ORIGINAL PAPER

INFLUENCE OF LEAD TOXICITY ON GROWTH AND ANTIOXIDANT ENZYME ACTIVITY FOR *SALIX ALBA* OFFSHOOTS IN HYDROPONIC CULTURES WITH DIFFERENT LEVELS OF POLLUTANTS

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Abstract. *The effect of the simultaneous presence of lead and magnesium in hydroponic culture on the growth of Salix alba offshoots was studied. Present study used biological material collected from area of Targoviste city, specifically from the bank of the Ialomita River. Willow offshoots, having the same number of internodes and same diameter, were collected.*

The hydroponic cultures were made with three water types as hydroponic substrate, tap water, flowing water and water, lead and magnesium being added in gravimetric ratios of 0.01%, 0.1% and 0.5% (w / w). Experiments were mounted so that each glass with a certain composition of hydroponic culture held three willow offshoots in the same conditions; environmental parameters (temperature and relative humidity %) were continuously monitored; sampling procedure was followed for further analytical investigations. As a general conclusion, there was a smaller number of leaves and roots when concentration of lead and magnesium was 0.5%. The type of water had an important influence on the development of Salix alba offshoots during experiments.

Guaiacol peroxidase activity was experimentally determined at the end of the fourth day of willow monitoring. This time was considered enough for the hydroponic system to reach equilibrium. The graphical representation of guaiacol peroxidase activity shows the same pattern with a small difference when tap water was used as solvent.

At the end of experiment, solid material (roots and leaves separately sampled) was dried at 60°C some hours and then digested in a mixture of oxygenated water (H_2O_2 30%) and nitric acid (HNO_3 67%). Liquid homogeneous samples obtained were measured by atomic absorption spectrometry (AAS), concentrations of zinc, cadmium, and selenium was determined. Thus, presence of significant quantities of magnesium and lead was tested for the influence on levels of cations with different reactivity reactivity, as zinc, cadmium and selenium.

Keywords: *phytoextraction, hydroponic cultures, cations translocation, Salix alba.*

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1. INTRODUCTION

Phytoextraction is a phytoremediation process, a low-cost technique, using plants and surrounding micro-environments to translocate metallic species from soil to different harvestable parts of vegetal organisms. Furthermore, plant species used in the phytoextraction of potentially toxic trace elements should possess good tolerance to relatively high concentration of pollutant, so that growing in contaminated environment may occur [1-5].

Translocation of toxic metallic ions from soil to plants is a complex physical-physiological equilibrium process, including a set of equilibrium reactions in soil and into plant metabolism [2-7]. In this process, different complexes between organic and inorganic anions and cations existing in fluids are transferred between soil and plants. Presence of certain different cations in soil a water could increase or decrease phytoextraction ability for potentially toxic trace elements [8-12].

To achieve an efficient phytoextraction process, trees have a range of characteristics, making them possible candidates. In particular, willow species have been used for phytoremediation due to their capacity to extract toxic metal ions from soil, the high growing rate and the good tolerance to contaminated soils [13]. In the same time, willow is suitable for hydroponic cultures, this being an efficient method to decontaminate water polluted with heavy metals.

Moreover, it is known that obtaining of willow water is a biological method to extract the rooting hormones indole-butyric acid and salicylic acid that are present in sufficient quantities in the Willow (*Salix*) trees to extract as a liquid that stimulates root growth.

Salicylic acid (SA) is a phenolic phytohormone and is found in plants with roles in plant growth and development, photosynthesis, transpiration, ion uptake and transport. Thus, salicylic acid plays an important role in establishing and signaling the response to defense against various pathogenic infections, but plays an important role in mediating the response of plants to abiotic stresses such as salinity, temperature, heavy metal concentrations [14-17]. Also, salicylic acid is a very good chelating agent for some transition metal cations, the process of phytoextraction including a general equilibrium with competitive processes involving differential affinity of salicylic acid for certain cations [18]. In these circumstances, concentration of some metals from plant material is modified as a response to the change of complexant concentrations, occured when plants came into contact with hydroponic solutions.

