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Response of the callus cells of fir (*Abies nordmanniana*) to *in vitro* heavy metal stress

Katarzyna Nawrot-Chorabik

University of Agriculture in Krakow, Faculty of Forestry, Department of Forest Pathology, Mycology and Tree Physiology, al. 29-Listopada 46, 31-425 Kraków, Poland, fax: +48 12 6625152, e-mail: rlnawrot@cyf-kr.edu.pl

Abstract

The aim of the presented research was to investigate the effect of three heavy metals – lead, cadmium and copper – on the callus cells of *Abies nordmanniana*. The toxicity degree and toxicity effect of the selected heavy metals was determined on the embryonic level. On the basis of the spectrometric analyses as well as macroscopic and microscopic observations, this research referred to the accumulation of heavy metals in tissues, assuming that this mechanism is related to the acquisition of tolerance by cells exposed to this type of abiotic stress. Moreover, the effect of the genotype of fir on the cell defence, that is, the induction of tolerance, was analysed. Understanding of the issues related to the heavy metal resistance of plant genotypes in future may contribute to the selection of genotypes of individuals that are more resistant to stress factors, particularly in the multi-directional and rational forest management. The results showed that lead (20 mg l^{-1}), which proved to be the most toxic amongst the three examined heavy metals, has the most severe negative effects on the tissue of fir trees. Copper (20 mg l^{-1}) was accumulated for a long time in the cells of fir trees, and it was not degraded or excreted outside the tissues even after three weeks of *in vitro* culture. Of the three tested genotypes, G14 had the greatest tendency to accumulate each of the examined metals, that is, it appeared to be the least tolerant genotype.

Key words

Abies, abiotic stress, callus, embryogenic suspension culture (ESC), metals, somatic embryogenesis

Introduction

Environmental pollution with heavy metals has increased greatly because of the rapid worldwide industrial development. Studies carried out on this subject include the effect of metals on humans, animals, and plants. Analyses of the effect of heavy metals on plants, including forest trees, were conducted by Lovrencic et al. (2008), Szymura (2009) and Zacchini et al. (2009) and the problem was discussed by Maksymiec (2007). Some studies also include the research on callus tissues carried out on the cellular level. Those studies allowed to understand the *in vitro* effect of various heavy metals on plant tissues including cell tolerance and the possibility of further cell growth (Israr et al. 2006; Nehnevajova et al. 2007; Iori et al. 2012). This issue was investigated in details in the presented study, which analysed the accumulation of copper, lead and cadmium in the cells of three genotypes of embryogenic callus of *Abies nordmanniana* (Steven) Spach. The *in vitro* culture and

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spectrometry allowed to solve three problems: the toxicity effect of the examined heavy metals on fir tissues, induction of stress tolerance in different genotypes and the effect of the time of metal accumulation on further organogenetic growth and development.

Caucasian fir is one of the most important species grown mostly in Christmas tree plantations, but its *in vitro* cultured embryogenic callus has become, because of this work, a point of reference for studies on the effect of heavy metals on gymnosperm trees, mainly from the genus *Abies*. The analyses included three heavy metals from two different groups, that is, metals necessary for proper growth and development of trees (Cu) and toxic metals (Cd and Pb). It needs to be pointed that only some heavy metals (i.e. those whose specific gravity of 1 cm^3 is lower than 5 g) present in the environment are necessary for proper functioning of plant cells. These metals include copper (Cu), iron (Fe), zinc (Zn), manganese (Mn), cobalt (Co) and nickel (Ni). The remaining heavy metals such as lead (Pb), cadmium (Cd), mercury (Hg) and aluminium (Al) are not used in cellular metabolism and can be toxic for plants even in very small quantities (Page et al. 2006).

Despite many years of studies on the reactions of plant cells to the impact of heavy metals, still several issues remain unexplained, mainly due to the fact that so far there is no detailed study on the biochemical changes caused by heavy metals in plant cells. There are both extensively studied issues, such as metal transport through membranes, heavy metal detoxification, oxidative stress and its neutralisation and those for which the available literature is scarce, such as the impact of metals on nucleic acids (Nowakowska and Oliver 2013; Woźny and Przybył 2004; Williams et al. 2000).

