African Journal of Biotechnology Vol. 11(78), pp. 14313-14319, 27 September, 2012
Available online at http://www.academicjournals.org/AJB
DOI:10.5897/AJB12.1004
ISSN 1684-5315 ©2012 Academic Journals

Full Length Research Paper

Response of *Salix alba* L. to heavy metals and diesel fuel contamination

Borišev Milan[1]*, Pajević Slobodanka[1], Nikolić Nataša[1], Krstić Borivoj[1], Županski Milan[1], Kebert Marko[2], Pilipović Andrej[2] and Orlović Saša[2]

[1]Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Trg Dositeja Obradovića 2, Novi Sad, Serbia.
[2]Institute of Lowland Forestry and Environment, University of Novi Sad, Antona Čehova 13, Novi Sad, Serbia.

Accepted 3 August, 2012

Pot experiment was set in a greenhouse in order to determine the physiological response of *Salix alba* grown on soil co-contaminated with individual and combination of Cd, Ni, Pb-ethylenediaminetetraacetic acid (EDTA) and diesel fuel treatments. EDTA enhanced the uptake of Cd and Ni, whereas the antagonism between Cd, Ni and Pb led to reduced accumulation of Pb. Presence of 5 g/kg of diesel fuel in soil significantly increased toxic influence of applied heavy metals by further reducing plant growth, photosynthetic rate, transpiration rate and water use efficiency. This concentration of diesel fuel also reduced the uptake and accumulation of Cd (soil concentration of Cd was 4.36 mg/kg), while Ni and Pb accumulation (soil concentrations of 118.7 and 186.7 mg/kg) remained at the same level, but with significant reduction of plant growth, thus reducing *S. alba* phytoextracion potential. At lower applied contaminant concentrations (3 g of diesel fuel/kg and Cd 2.15, Ni 70.8 and Pb 116.1 mg/kg), growth disturbances were low and diesel fuel presence reduced the uptake of Pb only in roots and old leaves, whereas accumulation capacity of Cd and Ni remained unaffected, indicating that *S. alba* plants have potential for remediation and re-development of co-contaminated sites with moderate levels of polluters.

Key words: Cd, Ni, Pb, phytoremediation, phytoextraction, willow, *Salix alba*.

INTRODUCTION

At polluted sites, organic and inorganic polluters are often combined at the same locality (Ensley, 2000). Remediation or re-development of sites with both types of contaminants represents specific technical and economic challenge. Phytoremediation is a complementary or alternative, cost-effective, environmentally friendly technology, that utilizes suitable plant species to remove or degrade organic and inorganic pollutants (Nwoko, 2010). In phytoremediation investigations, analyses are usually performed for specific type of pollution, organic or inorganic. To our knowledge, scientific informations available in the area of phytoremediation at sites co-contaminated with both organic and inorganic polluters, are still limited, although several papers have emerged in the past decade (Perronnet et al., 2003; Chen et al., 2004; Roy et al., 2005; Lin et al., 2008; Alisi et al., 2009; Atagana, 2011). Different species and genotypes of *Salix* proved to have considerable potential in phytoextraction of heavy metals (Klang-Westin and Eriksson, 2003; Lunácková et al., 2003; Dos Santos Utmazian et al., 2007; Liu et al., 2011; Wani et al., 2011), whereas in biological remediation of organic contaminants, willow trees are still not well investigated. Phytoremediation of areas polluted with organics, such as hydrocarbons from diesel fuel or crude oil, is mostly pursued by application of herbal plants, and it often strongly relies on microbial activity facilitated by root development (Merkl et al., 2005;

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*Corresponding author. E-mail: milan.borisev@dbe.uns.ac.rs. Tel: ++381642576166. Fax: +38121450988.

Abbreviations: PR, Photosynthetic rate; **TR,** transpiration rate; **WUE,** water use efficiency; **M,** metals; **D,** diesel fuel.

Table 1. Physico-chemical soil characteristics.

Large sand particles (%)	Small sand particles (%)	Powder (%)	Clay (%)	pH		$CaCO_3$ (%)	Humus (%)	Total N (%)
				in KCl	in H_2O			
2.24	76.38	13.68	7.70	7.74	8.44	18.74	1.48	0.13

Table 2. Total heavy metal and diesel fuel concentrations in the studied soil.

