about metals, especially Ni, Cd and Cu compartmentation inside tree phytoextractors is, however, lacking since micro-localization studies have so far been carried out using a rather small group of herbaceous species. Such evidence is needed to understand metal uptake in trees and design innovative applications targeting tree-relevant metal accumulation or tolerance mechanisms (Vollenweider et al. 2011). The white poplar (*Populus alba* L.) cv. "Villafranca" was used in the study of Balestrazzi et al. in 2009, in form of *in vitro* cell suspension cultures, to firstly monitor how different concentration of heavy metals can inflict on NO metabolism and its crucial enzymes, to gain biochemical properties of how white poplar cope with intoxication on molecular level in possible phytoremediation usage.

The visual symptoms of cadmium toxicity in all plant species are stunted growth (plant height and biomass), root length and reduced chlorosis in younger leaves. Poplar plants exposed to different concentrations of Cd^{2+} in growing nutrient media showed morphological, physiological and biochemical changes. In this case, decreases in biomass, leaf number, weight per plant, leaf area per plant, stem diameter and height have been reported. Sebastiani et al. (2004) studied woody cuttings of two clones of hybrid poplar (*Populus deltoides*×*maximowiczii Eridano-clone* and *P.*×*euramericanaand-clone I-214*) and reported morpho-physiological changes in these clones in response to intoxication induced by increased concentrations of industrial waste, including copper and cadmium. These poplar clones reacted by increasing plant growth and accumulation of metals, although the clones differed in response to pollutants, suggesting their potential adaptability to different polluted habitat. Differential responses between clones indicate the need for testing the increasing levels of industrial waste and developing programs that will include obtaining poplar clones that have high potential for accumulation of heavy metals in polluted habitats.

Also, in plants exposed to toxic levels of heavy metals is reported increased production of metal-binding proteins such as metallothioneins (MTs) and phytochelatins (Macovei et al. 2010).

The aim of study

The aim of this study was to determine the intensity of oxidative stress in three poplar clones from two species (*Populus euramericana*-M1, *Populus deltoides* 19/66 PE and B229), exposed to different concentrations of Cu^{2+}, Ni^{3+} and Cd^{2+}. The intensity of oxidative stress was screened *in vitro* by the following biochemical parameters: level of lipid peroxidation (LPx), total redox capacity of the samples by ferric reducing antioxidative power assay (FRAP), superoxide dismutase (SOD) activity and soluble protein content. The *in vitro* experiments are used to observe and screen the intensity of oxidative stress induced by heavy metals in the three different poplar clones, in order to obtain insights into the possibility of using these clones in phytoremediation of contaminated soil and theirs biochemical response to external contamination regarding future profiling of poplar clones in Serbia in possible phytoremediation usage.

Material and methods

Design of experiment and preparation of plant material

Experimental design was set in pots within greenhouse in semi-controlled conditions, at the Department of Biology and Ecology, Faculty of Science, University of Novi Sad. The experiment was a factorial design with three clones of two poplar species (*Populus euramericana*-M1, *Populus deltoides* B229 and PE 19/66), provided by the Institute of Lowland Forestry and Environment (Novi Sad, Serbia) collection.

Heavy metals (Cu^{2+}, Ni^{3+} and Cd^{2+}) were introduced to soil and all three clones were intoxicated with various concentrations of them. Concentrations of heavy metals were determined according to the maximum permissible concentration (MPC) for given heavy metals by implemented by: ***i)*** the Regulations on permitted amounts of hazardous and harmful substances in soil and methods of their testing published in the *Official Gazette* of the Republic of Serbia no.23, 1994, ***ii)*** the Ordinance on the methods of organic plant production and gathering wild fruits and medicinal plants as products of organic agriculture (from: *Official Gazette* of RS, 23/1994, SRJ, 51/2002) ***iii)*** and given the limits for the content of certain heavy metals in the soil. The values for the investigated metals concentrations were: MPC (Cu^{2+}) =100 mg/kg, MPC (Cd^{2+}) =3mg/kg, MPC (Ni^{3+}) =50 mg/kg, respectively.

Sandy Fluvisol soil in pots was contaminated with the heavy metal treatments as presented in Table 3. Each treatment was set up in three replicates. The land was treated and left for three months to form inner microbiological environment before official start of *in vitro* plants growing period. Metals were added as nitrate salts: $Cu(NO_3)_2$, $Cd(NO_3)_2$ and $Ni(NO)_3$, so they made the exact concentration calculated per 100 kg soil and were dissolved in deionized water and sprayed onto the soil, which was thoroughly homogenized by mixing and placed in pots of 10 kg. In each pot, 4 poplar clones were planted as two-year-old seedlings, so with a single treatment (one concentration of a metal) 12 plants (4 plants of each clone) were contaminated. Also, control poplars were planted in uncontaminated soil. Whole plant seedlings were set in pots in early spring (last week of February, 2012) and lasted for five months, until June, 2012. The soil was watered constantly to maintain optimal soil humidity. Every four weeks, 0.2 l of full-strength Hoagland solution was added to the pots. Leaves and roots of poplar clones were immediately sampled and series of extracts was prepared for *in vitro* analysis, at the end of five months growing period.

Soil characteristics, used in experimental design, through horizons are presented in the Table 1 and 2. These data presents characteristics of sandy Fluvisol soil which is commonly used in designing *in vitro* experiments for poplar species on Institute of Lowland Forestry and Environment, University of Novi Sad (Kebert et al. 2011).

Table 1. Chemical properties of soil through the horizons

Horizon	Depth Cm	pH In H_2O	Humus %	$CaCO_3$ %
1	2	3	4	5
Ap	0-30	7.55	2.64	17.08
I	30-58	7.91	1.58	19.56
II	58-72	8.08	1.00	16.30
III Gso	72-110	8.22	1.09	19.10
IV Gso	110-175	8.53	0.46	15.93

Table 2. Granulometric composition of soil through the horizons

Horizon	Depth	Particle size composition %						Textural class
		Coarse sand	Fine sand	Silt	Clay	Total sand	Total clay	
		> 0.2	0.2 – 0.02	0.02 – 0.002	< 0.002	> 0.02	< 0.02	
	Cm	Mm	mm	mm	mm	mm	mm	
1	2	3	4	5	6	7	8	9
Soil profile 1								
Ap	0-30	0.5	37.4	40.4	21.7	37.9	62.1	Loam
I	30-58	0.3	45.9	34.8	19.0	46.2	53.8	Loam
II	58-72	0.3	71.0	15.9	12.8	71.3	28.7	Sandy loam
III Gso	72-110	1.9	40.5	37.7	19.9	42.4	57.6	Loam
IV Gso	110-175	2.5	88.5	1.5	7.5	91.0	9.0	Sand

Table 3. Treatments of heavy metals used for contamination of poplar clones in experimental design

Treatment	Cd^{2+}(mg/kg)	Cu^{2+} (mg/kg)	Ni^{3+} (mg/kg)
0.5 MPC[1]	1.5	50.0	25.0
1 MPC	3.0	100.0	50.0
2 MPC	6.0	200.0	100.0
3 MPC	9.0	300.0	150.0

[1] MPC – maximum permissible concentration of heavy metal

Preparation of plant extracts

Plant extracts were obtained from 2 g of plant material (leaves and roots, separately) homogenized with quartz sand and suspended in 10 cm^3 0.1 mol/dm^3 K_2HPO_4 at pH 7.0 and placed into a cold porcelain mortar and macerated for 2 - 3 minutes (Kebert et al. 2011).