2. MATERIALS AND METHODS

2.1. MATERIALS

$FeSO_4 \cdot 7H_2O$ (powder, for analysis purity), $(CH_3COO)_2 Pb \cdot 3H_2O$ (powder, for analysis purity), guaiacol (99.5% purity) were purchased from Merck Millipore. H_2O_2 (30 % solution, for analysis putity), HNO_3 (55 % solution, for analysis purity), NaCl (powder, 99.5% purity), KH_2PO_4 (powder, 99.5 % purity), and K_2HPO_4 (powder, 99.5 % purity) were purchased from Chimopar. Redistilled water (with conductivity below 0.10 μS·cm−1 at 25 °C) was used for solutions used in the study and for supplementing in hydroponic cultures when water was evaporated.

2.2. METHODS

2.2.1. Cultivation and treatment of plant material

Biological material collected from area of Targoviste city, specifically from the bank of the Ialomita River. Willow stems with the same age, same number of internodes and approximately the same diameter were collected.

For hydroponic cultures, three sources of water were used: flowing water from Ialomita River, stagnant water from Chindia Lake and tap water. Different water sources for the hydroponic culture were used, to check if the liquid matrix had any influence on microelements content from plant the material.

Experiments were mounted in separated beakers with each beaker containing a certain composition of magnesium and lead (0.01%, 0.1% and 0.5%, w/w). Each hydroponic culture held three willow offshoots in same conditions with continuous monitoring of environmental conditions (temperature and relative humidity %). For each type of water samples, four different experimental setups were made. One is the blank and the other three have equal concentration of lead and magnesium. The polluted samples contained lead with magnesium added as competitive ions in order to draw conclusions about the phytoextraction mechanism of lead. The salts used for the preparation of the pollutants solution were lead acetate trihydrate ($Pb(CH_3COO)_2 \cdot 3H_2O$), and magnesium sulfate ($MgSO_4 \cdot 7H_2O$). All the substances were analytical grade from Merck Millipore.

The willow offshoots were placed in water to form roots. The harvested willow branches had leaves, and one of these had aments in the moment they were collected.
Each plant used in present experiment was monitored from start to end with regards to the number of leaves, roots, aments and internodes. These characteristics were recorded through the entire period of the experiment.

In the first stage of the experiment the willow offshoots were kept in tap water to form roots. Then, the rooted willow offshoots were introduced into hydroponic solutions containing lead and magnesium in equal concentrations, respectively 0.01%, 0.1% and 0.5% (w / w). The hydroponic solutions contained river water, lake water and tap water as solvents. The liquid level in the beakers was kept at 400 mL by adding bidistilled water throughout the experiment. After 35 days the willow offshoots were removed from the solution and the morphological changes resulting from the experiment were noted.

A pH-conductivity meter WTW Multi 9430 – Inolab equipped with Sentix 98 pH sensor (± 0.04 accuracy) with temperature compensation, was used to measure the pH value of hydroponic solutions.

2.2.2. Determination of zinc, cadmium and selenium content from plant material

Solid material (roots and leaves separately sampled) was dried (48 h at 40 □) and digested in a mixture of oxygenated water (H_2O_2 30%) and nitric acid (HNO_3 67%) at 180 □ for 30 minutes using a Berghof MWS-2 digestor. Obtained liquid homogeneous samples were measured by atomic absorption spectrometry using a GBC Avanta spectrometer with flame atomization system (FAAS). The instrument was equipped with an automatic eight-lamp turret and programmable hollow cathode lamp for each measured element. Calibration curves were realized using series of freshly prepared solutions from individual stock standard solutions of each studied element, with the concentration of 1000 $mg \cdot L^{-1}$ each.

Concentrations of zinc, cadmium and selenium were determined and conclusions about the effect of magnesium and lead composition from the hydroponic culture were drawn.