Treatment	Cd (mg/kg)	Ni (mg/kg)	Pb (mg/kg)	Diesel fuel (g/kg)
Control	0.35	29.41	26.57	-
Cd	4.68	28.28	27.20	-
Ni	0.30	118.10	27.41	-
Pb (Pb-EDTA)	0.36	30.02	188.5	-
M (Cd+Ni+Pb-EDTA– higher concentration)	4.48	106.61	191.8	-
m (Cd+Ni+Pb-EDTA– lower concentration)	1.94	62.22	113.5	-
D (diesel fuel– higher concentration)	0.33	29.02	25.95	5
d (diesel fuel– lower concentration)	0.35	28.97	26.71	3
M+D (Cd+Ni+Pb-EDTA+diesel fuel– higher concentration)	4.36	118.70	186.70	5
m+d (Cd+Ni+Pb-EDTA+diesel fuel– lower concentration)	2.15	70.80	116.1	3
MAC	3	50	100	-

MAC, Maximal allowed concentrations of heavy metals in soil, according to Serbian Law (Official Gazette of the Republic of Serbia, Issue No. 23/1994).

Ogbo et al., 2009).

Some target localities in Serbia are of special interest, due to specific co-pollution occurrence. During 1999, NATO bombing caused a spill of fossil fuels (mostly crude oil and diesel fuel) from several destroyed oil refineries. Localities with co-contamination of heavy metals and mineral oils were created. Also, in some old Serbian mines, sites were left with degraded land containing specific mixtures of both organic and inorganic contaminants. Planting pollutant resistant tree species on such sites could prove to be a cheap and effective way to decrease pollution content and start a new life cycle of such degraded land areas. The recultivation of these sites could be more efficient with improved landscape design achieved by controlled planting of new vegetation covers.

Experiment was set to simulate co-contamination of heavy metals and diesel fuel and to analyze mixed influence of these polluters on willow plant growth, photosynthetic, transpiration rates and heavy metal accumulation characteristics. *Salix alba* was selected as an autochthonous species which has already proved its potential for phytoextraction of heavy metals (Lunáčkova et al., 2003; Borišev et al., 2009).

MATERIALS AND METHODS

Stem cuttings (20 cm long) of one-year-old shoots of genotype *S. alba* – V 158, were placed in Micherlich pots filled with soil (six cuttings per pot, eight pots per treatment). Genotype was developed in the Institute of Lowland Forestry and Environment, University of Novi Sad, Serbia. Soil properties are shown in Table 1.

Plants were treated for 65 days with individual and combined treatments of Cd, Ni, Pb-ethylenediaminetetraacetic acid (EDTA) and diesel fuel. Pollutants were mixed with soil one month before planting. Cd was supplied as $CdCl_2 \cdot H_2O$, Ni as $NiSO_4 \cdot 6H_2O$ and Pb as Pb-EDTA. Soil was sprayed with diesel fuel from the distance of 10 cm, and thoroughly mixed. Smaller combined pollutant treatments (m and m+d) were projected to have approximately 50% heavy metal concentration in respect to larger combined concentration of pollutants (M and M + D). Concentrations of heavy metals (mg of metal / kg of dry soil) and diesel fuel (g of diesel fuel added to 1 kg of dry soil) are shown in Table 2.

Plant material was rinsed in deionized water, dried and prepared for analyses following standard methods for the examination of water and wastewater (APHA, 1995). Solutions of plant and soil samples were prepared using closed vessel high pressure microwave digestion (Milestone D series Microwave Digestion System). Concentrations of Cd, Ni and Pb in plants and soil samples were determined from prepared solutions by employing flame atomic absorption spectrophotometry (Varian, AAS240FS).

Photosynthetic and transpiration rates were measured using the LCpro+ portable photosynthesis system, manufactured by ADC BioScientific Ltd. Light conditions were set using the LCpro+ light unit, which emitted photosynthetic active radiation (PAR) at 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The air supply unit, provided a flow of ambient air to the leaf chamber at a constant rate of 100 $\mu mol \cdot s^{-1}$. Temperature, CO_2 concentration and humidity were at ambient levels.