The presented study aimed to explain the plant cell response to the accumulation of copper, lead and cadmium in plant tissues on the assumption that this mechanism is related to the acquisition of tolerance by cells exposed to abiotic stress. It is important that this tolerance is largely dependent on the plant genotype, as shown by spectrometric analyses and research on *in vitro* cultures, which represent a good model system for studies on plant response to stress factors.

Material and methods

Plant material

Mature open pollinated seeds of Caucasian fir (*A. nordmanniana* (Steven) Spach) acquired in the seed year 2009 were derived from Vallø region on the east coast of the island of Zealand in eastern Denmark (E: 55º24'; N: 12º15'), from seed plantations marked with numbers 13 and 19. Seeds of *A. nordmanniana* were subjected to a two-stage, 24-h disinfection process with the use of ethyl alcohol (30 s), 10% sodium hypochlorite (NaOCl) for 15 min, thorough rinsing in deionised water and polyvinylpyrrolidone water solution (PVP) (Sigma-Aldrich) for 24 h at 7 ± 1°C, before again rinsing in five changes of sterile deionised water in laminar flow cabinet (Nawrot-Chorabik 2016)

Embryogenic callus (ESC, embryogenic suspension culture) was initiated by somatic embryogenesis on zygotic embryos isolated from mature seeds of Caucasian fir.

The analyses of the impact of heavy metal were conducted on three selected genotypes of embryogenic callus (cell lines) characterised by a high proliferation rate, which were marked as G2, G14 and G21. About 300 mg of callus tissue of each genotype was placed in triplicate on Petri dishes with modified solid Schenk and Hildebrandt (SH) medium (Schenk and Hildebrandt 1972) containing 3.2 mM benzyladenine, 4.6 mM kinetin, 4.5 mM 2,4-dichlorophenoxyacetic acid and 2.0% sucrose. Heavy metals were added to the medium, composed as described above, and the final concentration of each heavy metal was 20 mg l^{-1}. Copper was used in the form of $CuSO_4 \times 5H_2O$, lead in the form of the standard solution of $Pb(NO_3)_2$ in 2% HNO_3 and cadmium in the form of the standard solution of $Cd(NO_3)_2$ in 2% HNO_3. The standard solutions were purchased from ULTRA Scientific. Heavy metals were not added into the media in control combinations. pH of the medium was set at 5.7. All reagents used for *in vitro* culture and spectrometric analyses were used from Sigma-Aldrich company.

The *in vitro* culture was conducted in the dark in a phytotron chamber at 24°C and 50% humidity.

Spectrometric analysis

After one, two and three weeks of culture, the calluses of Caucasian fir were collected from the medium and covered with 10 cm^3 of concentrated nitric acid (HNO_3).

The obtained partially digested suspension–solution was digested for 60 min in Microvela mineraliser in a microwave field until complete digestion. The wet-digested solution was transferred to a 50-cm^3 volumetric flask, and the content of individual heavy metals in subsequent samples was determined in the inductively coupled argon plasma emission spectrometer iCAP 6000 series (Thermo Scientific, MA, USA). The control callus tissue (cultured *in vitro* on a medium without heavy metals) was also subjected to spectrometric analysis. The measurements were carried out by using a two-dimensional CID (charge injection semiconductor detector), which allowed for carrying out emission measurements of all elements present in the samples simultaneously with the synchronous recording of all necessary emission lines.

Statistical analysis

Statistical analysis of data was conducted using two tests: the Tukey multiple comparison test preceded by the F-test – one-way analysis of variance (ANOVA) – as well as the Tukey multiple comparison test preceded by the Friedman test for repeated measure systems (Tab. 1–3). Because significant results of the F-test (analysis of variance) do not inform us between which of the examined metals there are differences (the test shows only which groups differ from each other); therefore, further multiple 'post-hoc' comparisons were conducted in order to determine between which metals there are statistically significant differences at the 0.05 significance level. These tests allowed to draw the objective conclusions from the resulting numerical data. All statistical analyses were conducted using STATISTICA version 7.1 (StatSoft Inc., Tulsa, OK, USA).

