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# THE EFFECT OF HEAVY METAL POLLUTION IN SOIL ON SERBIAN POPLAR CLONES

## UTJECAJ ONEČIŠĆENOSTI TLA TEŠKIM METALIMA NA KLONOVE TOPOLA IZ SRBIJE

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### Summary

Oxidative stress is known as disturbed balance between antioxidative protection mechanism and production of reactive oxygen species, which can negatively influence on normal biological and metabolical processes in living organisms, such as poplar species. Phytoremediation is promising biotechnical method of cleaning of polluted soils by various pollutants: heavy metals, organic contaminants, pesticides, oil etc. Until today, poplars showed potential for regenerating polluted soils during phytoremediation process. This study represents results of oxidative stress profiles of three poplar clones (M1, B229 and PE 19/66) shoots from Institute of Lowland Forestry and Environment, University of Novi Sad, Serbia, while being treated by different concentration of three heavy metals in soil: $Ni^{3+}$, $Cu^{2+}$ and $Cd^{2+}$. Biochemical parameters of oxidative stress which have been analyzed were: content of soluble proteins, intensity of lipid peroxidation, antioxidative capacity by ferric reducing antioxidative power assay and activity of superoxid dismutase. Results showed that the most acceptable phytoremediation response to heavy metal pollution in soil showed clone M1. Great differences between B229 and PE 19/66 clones were in response on soil heavy metal contamination, directly suggesting of not being suitable for possible phytoremediation application.

KEY WORDS: poplar clones, oxidative stress, phytoremediation, shoots

## Introduction
### Uvod

Two billion years ago, the appearance of oxygen in Earth's atmosphere created conditions for the development of aerobic organisms. Aerobic organisms use oxygen during respiration to obtain energy by oxidation of organic molecules such as carbohydrates, proteins and lipids.

Aerobic metabolism is accompanied by the production of different, partially reduced chemical forms of oxygen, often much more reactive then the oxygen molecule itself. These oxygen species can interact with basic cellular structures and biomolecules, lead to a number of physiological disorders (Popović and Štajner, 2008, Kebert et al., 2011).

In order to survive, aerobic organisms have acquired mechanisms of antioxidant protection, and among others include the activities of antioxidant enzymes such as catalase, peroxidase, and gluhation peroxidase and superoxide dismutase. In addition to many enzymes and low molecular

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weight compounds such as glutathione, vitamins, uric acid and bilirubin, those compounds contribute to the total antioxidant defense (hereinafter: *Antioxidant Defense System – ADS*). The role of ADS is of particular importance in terms of increased production of reactive oxygen species (hereinafter: *Reactive Oxygen Species – ROS*) and occurrence of oxidative stress (Popović and Štajner 2008).

In terms of global climate change, abiotic stresses, such as salinity, drought, temperature, chemical toxicity and oxidative stresses are the major causes for loss of agricultural production and natural vegetation. Abiotic stress causes various morphological, physiological, biochemical and molecular changes that affect plant growth and productivity. Managing abiotic stress is especially critical to the long-term growth of tree species. Tree species are also affected by mechanical stress such as wind and gravity. Many forest trees suffer from abiotic and biotic stresses under adverse environmental conditions, including heavy metal contamination as a folow-up process (Osakabe et al., 2011).

In plants, the appearance of small, toxic oxygen species causes disruption of cellular components and membrane lipid peroxidation, whereas the activated forms of oxygen damage the molecules of chlorophyll, which is manifested by the loss of green color and reduction of photosynthesis (Heath and Packer, 1968; Pallet and Dodge, 1979; Elstner and Osswald, 1980).

Studies have shown that plants reduce concentration of contaminants in soil and groundwater during that process, from which it was established the idea of the possibilities of their growing and treatment of contaminated habitats. This way of treating soil contamination is called phytoremediation. According to the Agency for Environmental Protection of the United States, phytoremediation is a set of techniques and technologies that use plants for the purpose of cleaning contaminated sites and soils. From a diverse range of plant species used for phytoremediation, poplars are commonly used woody plant species, due to their characteristics such as large leaf surface, the conductivity of water and minerals through the entire cross-section of the tree (diffuse porous species) and easy vegetative propagation by cuttings (Pilipović, 2005). Hybrid poplars were originally bred and grown as a cash crop for such uses as pulp wood and as renewable energy source, but because of their rapid growth rate and high evapotranspiration rates, they make ideal candidates for phytoremediation (Chappell, 1997). *Populus* makes an excellent subject for any bioremediation study since it is fast-growing (one of the fastest growing temperate trees) and because the use of clone material ensures that experiments are repeatable and with small error.

As environmental restoration of metal-polluted soils by traditional physical and chemical methods demands large investments of economic and technological resources, efforts are underway to involve *in situ* methods in environmental protection. Phytoremediation is an emerging technology which utilizes plants and rhizosphere microorganisms to remove and transform toxic chemicals in soils, sediments, ground water, surface water, and the atmosphere (Kumar et al., 1995; Susarla et al., 2002; Ghosh and Singh, 2005; Pajević et al., 2008). Heavy metal accumulation by plants is useful as a phytoextraction technique in phytoremediation, which refers to the use of plants that can extract and move contaminants to their harvestable parts (Marchiol et al., 2004). The efficiency of phytoextraction depends on the metal bioavailability, as well as on several characteristics of the plant-remediator: fast growth, a deep and extended root system, the capability of (hyper) accumulating essential and unessential metals, and the ability to translocate metals to the aerial parts (Zacchini et al., 2009).

Metal-accumulating woody species have been considered for phytoextraction of metal-contaminated sites. Apart from cleaning the environment, another advantage of using forest plants in this technology is their high production of biomass, which can eventually be used in producing energy (Laureysens et al., 2004).