2.2.3. Evaluation of guaiacol peroxidase activity in Salix alba leaves

Guaiacol peroxidase activity was experimentally determined at the end of the fourth day of willow monitoring. This time was considered enough for the hydroponic system to reach equilibrium. Harvested leaves were weighed using a Pioneer PA214C analytical balance with repeatability of 0.0001 g and a linearity of ± 0.0003 g. The samples were crushed and then treated with 25 mL of 2 % (w/w) aqueous NaCl solution. The broth was transferred to a graduated Berzelius beaker, and distilled water was added up to 100 mL. Resulting aqueous mixture was allowed to complete the enzyme extraction, under magnetic stirring for 30 minutes, and then filtered. A volume of 10 mL from the obtained filtrate was added to a volumetric flask of 50 mL and redistilled water was added up to mark. Aliquots from this solution were used to carry out enzymatic activity assays. Same procedure was applied to all studied willow plants. Determination of the extract peroxidase activity was performed by using guaiacol as a substrate and adding K_2HPO_4 / KH_2PO_4 pH buffer to maintain the pH value around 6.5.

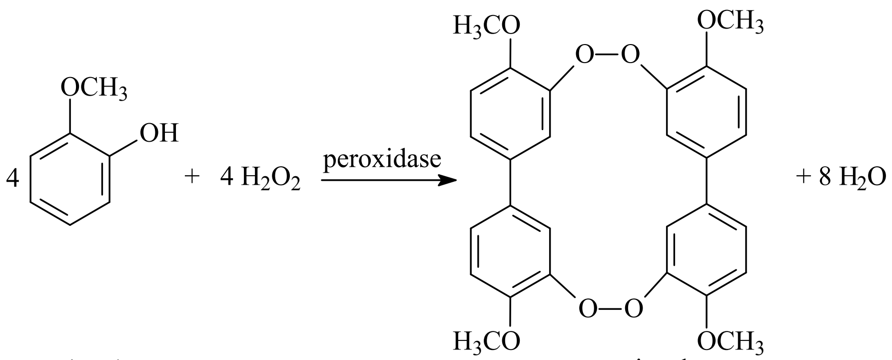

Figure 1. Conversion of guaiacol into tetraguaiacol in the presence of peroxidase.

A mixture containing 1 mL of 0.1 M K_2HPO_4 / KH_2PO_4 buffer (pH = 6.5), 1 mL of 15 mM guaiacol, and 1 mL of 3 mM H_2O_2 was homogenized well and 50 µL enzyme extract was added. After one-minute reaction time, the absorbance of resulted tetraguaiacol solution was measured at 470 nm using an ultraviolet-visible double beam Thermo-Evolution-260 Bio spectrophotometer and quartz cuvettes of 1 cm as sample support. For all measurements, a blank sample solution was prepared using buffer solution, hydrogen peroxide and enzyme extract were used, and 1 mL of redistilled water added instead of guaiacol solution. Enzymatic activity (U / mL) was determined according to the following equation:

$$\text{enzimatic activity } \left({}^{U}/_{mL}\right) = \frac{A}{\varepsilon \cdot V_e \cdot l} \cdot D_f \cdot 1000 \qquad (1)$$

where A is the measured aborbance, ε is molar exctinction coefficient for tetraguaiacol (26600 $L \cdot mol^{-1} \cdot cm^{-1}$ at 470 nm [19-20]), V_e is the volume of enzyme solution added in the mixture, l is the path length of the beam of light through the mixture (1 cm), and D_f is the dilution factor (ratio between the final and initial volume).

3. RESULTS AND DISCUSSION

The presence of lead, an important pollutant of the environment, is related to resulting effluents from different fields of activity. Lead is not a necessary element for metabolic processes but its availability at absorption can have negative effects on both animals and plants [21]. Like most toxic elements, lead interferes with a lot of processes that take place in living tissue. Lead can participate to protein structure destruction and can inhibit enzyme activity by binding to the sulfhydryl groups of different proteins, and may also cause displacement of essential elements resulting in deficiency effects in the plant cell [14, 22]. On the other hand, magnesium is needed for the plant physiology, and its presence in the growth environment is essential for plant development influencing the phytoextraction process of heavy metals like zinc and cadmium [23]. The study aimed to evaluate possible zinc and cadmium concentration changes in plant tissue, when the lead and magnesium concentration from the hydroponic solution varied in the range previously mentioned. Atomic absorption spectrometry technique was used to assess the concentration of metal accumulated in the plant tissue.

