EFFECT OF INCREASED COPPER CONCENTRATIONS ON DEFORMATIONS OF THE REGENERATES OF CARROT OBTAINED FROM ANDROGENETIC EMBRYOS

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Summary
The experiment aimed at determining the effect of high copper concentrations on the process of regeneration of carrot plants cv. Feria F1 from androgenetic embryos. The starting material consisted of green embryos obtained in anther cultures on a medium for the induction of androgenesis. Five B5 regeneration media of Gamborg et al. (1968) containing 0.1 (control), 1, 10, 100, and 1000 μM·L⁻¹ CuSO₄·5H₂O were used. The plant material was passaged onto fresh media 3 times after 4, 9, and 15 weeks from culture set-up date. During the passages, the regenerated structures were examined and categorized according to their growth, development and the course of regeneration in the in vitro culture.

The B5 regeneration medium that contained 1000 μM·L⁻¹ CuSO₄ was highly toxic for the growth and development of androgenetic embryos of carrot. The other concentrations of CuSO₄ that were higher than the level of the control medium, i.e. 1, 10, and 100 μM·L⁻¹, had a negative effect on the regeneration from androgenetic embryos of carrot in the second and third passages (after 9 and 15 weeks). They caused deformations of the leaves and the formation of an abnormal callus heel between the above-ground part and the root.

key words: androgenetic embryos, CuSO₄ concentration, regeneration, deformations of rosettes

INTRODUCTION

Copper is a microelement that is essential for normal growth and development of plants. In plant organisms it performs very important physiological and biochemical functions. It takes part in the processes of photosynthesis, respiration, conversion of nitrogen compounds, transport of carbohydrates, and also regulates the process of DNA formation (Podleśna & Wojcieska-Wyskupajtys 1996). Copper is a metal with diverse chemical properties. It has the ability to
form stable complex bonds with various organic compounds such as proteins, amino acids, cell wall components and others. These links play an important role in the uptake of copper ions by plant roots, their transport in vascular bundles, distribution in different tissues, and participation in metabolic functions. Copper can be taken up by the roots, and also by the leaves. Copper deficiency in plants causes chlorosis, lack of firmness in the shoots and leaves, and a serious drop in yield (Paluch et al. 2001). Most of the copper deficiency symptoms in plants are indirect or secondary effects (Kabata-Pendias 1996). Increased copper content in the soil is associated with the emission of pollutants produced by the copper smelting industry and electro-technical factories. Large amounts of copper find their way into the soil together with fertilizers, mainly phosphorus and calcium fertilizers, and pesticides. Plants are relatively resistant to copper poisoning, but excessive amounts of copper cause disturbances in metabolism resulting in limited growth and development. High concentrations of this element lead to the inhibition of the uptake by plants of important macro- and microelements, and block their transport (Bojarczuk 2002).

Many authors have pointed out the negative effects of high copper concentrations in in vitro cultures (Purnhauser & Gyulai 1993, Arnold et al. 1994, Prażak 2000a, Bojarczuk 2002). Such cultures have long been used to study plant responses to toxic metal ions. Using them makes it possible to study the mechanisms of resistance to metals in isolated cell lines with a view of regenerating tolerant plants (Gwoźdź & Kopyra 2003). Selection of plants in the natural conditions of high concentrations of metal ions, and also in in vitro conditions, can contribute to the obtaining of clones tolerant to toxic metal ions (Samanta-ray et al. 1999). By making use of in vitro cultures, plants have been obtained that tolerate increased concentrations of metal ions, including copper (Rout et al. 1998). Selection of plants for resistance to toxic ions contained in the soil can contribute to the obtaining of plant varieties with better adaptability under stress conditions, which will enable better development of degraded environments (Bojarczuk 2002).

Androgenesis in vitro is currently the most frequently used and the highest yielding method of obtaining haploids by stimulating the development of microspores or pollen grains into a callus or an androgenetic embryo (Malepszy 1979, Wang et al. 2000). By obtaining homozygous lines by means of in vitro anther cultures, the process of breeding heterozygous hybrids is several times shorter; moreover, the lines obtained by doubling the number of chromosomes in the haploids are fully homozygous. Regeneration of plants from embryos produced in anther cultures is an important stage of that process.