Results

Answers to the following questions were obtained: which of the selected heavy metals (Cu, Pb and Cd) is the most harmful to the cells of fir, whether there is the effect of the plant tissue genotype on the tolerance level to stress caused by heavy metals and whether the time of metal accumulation in plant cells (one, two and three weeks) induces their tolerance to this stress factor.

The results of this research indicated that lead has the strongest negative effects on the tissue of fir. The concentration of lead in cells is greater than that of the remaining metals (copper and cadmium). The difference between the mean content of copper and cadmium is statistically insignificant, but it is statistically significant in the case of lead (Tab. 1).

Table 1. Statistical analysis of data – multiple comparisons test (Tukey) preceded by the F-test of one-way ANOVA

Metal	Average metal content	Result of ANOVA test	Probability p in multiple comparisons test		
			Copper (Cu)	Cadmium (Cd)	Lead (Pb)
Copper (Cu)	0.123146	F = 5.574 p = 0.0055*		0.9976	0.0125*
Cadmium (Cd)	0.124294		0.9976		0.01506*
Lead (Pb)	0.173593		0.0125*	0.01506*	

* Statistically significant difference at the level of $\alpha = 0.05$.

On the basis of the results of spectrometric analyses, the statistical analysis was performed for each of the elements separately and showed that the genotypes 2 and 21 are statistically different from the genotype 14. This genotype had the greatest tendency to accumulation of each of the metals, which means that it is the weakest genotype. Genotypes 2 and 21 were resistant to all tested metals (Tab. 2). The values of accumulation in their cells had a downward trend (Fig. 1).

Table 2. The effect of the genotype of *A. nordmanniana* callus on the accumulation of copper, lead and cadmium confirmed by the statistical analysis of data

	Genotype	Average metal content	Friedman's test result	Probability p in multiple comparisons test		
				G2	G14	G21
1	2	3	4	5	6	7
Copper (Cu)	G2	0.1116	$X^2 = 12.667$ p = 0.0018*		0.0053*	0.4003
	G14	0.16537		0.0053*		0.0005*
	G21	0.09240		0.4003	0.0005*	

1	2	3	4	5	6	7
Cadmium (Cd)	G2	0.10920	$X^2 = 13.556$ $p = 0.0011$		0.0007*	0.9688
	G14	0.15689		0.0007*		0.0005*
	G21	0.10680		0.9688	0.0005*	
Lead (Pb)	G2	0.15825	$X^2 = 12.667$ $p = 0.0018$*		0.0176*	0.2719
	G14	0.25341		0.0176*		0.0008*
	G21	0.10912		0.2719	0.0008*	

* Statistically significant difference at the level of α = 0.05.

It was shown that only copper ($CuSO_4 \times 5H_2O$) showed an increase in the accumulation from one to three weeks. Statistical significance of differences between week 1 and 3 was proved by the Tukey test (Tab. 3). The presence of other examined heavy metals induced tolerance to this stress factor.

Table 3. The effect of time of accumulation of copper, lead and cadmium on the tolerance induction in the cells of *A. nordmanniana* confirmed by the statistical analysis of data

	Time (week)	Average metal content	Friedman's test result	Probability p in multiple comparisons test		
				week 1	week 2	week 3
copper (Cu)	1	0.09684	$X^2 = 9.556$ $p = 0.0084$*		0.1732	0.0094*
	2	0.12496		0.1732		0.3059
	3	0.14764		0.0094*	0.3059	
Cadmium (Cd)	1	0.10791	$X^2 = 9.556$ $p = 0.0622$	No statistically significant differences		
	2	0.12790				
	3	0.13707				
Lead (Pb)	1	0.14888	$X^2 = 0.667$ $p = 0.7165$	No statistically significant differences		
	2	0.17217				
	3	0.19973				

* Statistically significant difference at the level of α = 0.05.