Water use efficiency (WUE) was calculated as the ratio between photosynthetic and transpiration rate in μmol of $CO_2 \cdot m^{-2} \cdot s^{-1}$ / mmol of $H_2O \cdot m^{-2} \cdot s^{-1}$.

Table 3. Growth parameters of *S. alba* in relation to applied treatments.

Treatment	Height (cm)	Shoot mass (g)	Leaf mass (g)	Number of leaves/plant
Control	47.7 ± 3.4^{a}	4.1 ± 0.7^{a}	5.5 ± 0.4^{a}	43.3 ± 3.9^{a}
Cd	42.9 ± 2.2^{c}	1.2 ± 0.1^{c}	2.0 ± 0.2^{c}	29.2 ± 2.8^{c}
Ni	41.6 ± 2.5^{c}	1.0 ± 0.1^{cd}	1.9 ± 0.2^{c}	33.2 ± 2.9^{b}
Pb	46.1 ± 1.8^{ab}	0.9 ± 0.2^{d}	1.6 ± 0.1^{d}	29.2 ± 3.1^{c}
M	34.8 ± 2.0^{d}	0.7 ± 0.1^{de}	1.6 ± 0.2^{d}	27.5 ± 3.2^{d}
m	45.8 ± 1.7^{b}	1.7 ± 0.1^{b}	2.6 ± 0.3^{b}	29.8 ± 2.0^{c}
D	29.2 ± 1.9^{f}	0.5 ± 0.1^{ef}	1.1 ± 0.1^{e}	28.3 ± 3.3^{cd}
d	33.7 ± 1.8^{d}	1.6 ± 0.2^{b}	2.0 ± 0.3^{c}	28.3 ± 1.7^{cd}
M+D	18.9 ± 1.8^{g}	0.4 ± 0.1^{f}	1.0 ± 0.2^{e}	25.0 ± 1.8^{e}
m+d	31.5 ± 3.7^{e}	0.4 ± 0.1^{f}	1.0 ± 0.1^{e}	27.3 ± 2.5^{d}

Experimental data were expressed as mean ± SD (n = 6). Values followed by different letters in the same column are significantly different at $p<0.05$.

Statistical analyses were conducted using Duncan's multiple range test, at a level of significance of $p<0.05$, using 1-way factor analyses.

RESULTS

Negative impact of applied pollutants caused localized chlorotic and necrotic changes of leaves. These changes were visible mostly on younger leaves in small patches, on plants treated with higher concentrations of pollutant mixture (M + D and M) and on higher applied diesel fuel treatment (D).

All applied pollutants caused significant reduction of plant growth (Table 3). Mixing of both heavy metals and diesel fuel, further reduced plant growth in respect to individual pollutant treatments at the same concentration level. Concentration level of pollutants was a significant factor that determined the degree of growth reduction. The smallest negative impact on growth was determined by mixture of heavy metals applied at lower concentrations (m).

Combined treatment of higher concentration of heavy metals and diesel fuel (M + D) caused the strongest reduction of photosynthetic rate (PR), transpiration rate (TR) and WUE (Table 4). Smaller concentration of mixed pollutants (m + d), and diesel fuel (d) determined the weakest negative influence on these parameters. PR of plants exposed to Ni and Pb-EDTA was at the same level as in plants exposed to the mix of all heavy metals (M) in the same concentration range. Same relations are determined by WUE analyses.

Transpiration rate was significantly reduced by mixed treatments (M, m and M + D), with exception of lower co-pollutant concentration (m + d). PR was not reduced by Cd, but in relation to elevated TR. Therefore, WUE was significantly reduced by Cd, as compared to the control, suggesting that plants were ‘‘wasting water’’. Stabile WUE was determined at smaller concentration of heavy metals and diesel fuel (m and m + d), but also on combined treatment of metals applied at higher concentration (M). Correlation between photosynthetic rate and growth parameters was positive, but without statistic significance (Table 5).

