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In vitro SELECTION FOR LEAD TOLERANCE IN SHOOT CULTURE OF *Daphne* SPECIES

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Abstract *In vitro* culture may provide a suitable environment for selection of heavy-metal tolerant plantlets. Such clones of woody plants could be valuable material applicable to soil remediation. In *in vitro* culture conditions shoots of *Daphne jasminea* Sibth. & Sm. and *Daphne tangutica* Maxim. (Thymelaeaceae) were grown on media supplemented with 0.1, 0.5 and 1.0 mM lead nitrate. Level of lead bioaccumulation, growth parameters, content of photosynthetic pigments, and mineral status of cultured shoots were investigated. *D. jasminea* has grown vigorously on Pb^{2+}-containing media, with growth tolerance index reaching 73–89%, depending on concentration applied, and the highest growth value was obtained in the presence of 1.0 mM lead nitrate. *In vitro* propagation of *D. tangutica* shoots was slightly inhibited by lead ions, however the growth tolerance index has increased up to 152% on medium with 1.0 mM $Pb(NO_3)_2$. In both studied species the highest content of accumulated lead, as well as the value of bioconcentration factor, were found in shoots grown on 0.1 mM lead nitrate. *D. tangutica* accumulated over two times as much lead in comparison with *D. jasminea*. Chlorophyll content in *D. jasminea* was not affected by applied lead nitrate doses, while in *D. tangutica* stimulation of chlorophyll, as well as carotenoid, synthesis occurred. In tested concentrations lead nitrate had no toxic effect on the level of shoot nutrition. Detected levels of essential and trace elements were still high enough to maintain undisturbed growth and development of cultured shoots. This is first report confirming the suitability of *in vitro* selection for obtaining of vigorous, proliferative, tolerant to elevated lead concentration shoots of ornamental *Daphne* species.

Key words: bioaccumulation, heavy metal, *in vitro* culture, mineral nutrition, Thymelaeaceae

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INTRODUCTION

The concentration of toxic metals in numerous terrestrial and aquatic ecosystems is still exceeding permissible standards. Lead, cadmium and mercury ions are non-essential, and usually outstandingly adversely affecting plant metabolism. With regard to lead, it is a cumulative, rather slow acting element, and strongly poisonous to all known organisms [Wierzbicka 1998, Alkorta et al. 2004, Sharma and Dubey 2005, Wierzbicka et al. 2007]. The ability of numerous vascular plant species to accumulate particular chemical elements in organs, either roots or shoots, is well known [Zhera et al. 2009, Poorter et al. 2011]. In normal growth conditions metal uptake takes place through the roots, and to a certain level *via* the leaves, what makes especially trees perfect test plants to monitor the atmospheric pollution with lead [Demirayak et al. 2011]. Besides, it has been reported that some terrestrial woody ornamentals are able to accumulate considerable amount of lead ions [Ali et al. 2003, Trigueros et al. 2012]. In recent decades there have also appeared in print valuable publications regarding experiments focused on the response of such woody plants to radionuclide treatment [Bystrzejewska-Piotrowska et al. 2004, Soudek et al. 2004], and other chemicals like cyanide [Yu et al. 2005] or some herbicides [Baz and Fernandez 2002].

Significant progress has also been attained in remediation of environmental pollutants using plant genetic engineering technology [Dhankher et al. 2002, Alkorta et al. 2004, Martinez et al. 2006, Doran 2009], but effective micropropagation protocol is prerequisite to effectively develop genetically modified plants. Profitable alternative to genetic transformation would be obtaining the resistant plant material applying approach of *in vitro* selection [Toan et al. 2004, Gatti 2008]. Nonetheless, a necessary condition of making progress, using biotechnological techniques in breeding schedules, is to produce viable and healthy microcuttings. A few effective micropropagation protocols regarding numerous woody *Daphne* species have been recently published [Noshad et al. 2009, Hanus-Fajerska et al. 2012, Wiszniewska et al. 2013]. Species belonging to the genus *Daphne* are desirable ornamental plants of increasing exploitation in horticulture. In an urban environment the use of ornamental plants exhibiting tolerance to heavy metals would be especially substantiated. The utilization of woody species in remediation of hazardous wastes has several advantages over herbaceous plants, such as perennial habit, extensive root biomass and large transpirational rates [Stomp et al. 1993]. However, reports on identification of such plants applicable to remediation of contaminated soils are still sparse [Liu et al. 2008], and *Daphne* species have not been investigated in this respect to date. For this reason, the main aim of undertaken experimental work was to study the influence of various lead (II) nitrate concentrations on growth, the content of photosynthetic pigments, the level of accumulated lead, and the mineral status of two contrasting *Daphne* genotypes during *in vitro* selection using culture of proliferating shoots. The additional purpose of undertaken study was possible selection of clones tolerant to lead.