## The aim of study
### Cilj istraživanja

Different metals can disrupt metabolic processes and pathways in plants, especially in the thylakoid membrane, which could also result in increased production of free radical species, such as $RO^{\bullet}$, $OH^{\bullet}$, $O^{\bullet}{}_2^{-}$ etc. Heavy metals also inactivate antioxidant enzymes (peroxidases, catalase, superoxide dismutase, etc.). Responsible for detoxification of free radical species, peroxidases can also be activated by different metals in some cases.

Biochemical profiling of oxidative stress status in woody plant is not common in Serbia and therefore the aim of this study was to examine the effect of different concentrations of three heavy metal ions, $Ni^{3+}$, $Cu^{2+}$ and $Cd^{2+}$ on oxidative stress of three clones, two different species of poplar (*Populus euramericana*-M1; PE 19/66 and B 229-both *Populus deltoides* species). Biochemical parameters for indentifying the level of oxidative stress were: lipid peroxidation (hereinafter: LPx), ferric reducing antioxidative power assay (hereinafter: FRAP), superoxide dismutase (hereinafter: SOD) activity and soluble protein. Particularly, through *in vitro* experiment antioxidant potential of these three clones to different concentrations of heavy metal ions in the soil were determined. These results could give the guidelines in the selection of clones from Institute's collection, characterized by high (or low) phytoremediation potential, in commercial production of poplar genotypes.

## Materials and methods
Materijali i metode

### Plan of experiment – Plan eksperimenta

Greenhouse pot experiment in semi controlled conditions was conducted, of 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 (*Populusxeuramericana*-M1 clone and *Populus deltoides*-B229 and PE 19/66 clones), from the Institute of Lowland Forestry and Environment (University of 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 soil. The values for the investigated metals concentrations were: MPC ($Cu^{2+}$) =100 mg/kg, MPC ($Cd^{2+}$) =3 mg/kg, MPC ($Ni^{3+}$) =50 mg/kg, respectively.

Sandy fluvisol soil in pots was contaminated with the heavy metal treatments presented in table 1. Each treatment was set up in three replicates. The land was treated and left for three months to form inner microbiological environment. Metals were added as a nitrate salt, $Cu(NO_3)_2$, $Cd(NO_3)_2$ 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, four poplar plants were planted as two-year-old seedlings, so with a single treatment (one concentration of a metal) 12 plants (four plants of each clone) were contaminated. Also, control poplars plants of each clone were planted in soil not being treated with heavy metals. Shoots of poplar clones were sampled and a series of extracts was prepared for *in vitro* analysis.

**Table 1.** Treatments of heavy metals used for contamination of poplar clones in experimental design
Tabela 1. Tretmani teških metala koji se koriste za onečišćenje klonova topola u eksperimentalnom dizajnu

| Treatment | $Cd^{2+}$ (mg/kg) | $Cu^{2+}$ (mg/kg) | $Ni^{2+}$ (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 |
| 3 MPC | 9,0 | 300,0 | 150 |

### Preparation of plant extracts – Priprema biljnih ekstrakata

Plant extracts were made from 2 g of plant material (shoots) homogenized with quartz sand and suspended in 10 $cm^3$ 0.1 mol/$dm^3$ $K_2HPO_4$ at pH 7.0 placed into a cold porcelain mortar and macerated for 2–3 minutes.

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

### *In vitro* studies of extracts poplar clones – *In vitro* analize ekstrakata klonova topola

Total antioxidant capacity of shoots extracts was estimated according to the FRAP (Ferric Reducing Antioxidant Power) assay (Benzie and Strain, 1999). Total reducing power is expressed as FRAP units. FRAP unit is equal with 100 μmol/$dm^3$ $Fe^{2+}$. FRAP value was calculated using the formula:

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

Lipid peroxidation (LP) was determined by measuring amounts of malonyldialdehyde MDA which is one of its end-products and which is quantified by thiobarbituric acid (hereinafter: TBA) method (Placer et al., 1966). 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.

The total activity of superoxide dismutase was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (hereinafter: NBT) resulting in the blue reduction product of NBT with $O^{2-}$. Solution in test tubes were stirred for few seconds and set in front of the light source for 10 minutes (Auclair and Voisin, 1985). The unit of SOD was the quantity of enzyme that inhibited NBT reduction by 50 % at 25 °C and 560 nm. All absorbance reading for content of soluble proteins, SOD and FRAP were done using Janway UV / VIS spectrophotometer 6505 and for MDA reading, Multiscan Spectrum Thermo Corporation.

[1] MPC – maximum permissible concentration of heavy metal

### Statistical analysis – Statistička analiza

All determinations were performed in triplicates. Statistical comparisons between samples were performed in the program Statistica 9, using Duncan's test, with statistical significance $p<0.05$, comparing treated samples with proper control. Obtained results are presented graphically by histograms and above them are the letters denoting statistical difference between results and control (from **a** to **e**).

Comparable percentage was calculated by the formula: **Δ (%) = (100 * sample / control)–100;** where values may result with +, which means it comes to increase compared to control and resulted with –, which means it comes to decrease compared to control.

## Results
## Rezultati

The results of the content of soluble proteins shoots of our clones exposed to different concentrations of heavy metals in the soil are presented in figure 1. All comparisons were made to the control sample, representing the plant with no heavy metal treatment. The M1 clone first showed a slight downward trend starting from 10,79% in 0,5 MPC treatment and in 2 MPC treatment it showed a very slight decrease of 3.45 %.

In 3 MPC treatment there was an increase of 19,04 % in relation to control. There was an obvious trend of increasing soluble protein content by 106,4 % in clone B229, especially in 1 MPC treatment, and by 131,24 % in 3 MPC. PE 19/66 ranged from an increase of 20,20 % compared to the control sample in 0,5 MPC treatment, through more than 104,21 % in 2 MPC treatment to an increase of 40,03 % in 3 MPC treatment in relation to control.