The aim of the experiment presented here was to determine the effect of high copper concentrations on the process of regeneration of carrot plants from androgenetic embryos.
MATERIALS AND METHODS

The starting material for the experiment were embryos of carrot cv. Feria F1, made to turn green under continuous light, obtained on a medium for inducing androgenesis in anther cultures carried out according to the procedure described by Górecka et al. (2005).

The best medium, as determined by earlier experiments, for the regeneration of androgenetic carrot embryos – the B5 medium of Gamborg et al. (1968) without amino acids and growth regulators, and with a copper concentration of 0.1 μM·L⁻¹ recommended by the authors – served as the control. This medium was modified by increasing the concentration of copper, in the form of CuSO₄·5H₂O, to: Cu-1 – 1 μM·L⁻¹, Cu-2 – 10 μM·L⁻¹, Cu-3 – 100 μM·L⁻¹, Cu-4 – 1000 μM·L⁻¹. The pH level of the media was adjusted to 5.8.

Twenty embryos, obtained from anther cultures in the process of androgenesis, were placed onto each of the media with the different Cu²⁺ concentrations (1 embryo per 1 test tube). The test tubes with the embryos were then placed in a breeding room at a temp. of 20°C, with a photoperiod of 16 hrs light (approx. 30 μmol·m⁻²·sec⁻¹) and 8 hrs darkness.

The material produced was passaged 3 times: after 4, 9 and 15 weeks of culture.

The following categories of the deformed structures obtained were distinguished:
- non-rooted and rooted rosettes with a varied extent of leaf deformation
- abnormal rosettes, non-rooted and rooted, with a callus heel

The experiment was carried out in 8 replications for each medium (1 replication = 1 test tube). The structures formed in the 8 test tubes were categorized and then counted and weighed.

For the experiment to continue, a portion of the multiplied material was transferred into 20 test tubes with a fresh medium of the same composition. The number and weight values in the result tables represent the average number and weight of the plant material obtained from 8 test tubes expressed in terms of ‘per 1 test tube’. The experimental results were analyzed with an analysis of variance in a one-factorial system. The differences between the mean values were compared with Newman-Keuls test at a significance level of P ≤ 0.05.

RESULTS

The concentration of copper in the medium Cu-4 - 1000 μM·L⁻¹ CuSO₄·5H₂O, caused all the embryos placed in that medium to be destroyed.

In the first passage (after 4 weeks of culture), there were found no rooted rosettes with callused, thickened leaves, no rooted rosettes with the root separated from the shoot by a callus, nor any rosettes with severely callused, deformed leaves on any of the regeneration media with increased copper concentrations, nor on the control medium. Within the first 4 weeks, the concentration of 100 μM·L⁻¹ CuSO₄ in the medium had already caused the formation of the
highest number (2.4 per 1 embryo) of non-rooted rosettes with callused, thick-
ened leaves, whose weight was 6 times greater than the weight of the rosettes
obtained on the control medium, and the difference was proven statistically.
Both the number of these rosettes and their weight were 6 times as high as in
the control.

After a longer regeneration time, that is, in the second and third passages
(after 9 and 15 weeks, respectively), the increased copper concentrations caused
various deformations of the rosettes obtained from androgenetic embryos. De-
formed leaves were observed – thick, callused leaves in the rosettes and also in
plants (Table 1). There were also abnormal, callused structures of the heel be-
tween the above-ground part, i.e. the green shoot, and the root (Table 2).

