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Research article

The impact of Cu treatment on phenolic and polyamine levels in plant material regenerated from embryos obtained in anther culture of carrot

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Abstract

The influence of copper sulphate on the regeneration of carrot (*Daucus carota* L.) androgenic embryos and changes in the levels of phenolic substances and polyamines that might be indicative of the response to oxidative stress were investigated. The cultivation on the regeneration medium supplemented with Cu^{2+} at the concentrations 1 and 10 μM for 15 weeks resulted in significant dose-dependent inhibition of the growth and organogenic ability of carrot embryos. The total content of phenolic acids (represented by the sum of all soluble and insoluble fractions) in the Cu^{2+}-treated carrot cultures did not change in comparison with the control (0.1 μM Cu^{2+}). However, the levels of phenolic acids in the individual fractions showed significant differences. The cultivation in the presence of increased Cu^{2+} evoked first of all the rise of free chlorogenic and caffeic acids, and the increase in soluble ester-bound ferulic acid. Marked dose-dependent decline in the amount of ferulic acid incorporated into the cell walls of the Cu^{2+}-treated carrot cultures was partly compensated by the increase in the content of *p*-hydroxybenzoic acid. Decline in the total polyamine contents in the carrot tissues cultivated in the presence of increased Cu^{2+} concentrations was observed. The most abundant polyamine, both in a free and PCA-soluble conjugated forms, was putrescine, the least abundant was spermine, which occurred in free form only. While the levels of free polyamines slightly decreased in a dose-dependent manner in the Cu^{2+}-treated cultures, those of PCA-soluble conjugates markedly rose (enhancement to 135 and 170% in 1 and 10 μM Cu^{2+}, respectively, compared with the control). The decline in the total polyamine contents was caused mainly by the decline in the levels of PCA-insoluble conjugates. The decrease observed in this fraction was approximately to 70 and 50% in 1 and 10 μM Cu^{2+}-treated cultures, respectively, when compared with the control. The role of phenolic acids and polyamines in preventing Cu^{2+}stress in the carrot tissues is discussed.

Keywords: Carrot culture; Copper ions; Embryo regeneration; Phenolic acids; Polyamines

1. Introduction

Since the first embryos and haploid plants were obtained scientists have been interested in the possibility of deriving haploid plants from anther cultures. Haploidization through androgenesis and gynogenesis is a quick method of homozygotization [21]. This technique was used to speed up breeding of many modern plant cultivars. New cultivars of rice, barley, tobacco, rape and asparagus were created using haploid systems [43]. Treatment with mutagens may increase the number of these variations and selection of particular phenotypes allows obtaining quickly a wide range of variations of a given trait [29]. Mutation and transformation of the androgenetic plant material cultured in the medium supplemented with

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particular toxins lead to the selection of resistant forms in a relatively short time [20].

Carrot is one of the most important vegetables and therefore it has become an object of intensive research aiming to obtain new cultivars. The efficiency of androgenesis in anther carrot culture depends on several factors that have been described in our previous work [17]. Considerable differences in the efficiency of androgenesis not only between species but also between individual donor plants were observed in carrot cultivars [1]. The number of embryos obtained per 100 anthers for one cultivar varied between individual plants from 0.0 to 49.0 embryos [17].

Cu^{2+} in small doses is an important microelement involved in many physiological processes, especially chlorophyll synthesis and photosynthesis [26]. Nevertheless, redox-active metals (Cu and Fe) at higher concentrations catalyze the formation of harmful active oxygen species (AOS) and induce oxidative damage of important macromolecules such as DNA, proteins and lipids [10]. On the other hand, these metals belong to the essential trace elements taking part in redox reactions, electron transfers and many enzyme-catalysed reactions. However, as for many micronutrients, no clear optimal levels of Cu in different plant species are apparent. Differences in plant responses to Cu^{2+} seem to depend not only on its concentration but also on the ability of plants to increase the antioxidative protection against negative consequences of heavy metal stress. This toxic effect resulting the oxidative state may be allayed by several antioxidative systems to which phenolic substances and polyamines (PAs) also belong [4,6,23].

Phenolic substances play an important role in protecting plants against biotic and abiotic stresses [12] and the enhancement of their metabolism is one of the responses to heavy metal stress. Since they are reductants they may scavenge AOS or chelate heavy metals. In particular, their carboxyl or hydroxyl groups can strongly bind Cu^{2+} and Fe^{2+} and thus decrease heavy metal toxicity in cells [13]. The induction of shikimate pathway, the accumulation of soluble phenolics and the enhancement of lignin content in the pepper hypocotyls was observed in the copper treated plants [11].

During the last few years PAs have been reported as efficient antioxidants in many experimental systems and various kinds of environmental stresses [4]. The cationic nature of free PAs at physiological pH (especially of putrescine, spermidine and spermine) allows them to interact with negatively charged molecules such as nucleic acids, phospholipids and proteins and to protect them from metal-induced oxidative damage [33]. They may reduce the oxidative damage by increasing the activities of antioxidant enzymes [39] and they have also been suggested to function as metal chelators [24] and/or as direct or indirect free radical scavengers [18].

Aliphatic PAs, that have been classified as growth factors in plant cells, are implicated in the control of important developmental processes, including cell growth, cell division and morphogenesis [40] and stabilization of nucleic acids and membranes [41]. Several reports have shown the involvement of PAs, particularly of their free forms, in control of somatic embryogenesis [22]. The purpose of the current work has been to characterize the influence of copper sulphate on regeneration of androgenic embryos and to identify changes in the levels of phenolic substances and PAs that might be indicative of the response of carrot plants to oxidative stress generated by excess of Cu. Despite the beneficial influence of Cu on regeneration during *in vitro* culture of cereals and its importance during pollen development, its effect on androgenesis remains uncertain [44].