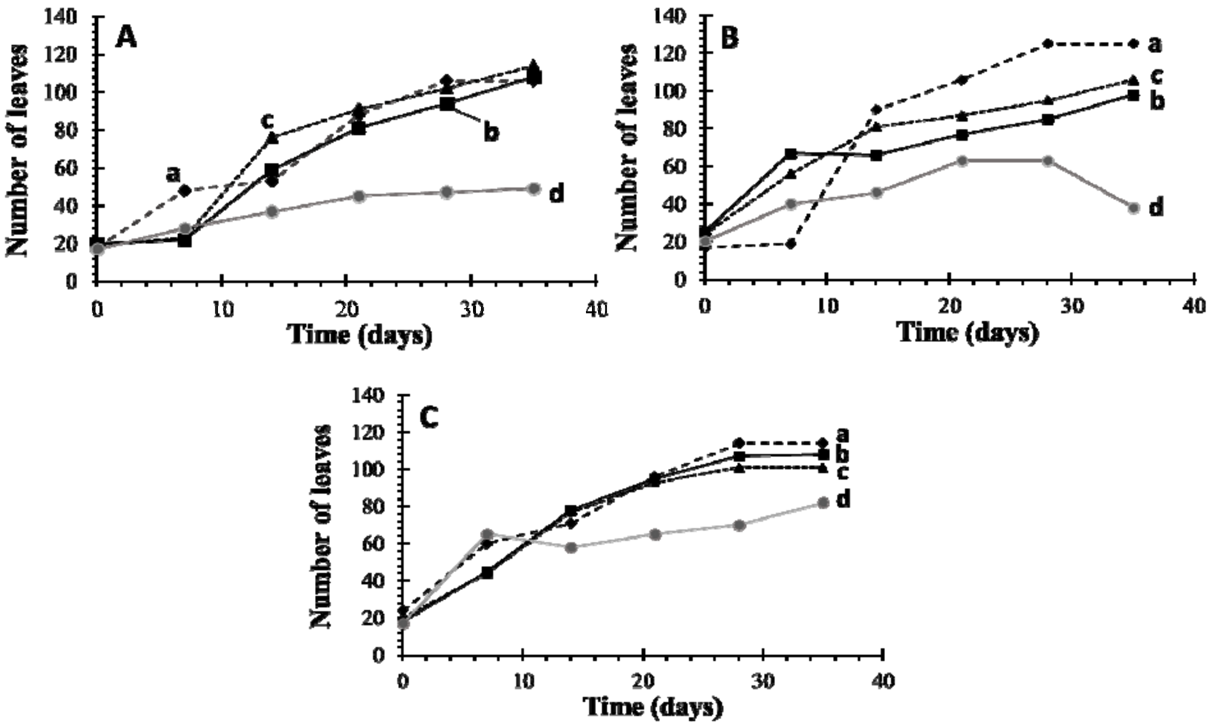

Figure 3. Number of leaves *vs.* time for *Salix alba* offshoots from hydroponic cultures with: A – tap water, B – flowing water, C – stagnant water (a – 0 %, b – 0.01 %, c – 0.1 %, d – 0.5 % added pollutant).

