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In vitro Evaluation of Copper Tolerance and Accumulation in *Populus nigra*

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Abstract: Phytoextraction is an efficient and cheap way to extract copper from soils in riparian zones. In this work five genotypes of the endangered tree species *Populus nigra* L. were tested for their copper tolerance and accumulation *in vitro* when cultivated on media with three Cu concentrations: 10^{-3}, 10^{-4} and 10^{-7} M (buffered with citric acid/Na-citrate buffer, pH 3 before sterilization). After five-weeks cultivation of rooted shoots, the highest increases in morphological and biomass parameters were observed at 10^{-7} M Cu^{2+}. As the medium with 10^{-3} M Cu^{2+} exhibited a toxic effect, the effect of 10^{-4} M Cu^{2+} and pH 3 was used for further genotype evaluation. According to the measured morphological and parameters of photosynthetic pigment contents, the best performance was achieved by the genotype *Populus nigra cl.* DN3. The highest copper accumulation on the same medium was achieved by genotype *Populus nigra cl.* BN5. The obtained data point to the considerable potential of the applied method in the evaluation of *Populus nigra* genotypes for use in projects of copper phytoextraction.

Key words: European black poplar; phytoextraction; low pH; tissue culture; microwave sterilization

INTRODUCTION

Heavy metals are among the most important pollutants threatening the ecosystem. Human habitable zones contain heavy metals that are potentially harmful to human health, as well as that of plants and other living organisms [1]. The toxic effect of most heavy metals is caused by their bonding to protein sulfhydryl groups, which leads to the inhibition of enzyme activity, compromises protein structure and causes the substitution of essential elements in biomolecules [2]; high concentrations of metal ions in the soil limit the assimilation of important micro- and macronutrients by plants [3,4].

Copper is an essential microelement, necessary for metabolism in all living organisms. However, excess concentrations of copper in plants can cause problems in the formation of the root system and plant growth in general. This effect is increased in soils with a fine texture and high cation exchange capacity because they contain more organic matter and have a low pH [5]. Recent research has described the accumulation of copper in alluvial zones [6], zones around mine sites, areas polluted after accidents in mine facilities [7] and agricultural soils treated with sewage sledge [8]. Thus, phytoremediation projects are of interest as they could improve cultivation in copper polluted areas by stabilizing this pollutant in the soil, thus preventing its excess in groundwater and its spread by wind; they could also be used to extract copper from the soil and thereby lower its presence. Research on the influence of different plant species on contaminated soils and underground water began in the early 1980s [9-11]. Trees have been suggested as a low-cost, sustainable and ecologically sound solution for the remediation of heavy metal-contaminated land [12], especially by phytoextraction [13].

The reaction of plants to excessive concentrations of copper was commonly tested on herbaceous species; however, recently there is an interest in the use of tree species (mainly hybrid poplars) in the management of copper contaminated areas [14]. Poplars are tree

species that are often used in phytoremediation due to their rapid growth, adaptability, well-developed root system that reaches underground waters, and the ability to transpire considerable amounts of water [15]. Poplars are not hyperaccumulators, but because of their large biomass production [12] and relatively high quantity of extracted metal per plant [13], potentially large quantities of heavy metals could be extracted. The European black poplar is of special interest as an endangered indigenous species that could be used in riparian areas under different protection regimes. The use of this species in phytoremediation projects in riparian zones could also contribute to improving the stability and biodiversity of this species and related ecosystems [16].

Aside from its importance as a rapid means of producing clonal planting stock, *in vitro* culture of tree species can also facilitate studies on the effects of elevated heavy metal concentrations and on the selection of tolerant genotypes. The molecular data obtained by Castiglione et al. [17] in the white poplar suggest that the *in vitro* model is a sensitive and reliable system for the study of responses to heavy metal stress. This is an important fact when considering the difficulties encountered in experiments on large long-lived organisms. Heavy metal tolerance and accumulation of the European black poplar has been poorly tested *in vitro* and needs further research.