The data presented here show changes in growth parameters, alterations in the pool of phenolic substances and detailed changes in the contents of free Put, Spd, Spm and Cad, their soluble and insoluble conjugates determined in the plant material regenerated from the androgenic embryos obtained in the anther culture of carrot (*Daucus carota* L.) and cultivated in the presence of different copper sulfate concentrations.

2. Results

2.1. Growth of carrot culture

Differences in the growth and root forming capacity were followed in the plant material (rosettes) regenerated from embryos obtained in the anther culture of carrot grown for 15 weeks on the medium containing 0.1 (control), 1, 10 or 100 μM Cu^{2+}. The highest concentration of Cu^{2+} (100 μM) caused severe malformations of the regenerated material in comparison with the control and therefore these plants were not further analysed (data not shown). Dose-dependent inhibition of the rosettes growth was observed at the higher concentrations of Cu^{2+} as compared with the control grown in the presence of 0.1 μM Cu^{2+} (Table 1). The organogenic ability of carrot embryos as well as the fresh weight of the regenerated rosettes decreased in the presence of higher Cu^{2+} concentrations. Rooting of the rosettes was completely blocked at 10 μM Cu^{2+} (Table 1).

2.2. Phenolic acid contents

The total contents of phenolic acids (represented by the sum of soluble and insoluble fractions of phenolic acids) in the 1 or 10 μM Cu^{2+}-treated carrot cultures did not change in comparison with the control grown in the presence of 0.1 μM Cu^{2+} (Fig. 1A). However, the contents of phenolic acids in individual fractions were different. In Cu^{2+}-treated

Table 1
Plant regeneration in anther culture of carrot grown for 15 weeks in the medium supplemented 0.1 (C), 1 and 10 μM $CuSO_4$

$CuSO_4$ (μM)	No. of rosettes with roots	Weight (g)	No. of rosettes without roots	Weight (g)
0.1 (C)	4.8	0.239	8.7	0.244
1	1.5*	0.106*	2.1*	0.013*
10	0.0*	0.0*	0.5*	0.024*

Data represent the number of rosettes formed from one embryo. Mean values of two experiments with 8 determinations each. Asteriks (*) indicate the results obtained from Cu^{2+}-treated carrot cultures that significantly differ from the corresponding control values at $p \leq 0.05$.

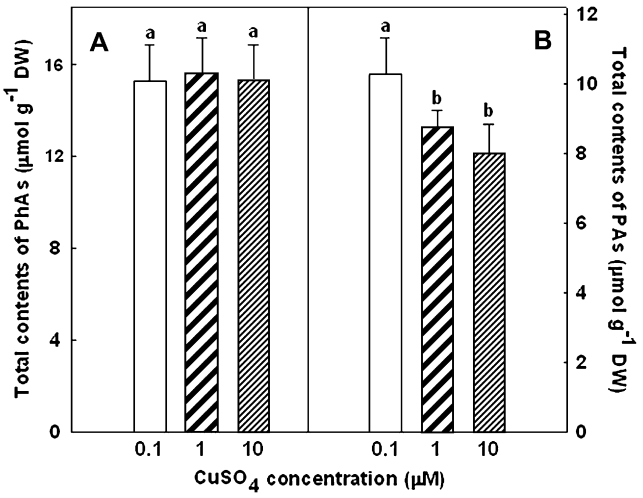

Fig. 1. Total contents of phenolic acids (represented by the sum of soluble and insoluble fractions of phenolic acids) and total contents of polyamines (represented by the sum of free polyamines and their PCA-soluble and PCA-insoluble conjugates) in the rosettes regenerated from the embryos obtained in the anther culture of carrot grown for 15 weeks on the medium with 0.1 (control), 1 and 10 µM Cu^{2+}. Mean values of two experiments with two to four parallel analyses ± SE. Different letters above the bars indicate significant differences from the controls ($P < 0.05$).

carrot culture the dose-dependent increase in soluble esterified forms of phenolic acids (F2) and slight increase in free phenolic acids (F1) were observed. The 10 µM Cu^{2+} concentration caused decrease in the levels of phenolic glycosides (F4). The amount of phenolic acids released after alkaline treatment of the cell wall material (F3) also decreased in Cu^{2+}-treated carrot tissue (Fig. 2).

The HPLC analyses indicated the presence of two families of phenolic acids in the carrot culture. Three derivatives of

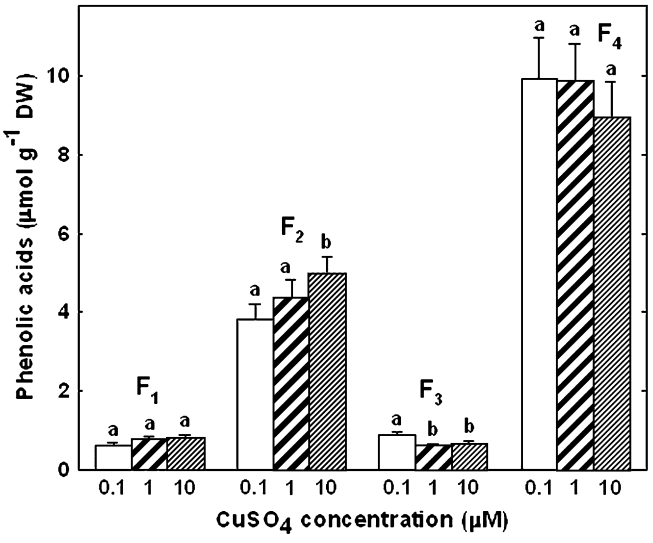

Fig. 2. Contents of free (F1), soluble ester-bound (F2), insoluble ester-bound (F3) and soluble glycoside-bound (F4) phenolic acids in the rosettes regenerated from embryos obtained in the anther culture of carrot grown for 15 weeks on the medium with 0.1 (control), 1 and 10 µM Cu^{2+}. Mean values of two experiments with two to four parallel analyses ± SE. Different letters above the bars indicate significant differences from the controls ($P < 0.05$).