When treated with cadmium clone M1 showed an increasing trend of soluble protein content in all treatments ranging from 43,65 % in 0,5 MPC to 88,27 % in 3 MPC treatment. B 229 showed a slight decrease compared to control in all treatments ranging from 3 % in 0,5 MPC to 23,34 % in 2 MPC treatments. Decrease of 8,3% was observed in 3 MPC treatment.

Clone PE 19/66 showed various responses regarding the trend of decreasing values of soluble protein content ranging from 17,63 % in 0,5 MPC to 36,97 % in 3 MPC treatments.

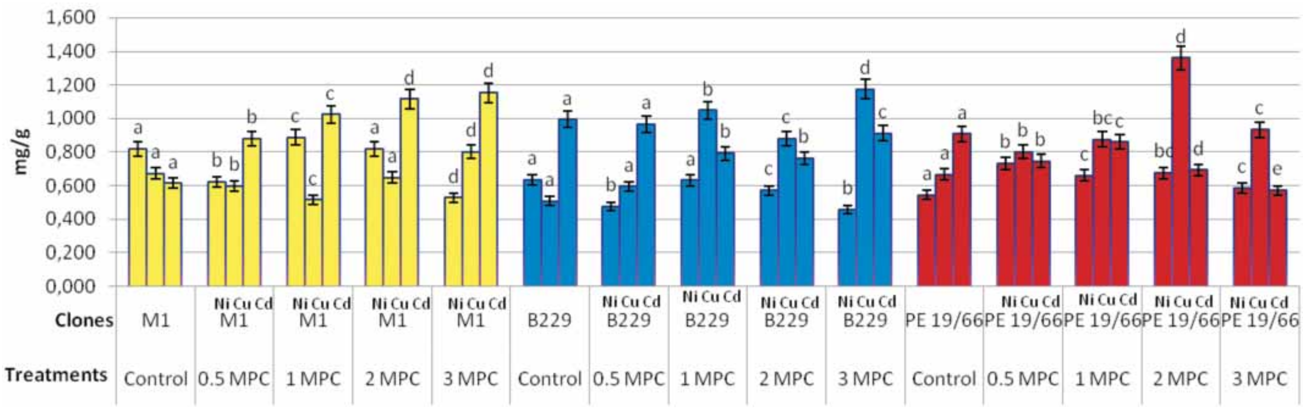


**Figure 1.** Content of soluble protein in shoots of three poplar clones
Slika 1. Sadržaj topivih proteina u izbojcima tri klona topola

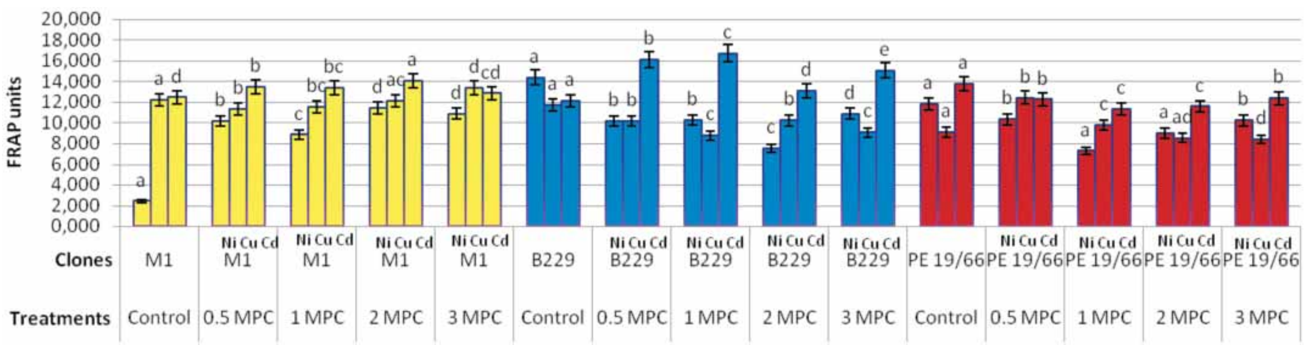


**Figure 2.** FRAP values in shoots of three poplar clones
Slika 2. FRAP vrijednosti u izbojcima tri klona topola

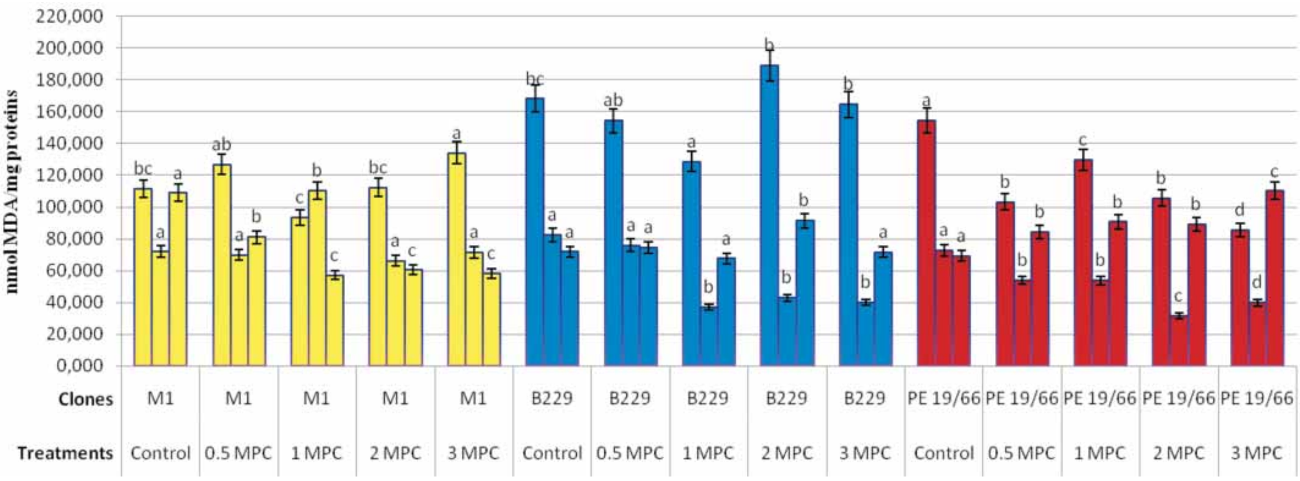

**Figure 3.** Intensity of lipid peroxidation in shoots of three poplar clones
Slika 3. Intenzitet lipidne peroksidacije u izbojcima tri klona topola