In this work, five different European black poplar (*Populus nigra* L.) genotypes were studied *in vitro* for their tolerance to copper based on morphological parameters, biomass accumulation and pigment content, as well as copper accumulation in aboveground plant parts. Also, the effect of the pH of the medium and copper concentration were examined in order to optimize the evaluation of copper tolerance *in vitro*. The aim of our work was to examine and select copper tolerant and accumulating genotypes, which are potentially interesting for copper phytoextraction in soils contaminated with copper.

MATERIALS AND METHODS

Plant material and shoot multiplication

The following five European black poplar (*Populus nigra* L.) genotypes from natural populations were used: DN3 form the population Deronje (45°26'N 19°12'E), PN2 from the population Padej (45°51'N 20°05'E), BN5 from the population Babatovo (45°26'N 20°13'E), TRN2 from the population Vražogrnac (44°00'N 22°21'E), and GN5 from the population Apatin (45°37'N 18°56'E). All examined genotypes were selected as vigorous and vital trees and introduced into tissue culture in spring as microcuttings, after sterilization in a 3.68 mM $HgCl_2$ solution for 5 min, followed by a 30-min rinse in sterilized distilled water. Micropropagation was performed by shoot tips and axillary buds to preserve clonal fidelity [18, 19]. ACM (Aspen Culture Medium), described by Ahuja [20], supplemented with 1 μM kinetin, 0.75 μM benzylaminopurine (BAP), 0.1 μM indolebutyric acid (IBA), 108.6 μM adenine-sulphate, 166.5 μM myo-inositol, 0.9 % agar, 58,43 mM sucrose, pH 4.5 adjusted before sterilization, was used for shoot multiplication. The cultures were kept at 26±2°C under white fluorescent light (3500 lux) with a 16-h photoperiod and subcultured at 4-week intervals.

Copper treatments

For the experiment, 1.5-2.0-cm-long shoot tips of the previously multiplied shoots were placed on a rooting medium based on ACM supplemented with 0.1 μM 2,3,5-triiodbensoic acid (TIBA), 0.1 μM IBA, 26.64 μM glycine and 1.2 mM citric acid, 0.9% agar and 58.43 mM sucrose. The effects of the following three Cu^{2+} concentrations were examined: 10^{-7}, 10^{-4} and 10^{-3} M, in media labeled C1, C2 and C3, respectively, pH 3 (adjusted before sterilization). The control medium (C0) contained 10^{-7} M Cu^{2+}, pH 5.5. Sterilization was performed using a microwave oven. The media were treated until they started to boil, and were then poured into sterilized jars in a laminar chamber. In this way, the jellification potential of agar was preserved in media with pH 3 [21]. A citric acid/Na-citrate buffer was used to provide pH stability [22]. Citric acid is expected to improve copper import into plants and in this way it provides a more critical test for copper tolerance and accumulation. Also, this buffer system provided a relatively wide pH spectrum for testing. The cultures were maintained in the same conditions as previously described for multiplication. Also, before placing into the experimental media, the plants were cultivated for two weeks on the control medium (C0)

in order to eliminate the influence of cytokinins from the multiplication medium. For the experiment, three jars with five plants per jar were set per each combination of genotype×medium in three repetitions. For pigment content determination, an additional three jars with five shoot per jar were established per each combination of genotype×medium.

Copper tolerance assessment

After 35 days of culture, the following characters were determined: morphological characters: number of roots per shoot and shoot height; characters describing the biomass and water content: fresh shoot mass per plant, dry shoot mass per plant, the shoot moisture content; characters describing the contents of photosynthetic pigments in fresh shoot mass: the contents of chlorophyll a (Chl a), chlorophyll b (Chl b), total carotenoids, chlorophyll a and b (Chl a+b), the chlorophyll a/b ratio. The concentrations of chloroplast pigments (Chl a, Chl b and total carotenoids) were determined spectrophotometrically [23]. The Chl a+b and chlorophyll a/b ratios were calculated.