benzoic acid: *p*-hydroxybenzoic (*p*HBA), syringic (SyA), vanillic (VA) and five of cinnamic acid: caffeic (CaA), chlorogenic (ChA), *p*-coumaric (*p*CA), ferulic (FA) and sinapic (SiA) acids (Table 2). The most abundant phenolic acids in F1 were ChA, *p*HBA and CaA; while FA > *p*HBA > VA > SiA > *p*CA dominated in the ester-bound fraction (Table 2). In the phenolic glycosides the relation was as follows CaA > *p*HBA > ChA > FA. High content of FA and *p*HBA occurred in cell wall-bound fraction (Table 2).

Treatments with 1 and 10 µM Cu^{2+} concentrations increased the levels of free ChA and CaA and of soluble ester-bound FA. Decline in phenolic glycosides was caused first of all by the decrease in the level of glycoside-bound CaA. Marked dose-dependent decline in the amount of FA incorporated into the cell walls of the Cu^{2+}-treated carrot cultures was partly compensated by the increase in the content of *p*HBA (Table 2, Fig. 3).

2.3. Polyamine contents

Remarkable decline in the total contents of PAs (represented by the sum of free PAs and their PCA-soluble and PCA-insoluble conjugates) extracted from the carrot tissues cultivated in the presence of increased Cu^{2+} concentrations and from the control is clearly seen in Fig. 1B. While the levels of free PAs decreased in a dose-dependent manner in the Cu^{2+}-treated cultures, marked accumulation of PCA-soluble conjugates occurred in these cultures (enhancement to 135 and 170% in 1 and 10 µM Cu^{2+}, respectively, compared with the control; Fig. 4). The decline in total PA contents was caused mainly by the decrease in the levels of PCA-insoluble conjugates (*i.e.* PAs bound to hemicelluloses and lignins and in small amounts also to proteins). The observed decrease in this fraction was approximately 70 and 50% in 1 and 10 µM Cu^{2+}-treated cultures, respectively, when compared with the control (Fig. 4).

The most abundant PA both in free and PCA-soluble conjugated forms was putrescine (Put). Spermidine (Spd) was detected in free form and PCA-insoluble conjugates, while spermine (Spm) occurred in a free form only. In the fraction of PCA-insoluble conjugates cadaverine (Cad) dominated, however, the content of this diamine decreased to 62 and 40% in 1 and 10 µM Cu^{2+}-treated cultures, respectively, when compared with the control (Table 3). As mentioned above, regeneration of the carrot cultures in the elevated concentrations of Cu^{2+} resulted in the increase in PCA-soluble conjugates with the concomitant decreases in free and predominately in PCA-insoluble conjugates. Interestingly, the total content of Put (the sum of three determined forms) was very similar in all studied cultures, however, its content in the individual PA fractions significantly changed (rise of PCA-soluble conjugates to 138 and 168% and the decrease in PCA-insoluble conjugates to 79 and 71% in 1 and 10 µM Cu^{2+}-treated cultures, respectively, when compared with the control; Table 3).

Table 2
Contents of individual phenolic acids in the carrot tissues cultured in the control and Cu^{2+} supplemented media; F_1 = free phenolic acids, F_2 = ester-bound methanol-soluble phenolic acids, F_3 = ester-bound cell wall phenolic acids, F_4 = glycoside-bound methanol-soluble phenolic acids, CaA = caffeic acid, *p*CA = *p*-coumaric acid, ChA = chlorogenic acid, FA = ferulic acid, *p*HBA = *p*-hydroxybenzoic acid, VA = vanillic acid, SiA = sinapic acid, SyA = syringic acid