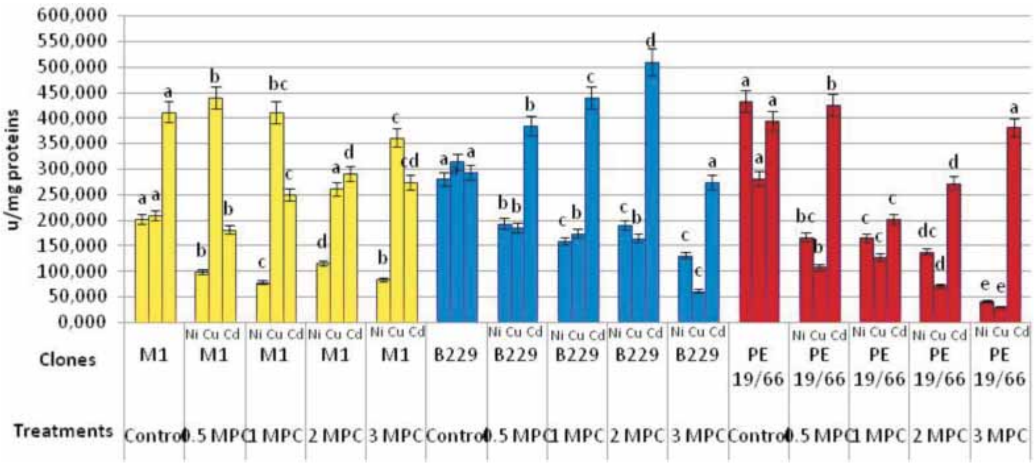

**Figure 4.** SOD activity in shoots of three poplar clones
Slika 4. Aktivnost SOD u izbojcima tri klona topola

Various responses of clone M1 to nickel treatment were observed compared to control. In 0,5 MPC treatment decrease of 23,98 % was observed in relation to control, and of 35,6 % in 3 MPC. In 1 MPC and 2 MPC treatments the values were similar to the control plant. A slight decreasing trend was observed in clone B 229 in relation to control – in 0,5 MPC decrease of 25,36 %, and in 3 MPC of 28,23 % was noticed. PE 19/66 unlike the previous two clones showed trend of increasing soluble protein content in relation to control, although a trend of decreasing values with increased nickel concentration in soil was observed. The values ranged from an increase of 34,91 % in 0,5 MPC treatment to an increase of 7,76 % in 3 MPC treatment.

The results of total antioxidative shoot capacity of our clones expressed through FRAP units are shown in figure 2. In regard to the copper treatment, M1 revealed high values of FRAP units, but there were no significant changes in regard to control. Clone B 229 showed a slightly decreasing trend of FRAP unit values ranging from 13,49 % (0,5 MPC) to 22,85 %. (3 MPC) compared to control. Clone PE 19/66 showed a sharp increase of 36,92 % (0,5 MPC), and thereafter a declining trend all the way up to 3 MPC treatment until it reached the value similar to that of the control. In regard to various cadmium treatment, clone M1 showed only very modest increases compared to control – the maximum of 12,68 % was reached in 2 MPC treatment. Although the changes were not significant, the values of FRAP units were very high. Clone B 229 also showed modest increases of 32,76 % (0,5 MPC), and 24,43 % (3 MPC) compared to control. Clone PE 19/66 showed a slight decline compared to control: 10,82 % (0,5 MPC), 17,81 % (1 MPC), and 10,06 % (3 MPC). In regard to the nickel treatment, M1 showed drastic increases in all applied treatments compared to control: 318,08 % (0,5 MPC), 265,14 % (1 MPC), 370,58 % (2 MPC), and 348,36 % (3 MPC). Clone B 229 showed a slight decline in all treatments compared to control: 29,42 % (0,5 MPC), 47,86 % (2 MPC), and finally a decline in 3 MPC treatment, where an increase of only 24,32 % was recorded. Clone PE 19/66 showed various responses and at the same time a decline in FRAP unit values: 12,64 % (0,5 MPC), 13,49 % (3 MPC), and 38,29 % and 23,99 % (1 MPC and 2 MPC).

Values of MDA products obtained after lipid peroxidation are shown in figure 3. In regard to the copper treatment in clone M1 only increase of 53,3 % (1 MPC) in MDA content was recorded, while the values in all other treatments were similar to those of the control. There was a significant decrease of 54,94 % (1 MPC) in clone B 229 compared to control, and this trend was recorded even in 3 MPC treatment, where a decrease of 51,32 was recorded. In clone PE 19/66 the MDA content was decreased in all treatments compared to the control, ranging from 25,65 % in 0,5 MPC, to 44,99 % (3 MPC). The greatest decrease of 56,38 % (2 MPC) was recorded compared to control. Treatment with different cadmium concentrations revealed a decreasing trend of MDA content in clone M1 in all treatments, and the values ranged from 54 % (0,5 MPC) to 46,33 % (3 MPC). The greatest decrease of 47,41 % was recorded in 1 MPC compared to control. Clone B 229 showed no significant differences compared to control, except in 2 MPC, where there was a slight increase of 27,15 % compared to control. An increasing trend in MDA content was observed in clone PE 19/66 in all treatment and it ranged from 21,4 % (0,5 MPC) to 58,79 % (3 MPC). In regard to different nickel treatment, clone M1 showed various responses to all treatments. Increase of 13,81 % was recorded in 0,5 MPC treatment and of 20,52 % in 3 MPC, while a decrease of 16,22 % was recorded in 1 MPC compared to control. Clone B 229 showed a slight decline compared to control in almost all applied treatments, except in 2 MPC where increase of 12,33 % was recorded. Clone PE 19/66 showed a decreasing trend compared to control ranging from 33,25 % (0,5 MPC) to 44,67 % (3 MPC).