Copper accumulation assessment

Two characters of copper accumulation were determined: copper accumulation (copper content per shoot dry mass) and copper content (copper content per shoot). In order to determine copper accumulation, i.e. the copper concentration in dry biomass (mg kg^{-1}), samples were mineralized by wet-washing in a microwave digester in 65% HNO_3 and 30% H_2O_2 (5:1 v/v). The copper content in a sample was determined by atomic absorption spectrometry (AA 240FS Fast Sequential Atomic Absorption Spectrometer, Varian, Australia).

Statistical analysis

The entire experiment was designed to be completely randomized. The data for the number of roots were transformed by square transformation ($\sqrt{X+1}$), and the data for the percentage of rooted shoots by arcsine transformation ($\arcsin\sqrt{X}$). These transformations were conducted in order to meet the normal distribution of frequencies of data required for the implementation of the used statistical methods. The obtained data were analyzed by two-way ANOVA and the LSD test with R statistical program [24].

RESULTS

According to the results of ANOVA, the differences among genotypes were significant only in the morphological and biomass characters, with the exception of dry root mass. However, the examined media had a significant effect on the variation of all examined characters, except dry shoot mass, whereas the interaction genotype×medium had a significant influence on copper accumulation characters and some characters of the photosynthetic pigment content (Table 1).

Morphological characters

There is a considerable difference in the effects of the media on the examined genotypes. Chlorosis, necrosis and absolute absence of root formation were

Table 1. F-test of ANOVA for the examined characters.

Examined characters	Source of variation		
	Genotype (A)	Medium (B)	Interaction A×B
Morphological characters			
Length of the longest root (mm)	5.25** [a]	18.14**	1.62
Number of roots	4.76**	40.60**	1.10
Shoot height (mm)	3.67**	17.47**	0.87
Rooting percentage	4.94**	39.71**	1.76
Biomass characters			
Dry root mass per plant (g)	1.52	5.04*	0.88
Dry shoot mass per plant (g)	2.81*	1.36	1.07
Shoot moisture content	2.63*	0.84	0.89
Root/shoot dry mass ratio	2.63*	7.31**	1.60
Content of photosynthetic pigments			
Chlorophyll a (mg kg^{-1})	1.82	12.75**	2.01
Chlorophyll b (mg kg^{-1})	1.45	4.38*	1.50
Chlorophyll a+b (mg kg^{-1})	1.76	9.98**	1.86
Carotenoids (mg kg^{-1})	0.88	12.05**	2.23*
Chlorophyll a/b ratio	1.12	4.24*	3.50**
Shoot copper accumulation characters			
Copper accumulation (mg g^{-1})	0.46	60.80**	14.82**
Copper content per plant (μg)	2.40	64.71**	30.70**

Labels for the F-test: * – significant at the level α=0.05; ** – significant at the level α=0.01

Table 2. Morphological characters of rooted shoots of *Populus nigra* grown on the examined media (LSD test).

