INFLUENCE OF COPPER IONS ON THE PLANT MATERIAL OBTAINED FROM THE ANther CULTURE OF CARROT

(Daucus carota L.)

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Summary

The influence of copper ions on the regeneration of carrot (Daucus carota L.) androgenic embryos, accumulation of copper in rosettes, soluble ester-bound phenolic acids and some parameters of oxidative stress were investigated. Two carrots: cv. Feria and 1014 breeding line were subjected to 1 μM, 10 μM and 100 μM Cu stress for 16 and 24 weeks. Under this stress, better growth, lower lipid peroxidation (TBARS level) and higher phenolic acid contents were observed in the cv. Feria. The rosettes of 1014 line accumulated less copper and produced smaller amount of TBARS after 24 weeks of incubation than after 16 weeks. Chlorogenic and caffeic acids were the main phenolic acids in both cultures. In the Feria rosettes the application of 10 μM Cu caused relatively high level of chlorogenic acid combined with low accumulation of copper in the tissues and unchanged levels of TBARS after both 16 and 24 weeks of incubation. On the other hand, despite the dose-dependent decline of chlorogenic acid in the rosettes of 1014 line, decrease in TBARS content was also observed after 24 weeks. The obtained results might suggest that the Feria carrot culture was able to develop more effective protection system/strategy against Cu excess in comparison to the 1014 line.

key words: copper ions; anther culture; lipid peroxidation; phenolic acids

INTRODUCTION

Copper (Cu) is a redox-active transition metal which at low concentration is an essential element for plants. Due to its redox abilities it is a cofactor of many enzymatic reactions necessary for normal plant growth and development. However, at supra-optimal concentrations Cu causes

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oxidative stress and interferes with a number of physiological processes such as photosynthesis, respiration and others. It is suggested that probably the primary site of its toxicity are the cell membranes (Maksymiec & Krupa 2006).

Accumulation of copper in soil comes from human activities such as overfertilization, excessive use of copper-containing herbicides and pesticides, dumping of waste and municipal sewage and industrial pollution.

It is known that different plant compounds such as phenolic acids (mainly chlorogenic and caffeic acids), flavonoids, polyamines, proline and many others have been reported to be antioxidant and anticancerogenous agents (Sroka & Cisowski 2003, Górecka et al. 2007, Janas et al. 2009). A numerous group of phenolics formed during L-phenylalanine deamination reaction catalysed by L-phenylalanine ammonia-lyase activity (PAL, EC 4.3.1.5) plays an important role in increasing metal resistance. Among thousands of compounds found in different plant organs only some are free-aglycons while most of them are bound with sugars, organic acids, polyamines and others.

The important role of phenolic acids in embryo development is their involvement in alterations of the cell wall composition during differentiation and morphogenesis (Cvikrová et al. 1998). It is worth noticing that phenolic acids influence also plant growth and development. These compounds may participate in auxin metabolism, change membrane permeability, influence respiration and oxidative phosphorylation or protein synthesis (Vaughan & Ord 1991).

Pollen (microspore)-derived haploid plants provide a rapid means of obtaining homozygous and homogeneous lines of agriculturally important plants. It should be pointed out that one of the most important factors influencing the efficiency of regeneration of androgenic embryos into plants is the applied medium producing embryos which are modulated by several factors including genotypes, growth conditions of donor plants, abiotic stress and others (Ochatt et al. 2009). Mutation and transformation of androgenetic plant material cultured in the medium supplemented with particular toxins include heavy metals can lead to the selection of resistant forms in a relatively short time. Selection of plants for resistance to toxic ions can contribute to the obtaining of plants with better adaptability under stress conditions, which will enable better development of degraded environments (Lee et al. 2003).

The aim of this study was to determine the impact of copper ions on growth, accumulation of copper in rosettes, soluble ester-bound phenolic acids and some parameters of oxidative stress in plant material obtained from androgenic embryos of carrots.