Homogenate was centrifuged for 10 min at 4000 g (QuyHai et al. 1975). The resulting supernatant was used for different antioxidant and scavenger determinations: SOD activity and the soluble protein content, total antioxidant activity FRAP method and lipid peroxidation through the determination of malonyldialdehyde (MDA).

In vitro studies of extracts poplar clones

Total antioxidant capacity of all extracts was estimated according to the Ferric Reducing Antioxidant Power (FRAP) assay (Benzie and Strain 1999). The absorbance reading was performed at 593 nm. Total reducing power was expressed as FRAP units. FRAP unit was equal to 100 $\mu mol/dm^3$ Fe^{2+} and the value was calculated using the given formula, where ΔA sample is the change in absorbance of the sample and ΔA standard is the change in absorbance of the standard (100 μmol/dm^3 Fe^{2+}) after 4 min incubation at 593 nm:

FRAP value=$\Delta A_{sample}/\Delta A_{standard}$

Lipid peroxidation (LPx) was determined by measuring the amounts of malonyldialdehyde (MDA) which is one of its end-products and is quantified by the bythiobarbituric acid (TBA) method at 532 nm (Placer et al. 1968). The values were given as nmol of MDA per mg of soluble proteins.

Soluble protein content was determined by the Bradford method (1976) and expressed as mg protein per g of dry weight. Absorbance reading was performed at 595 nm.

Activity of superoxide dismutase (SOD) was determined based on the inhibition of photochemical reduction of nitroblue tetrazolium (NBT), resulting in blue reduction product of NBT with O$^{2\bullet-}$. Solution in test tubes was stirred for a few seconds and set in front of the light source for 10 minutes (Auclair and Voisin 1985). The unit of SOD is represented by the quantity of enzyme that inhibits NBT reduction by 50% at 25°C and 560 nm. All absorbance readings for soluble proteins, SOD and FRAP were performed by using Janway UV/VIS spectrophotometer 6505, while for the MDA reading the Multiscan Spectrum Thermo Corporation was used.

Statistical analysis

All determinations were performed in triplicates. Statistical comparisons between samples were performed with the Statistica 9 program, using Duncan's test, with statistical significance $p < 0.05$, and comparing treated samples with proper control. Obtained results are presented graphically by histograms and above them are letters referring to statistical difference among results and control (from **a** to **e**). Values with the same letter, in each colon, are not significantly different according to Duncan test ($p < 0.05$).

Comparable percentage was done by the formula: **Δ (%) = (100 × sample / control) -100;** where values may result with +, which means it increases compared to the control and with – it decreases compared to the control. All percentage data are mentioned and discussed compared to the control.

Results and discussion

The effects of different heavy metals treatments on the content of soluble proteins in leaves of three poplar clones are presented on Figure 1. After treatments with copper ions, the M1 clones protein content showed decline to 25.51%, at 0.5 MPC treatments and an increase to 27.25% on 1 MPC treatment compared to the control. The content of soluble proteins continued to decrease in 3 MPC treatments. The B 229 clone showed an increase of 89.02% in 1 MPC treatment compared to the control. The content of soluble protein in PE 19/66 clone increased almost linearly in all treatments (from 27% to 204.61% compared to the control). The M1 clone showed significant reduction of soluble content protein after the first treatment with cadmium 0.5 MPC, but the changes occurred in similar manner in all treatments

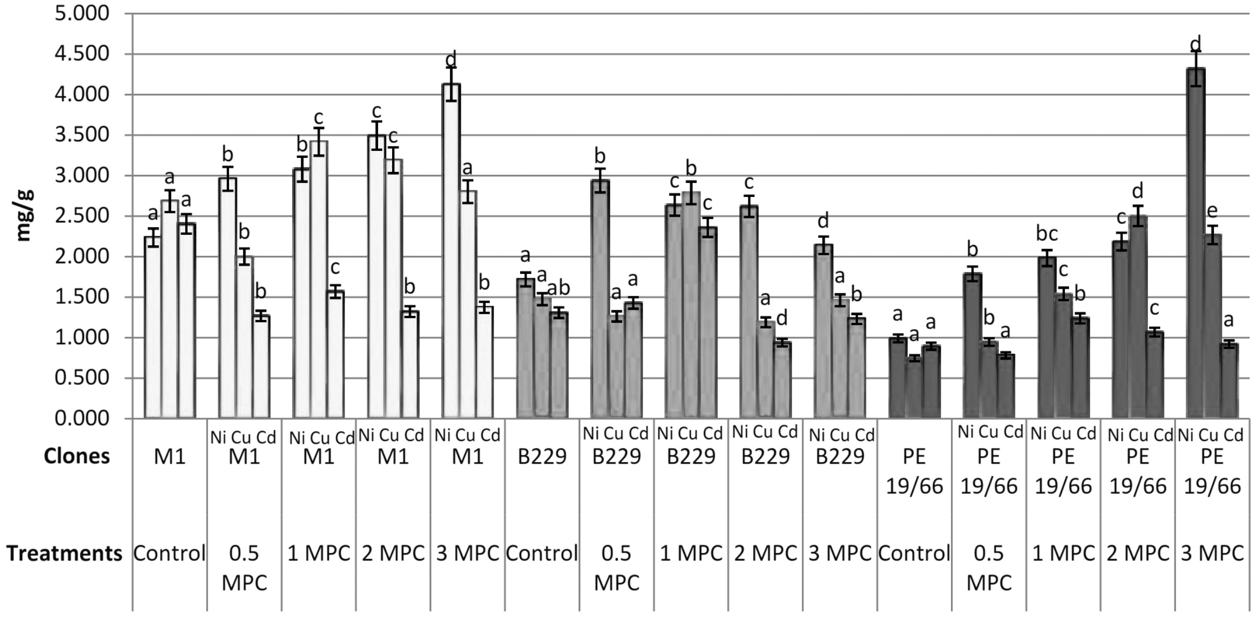

Figure 1. Content of soluble proteins in leaves of three poplar clones

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(from 47% for 0.5 MPC to 34.74% for 1 MPC, compared to the control). The B 229 clone showed a similar trend in changes after treatment with copper. The 1 MPC treatment of copper caused a significant increase in soluble proteins (80.6%) compared to the control. The PE 19/66- clone did not induce any significant difference compared to control. The M1 clone showed an increase in the soluble proteins content, up to 3 MPC (to 84.75%), compared to the control after nickel treatment. The B 229 clone showed no significant changes compared to the controls, while the PE 19/66 clone presented an increase in the soluble protein content compared to the control, ranging from 80.47% in 0.5 MPC to 336.28% at 3 MPC.