Genotype	Medium[1]	Length of the longest root (mm)		Number of roots		Shoot height (mm)		Rooting percentage	
BN5	C0	8.40	bcd [2]	1.68	cde	14.28	bde	24.05	cdef
BN5	C1	16.58	abc	4.55	ab	14.65	bcde	85.94	a
BN5	C2	3.13	de	1.38	de	16.07	bcd	17.91	def
BN5	C3	0	e	0	f	10.91	e	0	h
DN3	C0	14.69	b	3.98	a	16.51	bcd	79.99	a
DN3	C1	26.77	a	5.07	a	23.20	a	69.07	a
DN3	C2	14.35	b	3.47	ab	19.39	ac	53.73	abc
DN3	C3	0	e	0	f	11.09	e	0	h
GN5	C0	13.25	bc	3.16	abc	16.77	bcd	60.18	ab
GN5	C1	14.32	b	3.87	a	23.96	a	51.57	abcd
GN5	C2	8.68	bcd	1.97	bcde	19.52	abc	14.00	defg
GN5	C3	0	e	0	f	10.33	e	0	h
PN2	C0	0.83	bcde	0.67	cdef	9.17	de	1.85	efgh
PN2	C1	7.30	bcde	2.96	abcd	18.10	abcd	53.95	abcd
PN2	C2	3.80	cde	0.73	def	13.07	de	5.78	efgh
PN2	C3	0	de	0	f	9.93	e	0	gh
TRN2	C0	14.70	b	1.85	cde	13.67	de	33.29	bcde
TRN2	C1	11.50	bc	4.22	a	19.73	ac	51.92	abc
TRN2	C2	3.31	de	0.87	ef	16.74	bcd	7.02	fgh
TRN2	C3	0	e	0	f	12.55	de	0	h
BN5		4.70	b	1.12	b	13.68	c	13.61	b
DN3		14.82	a	2.88	a	18.02	a	38.42	a
GN5		7.88	b	1.71	b	16.69	ab	16.54	b
PN2		2.98	b	0.84	b	12.71	c	7.61	b
TRN2		6.68	b	1.35	b	15.48	bc	11.85	b
	C0	8.40	bcd	1.68	cde	14.28	bde	24.05	cdef
	C1	16.58	abc	4.55	ab	14.65	bcde	85.94	a
	C2	3.13	de	1.38	de	16.07	bcd	17.91	def
	C3	0	e	0	f	10.91	e	0	h

[1]Labels of the examined media: C0 – $10^{-7}Cu^{2+}$, pH 5.5, C1 – $10^{-7}Cu^{2+}$, pH 3, C2 – $10^{-4}Cu^{2+}$, pH 3, C3 – $10^{-3}Cu^{2+}$, pH 3 before autoclaving
[2]The differences among values marked with the same letter are not significant at the level α=0.05

Table 3. Biomass characters of rooted shoots of *Populus nigra* grown on examined media (LSD test).

Genotype	Medium[1]	Dry root mass per plant (g)		Dry shoot mass per plant (g)		Shoot moisture content		Root/shoot dry mass ratio	
BN5	C0	0.0009	bcd [2]	0.0127	abc	0.8589	a	0.0673	bcde
BN5	C1	0.0010	bcd	0.0132	abc	0.7962	abc	0.0791	bcde
BN5	C2	0.0008	bcd	0.0144	abc	0.7454	abc	0.0590	bcde
BN5	C3	0	cd	0.0077	bc	0.8197	ab	0	de
DN3	C0	0.0019	bc	0.0159	ab	0.8367	ab	0.1234	bcde
DN3	C1	0.0020	b	0.0206	a	0.7977	ab	0.1091	bcde
DN3	C2	0.0014	bcd	0.0130	bc	0.8526	a	0.1047	bcde
DN3	C3	0	cd	0.0084	bc	0.8367	a	0	de
GN5	C0	0.0013	bcd	0.0090	bc	0.8390	ab	0.1356	bcd
GN5	C1	0.0045	a	0.0083	bc	0.8372	ab	0.3919	a
GN5	C2	0.0009	bcd	0.0091	bc	0.8381	ab	0.0992	bcde
GN5	C3	0	d	0.0088	bc	0.7549	abc	0	e
PN2	C0	0	bcd	0.0097	abc	0.8446	abc	0	bcde