Severe deformations of plants in the second passage were already found on
the medium with 1 μM·L⁻¹ CuSO₄. After 9 weeks of culture, CuSO₄ at the con-
centrations of 1 μM·L⁻¹ and 10 μM·L⁻¹ in the media had contributed to the for-
formation of rooted and non-rooted rosettes with callused, thickened leaves
(Phot. 1). The highest number (5.9) of non-rooted rosettes with callused, thick-
ened leaves weighing 0.505 g had formed on the medium containing 10 μM·L⁻¹
CuSO₄. The highest number (14.3) of rooted rosettes with thickened, callused
leaves weighing 0.697 g per 1 test tube was obtained on the medium with
10 μM·L⁻¹ CuSO₄. A similar weight of 0.719 g per 1 test tube of rooted rosettes
with callused, thickened leaves was obtained on the medium with 1 μM·L⁻¹,
their number being 11.4. In the 9th week of culture, rosettes of this type had not
formed on the control medium and the medium containing 100 μM·L⁻¹ CuSO₄.
The differences were proven statistically significant, both for the number and
weight of the structures obtained.

After 15 weeks of culture, the highest number of rooted rosettes with se-
verely deformed leaves was found on the media containing 10 μM and 100
μM·L⁻¹ CuSO₄ (Phot. 2). The largest number of such rosettes (5.6) had formed
on the medium with 100 μM·L⁻¹, their weight also being the highest.

Rooted rosettes with an abnormal heel in the second passage were pro-
duced on the media containing 1 μM·L⁻¹ and 10 μM·L⁻¹ CuSO₄ (Phot. 3). The
highest number of them (2.3), weighing 0.053 g, had formed on the medium
with 10 μM·L⁻¹ CuSO₄, and the lowest number (0.8), weighing 0.046 g, were
obtained on the medium with 1 μM·L⁻¹ CuSO₄. In the third passage, rooted ro-
settes with a callus heel were found on the media containing 10 μM·L⁻¹ and 100
μM·L⁻¹ CuSO₄. On these media there were also plants with deformed leaves and
a heel of callus between the above-ground part and the root (Phot.4).
### Table 1. Effect of copper on the formation of rosettes with atypical, deformed leaves

<table>
<thead>
<tr>
<th>Passage</th>
<th>Medium</th>
<th>Non-rooted rosettes with callused, thickened leaves</th>
<th>Rooted rosettes with callused, thickened leaves</th>
<th>Rooted rosettes with severely callused, deformed leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>I 4 weeks after culture set-up</td>
<td>B5-K</td>
<td>0.4 b* 0.032 a*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cu-1</td>
<td>0.1 b 0.015 a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cu-2</td>
<td>0.0 b 0.000 a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cu-3</td>
<td>2.4 a 0.171 a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II 9 weeks after culture set-up</td>
<td>B5-K</td>
<td>2.3 a* 0.162 a*</td>
<td>0.0 b 0.000 b</td>
<td>0.0 a 0.000 a</td>
</tr>
<tr>
<td></td>
<td>Cu-1</td>
<td>2.8 a 0.228 a</td>
<td>11.4 a 0.719 a</td>
<td>4.0 a 0.277 a</td>
</tr>
<tr>
<td></td>
<td>Cu-2</td>
<td>5.9 a 0.505 a</td>
<td>14.3 a 0.697 a</td>
<td>0.0 a 0.000 a</td>
</tr>
<tr>
<td></td>
<td>Cu-3</td>
<td>2.4 a 0.374 a</td>
<td>0.0 b 0.000 b</td>
<td>0.5 a 0.067 a</td>
</tr>
<tr>
<td>III 15 weeks after culture set-up</td>
<td>B5-K</td>
<td>-</td>
<td>6.0 a 0.572 a</td>
<td>2.8 a 0.367 a</td>
</tr>
<tr>
<td></td>
<td>Cu-1</td>
<td>-</td>
<td>5.4 a 0.610 a</td>
<td>2.6 a 0.391 a</td>
</tr>
<tr>
<td></td>
<td>Cu-2</td>
<td>-</td>
<td>4.9 a 0.691 a</td>
<td>3.9 a 0.463 a</td>
</tr>
<tr>
<td></td>
<td>Cu-3</td>
<td>-</td>
<td>4.3 a 0.320 a</td>
<td>5.6 a 0.496 a</td>
</tr>
</tbody>
</table>