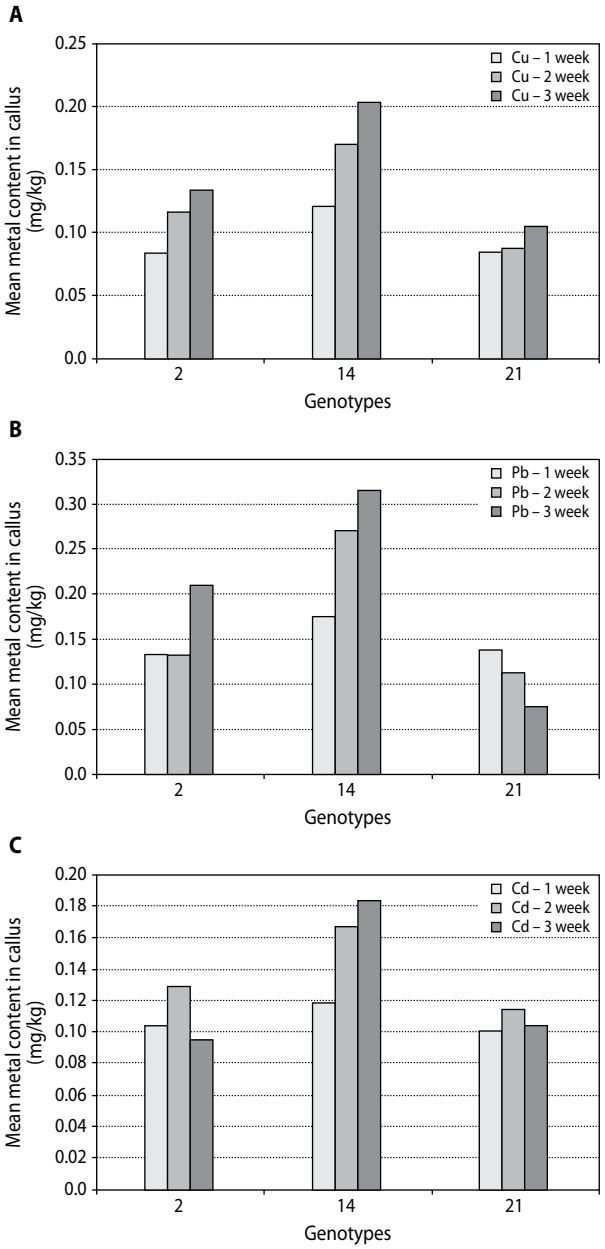

Figure 1. Concentration of copper, cadmium and lead in the cells of three genotypes (G2, G14 and G21) of *A. nordmanniana* callus within three weeks of *in vitro* culture

In conclusion, the results of spectrophotometric analysis and statistical analysis of data indicated the effect of heavy metals – copper, cadmium and lead – which is reflected in the morphological changes of the callus tissue. The final effect was evidenced by the transformations in the phenotype of embryogenic callus mass as a wound tissue, expressed as the gradual die-