Table 3. continued

PN2	C1	0.0010	bcd	0.0105	abc	0.8535	ab	0.0870	bcde
PN2	C2	0.0004	bcd	0.0114	abc	0.8086	ab	0.0419	bcde
PN2	C3	0	bcd	0.0062	bc	0.8324	ab	0	cde
TRN2	C0	0.0013	bcd	0.0067	c	0.8003	ab	0.1867	b
TRN2	C1	0.0015	bcd	0.0112	bc	0.6935	bc	0.1564	bc
TRN2	C2	0.0005	bcd	0.0068	c	0.8063	ab	0.0628	bcde
TRN2	C3	0	d	0.0114	bc	0.639	c	0	e
BN5		0.0006	a	0.0115	ab	0.8049	a	0.0424	b
DN3		0.0014	a	0.0148	a	0.8281	a	0.0833	ab
GN5		0.0015	a	0.0088	b	0.8092	a	0.1362	a
PN2		0.0004	a	0.0093	b	0.8305	a	0.0333	b
TRN2		0.0007	a	0.0091	b	0.7303	b	0.0925	ab
	C0	0.0013	ab	0.0108	ab	0.8327	a	0.1251	ab
	C1	0.0022	a	0.0142	a	0.7844	ab	0.1755	a
	C2	0.0009	b	0.0109	ab	0.8108	ab	0.0767	b
	C3	0	c	0.0089	b	0.7650	b	0	c

[1]Labels of examined media: C0 – 10^{-7} Cu^{2+}, pH 5.5, C1 – $10^{-7}Cu^{2+}$, pH 3, C2 – 10^{-4} Cu^{2+}, pH 3, C3 – 10^{-3} Cu^{2+}, pH 3 before autoclaving
[2]The differences among values marked with the same letter are not significant at the level α=0.05

observed on C3 medium in all genotypes. The best rooting performance was achieved on C1 medium, while there was no significant effect of media on shoot height (Table 2).

The best performance according to all four examined morphological characters was achieved by genotype DN3, while the poorest results for the morphological characters was achieved by PN2. Although the effect of the interaction genotype x medium was not significant, GN5 showed a specific response on C1 medium that was not significantly different from that obtained on the control (C0) (Table 2).

Other tested genotypes achieved significantly higher morphological characters on C1 than on C0. For medium C2, the results were considerably lower than on the control, while on medium C3 growth was almost absent. Only genotype DN3 attained significant results with regard to morphological characters on C2, which was at the level of the control.

Biomass characters

Although genotype DN3 had the highest values for dry root and shoot mass, differences among the examined genotypes in biomass characters were not statistically significant. However, there were differences in the reaction of the genotypes on the examined media. DN3 achieved a significantly higher shoot dry mass on C2 than on C1, while GN5 achieved higher dry root mass on C1 than on other media (Table 3).

In general, there were no significant differences between the treatments and the control for most of the examined biomass characters. Shoot moisture content and root/shoot dry mass ratio were significantly lower on the medium with the toxic copper concentration (C3). Furthermore, significantly greater root dry mass and root/shoot dry mass ratio were observed on the C1 medium than on C2 (Table 3).

Photosynthetic pigment content

The genotype DN3 had significantly higher photosynthetic pigment contents than the other genotypes. It also differed from the others by its reaction on the media. For this genotype, the pigment contents on C2 were significantly higher than on the control, while the differences were not significant in the other genotypes (Table 4). The photosynthetic pigment content calculated for fresh mass revealed almost the same relations among the genotypes and media (data not shown).

The highest content of photosynthetic pigments was achieved on C2, and the lowest on C3. However, only the content of Chl b on C2 significantly differed

Table 4. Photosynthetic pigments' content in dry shoot mass of rooted shoots of *Populus nigra* grown on examined media (LSD test).