MATERIAL AND METHODS

Experiments were carried out with carrot (Daucus carota L.) cv. Feria F1 and 1014 breeding line in 2007-2008. Detailed description of the anther culture procedure was previously given by Górecka et al. (2005). The anther cultures were kept in darkness at the temperature of 27°C. After emerging of the embryos, the cultures were transferred to con-
tinuous light and the temperature was kept the same. When the embryos become green they were transferred onto the B5 regeneration medium which was supplemented with CuSO₄ x 5 H₂O at concentrations: 1 μM, 10 μM, 100 μM. Concentration in the control medium was 0.1 μM and pH was set at 5.8. The plant material was incubated under light (30 μmol·m⁻²·sec⁻¹, 20°C, photoperiod 16/8) for 16 and 24 weeks and then counted and weighted.

The lyophilised carrot rosettes prepared according to Borowski (2003) were analysed for copper content with Perkin-Elmer ICP sequential spectrometer model Optima 2000 DV. This metal was detected at the 327.393 nm wavelength. The Merck ICP multi-element standard solution was used to prepare the calibration curve.

Lipid peroxidation in lyophilised carrot rosettes was evaluated by spectrophotometric measurements of thiobarbituric acid reactive substances (TBARS) levels, inter alia malondialdehyde (MDA), according to the modified Heath & Packer (1968) method.

For the determination of phenolic acids lyophilised carrot samples were prepared as described by Ama-rowicz et al. (2004). Phenolic acids were analyzed using a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) comprising a LC-10AD pump, SCTL 10A system controller, and SPD 10A photodiode array detector. Each sample was first filtered through a 0.45 μm nylon membrane and then injected onto a prepacked LiChrospher 100 RP-18 column (4 x 250 mm, 5 μm; Merck, Darmstad, Germany) (Lorenc-Kukula et al. 2009). The mobile phase consisted of water:acetonitrile:acetic acid (88:10:2; v/v/v), the flow rate was 1 ml·min⁻¹, and detection of phenolic acids was monitored at 320 nm. After HPLC analysis the content of phenolic acids in the injected sample was calculated from the plot of peak area vs. external standard concentration. All analyses were triplicated and the results are mean ± SD.

The experimental data were evaluated statistically according to Student’s test at a significance level of P=0.05.

RESULTS AND DISCUSSION

The adjustment of Cu concentration in culture media appears to be a key point in obtaining androgenic embryos and promoting plant regeneration (Dahleen 1995). These concentrations are different depending on the type of in vitro culture and the cultivar tested but when the optimal concentration is exceeded this may result in growth inhibition and other changes in metabolism. Growth of carrot rosettes as shown in Table 1 was adversely affected by Cu treatment particularly at the 100 μM where after 24 weeks of cultivation significant decreases in the total number and weight of regenerated rosettes were observed in both cultures. Similarly as in our experiments a significant inhibition of biomass accumulation of chamomile cultures was observed in the presence of 120 μM Cu by Kováčik et al. (2008). This is consistent with the reports of other authors about growth reduction in plants induced by Cu although its harmful concentrations vary considerably (Wiśniewski & Dickinson 2003, Alaoui-Sossé et al. 2004). On the other hand, some Cu
concentrations have a beneficial effect on anther cultures, improving both quantitative and qualitative yield of androgenesis (Wojnarowicz et al. 2002). This growth improvement was observed also in our study where in the 1014 line 10 μM of Cu caused significant increase in both total number and weight of rosettes (Table 1). The results presented above do not agree with our earlier study where Cu at 1 and 10 μM caused strong growth reduction of the carrot culture cv. Narbonne (Górecka et al. 2007) similarly as of radish seedlings (Chen et al. 2002). However, the highest Cu concentration (100 μM) decreased organogenic ability of embryos in both cultures but the effect was stronger in the 1014 line than cv. Feria (Table 1). Thus, it seems that these contrasting effects of Cu on carrot regeneration may be due to genotypic differences of individual carrot plants which were used to establish androgenic cultures similarly as described Ke et al. (2007).

Table 1. Effect of Cu on the growth of plant material regenerated from the embryos obtained in the anther culture of carrot (Daucus carota L.) cultivar: Feria and 1014 breeding line after 16 and 24 weeks of cultivation on B5 medium (Gamborg 1998). Control - 0.1 μM Cu.