Results of SOD values in all three clones and different heavy metal treatments are shown in figure 4. In regard to copper treatment, clone M1 revealed significant increase compared to control and the values ranged from 110,59 % (0,5 MPC) to 25,19 % (2 MPC). An increase of 72,45 % was recorded in 3 MPC treatment compared to control. Clone B 229 showed a decreasing trend compared to control. Decrease of 40,87 % was recorded in 0,5 MPC, 47,61 % in 2 MPC, and of 80,41 % in 3 MPC. PE 19/66 also revealed a decreasing trend in SOD values ranging from 61,88 % (0,5 MPC), and 89,28 % (3 MPC). In regard to cadmium treatment clone M1 showed decreasing trend in all treatments compared to control. Decrease of 55,86 % was recorded in 0,5 MPC, and of 33,29 % in 3 MPC. Clone B 229 showed significant increase in SOD values in all treatments except in 3 MPC where the values were very similar to those of the control. Almost linear increasing trend was observed ranging from 31,68 % (0,5 MPC) to 73,86 % (2 MPC). Clone PE 19/66 showed a noticeable decline of 48,63 % after 1 MPC treatment, and then it regained the values similar to those of the control in 3 MPC treatment. In regard to nickel treatment, clone M1 showed various responses to different concentrations of nickel in the substrate. Decrease of 33,06 % in 1 MPC treatment, and slight increase in 2 MPC treatment were observed and increase of 16,47 % was noticed in 3 MPC compared to control. Clone B 229 showed trend of decreasing values ranging from 28,62 % (0,5 MPC) to 58,33 % (3 MPC) compared to control. Clone PE 19/66 showed similar decreasing trend ranging from 61,63 % (0,5 MPC) to 79,53 % (3 MPC).

## Discussion
### Rasprava

Environmental stress is the major cause of crop and forest loss worldwide. Future issues such as the insufficiency of provisions, environmental conservation and production increase in biomass will depend on plant biotechnologies. An in-depth understanding of the physiological stress responses and the molecular events in woody plants, which are some of the major components of the global ecosystem and biomass resources, is now required (Osakabe et al., 2011).

Nickel is one of the essential micronutrients for plants, animals, and humans, but toxic at elevated concentrations. As we mentioned before, nickel belongs to heavy metals also. The aim of study of Krstić et al. (2007) was to analyze Ni concentration in certain plant species affected by Ni contamination of air and surface soil. *Ambrosia artemisifolia* and *Taraxum officinale* accumulated the greatest amounts of Ni (10.72 and 10.61 μg/g, respectively). It may be concluded that the analyzed plant species exhibit various phytoremediation potential for Ni under the same ecological conditions. It is necessary to have that fact in mind while observing any chemical impact of heavy metals on poplar clones, since it showed various antioxidative answers. Higher concentrations of cadmium and copper resulted in higher amount of soluble proteins in all clones and treatments of nickel showed only small changes in synthesis of soluble proteins in shoots. It may indicate that in young poplar shoots the synthesis of protein antoxidative system is starting while applying high concentration of cadmium and copper.

Cadmium effects often show its negative influence on biomass production, leaf number and area. Those are the symptoms that cadmium treatments usually cause and they are visually reported, but Pilipović et al. (2005) also measured photosynthetic and dark respiration rates, leaf concentration of photosynthetic pigments, nitrate reductase activity, as well as cadmium concentrations in leaves, stem, and roots in poplar clones PE 4/68, B-229, 665, and 45/51. Plants were grown hydroponically under controlled conditions and treated with two different cadmium (Cd) concentrations (10(–5) and

10(−7) M) in the same background solution (Hoagland's solution). Cd did not cause serious disturbance of growth and physiological parameters in the studied poplar clones. Within our results in shoots, Cd showed inhibitory effect on SOD activity, mostly on its high concentration applied in PE 19/66 and M1 clone. B 229 clone showed increasing level of SOD activity, indicating that this genotype has a significantly acceptable antioxidative answer to applied high concentration of heavy metal in soil. Previous investigations on species very close to poplars (Zacchini et al., 2009) showed that almost all selected willow clones, used to extract $Cd^{2+}$, accumulated half of the absorbed metal in the aerial parts exhibiting a translocation factor twice respect to poplar clones, that on the contrary, accumulated much more $Cd^{2+}$ than willow clones in the roots.

The high antioxidant activity was reported throughout the test period in specimens of species of *Populus alba* L. In this plant, 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. *P. alba* showed the highest antioxidant capacity in July, when the drought was the most severe and accumulation of antioxidant molecules was induced by stressful factor. (Štajner et al., 2011). This was a good example where *Populus* representatives showed acceptable antioxidative answer due abiotic stress. M1 and B229 clones showed their acceptable general anitoxidative activity within young shoots, despite they showed also variable results for different treatments. Antioxidative answer was noticeable indicating that it may range from moderate to high anitoxidative system activity within shoots, especially in M1 and B229 clones. Unfortunately, PE 19/66 in LPx parameter and decreased SOD activity showed weaker antioxidative answer to applied heavy metal treatments.