$CuSO_4$ (μM)		Phenolic acids (nmol g^{-1} DW)							
		*p*HBA	VA	CaA	ChA	SyA	*p*CA	FA	SiA
0.1 (C)	F_1	20.2 ± 1.9	1.7 ± 0.2	11.7 ± 1.3	572.7 ± 58.8	– ± –	7.1 ± 0.7	– ± –	– ± –
	F_2	287.2 ± 30.1	276.5 ± 29.2	– ± –	– ± –	33.5 ± 3.5	76.6 ± 7.8	3018.1 ± 308.9	136.0 ± 14.5
	F_3	352.4 ± 37.5	37.9 ± 3.9	– ± –	– ± –	– ± –	14.6 ± 1.5	459.5 ± 48.7	– ± –
	F_4	1067.4 ± 112.0	351.2 ± 37.2	6800.2 ± 705.0	860.8 ± 91.7	– ± –	47.5 ± 4.9	816.7 ± 84.5	– ± –
1	F_1	19.7 ± 1.8	2.3 ± 0.2	20.1 ± 1.8	707.1 ± 68.5	– ± –	8.2 ± 0.8	5.7 ± 0.6	– ± –
	F_2	282.7 ± 29.5	286.3 ± 29.6	– ± –	– ± –	37.5 ± 3.8	56.7 ± 5.9	3597.2 ± 374.5	99.9 ± 10.4
	F_3	410.0 ± 42.3	17.8 ± 1.9	– ± –	– ± –	– ± –	12.2 ± 1.1	156.1 ± 16.2	– ± –
	F_4	975.5 ± 96.8	345.0 ± 37.2	6792.4 ± 690.2	801.3 ± 82.4	– ± –	62.3 ± 6.3	871.4 ± 79.8	– ± –
10	F_1	19.3 ± 1.8	3.8 ± 0.4	31.2 ± 3.4	715.8 ± 74.6	– ± –	8.4 ± 0.7	7.4 ± 0.8	– ± –
	F_2	293.6 ± 31.0	296.2 ± 31.5	– ± –	– ± –	30.1 ± 3.1	55.5 ± 5.7	3615.8 ± 379.4	79.8 ± 8.1
	F_3	527.6 ± 54.3	15.8 ± 1.6	– ± –	– ± –	– ± –	13.1 ± 1.5	102.3 ± 12.1	– ± –
	F_4	1035.5 ± 110.5	401.4 ± 39.6	5787.4 ± 592.3	843.6 ± 83.9	– ± –	42.9 ± 4.4	807.4 ± 84.3	– ± –

Mean values of two experiments with two to four parallel analyses ± SE.

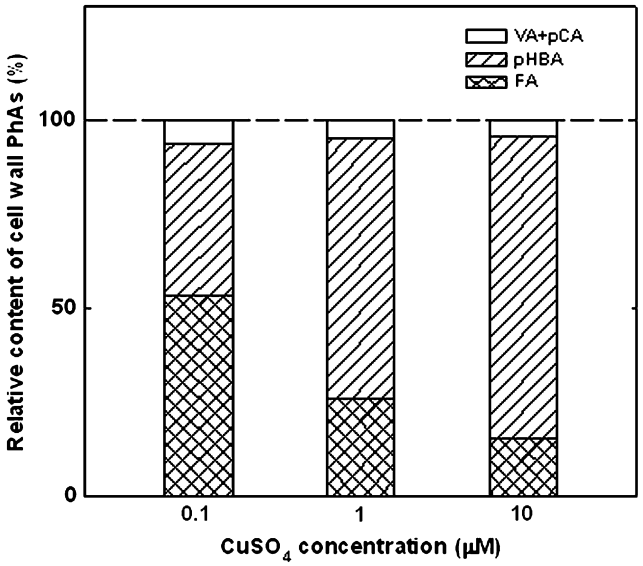

Fig. 3. The relative contents of insoluble cell wall-bound phenolics (F3) in the rosettes regenerated from the embryos obtained in the anther culture of carrot grown for 15 weeks on the medium with 0.1 (control), 1 and 10 μM Cu^{2+}. Mean values of two experiments with two to four parallel analyses ± SE.

3. Discussion

The results described in this work indicate that copper at 1 and 10 μM Cu^{2+} caused strong reduction of growth as well as shoot and root regeneration from embryos obtained in the anther culture of carrot (Table 1). Significant inhibition of growth of radish seedlings was observed at the low concentration of 1 μM Cu^{2+} [6]. However, a stimulatory effect of rather high concentrations of Cu^{2+}(25–100 μM) on shoot and root regeneration was reported in wheat and tobacco cultures

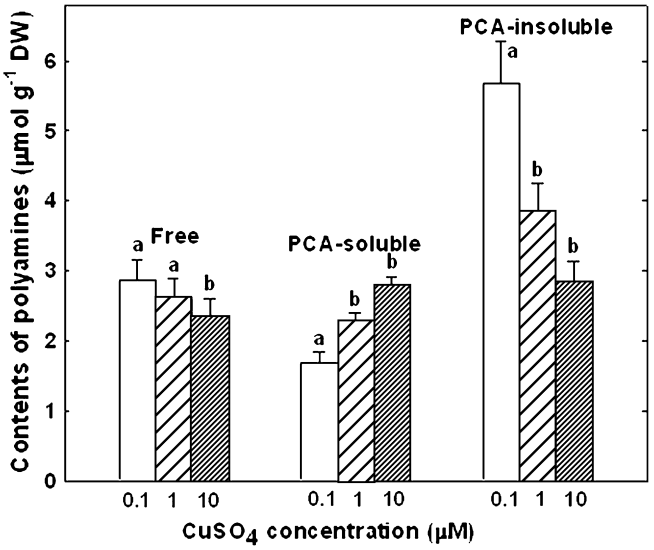

Fig. 4. Contents of free polyamines, their PCA-soluble and PCA-insoluble conjugates in the rosettes regenerated from the embryos obtained in the anther culture of carrot grown for 15 weeks on the medium with 0.1 (control), 1 and 10 μM Cu^{2+}. Mean values of two experiments with two to four parallel analyses ± SE. Different letters above the bars indicate significant differences from the controls ($P < 0.05$).