<table>
<thead>
<tr>
<th>Cu (μM)</th>
<th>Total no. of regenerated rosettes</th>
<th>Weight of regenerated rosettes (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FERIA 16 weeks</td>
<td>24 weeks</td>
</tr>
<tr>
<td>0.1</td>
<td>18.2</td>
<td>12.6</td>
</tr>
<tr>
<td>1</td>
<td>18.7</td>
<td>9.6*</td>
</tr>
<tr>
<td>10</td>
<td>15.1</td>
<td>10.6</td>
</tr>
<tr>
<td>100</td>
<td>14.8</td>
<td>8.4*</td>
</tr>
</tbody>
</table>

Note: The symbols (*, **, *** ) show that results obtained from Cu-treated carrot cultures are significantly different from controls (0.1μM) at 0.05 and 0.01 level of Student’s test.

One of the aims of this work has been to check how much copper is accumulated in carrot tissues over time of incubation and how Cu influences cell membrane integrity. As it is shown in Table 2 the Cu accumulation in both Feria and 1014 line significantly increased only in the rosettes exposed to 100 μM Cu. However, it is interesting that, in the Feria rosettes grown at 100 μM Cu, the prolongation of incubation time from 16 to 24 weeks caused increase in Cu accumulation, while in the 1014 line decline of Cu content was observed (Table 2). The most visible decrease in the Cu accumulation was found in the 1014 line treated with 10 μM Cu for 24 weeks which was 62% of copper content in 16-weeks old rosettes. Similar tendency was seen in moss cells of Scopelophila cataractae where at the initial phase of Cu-treatment (30 days) the significant increase in copper content was noticed, and then (60 days) the accumulation of this metal declined (Konno et al. 2010).
It is known that thiorbarbituric acid reactive substances (TBARS) can be a good marker of stress and plants which accumulate more TBARS are more sensitive to stressors (Garcia et al. 1999). Therefore, it seems that changes in TBARS concentration in a plant tissue can be a good indicator of the structural integrity of the membranes subjected to Cu stress. The TBARS content of the Cu-treated Feria rosettes did not differ statistically from the controls after both 16 and 24 weeks of incubation. These results are similar to those reported by Kováčik et al. (2008) in the leaf rosettes of Cu-treated Matricaria chamomilla but contradictory with those observed in Hydrilla verticillata (Srivastava et al. 2006).

In presented work, despite a higher copper accumulation in the Feria rosettes grown at 100 µM Cu, TBARS content remained at the same level as in the rosettes treated with the other Cu concentrations. This fact may be the evidence for the efficiency of carrot antioxidant machinery in quenching free radicals preventing lipid peroxidation induced membrane damage. On the contrary, in the 1014 breeding line the TBARS level increased substantially after 16 weeks in the rosettes treated with all Cu concentrations (especially 100 µM Cu) and decreased to the levels similar as in Feria after 24 weeks (Table 2). This phenomenon can be linked to an overall inhibition of plant metabolism similar to that in salt-sensitive rice cultivars (Lutts et al. 1996) and may indicate that the 1014 line is more sensitive than cv. Feria.

Table 2. Effect of Cu on copper accumulation and TBARS contents in the plant material regenerated from the embryos obtained in the anther culture of carrot (Daucus carota L.) cultivar: Feria and 1014 breeding line after 16 and 24 weeks of cultivation on B5 medium (Gamborg 1968). Control (C) - 0.1 µM Cu.

<table>
<thead>
<tr>
<th>Cu (µM)</th>
<th>Copper content (µg·g⁻¹DW)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16 weeks</td>
<td>24 weeks</td>
<td>16 weeks</td>
<td>24 weeks</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>0.279</td>
<td>0.244</td>
<td>0.351</td>
<td>0.333</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.272</td>
<td>0.248</td>
<td>0.319</td>
<td>0.283</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.258</td>
<td>0.274</td>
<td>0.376</td>
<td>0.234**</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.442**</td>
<td>0.537***</td>
<td>0.541**</td>
<td>0.486**</td>
<td></td>
</tr>
<tr>
<td>TRABS (µmol·g⁻¹DW)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>0.241</td>
<td>0.202</td>
<td>0.437</td>
<td>0.260</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.265</td>
<td>0.199</td>
<td>0.467</td>
<td>0.191*</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.265</td>
<td>0.218</td>
<td>0.461</td>
<td>0.244</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.271*</td>
<td>0.237</td>
<td>0.560**</td>
<td>0.271</td>
<td></td>
</tr>
</tbody>
</table>