High antioxidant activity was reported throughout the test period in specimens of *Populus alba* L (Štajner et al. 2011). In *Populus alba*, the process of lipid peroxidation and accumulation of free proline was intensified in order to protect the leaves from oxidative damage in dry periods, especially during July. Also, the drought was the most severe in summer period and accumulation of antioxidant molecules was induced by stressful factor. This indicates possibility of powerful influence of abiotic stress on plant ontogenesis (Štajner et al. 2011). High concentrations of heavy metals in the soil influences poplar clones to induce various antioxidant responses in order to cope with contamination. The results shown in Fig. 2 referred that the M1 clone showed no significant change in the soluble proteins content in roots compared to the control, but an increase in the content of soluble protein was present in the B 229 clone compared to the control. The most significant changes were noticed in 1 MPC treatment (35.65%) and 3 MPC treatments (63.54%). The PE 19/66 clone reported a slight increase in the protein content (2 MPC treatments for 49.09%) and a decrease after the 3 MPC treatments. Also, the B 229 clone decreased from 0.5 MPC down to 16.06% and at 1 MPC, when the highest decrease recorded 54.69%, while at 2 MPC and 3 MPC treatments the values returned to very near of the control. The PE 19/66 clone showed no significant changes compared to control. As far as the treatment with nickel is concerned, the M1 clone showed noticeable increase in the content of soluble protein compared to the control in 0.5 MPC by 25.82% and in 3 MPC treatments by 53.22%. The 3 MPC was the only treatment where there was a significant increase of 42.85% compared to the control. In PE 19/66 clone there was a slight increase of the soluble proteins content of 35.03% in 2 MPC treatments, and a slight decline of 30.41% in 3 MPC treatments compared to the control.

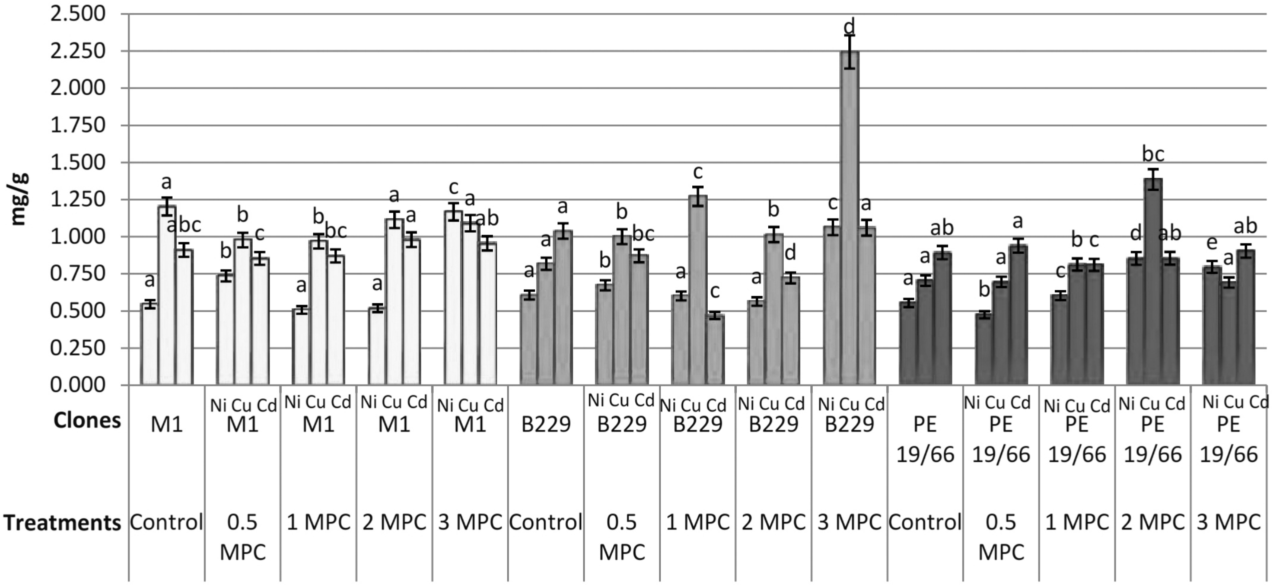

Figure 2. Content of soluble proteins in roots of three poplar clones

All species within the genus *Populus* share biochemical similarity in the metabolic routes of secondary metabolites biosynthesis. However, there are differences in the concentration and nature of these secondary compounds between species. One such analysis, which helped closer understanding of the chemical diversity within the genus *Populus* and contributed to the characterization of compounds that can directly participate in reducing oxidative stress in the plant, has been conducted by Dudone et al. (2010).

They examined the antioxidant properties and identification of secondary biomolecules in aqueous extracts of black poplar (*Populus nigra*) buds. The analysis showed a high content of phenolic compounds and strong antioxidant properties, determined by ORAC assay. These results may refer that antioxidant compounds may be responsible for strong antioxidative defense within clones B229 and M1, since they showed acceptable antioxidative answers against high concentration of heavy metals up taken from contaminated soil. The leaves showed highly variable changes and increase or decrease in the content of soluble proteins. This may indicate the possibility of antioxidant enzymes synthesis during oxidative stress induced by heavy metals. The increase in synthesis, and consequently in the soluble proteins content, may indicate that our poplar clones, especially clone B 229 and M1, have relatively rapid regulatory mechanisms for protection against oxidative stress.

Fig. 3 shows the results of the MDA content in leaves of the three poplar clones. The MDA content should be reduced if the clones have good antioxidant defense mechanisms (Strobawa and Lorenz-Plucinska 2007, 2008). Treatments with copper show significant changes in lipid peroxidation assay**.** An increase of MDA by 41% in M1 in 0.5 MPC treatments was observed. Values of reduction ranged from 21.02% (for 1 MPC treatment) to 25.61% (for 3 MPC treatments). All observed changes were compared to the control samples. The B 229 clone registered variable changes; it reacted differently to the increased concentration of copper in the soil. The largest decrease of 47% was observed in 1 MPC compared to control, while in 3 MPC the control values were regained. High concentration of MDA in the control plants of clone PE 19/66 was observed; 2 MPC and 3 MPC treatments reached the lowest value of MDA (76.55% decrease compared to the control of the 2 MPC and 77.15% for 3 MPC treatment). The cadmium treatment showed very pro-oxidative influence on clones**.** The M1 clone showed a large increase in the MDA content compared to the control in all treatments. The changes ranged from 121.89% in 0.5 MPC to 64.10% in 1 MPC treatment. Other treatments were in the range of these values. The responses regarding the B229 clone were variable and showed trend of reduction in the MDA content up to 32.57% in 1 MPC treatment compared to the control and rapidly increased to 74.19% at 2 MPC treatments, while the 3 MPC treatments presented baseline values. The PE 19/66 clone showed no significant changes compared to the controls. Slight decrease of 20.59% in the content of MDA in 2 MPC treatments was noted and then return to the control values similar to those in 3 MPC was also present. Treatments with nickel showed that clone M1 resulted in downward trend compared to the control in all treatments (from 32.08% in 1 MPC to 51.65% in 3 MPC treatments). For clone B 229, we observed a downward trend compared to the control in all treatments (from 41.58% in 0.5 MPC treatments to 25.54% in 3 MPC treatments). The PE 19/66 clone showed a downward trend compared to the control in all treatments (from 40.04% in 0.5 MPC treatments to 75.66% in 3 MPC treatments). The results of researchers in China have shown that other abiotic factors influence the effectiveness of the defense of *Populus* species to oxidative stress induced by drought. They performed experiments using *Populus kangdingensis* C. Wang et Tung and *Populus cathayana* Rehder, native to different parts of the eastern Himalayas. Those species were investigated