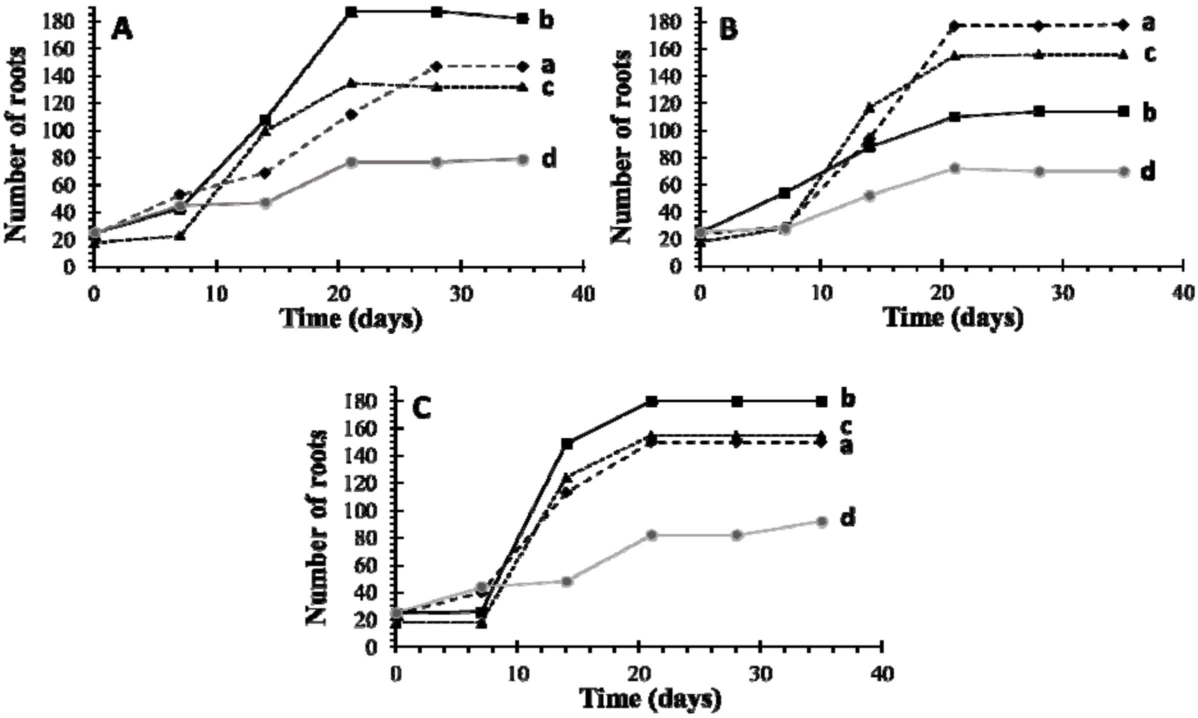

Figure 4. Number of roots *vs.* time for *Salix alba* offshoots from hydroponic cultures with: A – tap water, B – flowing water, C – stagnant water (a – 0 %, b – 0.01%, c – 0.1%, d – 0.5% added pollutant).

Fig. 3 illustrates the evolution of leaf number *vs.* time for three types of hydroponic substrates used with varied concentrations of lead and magnesium. It is noticeable that the addition of 0.5 % pollutant has effect of reducing the number of leaves grown on *Salix alba* offshoots. The most pronounced influence of pollutant concentration on *Salix alba* foliage was recorded when tap water was used as substrate for hydroponic solutions. In samples with flowing water as solvent for hydroponic solutions, it was observed that after 4 weeks some of the leaves were dried if concentration of the pollutant was 0.5 % (w / w). Foliar development seems not to be significantly influenced by the type of substrate when the added pollutant concentration was up to 0.1 % (w / w).

The evolution of number of roots developed on *Salix alba* offshoots in time is shown in Fig. 4. The presence of pollutants in concentration of 0.5% leads to the development of a smaller number of roots regardless to hydroponic substrate type. Thus, correlations of results presented in Fig. 3 and Fig. 4 indicate that the concentration limit of phytotoxicity, in the case of simultaneous addition of lead and magnesium, is between 0.1 % and 0.5 % (w / w).

Table 1. Pearson coefficients calculated for willow roots collected from experiments conducted with hydroponic substrate formed with three types of water.

	Water type					
	flowing water		stagnant water		tap water	
added metallic cation (Pb and Mg) % conc.	Conc. (ppm)	Pearson coeff.	Conc. (ppm)	Pearson coeff.	Conc. (ppm)	Pearson coeff.
Zinc						
blank	42.82 ± 1.03	0.12667	36.84 ± 0.53	0.99385	639.37 ± 9.78	-0.31173
0.01%	64.81 ± 0.67		53.68 ± 0.62		3099.81 ± 7.75	
0.10%	97.49 ± 2.18		62.55 ± 1.11		405.14 ± 2.47	
0.50%	65.91 ± 1.52		203.35 ± 2.36		818.49 ± 5.40	
Cadmium						
blank	6.48 ±0.32	-0.27630	5.89 ±1.06	-0.24860	6.33 ±0.22	-0.82468
0.01%	5.57 ±0.83		6.32 ±0.55		7.67 ±0.50	
0.10%	7.78 ±0.93		6.21 ±0.43		5.45 ±0.43	
0.50%	5.74 ±0.40		6.02 ±0.70		4.45 ±0.40	
Selenium						
blank	96.45 ±0.84	-0.96112	94.79 ±1.13	-0.88903	81.45 ±1.05	-0.42786
0.01%	78.96 ±0.67		62.81 ±1.10		94.05 ±0.77	
0.10%	90.91 ±1.98		77.64 ±1.85		17.53 ±0.45	
0.50%	10.18 ±0.14		26.22 ±0.73		46.77 ±0.98	