Accumulation of Cd, Ni and Pb was highest in roots (Tables 6, 7 and 8). Higher heavy metal concentrations (M and M + D treatments) determined higher uptake and accumulation of Cd and Ni with respect to m and m + d treatments. In roots, Cd and Ni content were significantly higher at M treatment, than in individual applications of Cd and Ni, whilst this correlation was not determined in shoots and leaves.

Presence of diesel fuel in the soil (treatments M + D and m + d) significantly reduced Cd content as compared to heavy metal mixtures without diesel (M and m). In some plant organs, this reduction was determined by Ni and Pb accumulation also, and if not, Ni accumulation was held at the same level, but in lower biomass due to growth reduction determined by diesel fuel presence, thus reducing the total Ni and Pb amount extracted from the soil (Table 7).

Individual treatment of Pb (Pb-EDTA) determined the highest accumulation level of Pb in all plant parts (Table 8). Presence of Cd and Ni in treated soil (treatment M) reduced Pb accumulation, with respect to individual Pb-EDTA treatment, indicating antagonism between heavy metals.

Pb accumulation in leaves was not significantly changed by decreasing the Pb content in the soil (treatments M and M + D when compared with m and m + d), even if uptake by roots was higher, indicating reduction of Pb translocation from roots to aboveground parts.

DISCUSSION

Metabolic processes describing toxic influence of heavy metals and some diesel fuel components on *Salix* and other plants species are well documented (Lasat, 2002;

Table 4. Photosynthesis, transpiration and water use efficiency in relation to applied treatments.

Treatment	Photosynthetic rate (PR) µmol m^{-2} s^{-1}	Transpiration rate (TR) mmol m^{-2} s^{-1}	Water use efficiency (WUE) µmol m^{-2} s^{-1}/mmol m^{-2} s^{-1}
Control	23.7±0.9^{a}	6.2±0.1^{bc}	3.8±0.1^{a}
Cd	22.7±2.0^{ab}	7.2±0.2^{a}	3.2±0.2^{bc}
Ni	16.2±1.2^{cd}	5.5±0.3^{cd}	2.9±0.2^{c}
Pb	18.7±3.0^{bc}	6.3±0.4^{b}	2.9±0.3^{c}
M	18.6±1.6^{bc}	5.4±0.2^{d}	3.4±0.3^{abc}
m	17.3±5.9^{cd}	4.6±1.0^{e}	3.7±0.5^{ab}
D	14.3±2.4^{d}	4.7±0.5^{e}	2.9±0.2^{c}
d	19.1±2.8^{bc}	5.6±0.4^{cd}	3.4±0.3^{abc}
M+D	6.2±3.2^{e}	3.1±0.6^{f}	2.0±0.6^{d}
m+d	22.5±2.9^{ab}	6.2±0.4^{bc}	3.7±0.3^{ab}

Experimental data were expressed as mean ± SD (n = 6). Values followed by different letters in the same column are significantly different at $p<0.05$.

Table 5. Linear correlations (r) between photosyntetic rate and growth parameters.

Growth parameter	Photosyntetic rate
Height	0.69
Shoot mass	0.46
Leaf mass	0.46
Number of leaves	0.50

Correlations above 0.75 are significant for $p < 0.05$.

Table 6. Accumulation of Cd (mg/kg).

Treatment	Root	Shoot	Old leaves	Young leaves
Control	7.0±1.68^{f}	3.3±0.3^{c}	3.3±0.4^{c}	3.9±0.2^{d}
Cd	104.4±11.4^{c}	24.1±4.8^{a}	22.4±3.7^{a}	18.7±1.9^{ab}
M	154.3±11.5^{a}	23.0±2.7^{a}	13.7±2.5^{b}	23.5±4.5^{a}
m	59.4±6.3^{d}	14.2±1.5^{b}	16.6±3.4^{b}	11.8±2.8^{c}
M+D	122.7±7.8^{b}	12.4±3.0^{b}	13.2±5.9^{b}	16.6±2.6^{bc}
m+d	27.0±1.6^{e}	4.3±0.6^{c}	4.3±0.8^{c}	5.8±1.6^{d}

Experimental data were expressed as mean ± SD (n = 3). Values followed by different letters in the same column are significantly different at $p<0.05$.