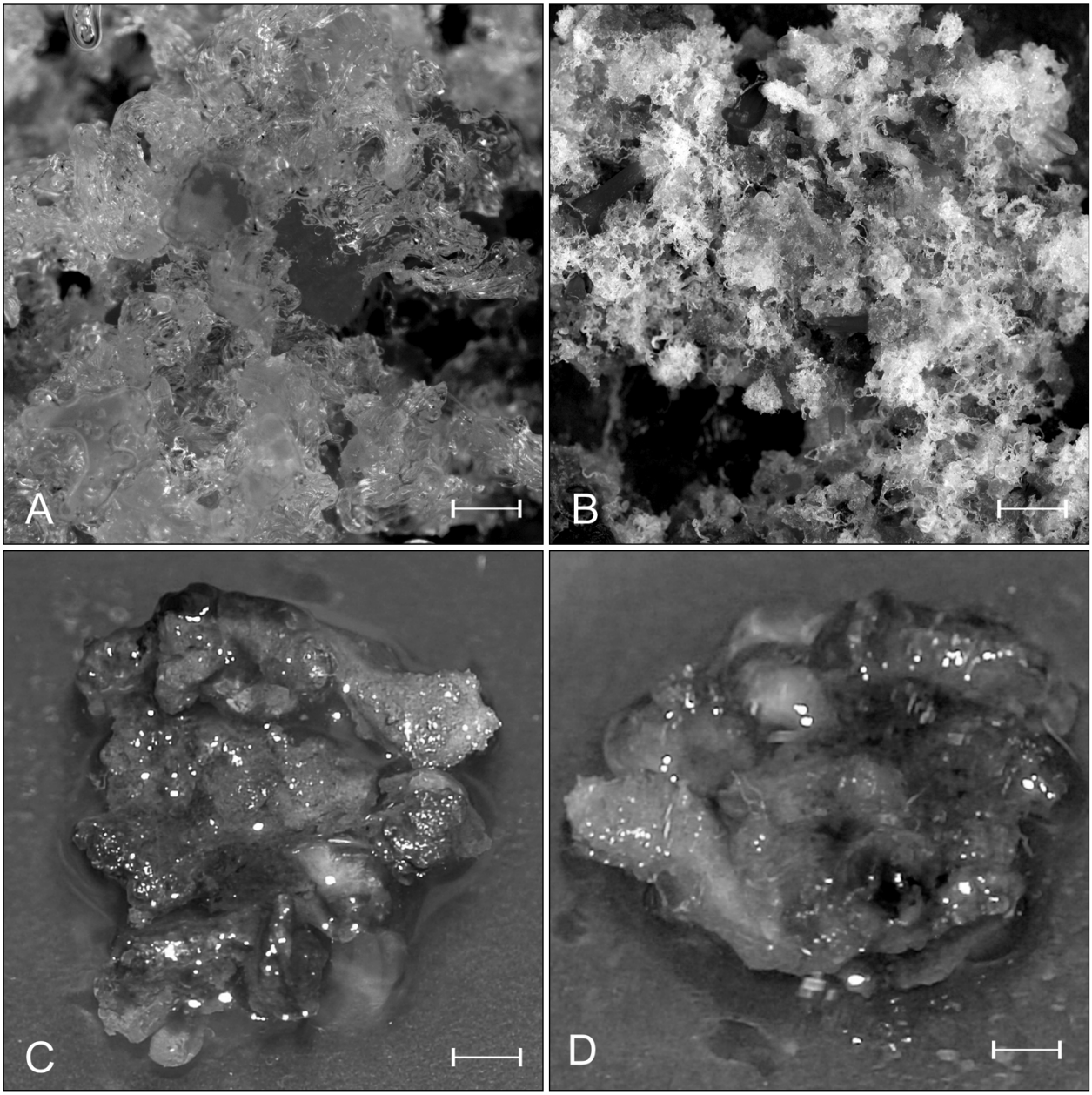

Figure 2. Organogenetic changes in the callus of *Abies nordmanniana* caused by heavy metals after three weeks of *in vitro* culture on SH medium: A – control embryogenic callus (heavy metal-free medium); B – callus with somatic embryos on medium containing 20 mg l^{-1} copper; C – darkening callus with necrotic changes, not producing embryos on medium containing 20 mg l^{-1} cadmium; D – dead, dehydrated callus with characteristic necrotic changes on medium containing 20 mg l^{-1} lead; bars indicate 5.0 mm

back of its cells. These changes resulted from negative, organogenetic transformations of cells, whose metabolism was disturbed by the introduction of heavy metals into the growth medium (Fig. 2). On the same type of medium in the case of cadmium (Fig. 2C) and lead (Fig. 2D), the tissue was dehydrated, was compact and did not undergo further morphogenesis. There were no green fragments, that is, those containing chlorophyll, which indicated a deficit of new cells, whilst in the existing cells, the synthesis of this pigment was inhibited.

After a few days, visible necroses and leakage of mucilaginous substance were recorded, which caused the death of the callus (Fig. 1C, D). Only in the case of copper (Fig. 2B), the necroses were fragmentary and only a small percentage of somatic embryos died. Although the callus tissue was slightly dehydrated compared to the control callus, it remained alive (Fig. 2A).