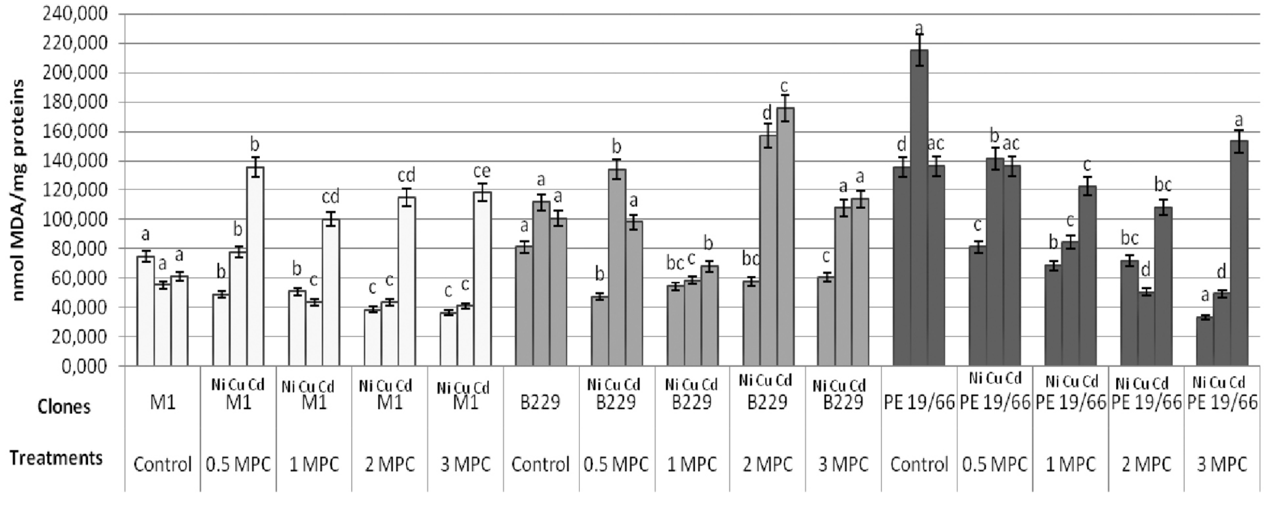

Figure 3. Intensity of lipid peroxidation in leaves of three poplar clones

during one vegetation period in the greenhouse, in order to determine the impact of progressive drought stress. The results are reflected in the fact, that the adaptive responses to this type of stress are different in these two species, originating from different altitudes. Significant changes in the heights of trees, development of leaves, the relative proportion of water (RWC), hydrogen peroxide and amount of MDA appeared earlier in *P. cathayana* than in *P. kangdingensis*, while the changes in soluble proteins, carbohydrates, proline and antioxidant enzymes occurred earlier in *P. kangdingensis*. In addition, changes in these parameters were becoming more and more significant and noticeable, as the drought progressed, especially in *P. cathayana*. Plant growth showed a significant positive correlation with soluble proteins and sugars and significantly negative correlation with RWC. In contrast to *P. cathayana, P. kangdingensis* was able to maintain a significant level of growth and development rate under conditions of severe drought. Also, *P. kangdingensis* showed a large increase in the content of soluble proteins and sugars, but much lower increase in the content of MDA and hydrogen peroxide, compared to *P. cathayana* cuttings, when they were exposed to progressive drought stress. Their results showed that *P. kangdingensis* originating from higher altitudes was more resistant to drought than *P. cathayana* originating from the lower altitudes (Yang and Miao 2010).

The results of MDA content in roots extracts from our poplar clones are shown in Fig. 4. The copper treatment resulted in the following types of changes within the clones: the M1 clone registered a sharp increase in the content of MDA in comparison to the control treatment at 2 MPC for 103.94% and a sudden drop of 81.2% in 3 MPC treatments; the B 229 clone showed variable responses to treatments and there was a significant increase of over 33.61% in 2 MPC treatments, while the PE 19/66 clone registered a sharp increase of 230.58% compared to the control, in the first 0.5 MPC treatments and the sudden decline of 29.80% in 2 MPC and a high increase of 280.67% in 3 MPC. In the cadmium treatment, no significant changes were observed compared to the control in the case of the M1 clone. The B 229 clone showed variable responses to increased content of generated MDA product when compared to the control. The largest increase of 246% was achieved with the 1 MPC treatment and it was followed by a decrease of 41.81% compared to the control in 3 MPC treatment. The PE 19/66 clone registered an increase of 33.74% in 0.5 MPC treatment compared to the control. The following changes were observed in the nickel treatment**:** the M1 clone showed variable responses, where 0.5 MPC treatments were reduced to 40.52% compared to the control, and a sudden increase of 23.91% in the content of MDA in 1 MPC treatment compared to the control and a decrease of 60.83% in 3 MPC treatment compared to the control; the B 229 clone showed a slight increase of 36% in the 1 MPC treatments and a decline similar to the values of the control in 3 MPC treatment; in the case of the PE 19/66 clone, variable responses