Table 3
Contents of free putrescine (Put), cadaverine (Cad), spermidine (Spd) and Spermine (Spm) and of their PCA-soluble and PCA-insoluble conjugates determined in the carrot tissues treated with 0.1 (control), 1 and 10 μM Cu^{2+}

$CuSO_4$ (μM)	Form	Polyamines (nmol g^{-1} DW)			
		Put	Cad	Spd	Spm
0.1 (C)	Free	1950.5 ± 202.6	42.0 ± 5.2	719.0 ± 73.5	157.3 ± 14.8
	PCA-sol	1480.2 ± 155.2	210.3 ± 20.8	− ± −	− ± −
	PCA-insol	1633.0 ± 140.4	3897.0 ± 405.6	161.7 ± 15.8	− ± −
1	Free	1655.2 ± 172.0	38.0 ± 4.3	757.1 ± 78.2	164.7 ± 17.2
	PCA-sol	2040.2 ± 212.8*	245.1 ± 25.3*	− ± −	− ± −
	PCA-insol	1298.9 ± 134.6*	2424.2 ± 253.3*	117.6 ± 12.5	− ± −
10	Free	1479.9 ± 155.6*	58.8 ± 6.2	693.5 ± 71.4	129.0 ± 13.6
	PCA-sol	2497.8 ± 254.8*	306.6 ± 31.7*	− ± −	− ± −
	PCA-insol	1162.0 ± 124.7*	1587.2 ± 165.8*	89.3 ± 9.3*	− ± −

Mean values of two experiments with two to four parallel analyses ± SE. Asterisks (*) indicate the results obtained from Cu^{2+}-treated carrot cultures that significantly differ from the corresponding control values at $p \leq 0.05$.

[31]. The inhibition of growth and regeneration observed in Cu^{2+}-treated carrot cultures might partly coincide with the accumulation of free phenolic acids. The presence of 1 and 10 μM Cu^{2+} in the cultivation media evoked first of all the elevation of free ChA, CaA and FA and the increase in soluble ester-bound FA (Table 2). The antioxidant properties mainly of ChA, CaA but also of other phenolic acids are well documented [37]. The scavenging activity of phenolic acids and their esters depends on the number of hydroxyl groups in the molecule [32]. However, on the other hand, a negative correlation between the content of hydroxycinnamic acids and mitotic activity in alfalfa suspension culture was observed [8].

An increased level of soluble phenolic acids, especially of ChA, VA, FA and *p*HBA, was observed in *Raphanus sativus* cultivated in the presence of Cu^{2+} [35]. These authors proposed that esterification of phenolics allows their transport to vacuoles where they may be used as substrates for vacuolar peroxidase in the peroxidase/phenolic/ascorbate system which protects cells against AOS [35]. The observed decline in phenolic glycosides in the Cu^{2+}-treated carrot culture was caused first of all by the decrease in the level of glycoside-bound caffeic acid (Table 2). Glycosylation can modulate redox properties of phenolics and hence their biological effect and/or glucosides may serve as a metabolic pool from which phenolics can be utilized during further cultivation [28].

Marked dose-dependent decline in the amount of FA and VA incorporated into the cell walls of the Cu^{2+}-treated carrot cultures was partly compensated by elevated levels of *p*HBA (Table 2, Fig. 3). However, we cannot exclude the negative effect of this alteration on further development of regenerated plants. We have found in the cell walls of alfalfa calli grown for a long- time on the medium containing glyphosate (an inhibitor of shikimate pathway enzyme, 5-enolpyruvyl-shikimate-3-phosphate synthase) that the extremely low level of FA and increased amounts of hydroxybenzoic acids were connected with changed morphology of cells [3]. Ester-linked phenolic acids (in addition to FA and *p*CA also VA, *p*HBA, SiA and CaA) are involved, besides lignin biosynthesis, in alterations of the cell wall composition during differentiation and morphogenesis [25].

PAs play multiple essential functions both in plant and animal cells. On one hand, they facilitate cell division and growth and on the other hand they support overexpression of some apoptotic genes, facilitating cell death. The basis of these diverse cellular responses is currently not known. Changes in PA homeostasis have been reported in cell death of nerve cells and in various *in vitro* models of apoptosis in plant systems [34,38]. PAs, similarly as phenolic acids, are rarely present in cells in the free form but create conjugates with hydroxycinnamic acids (PCA-soluble conjugates, *e.g.* feruolylputrescine, caffeoylputrescine, caffeoylspermidine) or with high molecular weight substances (PCA-insoluble conjugates). In our experiments, decrease in the levels of free PAs in the Cu^{2+}-treated carrot culture coincided with the marked dose-dependent increase in the levels of soluble conjugates (Fig. 4). The decline in total PA contents (Fig. 1B) was caused mainly by the decrease in the levels of PCA-insoluble conjugates (Fig. 4). The alterations in the pool of PAs were caused first of all by changes in the contents of Put in the individual fractions and by the decline in Cad and Spd insoluble conjugates (Table 3). The increased concentration of Cu^{2+} influenced the balance between free and conjugated forms of PAs to the detriment of free PAs.

The certain contents of free PAs are always associated with cell division and/or with re-programming of cells into new developmental patterns [42]. However, the levels of free Spd and Spm, PAs which appear to be essential for DNA replication and cell division [9], remained in Cu^{2+}-treated cultures nearly unchanged compared with the control (Table 3). In our experimental system the levels of free PAs do not seem to be the limiting factor for ongoing morphogenic processes.

PCA-soluble conjugates have been shown to occur at high levels in plants and are thought to be correlated with developmental processes [27]. Recently the role of conjugated PA forms in plants under environmental stress has drawn much interest [4]. The highest increase in Put in Cu^{2+}-treated carrot cultures was observed in the fraction of PCA-soluble conjugates (Table 3). Many types of environmental stresses caused significant accumulation of Put in plant tissues, while the levels of other PAs remained unchanged. It was suggested

that endogenous Put levels might affect the redox state of plant cells. Recently, it has been observed that Put was more effective in increasing the activities of antioxidant enzymes [30].