Genotype	Medium[1]	Chlorophyll (mg kg^{-1})						Carotenoids (mg kg^{-1})		Chlorophyll a/b ratio	
		a		b		a+b					
BN5	C0	4.92	bc 2)	1.46	b	6.38	bc	1.80	b	3.34	bcdef
BN5	C1	4.18	bcd	1.17	bcd	5.35	bcd	1.24	bcd	3.59	abcdef
BN5	C2	5.11	b	1.77	b	6.88	b	1.59	bc	2.92	defg
BN5	C3	1.29	e	0.32	d	1.61	e	0.54	de	4.11	abc
DN3	C0	3.85	bcd	1.30	bcd	5.14	bcd	1.28	bc	2.97	def
DN3	C1	4.96	bc	1.69	b	6.65	bc	1.51	bc	2.94	cdefgh
DN3	C2	8.72	a	3.02	a	11.74	a	2.59	a	2.91	efg
DN3	C3	2.20	bcde	0.60	bcd	2.80	bcde	0.79	cde	3.63	abcdef
GN5	C0	4.62	bc	1.25	bcd	5.88	bc	1.64	bc	3.73	abcdef
GN5	C1	4.13	bcd	1.12	bcd	5.25	bcd	1.36	bc	3.66	abcdef
GN5	C2	4.21	bcd	1.41	bc	5.62	bc	1.41	bc	2.98	def
GN5	C3	1.35	e	0.78	bcd	2.13	de	0.57	de	2.63	fgh
PN2	C0	3.15	bcde	0.67	bcd	3.82	bcde	1.30	bcde	4.72	ab
PN2	C1	4.99	bc	1.45	bcd	6.44	bc	1.75	bc	3.96	abcde
PN2	C2	1.76	cde	0.35	bcd	2.11	cde	0.80	cde	5.07	a
PN2	C3	1.88	de	1.15	bcd	3.03	cde	0.95	cde	1.62	h
TRN2	C0	4.6	bc	1.19	bcd	5.8	bc	1.62	bc	3.86	abcde
TRN2	C1	5.02	bc	1.41	bc	6.43	bc	1.61	bc	3.62	abcdef
TRN2	C2	4.26	bcd	1.37	bc	5.62	bc	1.36	bc	4.03	abcd
TRN2	C3	0.71	e	0.42	cd	1.13	e	0.34	e	1.84	gh
BN5		3.85	b	1.18	b	5.03	b	1.30	b	3.48	a
DN3		5.54	a	1.88	a	7.42	a	1.72	a	3.02	a
GN5		3.58	b	1.14	b	4.72	b	1.24	b	3.25	a
PN2		3.11	b	1.04	b	4.14	b	1.25	b	3.49	a
TRN2		3.65	b	1.10	b	4.74	b	1.23	b	3.34	a
	C0	4.39	a	1.25	b	5.65	a	1.56	a	3.57	a
	C1	4.64	a	1.35	ab	5.99	a	1.49	a	3.57	a
	C2	5.28	a	1.77	a	7.05	a	1.67	a	3.35	a
	C3	1.33	b	0.62	c	1.96	b	0.59	b	2.72	b

[1]Lables of examined media: C0 – 10^{-7} Cu^{2+}, pH 5.5, C1 – 10^{-7} Cu^{2+}, pH 3, C2 – 10^{-4} Cu^{2+}, pH 3, C3 – 10^{-3} Cu^{2+}, pH 3 before autoclaving
[2]The differences among values marked with the same letter are not significant at the level α=0.05

from C0. Also, only on C3 were the carotenoid content and chlorophyll a/b ratio significantly lower than on the other media.

Copper accumulation and content

There were no significant differences in copper accumulation and content between C0 and C1, while C2 and C3 differed significantly from C0 and C1 in both characters (Table 5). The highest value of copper accumulation and copper content in most genotypes was observed on C3 medium, except for BN5, which displayed the highest copper accumulation and content on C2.

DISCUSSION

High copper tolerance and the ability to accumulate this metal in the aboveground parts of plants are principal criteria in the evaluation of genotypes in order to be considered for use in phytoextraction [13]. Di Lonardo et al. [13] established that woody plant medium (WPM) with 10^{-3} M Cu^{2+} and pH 5.2 had no inhibitory effect on the growth and development of white poplar genotypes. In our work we conducted the test at pH 3, using citric acid to lower and stabilize the pH. It is well known that the pH of the media could be altered by many factors during both sterilization and cultivation *in vitro* [21]. Citric acid

Table 5. Copper accumulation and content in rooted shoots of *Populus nigra* grown on examined media (LSD test).