Table 1 and Table 2 show Pearson coefficients for willow root samples, and willow leaves respectively, calculated to perform an evaluation of the linearity of the zinc, cadmium and selenium concentrations evolution *versus* the variation of initial concentration in lead and magnesium concentrations in initial hydroponic substrate. Lead and magnesium concentrations (w/w percentages) in the substrate were varied simultaneously and in equal concentrations, from zero (blank) to 0.01 %, 0.1 %, and 0.5 % respectively.

Each of the cations discussed in present study belongs to a different analytical group [24], and consequently it exhibits different reactivity for different classes of complexation compounds. Thus, presence of significant quantities of magnesium and lead may influence behavior of cations with similar reactivity. In the physiological translocation processes of metallic cations occurring during phytoremediation, zinc and cadmium could exhibit minor changes of their concentration, this finding indicating the classification of cations in the five analytical groups as a possible way to explain the competitive processes involved in the phytoremediation process.

Table 2. Pearson calculated for willow leaves collected from experiments conducted with hydroponic substrate formed with three types of water.

	Water type					
	flowing water		stagnant water		tap water	
added metallic cation (Pb and Mg) % conc.	Conc. (ppm)	Pearson coeff.	Conc. (ppm)	Pearson coeff.	Conc. (ppm)	Pearson coeff.
Zinc						
blank	187.30 ± 1.05	0.81964	207.09 ± 3.70	-0.93627	277.38 ± 2.27	0.0301
0.01%	135.68 ± 2.45		225.85 ± 1.60		271.54 ± 2.63	
0.10%	228.12 ± 3.90		235.14 ± 4.82		1302.84 ± 7.94	
0.50%	178.57 ± 2.58		98.54 ± 0.77		460.04 ± 4.01	
Cadmium						
blank	8.23 ±0.91	-0.40980	7.84 ±0.59	-0.98180	4.76 ±0.80	0.57198
0.01%	7.05 ±0.77		8.05 ±0.55		8.70 ±0.63	
0.10%	7.22 ±0.33		7.77 ±0.52		6.04 ±0.70	
0.50%	8.18 ±0.31		6.90 ±1.00		8.96 ±0.57	
Selenium						
blank	43.10 ±0.69	0.47434	14.44 ±0.57	0.02100	95.24 ±0.25	-0.36786
0.01%	55.60 ±1.36		22.03 ±0.37		155.78 ±2.49	
0.10%	67.12 ±2.03		93.58 ±1.23		95.77 ±1.06	
0.50%	76.26 ±1.56		31.05 ±0.95		97.83 ±0.95	

Selenium is a metal having reactivity similar to sulphur, and borrowing of its metabolic pathways and transport systems was found to be possible [25]. The modification of selenium concentration may be associated with a change in the enzymatic activity corresponding to the lead phytoextraction process.

As may be observed from Table 1 and Table 2, zinc concentrations in roots sampled from willow offshoots maintained in tap water is higher than for root samples coming from flowing and stagnant water. In the case of leaves, it was found that zinc concentration in the dry matter was roughly the same, regardless of the substrate used. Also, as the calculated Pearson coefficients show, it may be concluded that no linear dependence was set between zinc concentrations in the roots and the concentration of lead and magnesium from the hydroponic cultures made with tap water and river water.

As concerns cadmium, measurements performed to find its concentration in dry mater show rather small variations from one sample to another, regardless of the hydroponics initial composition, with same behavior for both of roots and leaves.