Recently, we have found that pronounced accumulation of Put soluble conjugates in Cd^{2+}-treated tobacco cells coincided with the decline in the activity of diamine oxidase, an enzyme catalysing Put oxidative deamination [23]. This fact points to the important role of PA conjugation in controlling of free PA levels in cells under oxidative stress. However, the high level of PA conjugates was shown to inhibit cell division and to suppress differentiation in leaf explants of *Chrysanthemum morifolium* [2]. Therefore, according to the current knowledge we may suggest that on the one hand Put accumulation has a protective role in Cu^{2+}-treated cultures but on the other the high level of Put conjugates might negatively influence cell multiplication and suppress differentiation in treated carrot cultures.

The level of PCA-insoluble PAs decreased in the Cu^{2+}-treated carrot culture in dose-dependent manner. In the cell walls of Cu^{2+}-treated carrot diamines Cad and Put followed by triamine Spd were found. No clear relationship has yet been found between Cad and other PAs like Put, Spd and Spm. Cad is derived from lysine through lysine decarboxylase. In animal tissues Cad does not normally participate in any particular metabolic processes and it is completely excreted from the cells into the culture medium [19]. In plants Cad is most probably predominantely incorporated into the PA-insoluble fraction, as the levels of both free and soluble conjugated forms are very low (our results Table 3; [16]). In plant tissues under stress conditions lysine is catabolised into glutamate, an important signalling amino acid that regulates the responses to environment [14]. We may hypothesise that the marked decrease in bound Cad (to 62 and 40% in 1 and 10 μM Cu^{2+}-treated cultures, respectively, when compared with the control; Table 3), results from different metabolic channelling of lysine under Cu^{2+} stress. Considering that the cell-wall potential plays a central role in the regulation of plant cell-wall growth it is evident that the binding of PAs to negatively charged polygalacturonic acids lowers electrostatic potential of the wall. In this way PAs localized in cell walls could be involved in regulation of the growth processes [5].

The results presented here show that Cu^{2+} at 1 and 10 μM concentrations inhibited growth and rhizogenesis in the 15-week-old carrot rosettes regenerated from androgenous embryos. The accumulation of free and soluble ester-bound phenolic acids as well as the increase in soluble PA conjugates may be involved in a protective mechanism against heavy metal stress. On the other hand, high level of soluble Put conjugates and decreased content of cell wall bound phenolics and PAs may participate in the inhibition of growth and differentiation in the Cu^{2+}-treated carrot culture.

4. Materials and methods

4.1. Plant material

After harvesting the roots of donor carrot plants (*Daucus carota* L. cv. Narbonne) were kept in a chilling chamber at about +4 °C. After two months the roots were planted in pairs to 12-litter containers with a mixture of sand and peat (2:1, v/v) supplemented with a multicomponent complex fertilizer Azofoska containing 13.6% N, 1.9% P, 16.0% K with microelements Mg, Cu, Zn, Mn, B, Mo − 1.25 kg m^{-3} and chalk − 8.0 kg m^{-3}, and put into a greenhouse. The plants developed from the roots were kept in a greenhouse at about 20 °C. The detailed description of anther culture procedure was previously described by ref. [17]. The anther cultures were kept in darkness at the temperature of 27 °C. After emerging of the embryos, the cultures were transferred to continuous light and the temperature was kept the same. When the embryos become green they were transferred onto the regeneration medium B5.

B5 medium [15] with 20 g dm^{-3} sucrose and 6.5 g dm^{-3} agar and without aminoacids and growth regulators was supplemented with Cu^{2+}. The copper was added to the medium in the form of $CuSO_4 \times 5\ H_2O$ at concentrations: 1 μM, 10 μM, 100 μM. Cu^{2+} concentration in the control medium was 0.1 μM and pH was set at 5.6. Embryos were incubated under light (30 μmol m^{-2} sec^{-1}, 20 °C, photoperiod 16/8) for 15 weeks.

4.2. Phenolic acid analysis

Phenolic acids were extracted as described by ref. [7]. Briefly, free (F_1), ester-bound (F_2, released after alkaline hydrolysis) and glycoside-bound (F_3, released after acid hydrolysis) phenolic acids were obtained from a methanol extract of the tissue ground in liquid nitrogen. The 2,6-ditercbutyl β-cresol was used as an antioxidant to minimise the oxidation of phenolic acids during alkaline hydrolysis and nitrogen was immediately bubbled through the sample after NaOH addition. In spite of the antioxidant addition, the contents of chlorogenic and caffeic acids in the fractions of ester-bound phenolics (F_2, F_3) were lowered as indicated by the degradation of their standards. For this reason the values of ester-bound fractions of these two acids are only informative and are not mentioned in Table 1. Phenolic acids were analysed by means of HPLC using a Dionex Liquid Chromatograph (P660-HPLC Pump, ASI-100 Automated Sample Injector, TCC-100 Termostated Column Compartment, PDA-100 Photodiode Array Detector, Chromeleon Software 6.5) with C18 Spherisorb 5 ODS column (250 × 4.6 mm). Acetonitril and acetic acid gradient was used for elution. Phenolic acids were detected at their absorption maximum. λ_{max} was detected from the authentic compounds (Sigma-Aldrich, Prague, Czech Republic) that were used as references for quantitative analyses.