MATERIALS AND METHODS

Plant material and culture medium. *In vitro* shoots of *D. jasminea* Sibth. & Sm. and *D. tangutica* Maxim. (Thymelaeaceae Juss.) were kindly provided by Dr A. Riseman from the University of British Columbia Botanical Garden and Centre for Plant Research, Vancouver, Canada. Cultures were established using 15 mm (*D. tangutica*) or 5 mm long (*D. jasminea*) microcuttings. Basal medium for the shoot culture consisted of Woody Plant Medium (WPM) salts [Lloyd and McCown 1981], MS vitamins [Murashige and Skoog 1962], 12.3 µM N6-[2-isopentyl]adenine (2iP), 5.37 µM 1-naphthaleneacetic acid (NAA), 0.5 $g \cdot L^{-1}$ polyvinylpyrrolidone (PVP), 0.5 $g \cdot L^{-1}$ 2-N-morpholino-ethanesulfonic acid (MES), 0.6 $g \cdot L^{-1}$ activated charcoal, 0.65 $g \cdot L^{-1}$ calcium gluconate, and 20.0 $g \cdot L^{-1}$ sucrose, and was solidified with 0.8% Difco agar. The pH of the medium was adjusted to 5.6.

Lead treatment. *In vitro* selection was conducted using the basal medium supplemented with lead (II) nitrate (Sigma). Three different concentrations were tested: 0.1 mM, 0.5 mM and 1.0 mM $Pb(NO_3)_2$. Lead nitrate was added to the medium prior to autoclaving, and medium pH was adjusted to 5.6. Plant microcuttings were explanted on the respective media, for *D. jasminea* 10 explants/flask, and for *D. tangutica* 5 explants/flask. Cultures were maintained in a growth chamber at 24°C, under 16 h photoperiod (irradiance 80 $\mu mol \cdot m^{-2}s^{-1}$). The experiment lasted eight weeks, with a four-week subculture, and was repeated three times.

Evaluation of plant growth parameters. After 8 weeks of culture obtained shoots were counted and micropropagation coefficient was calculated using the formula: MC = (number of induced adventitious shoots/total number of explants). Shoots, as well as roots (if developed), were measured and weighted. For dry matter determination plant material was dried in 105°C in the oven for 24 h and weighted afterwards. Growth Tolerance Index (in %) was calculated on the basis of dry weight of shoots, using the formula: GTI = (mean dry weight of shoots developed on media with addition of lead/mean dry weight of shoots developed on medium without addition of lead) × 100. The content of photosynthetic pigments (chlorophyll a, chlorophyll b, carotenoids) in obtained plant material was determined according to Wellburn [1994] and expressed as $mg \cdot g^{-1}$ fresh weight of sample.

Determination of Pb, essential and another trace element content. Plant samples previously dried were mineralized in 65% super pure HNO_3 (Merck) in a CEM MARS-5 microwave oven in order to determine the content of P, K, Mg, Ca, S, Na, B, Cu, Fe, Mn, Mo Zn, and Pb using the ICP–OES technique with the use of a Prodigy Teledyne Leeman Labs USA spectrometer ICP-OES. The same procedure was applied to determine the Pb content in the freshly prepared media. The shoot Bio-Concentration Factor (BCF) for lead was calculated as follows: BCF = lead concentration in shoots ($mg \cdot g^{-1}$)/lead concentration in culture medium ($mg \cdot g^{-1}$).

Statistical analyses. The experiment was conducted in three replicates, with at least 30 explants (microcuttings) per treatment within one replicate (three culture flasks for *D. jasminea*, and six culture flasks for *D. tangutica*). Biometrical data concerning the efficiency of micropropagation (length/weight of organs, micropropagation coefficient), the accumulation of pigments, and the content of lead and of other chemical elements

were subjected to ANOVA analysis (STATISTICA, StatSoft, Tulsa, OK, USA). The post-hoc Duncan's test was used to study differences between treatments at $P < 0.05$.

RESULTS

Micropropagation. Multiplication rate of ***Daphne jasminea*** shoots obtained on media containing lead nitrate were proved to be rather similar to that from medium without Pb ions (control medium). Respective values of micropropagation coefficient varied from 3.4 (control) to 2.7, however the differences between treatments were found to be statistically insignificant (tab. 1). On Pb-supplemented media shoots were shorter in comparison with the control treatment (no Pb ions), irrespectively of given $Pb(NO_3)_2$ concentration. The presence of lead nitrate in the medium negatively affected fresh weight of shoots. The lowest value, which was about 72% of the control, was noted for shoots obtained on medium with 0.5 mM (tab. 1). The content of shoot dry biomass

Table 1. Effectiveness of *Daphne* spp. micropropagation on media containing various doses of lead (II) nitrate