Pulfford et al., 2002; Dos Santos Utmazian et al., 2007; Dickinson et al., 2009; Gerhardt et al., 2009). The results of this study suggest that individual toxic influence of heavy metals and diesel fuel, showed strong additive impact, combining high toxicity of bivalent heavy metal ions and hydrocarbon compounds in diesel fuel. Multiple inhibitory effects were especially strong when higher pollutant concentrations were applied (treatments M + D, M and D). Toxicity was manifested through leaf chlorosis and necrosis, reduced biomass of all plant organs and disturbances in photosynthetic CO_2 assimilation and water regime. Similar negative effect of a mixture of both organic and inorganic pollutants on physiology (assimilation and growth) of *S. alba* and some other plant species were previously verified (Roy et al., 2005; Pajević et al., 2009; Atagana, 2011).

Water use efficiency analysis provided a view of the interdependence between water balance and CO_2 assimilation. This parameter proved to be good tool for assessing genotypes and environment conditions in which lower water consumption is combined with sustained photosynthetic activity (Martin and Stevens, 2006). WUE was significantly reduced by higher concentrations of pollutant mixture (M + D). It was also reduced by Cd, which stimulated transpiration, thus increasing water loss. Such poor water usage could increase plant physiological disturbances at polluted sites, when water supply is limited. The success of

Table 7. Accumulation of Ni (mg/kg).

Treatment	Root	Shoot	Old leaves	Young leaves
Control	6.5±0.9^{d}	7.0±0.1^{d}	7.4±0.4^{c}	7.5±0.5^{d}
Ni	150.0±12.9^{b}	39.0±2.4^{b}	40.7±1.6^{a}	41.3±2.6^{a}
M	200.7±15.6^{a}	39.2±2.9^{b}	40.8±4.2^{a}	43.7±4.1^{a}
m	112.2±8.6^{c}	23.6±3.1^{c}	24.6±4.8^{b}	25.7±2.4^{c}
M+D	205.1±3.3^{a}	46.9±3.9^{a}	42.4±5.4^{a}	39.8±4.1^{a}
m+d	147.5±7.1^{b}	28.6±1.6^{c}	32.0±3.5^{b}	32.8±2.5^{b}

Experimental data were expressed as mean ± SD (n = 3). Values followed by different letters in the same column are significantly different at p<0.05.

Table 8. Accumulation of Pb (mg/kg).

Treatment	Root	Shoot	Old leaves	Young leaves
Control	11.8±3.9^{e}	6.8±0.4^{d}	6.0±1.0^{e}	7.4±0.4^{d}
Pb	162.7±6.8^{a}	29.9±4.4^{a}	27.4±6.2^{a}	28.7±4.3^{a}
M	114.4±14.5^{b}	19.1±3.4^{b}	18.1±3.0^{bc}	20.3±1.8^{b}
m	79.4±3.4^{c}	13.0±0.9^{c}	21.6±1.3^{b}	16.3±3.0^{bc}
M+D	111.3±12.1^{b}	16.0±1.3^{bc}	13.5±1.0^{cd}	15.8±1.3^{bc}
m+d	61.5±4.2^{d}	11.7±3.0^{cd}	12.4±2.5^{d}	13.2±1.1^{c}

Experimental data were expressed as mean ± SD (n = 3). Values followed by different letters in the same column are significantly different at p<0.05.

plant adaptation to pollutant stress depends both on stabile photosynthetic activity and water use efficiency, thus increasing remediation potential of such genotypes applied at each specific site.

According to the presented results, analysed *S. alba* genotype could have sufficient viability at sites with lower applied concentrations of pollutants (diesel fuel < 3 g/kg, Cd < 2.15 mg/kg, Ni < 70.8 mg/kg, Pb-EDTA < 116.1 mg/kg, respectfully), even if the pollutants are present as a mixture.

Accumulation of Cd, Ni and Pb (Tables 6, 7 and 8) confirmed previous findings that highest concentration of heavy metals in *Salix* species is always achieved in roots (Dos Santos Utmasian et al., 2007).