Note: see Table 1

Plants protect themselves against reactive oxygen species produced by Cu through synthesis of various small antioxidant molecules, including proline, phenolic acids, flavonoids and many others (Sharma & Dietz 2006). Thus, in the next experiment, the levels of phenolic acids were determined.
In the rosettes of cv. Feria and 1014 breeding line two derivatives of cinnamic acid (2 and 6 isoforms of chlorogenic and caffeic acid, respectively) were found. As reported by Niggeweg et al. (2004), chlorogenic acid acts as an antioxidant compound and protects against lipid peroxidation. Therefore, it can be assumed that relatively high level of chlorogenic acids in the Feria rosettes treated with 10 μM Cu (Fig. 1) and low accumulation of copper in tissues could contribute to the unchanged levels of TBARS after both 16 and 24 weeks of incubation (Table 2).

On the other hand, chlorogenic acids in the 1014 line seem to play a less important role because even if their level dose-dependently decreased, the membrane injury presented as TBARS level was also significantly reduced after 24 weeks of incubation. However, in this breeding
line dramatic increase in the proline content was also found after 24 weeks (data not shown) which can suggest a protective role of this amino acid. It was shown that various heavy metals induced accumulation of phenolic acids (Górecka et al. 2007), but in certain cases the reduction of these compounds was noted (Kováčik et al. 2008, Janas et al. 2009). The obtained results might suggest that the carrot culture of cv. Feria was able to develop more effective protection system/strategy against Cu excess in comparison to the 1014 line.

CONCLUSIONS

1. The cv. Feria seemed to be more resistant to Cu stress than the 1014 breeding line which was manifested by better regeneration capacity of embryos, lower TBARS level and higher phenolic acid contents.
2. The 16 weeks exposure of the 1014 line to all Cu concentrations had a deleterious effect on the cell membranes but the prolonged time promoted their repair and decreased TBARS level.
3. In the 1014 line prolongation of the Cu-treatment decreased copper accumulation in the rosettes, especially under 10 μM Cu stress.
4. Depending on cultivars, carrot cultures differently reacted to Cu treatment.

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REFERENCES


Górecka K., Cvikrová M., Kowalska U., Eder J., Szafranska K., Górecki R., Janas K.M. 2007. The impact of Cu treatment on phenolic and polyamine levels in plant material regener-


WPŁYW JONÓW MIEDZI NA MATERIAŁ ROŚLINNY UZYSKANY Z ZARODKÓW ANDROGENETYCZNYCH MARCHWI (Daucus carota L.)

Streszczenie

W niniejszej pracy zbadano wpływ różnych stężeń jonów miedzi na zdolności regeneracyjne zarodków androgenetycznych marchwi (Daucus carota L.), akumulację tego pierwiastka w rozetach, utlenianie lipidów błon komórkowych mierzonych stężeniem TBARS (substancje reagujące z kwasem tiobarbiturowym) oraz rozpuszczalnych estrów kwasów fenolowych. Do badań zastosowano pożywki B5 wg Gamborga i in. (1968), które zawierały 0,1 (kontrola); 1; 10 i 100 μM CuSO₄ x 5 H₂O. Materiał roślinny pasażowano na świeże pożywki i analizowano po 16 i 24 tygodniach. Odmiana Feria charakteryzowała się lepszym wzrostem, niższym stopniem peroksydacji lipidów błon oraz wyższą zawartością kwasów fenolowych w obecności Cu niż linia 1014. Po 24 tygodniach inkubacji rozety linii 1014 akumulowały mniej miedzi i TBARS niż po 16 tygodniach. Głównymi kwasami fenolowymi, zarówno w kulturze odmiany Feria jak i linii 1014, były kwas chlorogenowy i kawowy. W przypadku Ferii, rosnącej w obecności 10 μM Cu, stwierdzono relatywnie wysoką zawartość kwasu chlorogenowego a niską miedzi w tkankach oraz niezmieniony poziom TBARS zarówno po 16 jak i 24 tygodniach hodowli. Natomiast, w rozetach linii 1014 wraz ze wzrostem stężenia Cu w podłożu zaobserwowano spadek zawartości kwasu chlorogenowego oraz TBARS po 24 tygodniach inkubacji. Uzyskane wyniki mogą sugerować, że kultyury marchwi odmiany Feria posiadają lepszą strategię obrony przed nadmiarem miedzi niż linia hodowlana 1014.