4.3. Polyamine analysis

The carrot tissue was ground in liquid nitrogen and extracted overnight at 4 °C with 5% (v/v) perchloric acid (PCA) (100 mg fresh weight tissue cm^{-3} 5% PCA). 1,7-Diaminoheptane was added as an internal standard. The extracts were centrifuged at 21,000 × *g* for 15 min, and then free PAs were analyzed in one-half of the supernatant. The

remaining supernatant and pellet were acid hydrolysed in 6 M HCl for 18 h at 110 °C to obtain PCA-soluble and PCA-insoluble conjugates of PAs as described by [32]. Standards (Sigma-Aldrich, Prague, Czech Republic), PCA-soluble free PAs, and acid hydrolysed PA conjugates were benzoylated. HPLC analysis of benzoyl-amines was performed on a Beckman-Video Liquid Chromatograph equipped with a UV detector (detection at 254 nm) and C_{18} Spherisorb 5 ODS2 column (particle size 5 μm, column length 250 × 4.6 mm) according to the method of [36].

4.4. Statistical analyses

Means ± SE of two independent experiments with two replicates are shown in the tables and figures. Statistical tests were analyzed using the Student's *t* distribution criteria.

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References

[1] S.B. Andersen, J. Christiansen, B. Farestveit, Carrot (*Daucus carota* L.): *in vitro* production of haploids and field trials, in: Y.P.S. Bajaj (Ed.), Biotechnology in Agriculture and Forestry, 12, Springer Verlag, Berlin, 1990, pp. 393–402.

[2] M. Aribaud, M. Carré, J. Martin-Tanguy, Polyamine metabolism and *in vitro* cell multiplication and differentiation in leaf explants of *Chrysanthemum morifolium* Ramat, Plant Growth Regul. 15 (1994) 143–155.

[3] P. Binarová, M. Cvikrová, T. Havlický, J. Eder, J. Plevková, Changes of shikimate pathway in glyphosate tolerant alfalfa cell lines with reduced embryogenic ability, Biol. Plant. 36 (1994) 65–73.

[4] A. Bouchereau, A. Aziz, F. Larher, J. Martin-Tanguy, Polyamines and environmental challenges: recent development, Plant Sci. 140 (1999) 103–125.

[5] D. Charnay, J. Nari, G. Noat, Regulation of plant cell-wall pectin methyl esterase by polyamines-Interactions with effects of metal ions, Eur. J. Biochem. 205 (1992) 711–714.

[6] E.L. Chen, Y.A. Chen, L.M. Chen, Z.H. Liu, Effect of copper on peroxidase activity and lignin content in *Raphanus sativus*, Plant Physiol Biochem. 40 (2002) 439–444.

[7] M. Cvikrová, L. Meravý, I. Macháčková, J. Eder, Phenylalanine ammonia-lyase, phenolic acids and ethylene in alfalfa (*Medicago sativa* L.) cell cultures in relation to their embryogenic ability, Plant Cell Rep. 10 (1991) 251–255.

[8] M. Cvikrová, P. Binarová, J. Eder, M. Vágner, M. Hrubcová, J. Zoń, I. Macháčková, Effect of inhibition of phenylalanine ammonia-lyase activity on growth of alfalfa cell suspension culture: alterations in mitotic index, ethylene production, and content of phenolics, cytokinins, and polyamines, Physiol. Plant. 107 (1999) 329–337.

[9] E.H. Daoudi, S. Biondi, Metabolism and role of polyamines in plant development, Acta Bot. Gallica 142 (1995) 209–233.

[10] C.H. DeVos, H. Schat, R. Vooijs, W.H.O. Ernst, Cd induced damage to permeability barrier in roots of *Silene cucubalus*, J. Plant Physiol. 135 (1989) 164–169.

[11] J. Diaz, A. Bernal, F. Pomar, F. Merino, Induction of shikimate dehydrogenase and peroxidase in pepper (*Capsicum anuum* L.) seedlings in response to copper stress and its relation to lignification, Plant Sci. 161 (2001) 179–188.

[12] R.A. Dixon, N.L. Paiva, Stress-induced phenylpropanoid metabolism, Plant Cell 7 (1995) 1085–1097.

[13] M.T. Fernandez, M.L. Mira, M.H. Florencio, K.R. Jennings, Iron and copper chelation by flavonoids: an electrospray mass spectrometry study, J. Inorg. Biochem. 92 (2002) 105–111.

[14] G. Galili, New insights into the regulation and functional significance of lysine metabolism in plants, Annu. Rev. Plant Biol. 53 (2002) 27–43.

[15] O.L. Gamborg, R.A. Miller, K. Ojima, Nutrient requirements of suspension culture of soybean root cells, Exp. Cell Res. 50 (1) (1968) 151–158.

[16] L. Gemperlová, J. Eder, M. Cvikrová, Polyamine metabolism during the growth cycle of tobacco BY-2 cells, Plant Physiol. Biochem. 43 (2005) 375–381.

[17] K. Górecka, D. Krzyżanowska, R. Górecki, The influence of several factors on the efficiency of androgenesis in carrot, J. Appl. Genet. 46 (2005) 265–269.

[18] H.C. Ha, N.S. Sirisoma, P. Kuppusamy, J.L. Zweier, P.M. Woster, R.A. Casero, The natural polyamine spermine functions directly as a free radical scavenger, Proc. Natl. Acad. Sci. USA 95 (1998) 11140–11145.

[19] L. Hawel III, C.V. Byus, A streamlined method for the isolation and quantitation of nanomole levels of exported polyamines in cell culture media, Anal. Biochem. 311 (2002) 127–132.