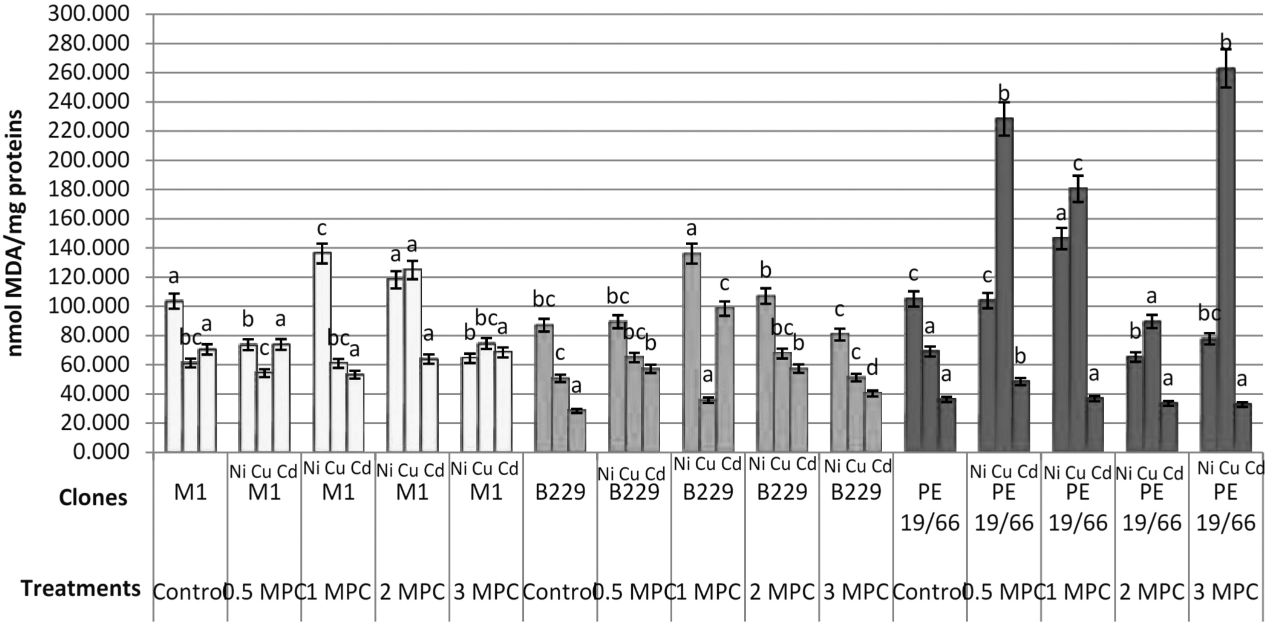

Figure 4. Intensity of lipid peroxidation in roots of three poplar clones

were registered, from an increase of 28.27% of the MDA content in 1 MPC treatment compared to the control to a sudden decline of 60.89% in 2 MPC treatment, and a decline of 35.03% in 3 MPC treatment.

He et al. (2011) studied the influence of large amounts of Cd^{2+} accumulated in buds. Accumulation of Cd^{2+} led to a reduction in the photosynthetic assimilation of carbon dioxide. Increased levels of ROS were present in all tissues, except in the bark. To deal with the Cd^{2+}-induced superoxide radicals and hydrogen peroxide, the specimens of *P.* × *canescens* relied mainly on the formation of phenols, accumulated mostly in the bark and least in the core of the tree. Other potential free radical scavengers, such as proline, sugars, alcohol and antioxidant enzymes showed tissue-specific and temporal responses to Cd^{2+}. These results showed that different parts of different plants are able to fight against oxidative stress induced by Cd^{2+}, probably due to the different capacities of plant tissue in poplar, to produce protective phenols. Our results showed that the MDA values were less in roots for all three heavy metals, indicating differential responses of plant organs to abiotic stress. The M1 and B229 clones showed decreased values of MDA in both organs then the PE 19/66 clone, indicating their better mechanisms to overcome oxidative stress induced by heavy metals. Higher concentrations of copper and nickel especially showed pro-oxidative activity in roots, while cadmium presented similar activity in leaves. An intensive study of oxidative stress in *Populus* species on different types of soil contaminated with heavy metals and organic fuels were performed by Stobraw and Lorenz-Plucinska (2007, 2008); all biochemical and physiological tests were conducted on fine roots taken from cuttings of black poplar. They referred an increase in MDA and a reduction in SOD activity at higher heavy metals concentrations.

The SOD activity from leaf extracts in different treatments is shown in Fig. 5. SOD activity should be increased if the clones that have good antioxidant defense mechanisms. SOD and LPx are inversely proportional parameters. The copper treatment applied to our clones gave different results: for M1 clone, an increase of 72.47% in SOD activity was observed in all 1 MPC treatments compared to the control, and of 102.04% in 3 MPC treatments; the B 229 clone showed only a decrease of 30.18% in 1 MPC treatment; the PE 19/66 clone also presented a decrease of 33.15% in SOD activity compared with the control in the case of the 0.5 MPC treatment, and 75.51% in 3 MPC treatment. In the cadmium treatment, within M1 clone, there was a slight increase of 39.28% in SOD values in the 2 MPC treatment and 15.45% in 3 MPC treatments.

The B 229 clone revealed a slight downward trend compared to the control, with 15.35% in 0.5 MPC and 22.1% in 1 MPC treatment, while a sudden increase of 143.56% in the 2 MPC treatments compared to the control occurred at the end. The PE 19/66 clone showed a noticeable decrease of 44.67% in 0.5 MPC treatment, and of 20.6% in 3 MPC compared to the control. Treatment with nickel showed an obvious more toxic influence to SOD activity than cadmium; the results for all clones showed decreasing trend following the intensity of the treatments. In all clones and

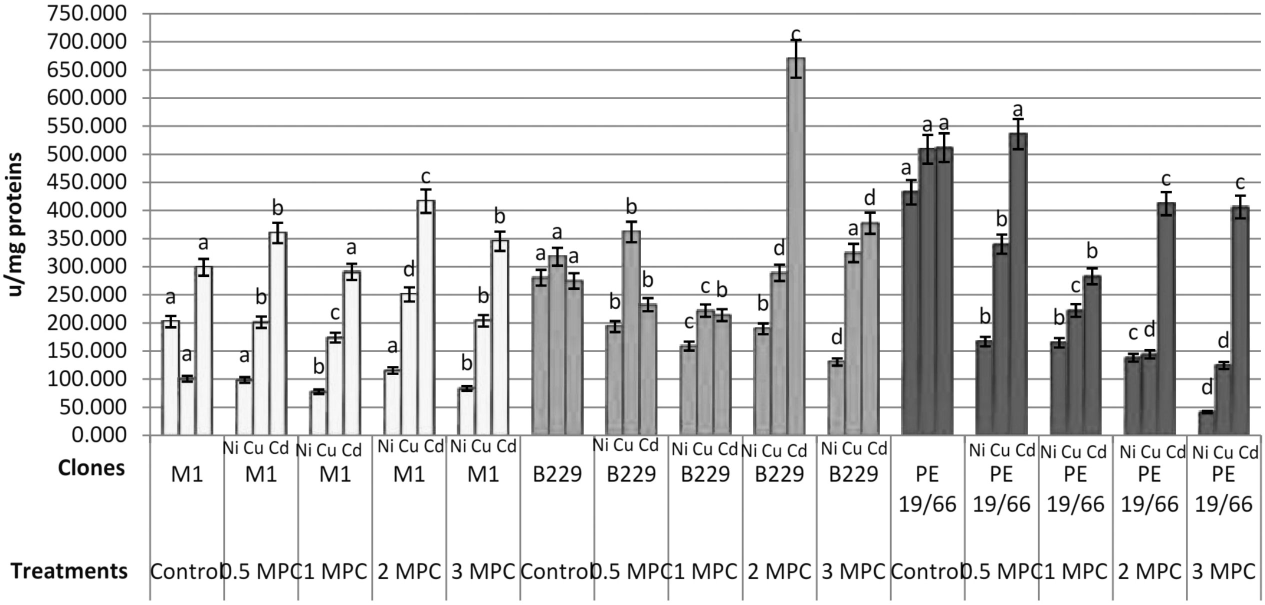

Figure 5. SOD activity in leaves of three poplar clones

in all treatments, the observed trend of decreasing SOD values compared to the control were the following: 61.51% of M1 in 1 MPC and 42.92% in 2 MPC; 30.94% of B 229 in 0.5 MPC and 53.38% in 3 MPC; 61.38% of PE 19/66 in 0.5 MPC, and 90.36% in 3 MPC.