Considering the experimental findings commented above and the data presented in Table 1 and Table 2, it may be concluded that a change in the concentration of lead and magnesium in hydroponic solutions does not influence the concentration cadmium in the analyzed plant samples. Thus, it can be assumed that cadmium show transportation pathways through the vegetal material different from magnesium and lead. On the other hand, zinc concentration varies with pollutant concentration, but no linear pattern was recorded.

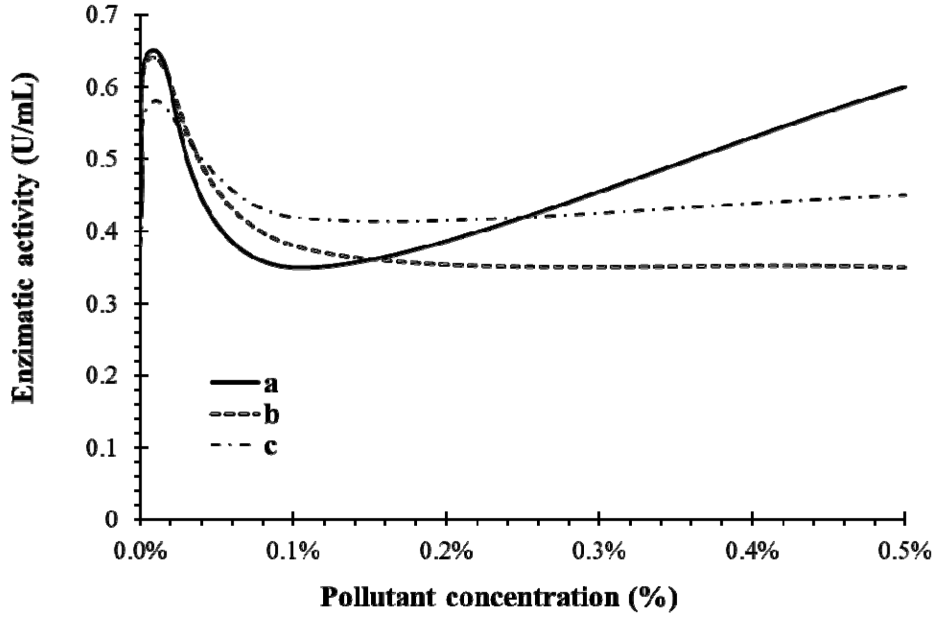

Figure 5. Variation of guaiacol peroxidase activity (U/mL) with pollutant concentration (Pb^{2+}, in w/w - %) in hydroponic solutions with: a) tap water, b) flowing water, c) stagnant water.

The pH measurements showed an increase of the hydroponic solutions pH for all studied situations, from initial values in the range of 6.00 to 7.00, towards higher values, around 7.90. This behavior may indicate that some chemical species possessing buffering capacity were transferred in the aqueous phase during the 35 days of experiment.

On the other hand, addition of lead and magnesium could modify the activity of guaiacol peroxidase. Guaiacol peroxidases are heme-containing proteins that preferably oxidize aromatic electron donors such as guaiacol and pyrogallol at the expense of oxygenated water [26]. Guaiacol peroxidase is associated with many important biosynthetic processes and defense against abiotic and biotic stresses. The guaiacol peroxidases are widely accepted as stress “enzymes” [26]. Various stressful conditions of the environment, including different pollutants such as heavy metals [27, 28], herbicides [29], ozone [30], and polycyclic aromatic hydrocarbon [31], have been shown to induce the activity of guaiacol peroxidase. Peroxidases can contain in their active sites redox-active cysteine or selenocysteine residues.

Figure 5 shows the influence of lead and magnesium concentration on guaiacol peroxidase activity (U/mL) calculated according to equation 1. In all cases the increase of lead concentration up to 0.01 % leads to the increase of peroxidase activity followed by a decrease of enzymatic activity if the concentration exceeds the value of 0.01 %. If hydroponic solutions containing flowing water or stagnant water were used, the increase of pollutant concentration over a certain value did not lead to significant changes in enzyme activity. This could indicate an equilibrium phase, which is not influenced by modifications in the amount of lead or magnesium in the sample. If tap water was used as solvent, the addition of lead and magnesium ions leads to a gradual increase in enzymatic activity after the concentration exceeds 0.1 % (w/w) of pollutant added to hydroponic solution.