SOD activity was also measured by Nikolić et al. (2008). It showed decrease in roots and increased in the leaves of treated plants of hybrid poplar (*Populus nigra × maximowitzii × P.nigra var. Italica*), clone 9111/93. Cd-induced toxic effects (stunted growth, leaf chlorosis, oxidative stress) were observed, indicating that this clone was vulnerable to the pollutant. High amounts of Cd accumulated in roots, but in view of its low translocation from roots to aboveground parts, along with the disturbances in plant growth, this hybrid poplar showed little potential for use in remediation of sites contaminated with Cd. There is possibility that 9111/93 and PE 19/66 clone share similar genetic profile, because both of them showed unacceptable antioxidative answer on applied cadmium treatment, since it is well-known pollutant in soil. Poplar trees (*Popullus deltoides x Populus cv caudina, NE clone 353*) were used in studies of Sen Gupta and Alscher (1991) and showed both glutathione levels and superoxide dismutase activity increased before there was any observable ozone effect on photosynthesis.

Cd was found to produce oxidative stress (Hendry et al., 1992; Somashekaraiah et al., 1992), but, in contrast with other heavy metals such as Cu, it does not seem to act directly on the production of oxygen reactive species (via Fenton and/or Haber Weiss reactions) (Salin, 1988). On the other hand, Cd ions can inhibit (and sometimes stimulate) the activity of several antioxidative enzymes. In species such as *Helianthus annuus* leaves, Cd enhanced lipid peroxidation, increased lipoxygenase activity and decreased the activity of the following antioxidative enzymes: superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase and dehydroascorbate reductase (Gallego et al., 1996). In *Phaseolus aureus*, Cd ions produced lipid peroxidation, decrease of catalase activity and increase of guaiacol peroxidase and ascorbate peroxidase activity (Shaw, 1995).

Kebert et al. (2011) were also examining the effects of different types and concentrations of contaminants on the oxidative stress of several poplar clones. They analyzed the antioxidant capacity of poplar clones PE 19/66, 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 controls. Our results for B229 clone showed mostly decreased values of FRAP units on applied treatments, in shoots. PE 19/66 showed similar answer, but M1 showed increased FRAP values especially during nickel treatments. Those studies indicate that different anitoxidative answers may be gained regarding the analyzed organ. Leaves are metabolically very active organs, but shoots are mostly known of transporting the metabolites and nutrients on two ways and that may show less need of plants for antioxidative defense in shoots since the nutrients and other substances taken from soil rarely stayed inside it.

Trudić et. al 2012., showed variable responses within leaves and roots (taken from the same clones mentioned in our study) in response to oxidative stress induced by heavy metals (also $Ni^{3+}$, $Cu^{2+}$ and $Cd^{2+}$) and the most promising clone for phytoremediation of contaminated soils is B229 clone, while M1 and PE 19/66 showed variable antioxidant response. The M1 clone showed a decrease in 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 FRAP content, while the SOD activity in various organs was different.

In research of Pilipović et al. 2012, biomass production, together with: nitrate reductase activity, net photosynthesis/dark respiration, proline content, chlorophyll fluorescence and pigments contents were studied as a possible markers for crude oil phytoremediation processes with poplars clones. Investiagted clones (*Populus* × *euramericana* clone 'Pannonia', *Populus deltoids*-clone 'Bora' and *Populus nigra* × *P. maximowitzii*× *P. nigra var. Italica* clone '9111/93') showed various reactions to the different levels of soil contamination. The effect of crude oil contamination on physiological processes of poplar clones was observed in all investigated parameters with exception of the carotenoids concentration. On the basis of these results, poplar clones 'Bora' and 'Pannonia' showed potential for growth on crude oil contaminated soils.

Micropropagated poplar (*Populus jacquemontiana var. glauca (Haines) cv. 'Kopeczki'*) was estimated on the basis of Cd stress induced oxidative stress parameters. Cd stress caused acute damage evidenced by the increased MDA content and the elevated ratio of quenching by inactive PSII reaction centers. By the end of the third week, MDA content didn't differed significantly from the control values, the MDA content of stressed plants was even lower than that of the controls. Nevertheless, both of the beta-carotene content and ascorbate peroxidase activity remained elevated at the 4th week, though they reached their maximum by the third week (Solti et al., 2011). Further in-depth antioxidant analysis of clones M1, B229 and PE 19/66 are needed because LPx parameter showed variable answers on different treatments on applied heavy metals.

## Conclusions
### Zaključci

The availability of selected species and genotypes adapted to a given ecophysiollogical condition is a fundamental perquisite to successful application of *Salicaceae* to extract heavy metals from polluted waters or humid soils.

Trudić et al. 2012 analyzed through the same oxidative stress methods extracts of leaves and roots of mentioned poplar clones. Comparing these results, B229 clone showed through leaves and roots more acceptable antioxidative answer, while in this study, M1 clone was the most promising through shoots. Taken into account of parameters from Pilipović et al. 2012, Trudić et al. 2012. research and our study, in those way used joint oxidative and physiological markers system in selection of poplar clones for crude oil phytoremediation and heavy metal contamination, it shall be a future stress 'metabolical fingerprint' strategy profiling for any possible woody plant species phytoremediation application.

Our results may indicate genotypic specificity of all investigated biochemical parameters and mark some of the poplar clones, such as M1 as the primary, and B229 clone as the secondary clone in application of phytoremediation of heavy metal polluted soils. Still, further *in vitro* antioxidative analysis are needed for gaining new, deeper results regarding oxidative stress level due heavy metal pollution in soil.