	Treatment	MC	Shoot length (mm)	Shoot fresh weight (g)	Shoot dry weight (% f.w.)	GTI (%)
D. jasminea	control – no $Pb(NO_3)_2$	3.4 [a*]	23.90 ±7.6 [a]	0.0678 ±0.068 [a]	15.48 ±1.5 [c]	n/a
	0.1 mM $Pb(NO_3)_2$	3.1 [a]	13.54 ±3.9 [b]	0.0544 ±0.019 [b]	17.01 ±1.7 [a]	73.7 ±2.4 [b]
	0.5 mM $Pb(NO_3)_2$	2.7 [a]	12.36 ±3.6 [b]	0.0488 ±0.016 [c]	18.13 ±1.8 [a]	72.8 ±2.7 [b]
	1.0 mM $Pb(NO_3)_2$	2.8 [a]	15.04 ±4.2 [b]	0.0531 ±0.019 [b]	16.82 ±0.8 [b]	89.5 ±9.1 [a]
D. tangutica	control – no $Pb(NO_3)_2$	3.9 [a]	49.00 ±5.3 [a]	0.260 ±0.03 [b]	14.40 ±0.2 [a]	n/a
	0.1 mM $Pb(NO_3)_2$	2.4 [c]	25.75 ±3.9 [c]	0.212 ±0.04 [c]	15.10 ±1.6 [a]	84.8 ±14.2 [b]
	0.5 mM $Pb(NO_3)_2$	3.2 [b]	36.50 ±4.7 [b]	0.275 ±0.13 [b]	14.26 ±1.1 [a]	101.6 ±12.7 [b]
	1.0 mM $Pb(NO_3)_2$	3.3 [b]	36.40 ±3.2 [b]	0.395 ±0.15 [a]	14.24 ±1.4 [a]	152.7 ±8.1 [a]

MC – micropropagation coefficient; GTI – growth tolerance index
* – values are means of three replicates, means indicated by the same letter within the columns do not significantly differ at $P < 0.05$ according to Duncan's test

significantly increased on media with Pb ions. Interestingly, growth tolerance index (GTI) was the highest on medium containing 1.0 mM lead nitrate and reached 89.5%, lower doses of the salt resulted in significant inhibition of shoot dry biomass production (to approx. GTI = 72–73%) (tab. 1). Although biomass production decreased on all tested lead nitrate concentrations in comparison to control treatments, in the course of experiment proliferative shoot cultures were established on all media containing $Pb(NO_3)_2$. Irrespective of applied medium, shoots were properly colored, with no signs of leaf chlorosis. Development of root system was observed on propagation medium

without addition of lead nitrate. Mean number of roots per microcutting accounted to 8.9 ±1.8 (data not shown). Regenerated roots were short (mean length = 8.2 mm) and had approximately 12% of dry biomass content. During the experiment, on medium containing 0.1 $Pb(NO_3)_2$ small root primordia (≤ 1 mm) developed on the shoot base. On the remaining media no macroscopic stages of rhizogenesis were observed.

The second of examined *Daphne* species, ***D. tangutica***, proliferated less vigorously on the media containing various doses of $Pb(NO_3)_2$ than on the control medium (tab. 1). Interestingly, lead nitrate in its lowest concentration tested occurred to be the most inhibitory. On medium with 0.1 mM $Pb(NO_3)_2$ micropropagation coefficient decreased to 2.4, in comparison to MC = 3.9 on control medium. Also, on the medium with 0.1 $Pb(NO_3)_2$ shoots have been the shortest (approx. 50% of shoot height in control) and had the lowest fresh weight. Higher doses of lead nitrate slightly inhibited shoot proliferation (obtained MC ranged from 3.2 to 3.3), as well as growth of shoots (approx. 73% of control shoot height) (tab. 1). Shoots obtained on tested media differed significantly in fresh matter content, and the highest amount was noted in shoots from medium with 1.0 mM $Pb(NO_3)_2$. On the other hand, the content of dry matter in shoots did not significantly differ between tested media, and amounted to 14–15% (tab. 1). Growth tolerance index (GTI) for shoots obtained on media with 0.1 and 0.5 mM $Pb(NO_3)_2$ came to 84.8–101.6%, with differences statistically insignificant. In the case of the highest concentration of $Pb(NO_3)_2$, GTI reached 152.7%, and on this medium, proliferative and Pb-tolerant shoot cultures were obtained. During the experiment *D. tangutica* shoots did not regenerated roots spontaneously on any propagation medium, both supplemented and non-supplemented with Pb ions.