B5-K 0.1 μM·L⁻¹ CuSO₄·5H₂O; Cu-1 1 μM·L⁻¹ CuSO₄·5H₂O; Cu-2 10 μM·L⁻¹ CuSO₄·5H₂O; Cu-3 100 μM·L⁻¹ CuSO₄·5H₂O

Note: Values marked with the same letter within a column are not significantly different according to Newman-Keuls test at n=8, P≤0.05. The mean values are given in terms of ‘per 1 test tube’

<table>
<thead>
<tr>
<th>Passage</th>
<th>Medium</th>
<th>Non-rooted rosettes with callus heel</th>
<th>Rooted rosettes with root separated from shoot by callus</th>
</tr>
</thead>
<tbody>
<tr>
<td>I 4 weeks after culture set-up</td>
<td>B5-K</td>
<td>0.9 a* 0.032 a*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cu-1</td>
<td>0.0 a 0.000 a</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cu-2</td>
<td>0.5 a 0.028 a</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cu-3</td>
<td>0.5 a 0.046 a</td>
<td>-</td>
</tr>
<tr>
<td>II 9 weeks after culture set-up</td>
<td>B5-K</td>
<td>4.4 a* 0.166 a*</td>
<td>0.0 a 0.000 a</td>
</tr>
<tr>
<td></td>
<td>Cu-1</td>
<td>0.0 b 0.000 b</td>
<td>0.8 a 0.046 a</td>
</tr>
<tr>
<td></td>
<td>Cu-2</td>
<td>0.0 b 0.000 b</td>
<td>2.3 a 0.053 a</td>
</tr>
<tr>
<td></td>
<td>Cu-3</td>
<td>0.0 b 0.000 b</td>
<td>0.0 a 0.000 a</td>
</tr>
<tr>
<td>III 15 weeks after culture set-up</td>
<td>B5-K</td>
<td>-</td>
<td>0.0 a 0.000 a</td>
</tr>
<tr>
<td></td>
<td>Cu-1</td>
<td>-</td>
<td>0.0 a 0.000 a</td>
</tr>
<tr>
<td></td>
<td>Cu-2</td>
<td>-</td>
<td>0.6 a 0.179 a</td>
</tr>
<tr>
<td></td>
<td>Cu-3</td>
<td>-</td>
<td>1.3 a 0.105 a</td>
</tr>
</tbody>
</table>

Note: see Table 1.
Phot. 1. Non-rooted rosettes with callused leaves after passage II, Cu-2 10 μM·L⁻¹ CuSO₄·5H₂O

Phot. 2. Rooted rosettes with severely callused leaves after passage III, Cu-2 10 μM·L⁻¹ CuSO₄·5H₂O

Phot. 3. Rooted rosettes with a callus heel after passage II, Cu-2 10 μM·L⁻¹ CuSO₄·5H₂O

Phot. 4. Rooted rosettes with a callus heel and callused, thickened leaves after passage III, Cu-2 10 μM·L⁻¹ CuSO₄·5H₂O
DISCUSSION

In the experiment presented here it was evident that there was a negative effect of high copper concentrations of 1, 10, and 100 μM·L⁻¹ CuSO₄ on the development of androgenetic embryos of carrot in in vitro cultures after 9 and 15 weeks. However, in the first 4 weeks of culture, these copper concentrations had a positive effect on the process of regeneration. They stimulated the formation of normal rosettes and plants, but in the second and third passage (after 9 and 15 weeks) they inhibited the formation of normal structures. The increased copper concentrations also had a negative effect on the formation of secondary embryos (Kowalska et al. 2009).