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## References
### Literatura


- Auclair, C. and Voisin, E. 1985. Nitroblue tetrasolium reduction, In: *CRC Handbook of Methods for Oxygen Radical Research* (Ed. Greenwald R.A.) p. 123–132
- Benzie, I.F.F. and Strain, J.J. 1999. Ferric reducing antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods in Enzymolology,* 299: 15–27
- Bradford, M.M. 1976. A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248–254.
- Chappell, J. 1997. Phytoremediation of TCE using *Populus*. Status Report prepared for the U.S. EPA Technology Innovation Office under a National Network of Environmental Management Studies Fellowship
- Elstner, E. F., Osswald, W. 1980. Chlorophyll Photobleaching and Ethan Production in Dichlorophenyldi-methylurea-(DCMU) or Paraquat-Treated *Euglena Gracilis Cells, Z Naturforsch,* 35c:129–135
- Gallego, S.M., Benavides, M.P., Tomaro, M.L., 1996. Effect of heavy metal ion excess on sunflower leaves: evidence for involvement of oxidative stress. *Plant Science*, 121: 151–159.
- Ghosh, M., and S. P. Singh 2005. A review on phytoremediation of heavy metals and utilization of its byproducts. *Appled Ecology and Environental Research,* **3(1):** 1–18
- Heath, R. L. and Packer, L. 1968. Photoperoxidation in isolated chloroplast. I. Kinetics and Stechiometry of Fatty Acid Peroxidation. *Archives of Biochemistry and Biophysics*, 125: 189–198
- Hendry, G.A.F., Baker, A.J.M., Ewart, C.F., 1992. Cadmium tolerance and toxicity, oxygen radical processes and molecular damage in cadmium-tolerant and cadmium-sensitive clones of *Holcus lanatus*. *Acta Botanica Neerlandica*, 41: 271–281
- Kebert, M., Trudić, B., Stojnić, S., Orlović, S., Štajner, D., Popović, B. and Galić, Z. 2011. Estimation of antioxidant capacities of poplar clones involved in phytoremediation processes. In: *STREPOW workshop: book of proceedings,* p. 273–281

- Krstić, B., Stanković, D., Igić, R., Nikolić, N. 2007. The potential of different plant species for nickel accumulation. *Biotechnology & Biotechnology,* p: 431–436
- Kumar, N., Dushenkov, V., Motto, H., and Raskin, I. 1995. Phytoextraction: The use of plants to remove heavy metals from soils. *Environmental Science and Technolology*, **29:** 1232–1238
- Laureysens, I., Bogaert, J., Blust, R., and Ceulemans, R. 2004. Biomass production of 17 poplar clones in a short-rotation coppice culture on a waste disposal site and its relation to soil characteristics. *Forest Ecology and Management*, 187: 295–309
- Marchiol, L., Assolari, S., Sacco, P., and Zerbi, G. (2004). Phytoextraction of heavy metals by canola (Brassica napus) and radish (Raphanus sativus) grown on multicontaminated soil. *Environmental Pollution*, 132(1): 21–27
- Nikolić, N., Kojić, D., Pilipović, A., Pajević, S., Krstić, B., Borišev, M. and Orlović, S. 2008. Responses of hybrid poplar to cadmium stress: photosynthetic characteristics, cadmium and proline accumulation, and antioxidant enzyme activity. *Acta biologica cracoviensia*, *Series Botanica,* 50/2: 95–103
- Kajitab, S., Osakabe, K. 2011. Genetic engineering of woody plants: current and future targets in a stressful environment. *Physiologia Plantarum*, 142: 105–117
- Pajević, S., Borišev, M., Rončević, S., Vukov, D., and Igić, R. (2008). Heavy metal accumulation of Danube river aquatic plants – indication of chemical contamination. *Central European Journal of Biology*, **3(3):** 285–294
- Pallet, K. E. and Dodge, A. D. 1979. Role of Light and Oxygen in the Action of the Photostyntetic Inhibitor Herbicide Monuron. *Z Naturforsch,* 34 c: 1058–1061
- Pilipović, A. 2005. The role of poplar (*Populus sp.*) In the phytoremediation of water contaminated with nitrates. MSc Thesis, Faculty of agriculture, Novi Sad
- Pilipović, A., Nikolić, N., Orlović, S., Petrović, N., and Krstić, B. 2005. Cadmium Phytoextraction Potential of Poplar Clones (*Populus* spp.). *Z Naturforsch,* 60 c: 247–251
- Pilipović, A., Orlović, S., Nikolić, N., Borišev, M., Krstić, B., Rončević, S. 2012. Growth and plant physiological parameters as markers for selection of poplar clones for crude oil phytoremediation. *Šumarski list,* 5–6: 273–281
- Placer, Z.A., Cushman, L.L. and Johnson, B.C. 1966. Estimation of product of lipid peroxidation (malonyldialdehyde) in biochemical systems. *Analitical Biochemistry,* 16: 359–364
- Popović, B. and Štajner, D. 2008. Oxidative stress in plants, Agricultural Faculty, University of Novi Sad
- Trudić, B., Kebert, M., Popović, B.M., Štajner, D., Orlović, S., Galović, V. 2012. The Level of Oxidative Stress in Poplars due to Heavy Metal Pollution in Soil. *Baltic Forestry*, 18(2): 214–227.
- Quy Hai, D., Kovacs, K., Matkovics, I. and Matkovics, B. 1975. Properties of enzymes X. Peroxidase and superoxide dismutase contents of plant seeds. *Biochemic Physiologie f⊠r Pflanzen (BPP),* 167: 357–359
- Salin, M.L. 1988. Toxic oxygen species and protective systems of the chloroplasts. *Physiology of Plants*, 72: 681–689
- Sen Gupta, A., Grene, Alscher, R., McCune, D. 1991. Response of Photosynthesis and Cellular Antioxidants to Ozone in Populus Leaves. *Plant Physiology*, 96(2): 650–655
- Shaw, B.P. 1995. Effects of mercury and cadmium on the activities of antioxidative enzymes in the seedlings of *Phaseolus aureus*. *Biologica Plantarum*, 37: 587–596
- Solti, Á., Szôllôsi, E., Gémes, Juhász, A., Mészáros, I., Fodor, F., Sárvári, É. 2011. Significance of antioxidative defence under long-term Cd stress. *Acta Biologica Szegediensis*, 55(1):151–153
- Somashekaraiah, B.V., Padmaja, K., Prasad, A.R.K. 1992. Phytotoxicity of cadmium ions on germinating seedlings of mung bean (*Phaseolus vulgaris*): involvement of lipid peroxides in chlorophyll degradation. *Physiology of Plants*, 85: 85–89
- Štajner, D., Orlović, S., Popović, M. B., Kebert, M. and Galić, Z. 2011. Screening of drought oxidative stress tolerance in Serbian melliferous plant species. *African Journal of Biotechnology* 10 (9):1609–1614
- Susarla, S., Medina, V. F., and S. C. McCutcheon 2002. Phytoremediation: an ecological solution to organic chemical contamination. *Ecological Engineering*, 18: 647–658
- Zacchini, M., Pietrini, F., Scarascia Mugnozza, G., Iori, V., Pietrosanti, L., Massacci, A. 2009. Metal tolerance, accumulation and translocation in poplar and willow clones treated with cadmium in hydroponics. *Water Air Soil Pollution*, 197: 23–34