Pigment analysis. ***D. jasminea*** shoots contained approximately threefold higher content of chlorophyll a, than chlorophyll b (fig. 1). The proportion of chlorophyll *a*/chlorophyll *b* content was similar in all tested medium variants and ranged from 3.4 to 3.7. Applied doses of lead nitrate did not influenced considerably the content of both types of chlorophyll and carotenoids (fig. 1). Only on medium with 0.5 mM $Pb(NO_3)_2$ the content of chlorophyll *a* and carotenoids slightly decreased. In contrast to *D. jasminea*, ***D. tangutica*** shoots contained higher amounts of photosynthetic pigments (fig. 1). The ratio of chlorophyll *a*/chlorophyll *b* was similar to the ratio in *D. jasminea* and reached 3.2–3.5, depending on medium variant. The content of both chlorophyll types, as well as carotenoids, was the highest in shoots obtained on the medium with 0.5 mM $Pb(NO_3)_2$. The most pronounced differences between applied media were detected in the chlorophyll *a* content, which significantly increased on media with low doses of $Pb(NO_3)_2$ (0.1 and 0.5 mM) (fig. 1). There was no decrease detected in the content of analyzed pigments on media containing lead nitrate in comparison with the Pb-free medium.

Accumulation of lead in cultured shoots. Accumulation of lead in the shoots after eight week culture of ***D. tangutica*** increased significantly with the increase in $Pb(NO_3)_2$ in the medium (fig. 2). Shoots accumulated up to 0.198 mg Pb per g of dry weight on medium supplemented with 1.0 mM of lead salt. On the other hand, in ***D. jasminea*** the highest accumulation of lead occurred on medium with the lowest concentration of lead nitrate, where the amount of Pb in shoots reached 0.107 $mg \cdot g^{-1}$ d. w. On the remaining media Pb accumulation in *D. jasminea* was significantly lower, not exceeding

0.06 mg·g^{-1} d. w. (fig. 2). Bioconcentration factor (BCF) was calculated to express the potential of cultured shoots for Pb extraction from the medium (the formula was described in Materials and methods section). For both tested species, the highest value of BCF was obtained on the medium containing 0.1 mM $Pb(NO_3)_2$ (fig. 3). Bioconcentration factors calculated for media with 0.5 and 1.0 mM $Pb(NO_3)_2$ were significantly lower from that from 0.1 mM medium, however did not differ from each other.

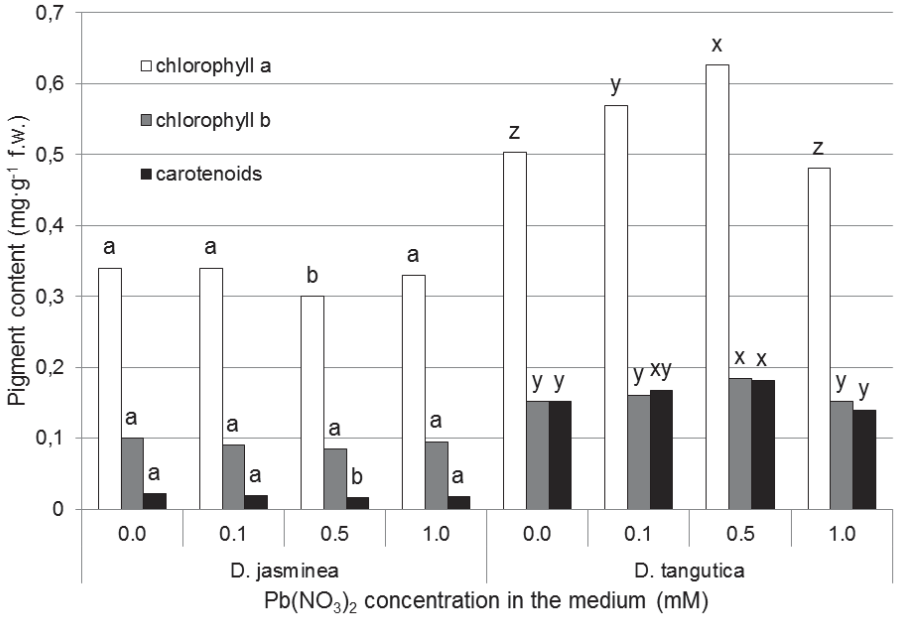

Fig. 1. The content of photosynthetic pigments: chlorophyll *a*, chlorophyll *b*, and carotenoids in shoots of two *Daphne* species cultured on media containing various doses of lead (II) nitrate. Different letters indicate means that are significantly different at $P < 0.05$ (a–b for *D. jasminea*, x–z for *D. tangutica*)

Analysis of lead concentration expressed as BCF revealed that *D. tangutica* shoots can accumulate 1.78-fold, 7.87-fold and 2.65-fold higher amounts of Pb ions than *D. jasminea* on the media containing 0.1, 0.5 and 1.0 mM $Pb(NO_3)_2$, respectively.