Application of EDTA yielded positive results. Cd and Ni were more accumulated when mixed heavy metal treatment was applied (M) compared with individual heavy metal treatments. This is most likely explained by the presence of EDTA in the combined heavy metal treatment (M). EDTA is a chelating molecule that can further increase uptake and translocation of heavy metals (Hernández-Allica et al., 2007; Bianchi et al., 2008; Tsetimi and Okieimen, 2011). Bioavailability of heavy metals is lower in alkaline soils (soil pH was slightly alkaline) (Table 1). EDTA application can reduce pH value, thus improving solubility of heavy metals (Karami and Shamsuddin, 2010). Roy et al. (2005) confirmed that in co-contaminated sites, EDTA could improve metal translocation in plant tissue as well as overall phytoextraction potential, especially in alkaline soil, also used in their research. However, Mühlbachová (2009) determined that the application of EDTA in heavy metal contaminated soil, at early stages can produce toxic effects on soil microorganisms, thus reducing their overall biomass. Further research is required to explain EDTA role in co-contaminated environment, but possible reduction of microbial biomass in the root area could badly affect *S. alba* growth, especially on sites with elevated levels of organic contaminants.

Ability of analyzed *S. alba* genotype to uptake and accumulate heavy metals was significantly reduced by presence of diesel fuel. This was determined by reduced concentrations of heavy metals in the plant organs and by reduced biomass accessible for heavy metal accumulation. Similar response was determined in *Zea mays* with Cu phytoextraction ability, reduced by the presence of pyrene in contaminated soil (Lin et al., 2008). Changed and reduced bioavailability of heavy metals in the presence of organic co-contamination in soil is also confirmed by Chen et al. (2004).

Lower accumulation of Pb, was correlated with presence of Cd and Ni in applied soil, indicating antagonism between heavy metal ions. Similar PR level measured on individual Ni, Pb-EDTA and mixed M treatment indicates that the antagonism between heavy metal ions could reduce the individual toxic impact of each ion. Antagonism between heavy metals is specifically dependent on the heavy metal species and chemical form. It is confirmed between Cd and Zn (Vasiliadou and Dordas, 2009), Se and Zn with respect to

Pb and Cd in vegetables (He et al., 2004), between Cd and Pb in spinach (Xin et al., 2010) and is believed to be strongest between heavy metal ions similar in radius diameter (Seregin and Kozhevnikova, 2008). In phytoextraction, antagonism between heavy metal ions reduces their toxic effect, enabling higher growth, but at the same time, it decreases their accumulation level in plant tissues.

Reduced translocation of Pb was determined at higher concentration of heavy metal mix (M). Pb ion is known for its low mobility in plants. Malkowski et al. (2005) suggest that Pb, as a large ion has a high potential for apoplast adsorption. At some point, this Pb adsorption will reach its maximum capacity. Further increase of Pb content in the root medium will not increase Pb translocation due to its limited mobility through simplast and maximum adsorption capacity reached in the root apoplast, thus constraining the Pb translocation ratio from root to aboveground plant parts.

In general, combination of heavy metal and diesel fuel stress on analyzed genotype, significantly reduced phytoextraction potential, through reduction of plant growth, decrease in heavy metal accumulation ability, and at higher applied pollutant concentrations, there was decrease of photosynthetic rate and water use efficiency. Application of analyzed *S. alba* genotype in phytoremediation is expected to be practical only on sites moderately contaminated with combined heavy metal (Cd + Ni + Pb) and diesel fuel pollution (Cd + Ni + Pb + diesel fuel), or as a polishing technology for small scale pollutions, if no additional biotechnical measures are applied. The use of *Salix* as a heavy metal phytoextraction tool in several papers is also proposed for remediation of soils moderately contaminated with heavy metals, with concentrations slightly elevated above maximum allowed concentrations (Pulford et al., 2002; Dickinson et al., 2009).

Obtained results from this paper support the possibility of application of *S. alba* which could upgrade its general economical potential and diversify its practical use. *S. alba* could be a suitable tool which is able to provide restoration and new management of sites co-contaminated with moderate levels of both heavy metals and diesel fuel (with respect to analyzed concentrations).

ACKNOWLEDGEMENT

This paper was financed by the Ministry of Education and Science of the Republic of Serbia within the framework of integrated and interdisciplinary research as a part of the project number 043007.

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