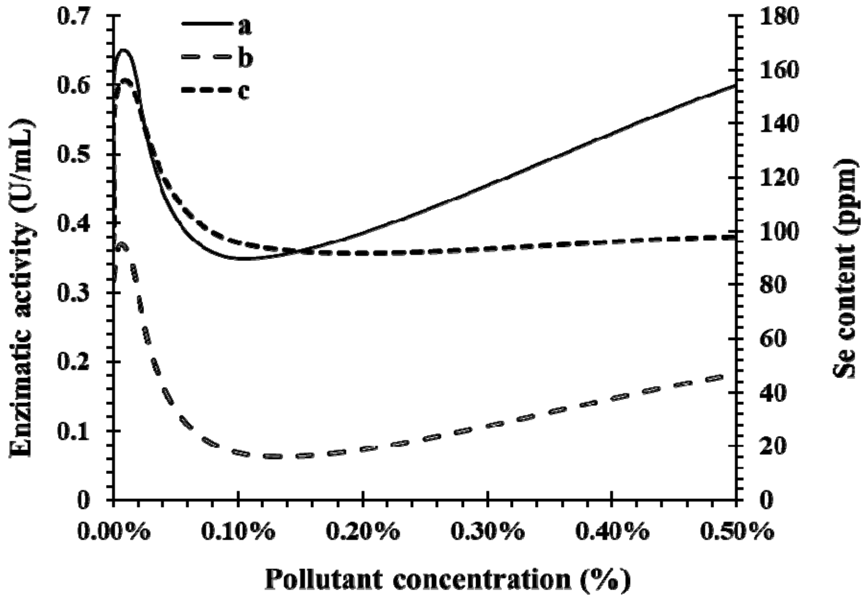

Figure 6. Graphical representation profile for: a) guaiacol peroxidase activity, b) Se content (ppm) in roots, and c) Se content in leaves *vs.* pollutant concentration Pb (w/w %) in hydroponic solutions with tap water.

Fig. 6 presents selenium concentration in dried vegetable material samples *vs.* pollutant concentration and guaiacol peroxidase activity vs. pollutant concentration when tap water was used as solvent for hydroponic solutions showing a similar pattern of variation. When stagnant water or flowing water were used as substrates for hydroponic cultures there was no similar variation between selenium content and peroxidase activity.

4. CONCLUSIONS

The study presented the influence of simultaneous variations of lead and magnesium concentration on development of radicular and foliar system of *Salix alba* offshoots grown in hydroponic cultures when the water type used as substrate for hydroponic solutions was varied. Hydroponic solutions were made with lead and magnesium added in equal concentrations (0.01%, 0.1% and 0.5%, w / w), aiming to obtain informations on phytotoxicity limit for *Salix alba*. Willow offshoots were kept in hydroponic solutions with different types of water and different concentrations of pollutant for 35 days. After 35 days the experimental data that present the evolution of leaves and roots number in time show that phytoxicity limit for simultaneous adding of lead and magnesium ranges from 0.1 % to 0.5 % (w / w).

Dried plants were digested and concentration of cadmium, zinc, and selenium ions were measured by atomic absorption spectrometry. Cadmium, zinc, and selenium ions were chosen due to their different complexation properties compared to lead and magnesium. The existence of competitive processes involving cadmium, zinc or selenium in the process of lead and magnesium translocation from the hydroponic solution to plant tissue were proved by modification of their concentrations in the dried plant. Thus, zinc and selenium concentrations varied when different types of hydroponic solutions were used, however calculated Pearson coefficients did not show linear patterns. In the same time, cadmium concentrations from dried samples presented small variations from one type of hydroponic substrate to another indicating no significant interaction with metabolic pathways of lead and magnesium.

The activity of guaiacol peroxidase was determined four days after contacting the offshots with the tested hydroponic solutions, this period of time being enough for the system to reach the equilibrium of metabolic processes after moving the plants into the hydroponic substrates with pollutants added at different concentrations. Experimental data showed a pattern for guaiacol peroxidase activity evolution with respect to added pollutant concentration, regardless of water type used so that pollutant addition over 0.01% (w / w) leads to a sharp decrease in enzyme activity values.

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