## Sažetak


Oksidacijski stres je poznat kao narušena ravnoteža između antioksidacijskog mehanizma zaštite i proizvodnje reaktivnih kisikovih vrsta, što može negativno utjecati na normalne biološke i metaboličke procese u živim organizmima, kao na primjer kod topola. U cilju opstanka, aerobni organizmi su stekli mehanizme antioksidacijske zaštite, gdje su od enzimskih najistraženije aktivnosti antioksidativnih enzima kao što su katalaze, peroksidaze, glutation peroksidaze i superoksid dismutaza. Fitoremedijacija je obećavajuća biotehnička metoda čišćenja zagađenih tala raznim onečišćujućim tvarima poput: teških kovina, organskih kontaminanata, pesticida i dr. Do danas, topole su pokazale potencijal za regeneraciju onečišćenih tala tijekom fitoremedijacijskog procesa.

Biokemijsko profiliranje statusa oksidacijskog stresa u drvenastim biljkama nije često istraživana u Srbiji i stoga je cilj ovoga pokusa bio ispitati utjecaj različitih koncentracija tri jona teških kovina, $Ni^{3+}$, $Cu^{2+}$ i $Cd^{2+}$ na razinu oksidacijskog stresa tri klona, dvije različite vrste topole (*Populus euramericana*-M1; PE 19/66 i B-229 oba *Populus deltoides* vrsta). Biokemijski parametri za određivanje razine oksidacijskog stresa su: lipidna peroksidacija (LPx), test redukcijske snage željeza (FRAP), superoksid dismutaze (SOD) aktivnost i sadržina topivih bjelančevina. Ovi rezultati mogli bi dati smjernice u odabiru klonova iz kolekcije Instituta za nizijsko šumarstvo i životnu sredinu Univerziteta u Novom Sadu za buduće fitoremedijacijske uporabe ovih genotipova topola. Pješčano fluvisol tlo u pokusnim posudama je zagađeno različitim tretmanima teških kovina, koji su prikazani u tablici 1. Biljni ekstrakti izrađeni su od 2 g biljnog materijala (izbojci) i homogenizirani s kvarcnim pijeskom i suspenzirani u 10 $cm^3$ 0,1 $mol/dm^3$ $K_2HPO_4$ pH 7,0. Homogenati su centrifugirani za 10 min na 4000 g (Quy Hai sur., 1975). Sva očitavanja apsorbancija za sadržaj topivih proteina, SOD i FRAP odrađeno pomoću Janway UV / VIS spektrofotometra 6505 i za čitanje količine nastalog MDA korišten je višefrekvencijski Spectrum Termo Corporation.

Sve analize izvedene su u tri ponavljanja. Statistička usporedba između uzoraka izvedena je u programu Statistica 9, koristeći Duncan test, s statističkom značajnosti $p < 0,05$. Dobiveni rezultati prikazani su grafički preko histograma (slike 1. do 4.) i iznad njih su slova koja označuju statistički značajne razlike između rezultata i kontrola (od a do e).

Usporedni postotak je izračunat prema formuli: $\Delta$ (%) = (100 * uzorak / kontrola) –100, gdje vrijednosti mogu rezultirati pozitivno (+) ako je došlo do povećanja u odnosu na kontrolu i rezultiralo negativno (–), ako je došlo do smanjenja u odnosu na kontrolu.

Trudić i sur. (2012) analizirali su kroz iste metode okdisacijskog stresa ekstrakte lišća i korijena spomenutih klonova topola. Uspoređujući ove rezultate, B229 klon je kroz lišće i korijenje pokazao više prihvatljiv antioksidacijski odgovor, dok je, u ovom istraživanju, M1 klon pokazao najprihvatljivije antioksidacijske odgovore kroz ekstrakte izbojaka. Parametri iz Pilipović i sur. (2012), Trudić i sur. (2012) i naše studije, mogu predstavljati udružen fiziološki i antioksidacijski marker sustav za buduće različite fitoremedijacijske primjene ovih klonova na tlima onečišćenim raznim tvarima.

Rezultati ovog istraživanja mogu ukazati na genotipske specifičnosti svih ispitivanih biokemijskih parametara i obilježava klonove topola, kao što je M1 kao primarni, a B229 klon kao potencijalno sljedeći u primjeni fitoremedijacije tala onečišćenih teškim kovinama. Ipak, za stjecanje novih, dubljih rezultata u vezi s oksidacijskim stresom izazvanim onečišćavanjem tala teškim kovinama, potrebne su dalje *in vitro* antioksidacijske analize.

**KLJUČNE RIJEČI**: klonovi topola, oksidacijski stres, fitoremedijacija, izbojci