Among the parameters that were measured in the poplar system by Sen Gupta et al. (1991), photosynthesis was the least sensitive to ozone. Ozone showed to be one of the powerful abiotic factors which may influence directly on plant physiology. SOD activity increased before there was any observable ozone effect on photosynthesis. Total SOD activity was initially unaffected by ozone, but after four hours exposure it increased by 34% and this increased level of activity was maintained 21 h later. SOD activity decreased in roots and increased in the leaves of the treated hybrid poplar *Populus nigra × maximowitzii × P.nigra* var. *Italica* clone 9111/93. Cd-induced toxic effects (stunted growth, leaf chlorosis, oxidative stress) were observed and it was indicating that this clone was vulnerable to these pollutant levels. High amounts of Cd accumulated in roots, but from perspective of its low translocation from roots to aboveground parts, along with the disturbances in plant growth, this hybrid poplar showed week potential for use in remediation of contaminated soils with heavy metal (Nikolić et al. 2008).

Regarding SOD activity, a clear increase in enzyme activity was observed only in *P. nigra* L. under drought conditions, and decreased or no changes under pollution by heavy metals (Stobraw and Lorenz-Plucinska 2007). Bole and Polle (2004) studied the oxidative stress induced by different concentrations of NaCl in the hybrid poplar *P. nigra x alba* clone INRA 717-1B4, obtained by micropropagation. They found that, under conditions of high salinity, the concentration of MDA in the roots started to increase after 7 days of exposure to NaCl and was approx. two times higher than in the control after three weeks of exposure. At low concentrations of NaCl, in the tested plants, SOD activity began to increase prior to the end of the experiment. At the high levels of salinity, the SOD activity immediately increased, and the elevated levels remained unchanged for about two weeks and then started to increase, corresponding to increasing concentrations of MDA, protein degradation and loss of biomass production. However, the time dependence on SOD activities in this study showed two distinct stages: first a rapid increase in SOD activity when oxidative damage was not noticeable, and the second an increase in MDA. The data indicate that, although the roots were in direct contact with NaCl, they demonstrated their capability to deal with oxidative stress better than shoots.

The roots reaction to all heavy metal treatments are shown in Fig. 6. In the case of Cu-treatment in M1 clone we observed an increase of 86.79% in 1 MPC and 62.09%in 3 MPC treatments compared to the control. The B 229 clone showed variable responses; first a slight increase in 0.5 MPC treatments and 2 MPC was noticed, and a significant decrease of 44.7% in 1 MPC treatments and of 59.28% in 3 MPC compared to the control. The PE 19/66 clone also showed variable responses and the most significant changes presented a SOD increase of 29.91% in 0.5 MPC, and of 40.2% in 3 MPC. Cd-treatment for M1 clone showed variable