The influence of lead nitrate in the medium on the content of essential and trace elements in shoots. The determination of macroelements in shoot samples revealed that the presence of lead nitrate in the medium differently affected the content of macroelements in tested species. In *D. jasminea* there was no effect of applied Pb^{+2} ions on the content of calcium and magnesium, while in *D. tangutica* the content of these two elements significantly decreased in media with lead nitrate (tab. 2). Moreover, in *D. jasminea* significant decrease in phosphorus, potassium and sodium content was observed, while in *D. tangutica* there were no differences in the level of these elements (tab. 2). In both species the decrease in sulphur content was detected in media containing

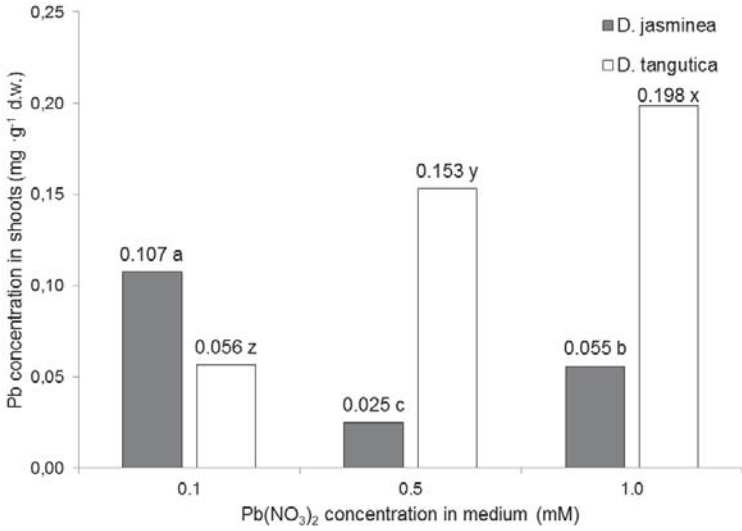

Fig. 2. Lead concentration in shoots of two *Daphne* species cultured on media containing various doses of lead (II) nitrate. Different letters indicate means that are significantly different at $P < 0.05$ (a–b for *D. jasminea*, x–y for *D. tangutica*)

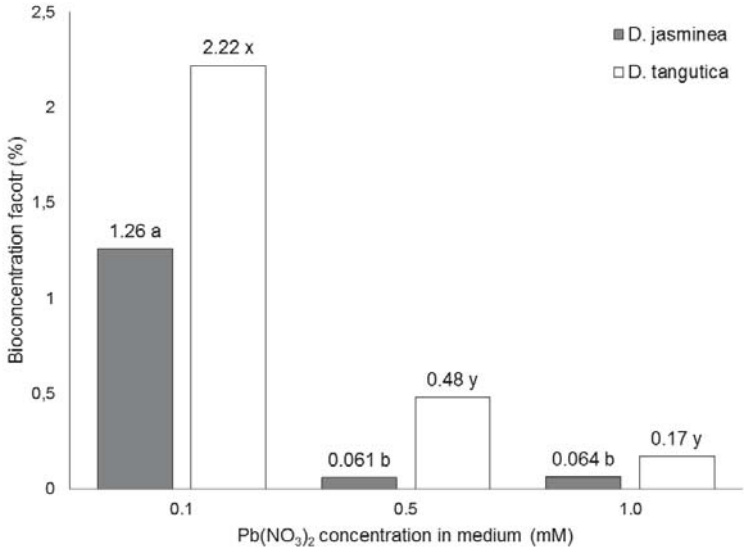

Fig. 3. Lead bioconcentration factor for two *Daphne* species cultured on media containing various doses of lead (II) nitrate. The bioconcentration factor was calculated as follows: BCF = lead concentration in shoots ($mg \cdot g^{-1}$)/lead concentration in culture medium ($mg \cdot g^{-1}$). Different letters indicate means that are significantly different at $P < 0.05$ (a–b for *D. jasminea*, x–z for *D. tangutica*)

Table 2. The content of chosen essential elements in *Daphne* spp. shoots after *in vitro* selection

Treatment		Essential elements (% d.w.)					
		Ca	P	K	S	Mg	Na
D. jasminea	no $Pb(NO_3)_2$	0.331 [a]	0.465 [a]	2.512 [a]	0.667 [a]	0.134 [a]	0.217 [b]
	0.1 mM $Pb(NO_3)_2$	0.264 [a]	0.421 [a]	2.159 [b]	0.518 [b]	0.122 [a]	0.153 [d]
	0.5 mM $Pb(NO_3)_2$	0.261 [a]	0.347 [c]	2.030 [b]	0.503 [b]	0.117 [a]	0.196 [c]
	1.0 mM $Pb(NO_3)_2$	0.330 [a]	0.285 [b]	2.212 [b]	0.576 [b]	0.116 [a]	0.250 [a]
D. tangutica	no $Pb(NO_3)_2$	0.531 [a]	0.369 [a]	2.583 [a]	0.698 [a]	0.136 [a]	0.173 [a]
	0.1 mM $Pb(NO_3)_2$	0.463 [b]	0.365 [a]	2.405 [a]	0.639 [b]	0.118 [b]	0.158 [a]
	0.5 mM $Pb(NO_3)_2$	0.335 [c]	0.314 [a]	2.413 [a]	0.595 [c]	0.110 [c]	0.178 [a]
	1.0 mM $Pb(NO_3)_2$	0.310 [c]	0.300 [a]	2.240 [a]	0.550 [c]	0.104 [c]	0.198 [a]