Genotype	Medium[1]	Copper accumulation (mg g^{-1})		Copper content (µg plant^{-1})	
BN5	C0	0.257	d 2)	3.271	d
BN5	C1	0.080	d	1.048	d
BN5	C2	2.182	c	31.366	a
BN5	C3	0.391	d	3.004	d
DN3	C0	0.103	d	1.639	d
DN3	C1	0.020	d	0.416	d
DN3	C2	0.180	d	2.336	d
DN3	C3	2.987	b	25.200	b
GN5	C0	0.069	d	0.624	d
GN5	C1	0.105	d	0.877	d
GN5	C2	0.177	d	1.612	d
GN5	C3	3.066	ab	27.047	ab
PN2	C0	0.081	d	0.785	d
PN2	C1	0.412	d	4.343	cd
PN2	C2	0.239	d	2.725	d
PN2	C3	1.719	c	10.706	c
TRN2	C0	0.072	d	0.481	d
TRN2	C1	0.032	d	0.353	d
TRN2	C2	0.519	d	3.535	d
TRN2	C3	2.227	ac	25.332	b
BN5		1.007	a	13.429	a
DN3		0.975	a	8.672	b
GN5		0.854	a	7.540	bc
PN2		0.735	a	5.332	c
TRN2		0.933	a	9.761	b
	C0	0.114	c	1.407	c
	C1	0.112	c	1.242	c
	C2	0.798	b	10.476	b
	C3	1.917	a	16.703	a

[1]Labels of examined media: C0 – 10^{-7} Cu^{2+}, pH 5.5, C1 – 10^{-7} Cu^{2+}, pH 3, C2 – 10^{-4} Cu^{2+}, pH 3, C3 – 10^{-3} Cu^{2+}, pH 3 before autoclaving
[2]The differences among values marked with the same letter are not significant at the level α=0.05

is also known as a low molecular weight organic acid (LMWOA) capable of forming chelates with heavy metals, improving their mobility and bioavailability without causing leaching of heavy metals into lower soil strata [25,26]. According to Evangelou et al. [26], the effect on copper bioavailability in research on *Nicotiana tabacum* was even better with citric acid than with EDTA. Chen et al. [27] showed that the effect of citric acid on the uptake of lead and cadmium, anions that have even less mobility, is strongly related to pH lowering. In our work, in presence of citric acid (1.2 mM) and low pH, the concentration of 10^{-3} M Cu^{2+} in medium C3 was sufficient to produce a toxic effect in the examined European poplar genotypes. Therefore, a copper concentration of 10^{-4} M in media with pH 3 should be recommended in future work on copper tolerance and the evaluation of its accumulation in *Populus nigra in vitro*. In addition, these results should be taken into consideration in further research in field conditions on acidic soils and in cases when citric acid is used to improve copper availability.

The effect of low pH was tested by comparing the results of C1 and C0. The number of roots and the percentage of rooted shoots were significantly higher on C1; however, the shoot height and shoot dry mass on this medium were similar to those on C0. Kovačević et al. [21] observed significant growth and improved development of white poplar with regard to both shoot and root in plants grown *in vitro* on medium with an initially low (before sterilization) pH of 3.0. The authors did not use citric acid nor any additional buffer system. At the end of cultivation, the final pH of the media differed significantly from the initial value. The Na-citrate/citrate buffer system together with microwave sterilization could be a useful approach for further studies of the effect of low pH *in vitro*.

Among the examined morphological characters, the best differentiation among genotypes on C2 medium was observed on root formation, in particular the rooting percentage. Genotype DN3 had the highest scores for rooting characters, which points to a high tolerance to increased copper concentration of this genotype. Greater differences in rooting characters than in shoot height between the among genotypes could be related to the fact that the roots of poplar genotypes *in vitro* seem to be more sensitive to high heavy metal concentrations in media compared to the shoots [13]. It seems that morphological differences in the response to C2 medium among the genotypes were most intensively expressed by their most sensitive organ, the root. Thus, we assumed that the rooting characters could be proposed for quick copper tolerance *in vitro* tests in *Populus nigra* in the future.