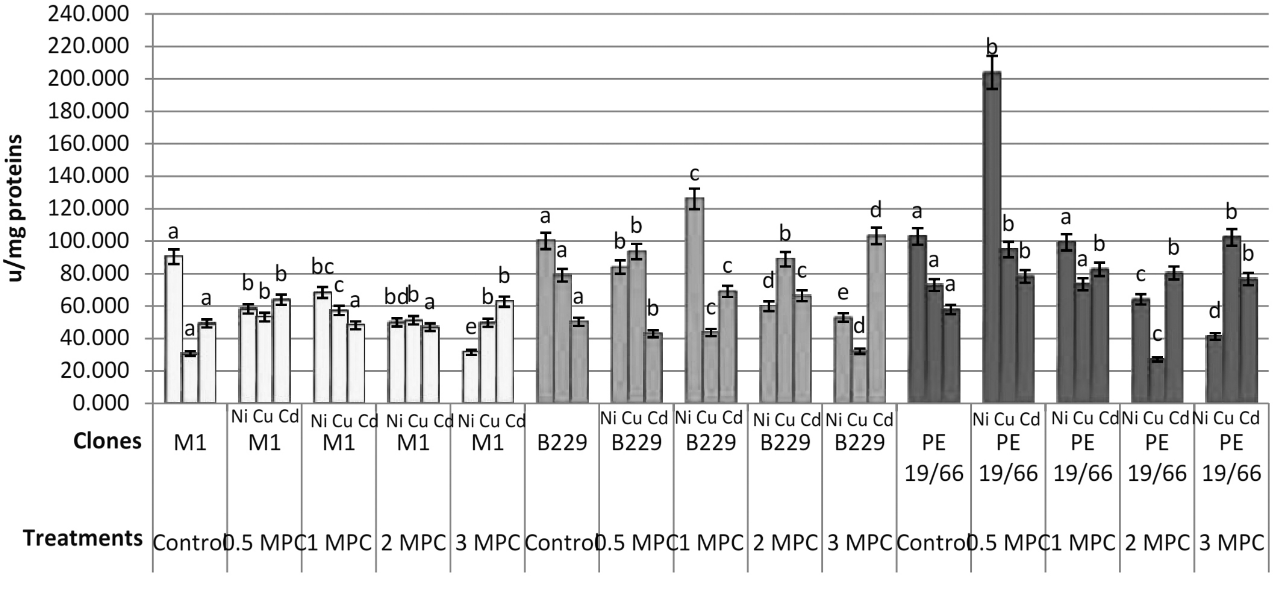

Figure 6. SOD activity in roots of three poplar clones

responses and the most significant changes were noticed in the 29.08% increase in SOD activity in 0.5 MPC, and 27.18% in 3 MPC compared to the control. There was an increasing trend compared to the control in all treatments for B 229 clone, with the highest value of 105.64% in 3 MPC treatments. There had been an increasing trend in all treatments compared to the control for PE 19/66 clone and the most significant changes were noticed in 1 MPC treatment - 42.75% and in 3 MPC treatments - 32.46% compared to the control. Treatment of nickel again showed very toxic influence to the SOD activity in roots; a decreasing trend was noticed in SOD activity compared to the control in all clones and in all applied treatments; it ranged from 16.86% in M1 in 2 MPC to 186.53% in 3 MPC; B229 - from 19.13% in 0.5 MPC to 89.03% in 3 MPC;PE 19/66- from 3.59% in 1 MPC to 149.2% in 3 MPC. Comparing literature data with other abiotic stresses, our results showed that higher concentration of heavy metals can produce oxidative stress in plant, but not necessarily in the same pattern. Variability of antioxidant responses was unpredictable, but still acceptable for species in which the full functional genomics is unknown. Kebert et al. (2011) were also examining the effects of different types and concentrations of contaminants on the oxidative stress in several poplar clones. They analyzed the antioxidant capacity of poplar clones PE19/66, B229 (*Populus deltoides*) clone and Pannonia (*Populus x euramericana*) in leaves after treatment with heavy metals, herbicides, diesel fuel and the combination of heavy metals and diesel fuel in the experimental field. They measured the total antioxidant capacity using the FRAP method and all three clones showed an increased total antioxidant capacity under conditions of increased quantities of pollutants compared to the controls.

The results of FRAP units for different treatments using our three poplar clones are presented in Fig. 7. Cu-treatment showed that M1 clone had an increase in FRAP values in all treatments compared to the control, and the largest increase of 94.2% was recorded in 2 MPC treatments, while a decreased of 37% was observed in 3 MPC treatments. No significant change in FRAP units were found compared to the control in B 229 clone.

Small changes in the reduction of FRAP values ranging from 28.25% in 0.5 MPC, to 18.28% in 3 MPC treatments were recorded in the PE 19/66 clone. Within the Cd-treatment, the M1 clone showed a decrease of 43.44% in FRAP values in relation to the control and 1 MPC treatment and of 31.58% in 3 MPC treatments. The B 229 clone showed a dramatic trend of increasing values in FRAP units in following treatments, as follows: 97.86% in 0.5 MPC, and 246.97% in 3 MPC. The PE 19/66 clone showed a slight decrease of 33.1% in 0.5 MP FRAP units compared to the control, 40.3% in 1 MPC treatment, and 25.46% in 3 MPC treatment when compared to the control. Ni-treatment in M1 clone showed an increasing of 31.31% in 0.5 MPC and 68.34% in 3 MPC in FRAP values in com-

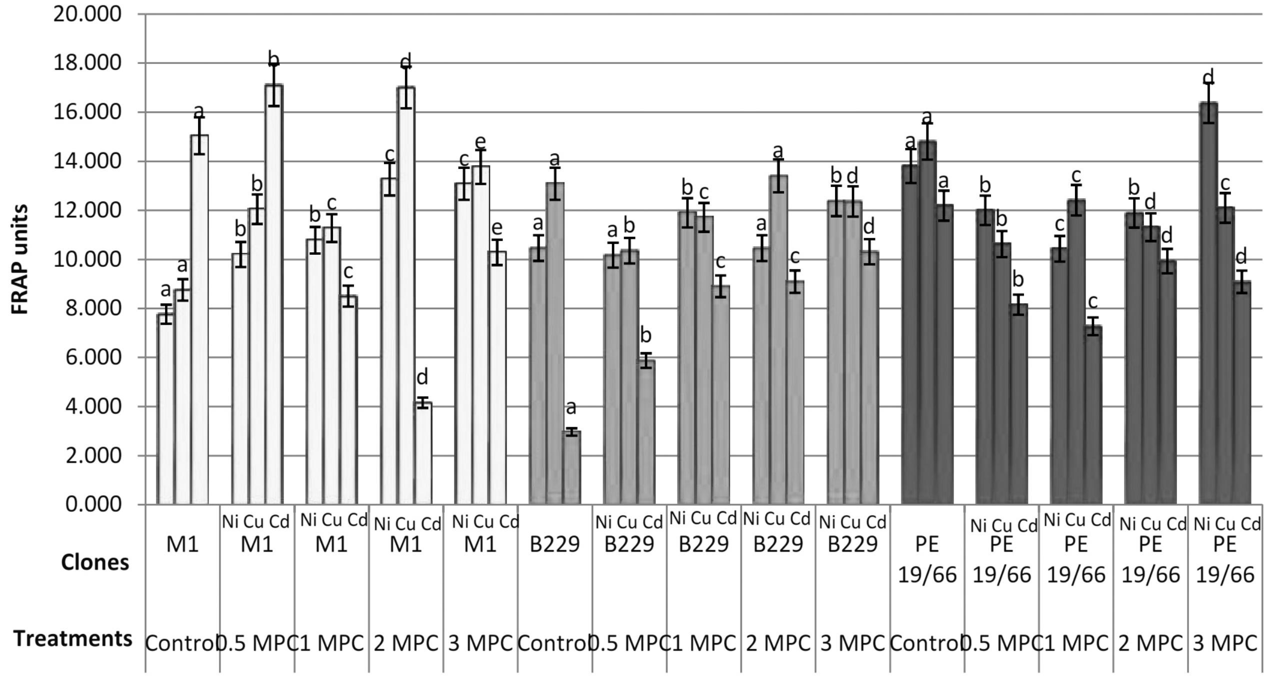

Figure 7. FRAP values in leaves of three poplar clones

parison to the control. The B 229 clone presented no significant changes compared to control. The PE 19/66 clone had noticeable small and variable changes, where only the 3 MPC treatments had increased values of 18.65%.

The results for FRAP units in extracts of root from our 3 poplar clones, with different treatments are shown in Fig. 8. Cu-treatment for the M1 clone showed abrupt changes, with a maximum increase of 26.65% in 1 MPC treatment compared to the control. The largest decrease of 38.61% was registered in 2 MPC treatments compared to the control. The B 229 clone showed no significant changes compared to the controls. The PE 19/66 clone registered only a reduction of 35.78% in 3 MPC treatment compared to the control. Cd-treatment in M1 clone revealed an increase of 50.80% in FRAP values in 0.5 MPC treatments compared to the control and it slowly decreased by 24.01% in the other treatments. The B 229 clone showed a noticeable decline ranging from 41% in 1 MPC treatment to 26.53% in 3 MPC treatment compared to the control. The PE 19/66 clone showed noticeable trend of decreasing FRAP values in relation to the control, although not significantly strong; it slightly increased by 20.67% in 1 MPC and decreased by 30.06% in 3 MPC treatments, compared to control. Ni-treatment in the M1 clone presented no significant changes, but the responses were variable and reduction ranging from 3.2% in 0.5 MPC treatments to 18.27% in 3 MPC was evident. In the B 229 clone, there were no significant changes compared to controls, and the results varied in the trend of decreasing values from 7.07% in 0.5 MPC to 29.35% in 2 MPC treatments. In the PE 19/66 clone, there were no significant changes compared to the controls, while the results varied and the only significant change was a decrease of 15.56% in the 1 MPC treatment.