* – values are means of three replicates. Superscript letters within columns indicate means that are significantly different at $P < 0.05$ according to Duncan's test

Table 3. The content of trace elements in *Daphne* spp. shoots after *in vitro* selection

Treatment		Trace elements (mg $\cdot g^{-1}$ d.w.)					
		B	Cu	Fe	Mn	Mo	Zn
D. jasminea	no $Pb(NO_3)_2$	0.0909 [a]	0.008 [a]	0.193 [a]	0.124 [a]	0.002 [a]	0.085 [a]
	0.1 mM $Pb(NO_3)_2$	0.0620 [b]	0.004 [b]	0.119 [b]	0.063 [b]	0.002 [a]	0.066 [ab]
	0.5 mM $Pb(NO_3)_2$	0.0675 [b]	0.004 [b]	0.094 [b]	0.064 [b]	0.001 [b]	0.058 [b]
	1.0 mM $Pb(NO_3)_2$	0.0770 [ab]	0.001 [c]	0.089 [b]	0.064 [b]	0.001 [b]	0.062 [b]
D. tangutica	no $Pb(NO_3)_2$	0.0844 [a]	0.008 [b]	0.144 [a]	0.144 [a]	0.002 [a]	0.109 [a]
	0.1 mM $Pb(NO_3)_2$	0.0845 [a]	0.014 [a]	0.153 [a]	0.094 [b]	0.001 [b]	0.081 [b]
	0.5 mM $Pb(NO_3)_2$	0.0812 [a]	0.007 [b]	0.125 [a]	0.077 [c]	0.001 [b]	0.071 [b]
	1.0 mM $Pb(NO_3)_2$	0.0665 [b]	0.006 [b]	0.166 [a]	0.080 [c]	0.001 [b]	0.071 [b]

* – values are means of three replicates. Superscript letters within columns indicate means that are significantly different at $P < 0.05$ according to Duncan's test

$Pb(NO_3)_2$. In *D. jasminea* shoots obtained on media with lead nitrate the content of all analyzed microelements decreased significantly in comparison with the control medium (tab. 3). The effect of applied concentration of $Pb(NO_3)_2$ depended on the specific element. In *D. tangutica* the level of majority trace elements decreased, with the exception of iron and, partly, boron. The content of Fe remained constant regardless tested medium variants, while in the case of boron a significant decrease occurred only in medium with the highest amount of Pb^{+2} ions (tab. 3).

DISCUSSION

Numerous publications reported on the toxic effect of lead on numerous plant species [Alkorta et al. 2004, Sharma and Dubey 2005, Zhera et al. 2009]. The non-toxic lead concentration for most vascular plants is usually lower than 0.01 $mg \cdot g^{-1}$, however in tolerant material the amount can be considerably elevated, and species capable to accumulate above-average level of heavy metals are useful for biological soil reclamation [Ali et al. 2003, Trigueros et al. 2012]. For both studied *Daphne* species the highest bioconcentration factor (BCF) has been found on the medium containing the lowest dose of lead ions, that is 0.1 mM $Pb(NO_3)_2$, whereas BCF calculated for other media were significantly lower. It can be stated that the effectiveness of lead concentration in tissues was the highest in the least contaminated medium, and decreased with the increasing level of toxic ions. Obtained values should be considered low, since in callus cultures of tolerant poplar clones bioconcentration factor exceeded 15 [Iori et al. 2012], in contrast to max. 2.2 obtained in our experiment. On the other hand, the physiology of callus tissue differs considerably from the entire shoots. For example, ion uptake by callus clusters is not affected by the presence of epidermis, endodermis or waxes [Doran 2009], so accumulative properties of callus may be significantly higher than of more complex plant material. Although the lead accumulation was considerably low, in both *D. jasminea* and *D. tangutica* growth tolerance index was the highest on medium containing the highest tested dose of lead nitrate. What is more, irrespective of applied medium, *Daphne* shoots were viable, with neither chlorotic, nor necrotic spots on leaves. In the course of selection experiment healthy looking, proliferative shoot cultures were established on all media containing $Pb(NO_3)_2$. This in turn suggests potential of tolerance in these genotypes, taking into account fact, that inorganic salts of lead, among them lead nitrates, can induce the aberration of mitosis what frequently result in strong inhibition of growth and development, manifested on the whole plant level [Patra et al. 2004, Sytar et al. 2013]. Furthermore, the *D. jasminea* multiplication coefficients obtained on all tested media, both without lead ions and with their various amounts, were similar to each other, suggesting that developmental competencies in this genotype were not inhibited by lead.