There were significant differences among genotypes in photosynthetic pigment contents, especially on the C2 medium. On this medium, genotype DN3 differed significantly from the others, with higher chlo-

rophyll and carotenoid contents, while the genotype PN2 had the highest chlorophyll a/b ratio (Table 4).

In our study, chlorosis was observed on C3 medium in all genotypes. The loss of photosynthetic pigments is a common reaction of plants to excessive copper, and is related to disturbances in the chloroplast inner structure caused by alterations in lipoproteins in thylakoid membranes [29]. No significant difference in the content of examined photosynthetic pigments were observed on C2 compared to C0 and C1, whereas the chlorophyll a/b ratio was significantly lower in samples grown on the C3 medium than on C1. In contrast to our results, Borghi et al. [14] observed an increment in the chlorophyll a content in treatments with copper concentrations ranging from 0.4 to 500 10^{-6} M Cu^{2+}, which was followed by a significant decline in *Populus* × *euramericana cl.* Adda leaves after growth in 10^{-3} M Cu^{2+} in hydroponic culture. The same author found that the excess of copper produced a significant difference in the chlorophyll a/b ratio of the rooted cuttings.

In general, copper accumulation increased with the increment of copper concentration in the medium. In the Euramerican poplar clone Adda grown in hydroponics, Borghi et al. [14] found such an increment only in roots, but not in the stem and leaves. The highest copper accumulation and copper content in shoot tissue of the examined European black poplar genotypes was observed on C2 and C3 media.

We propose the C2 medium for application in further tests of copper tolerance and accumulation in *Populus nigra* tissue culture, since the C3 medium had a toxic effect on all tested genotypes. The C2 medium had an inhibitory but not toxic effect, and significant differences in most of the examined characters as compared to the control medium. Furthermore, it provided the best differentiation among the genotypes.

The examined genotypes considerably differed in copper tolerance and accumulation. Genotype DN3 achieved the best performance according to morphometrics, biomass and photosynthetic pigment contents. High scores in morphometrics, especially in rooting characters and in the photosynthetic pigment contents, on C2 suggest that this genotype could tolerate the presence of copper in the substrate at increased concentrations. The highest copper accumulation on C2, the medium preferred for copper tolerance evaluation tests, was achieved by BN5, while all the other genotypes had the highest copper accumulation when grown on the C3 medium. These results favor BN5 to be tested beside DN3 in phytoextraction projects on soils where the copper content is low enough to be sufficiently tolerated by this genotype. Here it could achieve a higher copper accumulation than the other genotypes and would be more appropriate for copper phytoextraction on soils with a near to toxic copper concentration. Further field tests should be performed in order to examine the effect of copper on biomass accumulation, which is the other component of a plant's phytoextraction potential. The use of *Populus nigra* genotypes on highly contaminated soils should be done with caution, especially considering the inhibitory effect of high copper concentration on root formation.

The general opinion is that differences in the bioavailability of contaminants and the processes of pollutant uptake and metabolite distribution are likely to be substantial in tissue culture and field conditions. In *Salix* sp. it was shown that the results obtained in hydroponics and in the field are comparable [29,30]. Also, in [31] and [32], the authors support the idea that the response of plants to environmental contaminants can be predicted based on results from tissue cultures, reducing the cost of subsequent conventional whole-plant experiments.

Considering the observed differences among the examined genotypes with regard to copper tolerance and accumulation, *in vitro* tests can serve to narrow the group of candidate genotypes for copper phytoextraction projects. However, for the final evaluation of a particular genotype, research should be performed in the field, considering the lower availability of lead in soil, higher juvenility of the material *in vitro* and complexity of the interaction between plant and the habitat.

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planned and organized the research; Dragana Miladinović took part in the writing of the manuscript.

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