Conclusions

The mechanisms of protection against oxidative stress can cause a variety of physiological and biochemical changes, such as the activation of antioxidant systems and the accumulation of some secondary biomolecules with antioxidant activity (Pietrini 2008, Macovei et al. 2010, Štajner et al. 2011). The induction of antioxidant molecules biosynthesis was observed in many important agricultural plant species, such as wheat, in response to nickel toxicity (Diaz et al. 2001) and in maize, in response to aluminium (Winkel-Shirley 2002). *Phaseolus vulgaris* exposed to Cd^{2+} acccumulate soluble and insoluble phenolics and *Phyllantus tenellus* leaves contain more antioxidant than the control plants after being sprayed with copper sulphate (Diaz et al. 2001).

Presented results suggest that the antioxidant response varied among the clones and the most intense changes were occurring mostly in the leaves. This is consistent with the fact that leaves are metabolically the most active organs in the plant, where the crucial phase of photosynthesis, necessary for basic life processes in plants occurs (Stanković et al. 2006, Štajner et al. 2009). Our results are referring the negative effect of Cd^{2+} and high concentration of Cu^{2+} on the growth and development of plants, as the potent inducers of oxidative stress. Copper ion is a weak intoxicant at low concentrations, mostly because it has

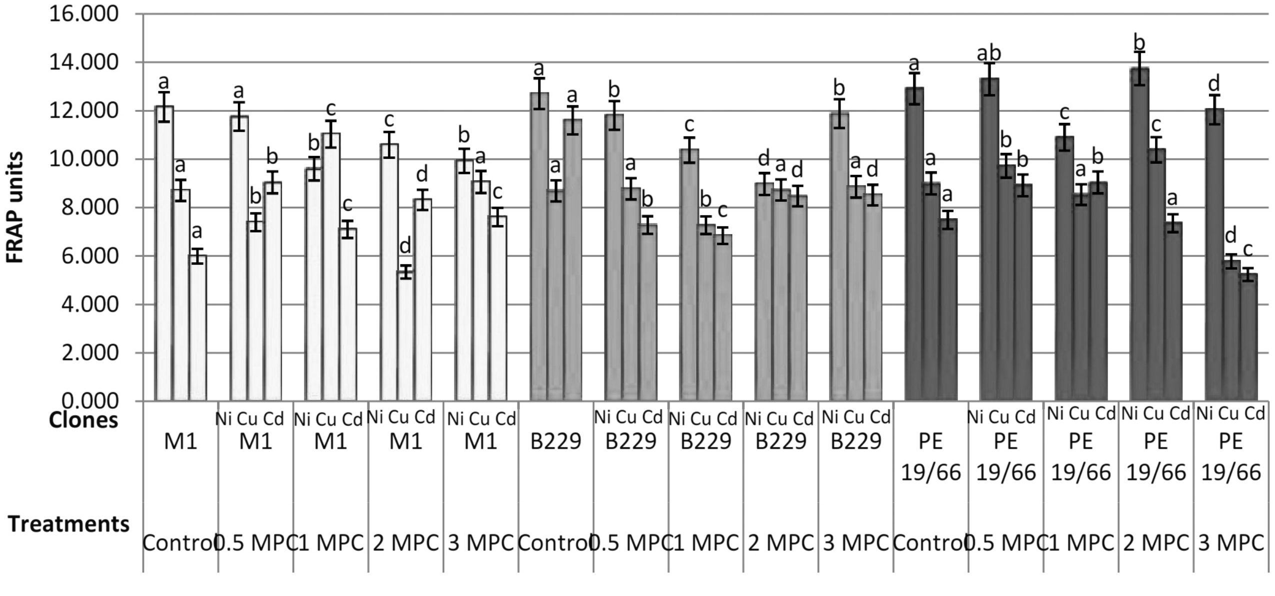

Figure 8. FRAP values in roots of three poplar clones

a positive influence as a biogenic trace element in plant and as a cofactor for a number of enzymes, although higher concentrations of Cu in the soil can induce oxidative stress, as our results confirmed. Treatments with different applied concentration of Ni ion showed strong suppressing influence on the activity of SOD in both analyzed organs, in all three clones. Clones B229 and M1 showed greater resistance to metabolic oxidative stress then clone PE 19/66. However, at higher concentrations of heavy metals, the antioxidative response of these two clones also varied depending on the examined organs. The M1 clone showed a decrease in the SOD values, FRAP and protein values, as well as an increase in MDA production at higher concentrations of heavy metals, indicating its weak resistance to greater contamination by heavy metals. Clone B229 mostly varied in response to oxidative stress, although its response to stress induced by high concentrations of heavy metal ions indicated that it was the most resistant to their presence, especially Cd. Compared to their control samples, the B 229 clone showed small changes in total protein and the FRAP content, while the SOD activity in various organs was different. This indicates the further need for intensive metabolic and biochemical testing for the observed clones in order to accurately determine which clone has a better adaptive response at the metabolic level in relation to heavy metals soil contamination.

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УРОВЕНЬ ОКИСЛИТЕЛЬНОГО СТРЕССА У ТОПОЛЕЙ ВСЛЕДСТВИЕ ЗАГРЯЗНЕНИЯ ТЯЖЕЛЫМИ МЕТАЛЛАМИ

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Резюме

Тополь (*Populus* spp.) обычно используется в фиторемедиации благодаря своим специфическим характеристикам, таким как большая поверхность листьев, проводимость воды и минералов по всей секции дерева (диффузные пористые виды) и легкая вегетативная репродукция через отростки. Использование тополей для детоксикации почвы и воды, загрязненных тяжелыми металлами, было продемонстрировано для различных видов.

В данной работе проанализировано влияние различных концентраций трех ионов тяжелых металлов, Cu^{2+}, Ni^{3+} и Cd^{2+}, на окислительный стресс в трех клонах, двух разных видах тополя (ПЭ 19/66 и B229), принадлежащих к *Populus deltoides* (Marshall) и к (клон M1) *Populus euramericana* (Dode-Guinier). Биохимические показатели, примененные для определения уровня окислительного стресса в этих трех клонах, были следующие: содержание растворимых белков, перокисление липидов, железо, снижение антиоксидантного влияния анализа и активности супероксид дисмутазы. Было измерено возможное содержание антиоксидантов в этих трех клонах в ответ на различные концентрации ионов тяжелых металлов в субстрате, с целью выявить, какой из клонов будет наиболее подходящим для процессов фиторемедиации. Результаты показали изменчивые реакции в листьях и корнях тополей на окислительный стресс, вызванный тяжелыми металлами, но наиболее перспективным клоном для фиторемедиации загрязненных почв является клон B229, в то время как M1 и ПЭ 19/66 показали изменчивые реакции на антиоксидант.

Ключевые слова: тяжелые металлы, тополь, окислительный стресс, антиоксидантная активность, фиторемедиация