Discussion

This study presents the results and conclusions from three areas of research on heavy metals on the embryonic level. The numerical data obtained from the spectrometric analysis, illustrated in the graphs, showed the relationships between different metals and genotypes (Fig. 2). Macroscopic and microscopic observations of phenotypic changes in the callus tissue of *A. nordmanniana* and finally the statistical analysis of the obtained data confirmed the final conclusion: lead (20 mg l^{-1}) has the most toxic effects on the tissue of fir (Fig. 2D; Tab. 1–3). The results obtained in this study indicate that lead proved to have the strongest negative impact on the tissue of fir. Its concentration in plant cells exceeded the concentration of the other tested heavy metals (copper and cadmium). What is more, the difference between the mean content of copper and cadmium is statistically insignificant, whilst it is statistically significant between lead and the remaining metals (Tab. 1). Maksymiec (2007) emphasises that the effect of toxic lead influence on plants is strong and fast inhibition of growth processes of the above- and underground parts, as well as the decrease in the activity of the photosynthetic apparatus, often correlated with progressing senescence processes. The *in vitro* studies on the effect of lead were conducted by numerous authors, amongst others, Agrawal and Sharma (2006) on the shoots of *Holarrhena antidysenterica* flowering plant of the genus *Wrightia* based on the concomitant changes in protein. They concluded that the rate of inhibition in morphogenesis was in the order of Cd > Pb > Cu > Zn. Lead, being the second most inhibitive metal, was responsible for phytotoxic effects of *H. antidysenterica* on *in vitro* regeneration. As shown in the literature, lead deposits occupy a significant part of vacuoles and, in extreme cases, fill it almost entirely. Lead causes disturbances in cellular metabolism, which is manifested in the form of intensive vacuolisation, concentric endoplasmic reticulum, lobed nucleus and numerous multivesicular bodies present in the cell (Woźny and Przybył 2004). The presented studies on this metal emphasise the significant role of lead in the cellular morphogenesis based on the example of callus of *A. nordmanniana*. Callus treated with lead died after 30 days, which was particularly evident in the genotype 14. It was shown that the addition of 20 mg l^{-1} of this metal to the medium was toxic to cells of fir. Strong necrotic changes, rapid dehydration of cells and no induction of somatic embryos on the callus were observed microscopically (Fig. 2D).

Toxic effects of metal ions can be observed not only in the ultrastructure of cells but also on a molecular level, that is, in the expression or inactivation of certain genes. Changes in the DNA have further consequences, such as robust transcription or biosynthesis of some proteins, and cause induced imbalances in activated oxygen metabolism as well as antioxidant enzymes (Schröder et al. 2003). Analysing of those mechanisms is legitimate but not always necessary to obtain answers to some questions.

In *Brassica juncea*, studies were conducted on the accumulation of lead, cadmium and zinc in *in vitro* cultures, with regard to somaclonal variation. In this research, the emphasis was put on shoot regeneration with callus. It appeared that whilst more than 90% of hypocotyls formed callus on the control medium, only 50% of all hypocotyl explants formed callus in the presence of 40 μM Cd. The best regenerants were chosen under hydroponic conditions, which, after selection, could be used for the purposes of phytoremediation (Nehnevajova et al. 2007).

This shows that research on heavy metals is multilevel and multi-directional; however, there are few studies conducted on trees in which the effect of genotype on the tolerance to heavy metals was verified. Therefore, this problem was addressed in the presented study, and it has become undoubtedly one of the most important aspects of this research. Solution to the issue related to the genotypes resistant to heavy metals in future could contribute to the selection of genotypes of individuals more or less resistant to stress factors, especially with the assumption of multi-directional and rational forest management.

The statistical analysis, based on the results of spectrometric measurements, indicated that the genotypes 2 and 21 are statistically different from the genotype 14.

This genotype accumulated the greatest amounts of each of the analysed metals, indicating that this is the weakest genotype. In contrast, genotypes 2 and 21 were resistant to all of the examined metals (Tab. 2). The values of accumulation in their cells had a downward trend (Fig. 1). Probably those genotypes formed defence strategies against stress factors allowing either to avoid stress or to tolerate it. The first strategy involves the development and launching the cell mechanisms that prevent or hinder the metal ion transgression of a barrier formed by the cellular membrane, that is, to prevent their entry into the protoplast. The second – and more likely – strategy is based on the development and launching of mechanisms inside the cell that neutralise the metal ions and the corrective mechanisms that remove the damage caused by the heavy metals. Therefore, the second strategy allows for the growth and development of a cell after the penetration of metal into the protoplast, which was noticed in the case of the genotypes 2 and 21 (Fig. 2).