The plant material appropriate for phytoremediation should have ability to produce a large biomass in stress conditions [Di Lonardo et al. 2011]. In our experiment the fresh biomass production in the presence of lead was inhibited in *D. jasminea*, while in *D. tangutica* slight decrease occurred only on the medium containing intermediate amount of lead nitrate. The decrease in fresh weight of plant tissues is a common reaction in the presence of lead, probably due to decrease in water potential in cells resulted from osmotic stress [Sharma and Dubey 2005]. In contrast, dry biomass production was not affected by lead in *D. tangutica*, and even increased in *D. jasminea*. Moreover, dry matter content in plant organisms strongly depends on nutrient uptake, since it may be concluded that in tested concentrations lead nitrate had no toxic effect on the level of shoot nutrition. Detected lower levels of essential and trace elements were probably still high enough to maintain undisturbed growth and development of cultured shoots. These results are interesting, since in numerous cases plant biomass production is negatively affected by heavy metal ions [Fernandez et al. 2008, Ghnaja et al. 2010, Čabala et al.

2011]. In *Calendula officinalis* selected for cadmium tolerance the increase in dry biomass was also noted and due to such contrasting reaction the plant was judged as being worthy of further studies concerning phytoremediation [Liu et al. 2010]. Our results may indicate that in both *Daphne* species some, yet unknown, defense mechanisms against water/osmotic stress may be active under lead stress.

The another aspect of applied *in vitro* selection is limited spontaneous rooting of *D. jasminea* in the presence of lead. It is interesting, since this species is quite easily rooting plant [Wiszniewska et al. 2013]. As we have found during supplementary observations, rhizogenesis on media containing lead nitrate was delayed in comparison with control medium (unpublished data). It is known that lead uptake via root system is much more effective than via shoot base [Patra et al. 2004]. To test the effect of lead on *D. jasminea* microplantlets (shoots and roots) selection time should be elongated (unpublished data). It is possible that in the presence of fully expanded root system lead accumulation would be higher. There is also a need to verify if in the presence of roots toxic effects of lead on shoot growth and development would be manifested. For *D. tangutica*, which is difficult-to-root species, the rooting protocol has been recently elaborated [Wiszniewska et al. 2013], so similar observations could be also made in the future.

There are evidences that in plants Pb ions negatively influence the uptake of P, K, Ca, Cu, Fe, Mn and Zn [Kabata-Pendias and Mukherjee 2007, Kabata-Pendias 2011, Ahmad et al. 2013]. The determination of chemical composition of sampled material revealed that the lead concentration diversely affected the content of chemical elements in *Daphne* shoots. In both species, an antagonistic activity of Pb was confirmed in relation to manganese and zinc. The influence of lead ions on the content of remaining elements seems to be species-dependent. In *D. tangutica* antagonistic action of Pb was observed also against Ca and Mg uptake/content, while a contrary effect was found for Cu. The presence of lead ions did not influence P and K content. Although the level of majority elements in *D. jasminea* decreased in the presence of Pb ions, for Ca and Mg no antagonistic effect occurred. Obtained results are probably due to unique species-dependent reaction on Pb^{2+} in the culture medium. Mineral status of a plant may be related to its tolerance to lead. It is postulated that Pb-tolerant species achieve considerably high status of calcium content and develop a characteristic metabolism of this element [Antosiewicz 1995]. Trigueros et al. [2012] also report on occurring interdependence between the level of lead tolerance and accompanied tolerance to calcium deficit. Additionally, calcium acts as a secondary messenger in plant response to most external stimuli [Liu et al. 2010]. Constant calcium level in cultured shoots of *D. jasminea* may be another premise of potential tolerance of this species to lead contamination.

In both studied species the decrease in sulphur content was detected in media containing $Pb(NO_3)_2$. Most likely it is a result of lead toxicity mechanism, deriving from lead ability to bind strongly to sulphur atoms [Schützendübel and Polle 2002]. This contributes to heavy metal-induced reduction in glutathione content and activity of glutathione reductase, and consequently affects antioxidative response of a plant. Reduction in sulphur content observed in *Daphne* shoots may indicate on inhibitory action of lead against antioxidant machinery.