[20] C.A.P. Henderson, K.P. Pauls, The use of haploidy to develop plants that express several recessive traits using light-seeded canola (*Brassica napus*) as a example, Theor. Appl. Genet. 83 (1992) 476–479.

[21] Y. Hervé, Le Développement des variétés Hybrides F_1 chez plants léqumiérés, Sciences Agronomiques Rennes 6 (1977) 93–100.

[22] C. Kevers, N. Le Gal, M. Monteiro, J. Dommes, Th. Gaspar, Somatic embryogenesis of *Panax ginseng* in liquid cultures: a role for polyamines and their metabolic pathways, Plant Growth Regul. 31 (2000) 209–214.

[23] A. Kuthanová, L. Gemperlová, S. Zelenková, J. Eder, I. Macháčková, Z. Opatrný, M. Cvikrová, Cytological changes and alterations in polyamine contents induced by cadmium in tobacco BY-2 cells, Plant Physiol. Biochem. 42 (2004) 149–156.

[24] E. Lovaas, Antioxidant and metal-chelating effects of polyamines, in: H. Sies (Ed.), Advances in Pharmacology, Antioxidants in Disease Mechanisms and Therapy, vol. 38, Academic Press, New York, 1996, pp. 119–149.

[25] V. Lozovaya, T. Gorshkova, E. Yablokova, O. Zabotina, M. Ageeva, N. Rumyantseva, E. Kolesnichenko, A. Waranyuwat, J. Widholm, Callus cell wall phenolics and plant regeneration ability, J. Plant Physiol. 148 (1996) 711–717.

[26] W. Maksymiec, Effect of copper on cellular processes in higher plants, Photosynthetica 34 (1997) 321–342.

[27] J. Martin-Tanguy, Metabolism and function of polyamines in plants: recent development (new approaches), Plant Growth Regul. 34 (2001) 135–148.

[28] J.F. Moran, R.V. Klucas, R.J. Grayer, J. Abian, M. Becana, Complexes of iron with phenolic compounds from soybean nodules and other legume tissues: prooxidant and antioxidant properties, Free Radic. Biol. Med. 22 (1997) 861–870.

[29] R.A. Morrison, D.A. Evans, Haploid plants from tissue culture: new plant varieties in a shortened time frame, Bio/Technology 6 (1988) 684–690.

[30] A.K. Papadakis, K.A. Roubelakis-Angelakis, Polyamines inhibit NADPH oxidase-mediated superoxide generation and putrescine prevents programmed cell death induced by polyamine oxidase-generated hydrogen peroxide, Planta 220 (2005) 826–837.

[31] L. Purnhauser, G. Gyulai, Effect of copper on shoot and root regeneration in wheat, triticale, rape and tobacco tissue cultures, Plant Cell Tiss. Org. Cult. 35 (1993) 137–139.

[32] C.A. Rice-Evans, N.J. Miller, G. Paganga, Structure-antioxidant activity relationships of flavonoids and phenolic acids, Free Radical Biol. Med. 20 (1996) 933–956.

[33] D.R. Roberts, E.B. Dumbroff, J.E. Thompson, Exogenous polyamines alter membrane fluidity in bean-leaves-A basis for potential misinterpretation of their true physiological role, Planta 167 (1986) 395–401.

[34] R.G. Schipper, L.C. Penning, A.A.J. Verhofstad, Involvement of polyamines in apoptosis. Facts and controversies: effectors or protectors? Seminars Cancer Biol. 10 (2000) 55–68.

[35] C. Sgherri, E. Cosi, F. Navari-Izzo, Phenols and antioxidative status of *Raphanus sativus* grown in copper excess, Physiol. Plant. 118 (2003) 21–28.
[36] R.D. Slocum, H.E. Flores, A.W. Galston, L.H. Weinstein, Improved method for HPLC analysis of polyamines, agmatine and aromatic monoamines in plant tissue, Plant Physiol 89 (1989) 512–517.
[37] Z. Sroka, W. Cisowski, Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids, Food Chem. Toxicol. 41 (2003) 753–758.
[38] K. Takao, M. Rickhag, C. Hegardt, S. Oredsson, L. Persson, Induction of apoptotic cell death by putrescine, Int. J. Biochem. Cell Biol. 38 (2006) 621–628.
[39] W. Tang, R.J. Newton, Polyamines reduce salt-induced oxidative damage by increasing the activities of antioxidant enzymes and decreasing lipid peroxidation in *Virginia pine*, Plant Growth Regul. 46 (2005) 31–43.
[40] C. Theiss, P. Bohley, J. Voigt, Regulation by polyamines of ornithine decarboxylase activity and cell division in the unicellular green alga *Chlamydomonas reinhardtii*, Plant Physiol. 128 (2002) 1470–1479.
[41] T. Thomas, T.J. Thomas, Polyamine in cell growth and cell death: molecular mechanisms and therapeutic applications, Cell Mol. Life Sci. 58 (2001) 244–258.
[42] P. Torrigiani, D. Serafini-Fracassini, N. Bagni, Polyamine biosynthesis and effect dicyclohexylamine during the cell cycle of *Helianthus tuberosus* tuber, Plant Physiol. 84 (1987) 148–152.
[43] R.E. Veilleux, Development of new cultivars *via* anther culture, Hort. Sci. 29 (1994) 1238–1241.
[44] G. Wojnarowiez, C. Jacquard, P. Devaux, R.S. Sangwan, C. Clément, Influence of copper sulfate on anther culture in barley (*Hordeum vulgare* L.), Plant Sci. 162 (2002) 843–847.