Another aspect addressed in this study concerned the time of accumulation of copper, lead and cadmium in the cells of *A. nordmanniana*. It was demonstrated that amongst the tested metals, only the accumulation of copper increased from one to three weeks. The Tukey test confirmed the statistical significance of differences in the concentrations between week 1 and 3 (Tab. 3). The tolerance to this stressor was generated by the presence of other examined heavy metals. Copper is treated as an essential microelement for living organisms, and it is a relatively low toxic metal. Unlike other heavy metals, it is accumulated in chloroplasts and vesicles of various origin (endoplasmic reticulum (ER) and Golgi apparatus (GA)). This metal, together with iron, is one of the elements that participate in the redox processes. Spectrometric analyses indicate that too high concentrations of copper (20 mg l^{-1}) can cause negative effects in plant cells. This can possibly result from the generation of a large excess of reactive oxygen species (ROS) and free radicals (FR), which ultimately leads to oxidative stress in a cell, that is, a situation in which more ROS and FR are produced than metabolised. Copper in low concentrations is well metabolised by plants and its high concentrations result in long-term accumulation of this element in plant tissues and thus the inhibition of growth and development and adverse changes in organogenetic metabolism. The reason for long-term accumulation of copper can be either the mentioned oxidative stress or the fact that copper is transported not to vacuoles but to ER and GA, and the mechanism of disposal of copper from the cells has not been fully elucidated yet. This may also be caused by the transport proteins, but this hypothesis requires further advanced research leading to the identification of such proteins. Similar to the presented study, Agrawal and Sharma (2006), in their studies conducted on medicinal herbaceous species *H. antidysenterica*, analysed the toxicity effect of different metals, including copper. The conclusions were drawn based on the protein analysis carried out by sodium dodecyl sulphate polyacrylamide gel electrophoresis. These authors showed that two new peptides were synthesised (29 and 20 kDA) in response to both copper and zinc. In their research, low concentration of copper (1 mg l^{-1}) resulted in good organogenesis in shoots of *H. antidysenterica*. On the other hand, in higher concentrations of copper, the induction of multiple shoots buds was successful but the growth was limited. Whilst zinc *in vivo* was the only metal with concentrations significantly higher in the leaves than that in the root and stem (Milić et al. 2012). Patterson III and Olson (1983) studied the effect of heavy metals on variety of substrates. Copper, nickel and cobalt solutions were added to a filter paper, mineral soil and organic soil substrates to test the effects of these metals on the germination and radicle elongation of woody species to eastern North America. For all species, toxicity followed the pattern Ni > Cu > Co for filter paper and Ni > Co > Cu for mineral and organic soils. Deciduous species were more readily damaged by these metals than the coniferous species. This study carried out on the medium as a substrate broaden the knowledge of, amongst others, copper and cadmium in the species of gymnosperms. It turns out that these metals more adversely affect the tissue of trees.

To summarise, it needs to be emphasised that *in vitro* culture is considered to be a valuable tool in micropropagation of trees (Pospíšilová et al. 1999; Nawrot-Chorabik 2008), genetic studies of, for example, somaclonal variation (Nawrot-Chorabik 2009) and cryopreservation (Charne et al. 1988), as well as in biotechnology in, for example, studies on pathogenicity on embryonic level in dual cultures (Nawrot-Chorabik 2014; Nawrot-Chorabik et al. 2016; Sieber et al. 1990; Vookova et al. 2006). Moreover, as evidenced by the presented paper,

the *in vitro* culture can also be used in the selection of plant material being tolerant to stress factors, such as toxic concentration of heavy metals. The latter application is significant especially in the case of woody species characterised by long reproductive cycles.

Conclusion

The toxicity degree and toxicity effect of the selected heavy metals were determined on the embryonic level – lead (20 mg l^{-1}) has the most toxic effects on the fir tissue.

There are genotypes of trees that are resistant to the effects of heavy metals, and there are also those genotypes that do not show any resistance.

The *in vitro* culture can be used in the selection of plant material being tolerant to stress factors, such as toxic concentration of heavy metals.

It should be cultured *in vitro* the callus genotypes that are resistant to heavy metals in future that could contribute to the selection of genotypes of individuals more or less resistant to stress factors, especially with the assumption of multi-directional and rational forest management.

The results of these studies can be useful to solve the problems of tolerance to stress, the effect of heavy metals on plants at the cellular level, a point genotype plants in the immune response, remediation and so on.

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