Analysis of photosynthetic pigments was aimed at the estimation of shoot physiological condition. It revealed that in *D. jasminea* the content of chlorophyll was not

affected by lead nitrate, and in *D. tangutica* an unexpected increase of chlorophyll content occurred. It is especially interesting, because while the chlorophyll content was increasing in shoots cultured on 0.1 and 0.5 mM $Pb(NO_3)$, the content of Mg decreased with increasing concentration of lead. It seems that during selection low doses of Pb stimulated chlorophyll synthesis regardless an unbalanced uptake of essential elements. Similar tendency was observed by Liu et al. [2010] in wheat growing under lead stress. Chlorophyll synthesis was there stimulated by both low and high concentrations of Pb during 21 days. Such phenomenon may indicate the involvement of processes leading to the plant accommodation to the growth in a contaminated environment [Liu et al. 2010]. The content of carotenoids was only slightly affected by the lead treatment. The application of 0.5 mM $Pb(NO_3)$ caused a decrease in carotenoids content in *D. jasminea*, and the increase in *D. tangutica*. In other plants treated with lead the synthesis of carotenoids also diminished [Bibi and Hussain 2005]. Its acceleration may be related to protective role of carotenoids against antioxidative stress and Pb accumulation in thylakoid membranes [Kastori et al. 1998]. Observed species-dependent reactions in *Daphne* shoot cultures still need to be clarified.

It is worthwhile to point at the potential usefulness of Pb-tolerant *Daphne jasminea* clones as appropriate plant material to be planted in urban areas. This species is heat resistant, can be exploited in elimination of secondary dusting due to its decumbent habit and has decidedly ornamental character. Presented reproducible selection protocol together with subsequent rooting protocol would allow future utilization of this plant in polluted urban environment. Moreover, shoots of both studied *Daphne* species displayed some interesting physiological features when selected in the presence of elevated lead concentration. Thus apart from practical approach and production of Pb-tolerant plantlets, an insight into defense mechanisms, especially against osmotic and oxidative stress, is an important task to be held in the future.

CONCLUSIONS

1. In tested concentrations $Pb(NO_3)_2$ had no toxic effect on the growth and multiplication of studied *Daphne* shoots, as well as on pigment content and the level of shoot nutrition.

2. *D. tangutica* shoots proved to accumulate higher amounts of lead than *D. jasminea*. Moreover, several tested parameters indicated on certain potential of *D. tangutica* to tolerate elevated concentrations of lead ions.

3. The study confirmed the suitability of *in vitro* selection for obtaining of vigorous, proliferative and tolerant to elevated lead concentration shoots of two *Daphne* genotypes.

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SELEKCJA *in vitro* PĘDÓW WAWRZYNKA W KIERUNKU TOLERANCJI NA OŁÓW

Streszczenie. W kulturze *in vitro* można stworzyć odpowiednie środowisko do selekcji tolerancyjnych względem podwyższonych stężeń metali ciężkich roślin drzewiastych, mogących znaleźć zastosowanie w remediacji gleby. W takich warunkach mnożono pędy wawrzynków *Daphne jasminea* Sibth. & Sm. i *Daphne tangutica* Maxim. (Thymelaeaceae), na pożywkach zawierających 0,1, 0,5 lub 1,0 mM azotanu ołowiu. Oceniano poziom bioakumulacji ołowiu, parametry wzrostu, zawartość barwników fotosyntetycznych oraz stan odżywienia mineralnego pędów. Na pożywkach zawierających jony ołowiu zanotowano intensywny wzrost gatunku *D. jasminea*, którego pędy charakteryzowały się dużą witalnością, a współczynnik tolerancji względem ołowiu wyniósł 73–89%, największą wartość osiągając na pożywce z dodatkiem 1,0 mM azotanu ołowiu. Obecność jonów ołowiu w pożywce ograniczyła namnażanie pędów *D. tangutica*, jednakże współczynnik tolerancji na pożywce zawierającej 1,0 mM $Pb(NO_3)_2$ osiągnął 152%. Dla obydwu badanych gatunków największy współczynnik bioakumulacji stwierdzono na pożywce z dodatkiem 0.1 mM azotanu ołowiu. Pędy *D. tangutica* akumulowały ponad dwukrotnie więcej ołowiu w porównaniu z pędami *D. jasminea*. Zawartość chlorofilu w pędach *D. jasminea* nie zmieniała się znacząco pod wpływem jonów ołowiu, natomiast w kulturach *D. tangutica* stwierdzono stymulację syntezy chlorofilu i karotenoidów na pożywkach zawierających Pb^{2+}. Nie obserwowano toksycznego wpływu jonów ołowiu na poziom odżywienia mineralnego pędów. Jest to pierwsza praca dotycząca perspektywicznych gatunków ozdobnych z rodzaju wawrzynek, przedstawiająca uzyskanie żywotnych, proliferujących kultur *in vitro* pędów, które wykazują tolerancję na podwyższoną zawartość jonów ołowiu w pożywce.

Słowa kluczowe: bioakumulacja, kultura *in vitro*, metale ciężkie, Thymelaeaceae, żywienie mineralne

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