In the second and third passages, an increase in copper concentration was observed to contribute to the deformations of the leaves of the non-rooted rosettes and plants of carrot obtained from androgenetic embryos. The results of this experiment coincide with the results obtained by other authors in their studies of various plant species regenerated on media with increased copper concentrations. Observations similar to ours had been made by Prażak (2000a) in relation to the regeneration of shoots in Triticum aestivum L. Copper at concentrations higher than those recommended for the MS medium had an unfavourable effect on the formation of shoots in wheat. The concentration of 100 μM·L⁻¹ CuSO₄ in the MS medium also had a negative effect on the formation of shoots in the hybrid Triticum x Aegilops (Prażak 2004). In yet another paper, the same author (Prażak 2000b) had stated that 100 μM·L⁻¹ CuSO₄ in Dendrobium kingianum BIDWILL caused the underside of the leaf blade to turn purple, indicating the appearance of anthocyanins and chlorosis of the leaves. These results suggest that plants try to protect themselves against too high a concentration of copper by synthesizing anthocyanins, which are known to be strong antioxidants (Hall 2002). Arnold et al. (1994), while carrying out in vitro cultures of birch, showed that 79 μM·L⁻¹ CuSO₄ caused the leaves to age and the shoots to wither away, whereas on a medium containing 157 μM·L⁻¹ CuCl₂ numerous deformations of the above-ground part appeared.

Purnhauser and Gyulai (1993) found that the concentrations of 300 μM and 1000 μM·L⁻¹ CuSO₄ markedly inhibited the regeneration of shoots from leaf fragments of Nicotiana tabacum, and in Brassica napus treatments with 0.1-100 μM CuSO₄ were not only ineffective but even inhibited growth. Bojarczuk (2002) showed that Cu at a concentration of 30 μM·L⁻¹ markedly impaired differentiation of cultures, almost completely inhibited shoot development in poplar, and reduced the quality of cultures by increasing chlorosis and making the leaves turn brown.

In our experiment, higher copper concentrations in the media affected the formation of a callus heel between the above-ground part and the root. Prażak (2000 a,b) had observed that higher levels of copper inhibited the regeneration of roots in Triticum aestivum L. in comparison with the control, and in Dendrobium kingianum BIDWILL caused the roots to become shorter and thicker. Growth inhibition and severe deformations of the roots in in vitro cultures of birch on a
medium with a high copper content of 179 μM·L⁻¹ CuSO₄ were also demonstrated by Arnold et al. (1994). Poplar roots proved to be even more sensitive to increased copper concentrations in nutrient media. At the concentration of 30 μM·L⁻¹ their growth was already almost completely inhibited (Bojarczuk 2002).

In our experiment, the concentration of 1000 μM·L⁻¹ CuSO₄ turned out to be highly toxic to androgenetic embryos of carrot. All of the embryos had already died in the first passage. Prażak (2000a) had demonstrated that 100 μM·L⁻¹ CuSO₄ was so toxic to Triticum aestivum L. that no shoots were obtained.

CONCLUSIONS

After 9 and 15 weeks of culture, the increased copper concentrations of 1, 10, and 100 μM·L⁻¹ CuSO₄·5H₂O caused deformations of the leaves of the rosettes of carrot obtained from androgenetic embryos and contributed to the formation of an abnormal callus heel at the base of the shoot.

The concentration of 1000 μM·L⁻¹ CuSO₄·5H₂O in the regeneration medium was toxic to carrot embryos.

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REFERENCES


WŁYW PODWYŻSZONYCH STĘŻEŃ MIEDZI NA DEFORMACJE REGENERANTÓW MARCHWI OTRZYMANYCH Z ZARODKÓW ANDROGENETYCZNYCH

Streszczenie

Badania miały na celu określenie wpływu wysokich stężeń miedzi na proces regeneracji roślin marchwi odmiany Feria F 1 z zarodków androgenetycznych. Materiałem wyjściowym były zazielenione zarodki uzyskane w kulturach pyłnikowych na pożywce do indukcji androgenesy. Zastosowano 5 pożywek regeneracyjnych B5 według Gamborga i in. (1968) zawierających 0,1 (kontrola); 1; 10; 100; 1000 μM.L-1 CuSO4.5H2O. Materiał roślinny pasażowano na świeże pożywki 3-krotnie po 4, 9 i 15 tygodniach od założenia kultury. Podczas pasaży przeprowadzono obserwacje zregenerowanych struktur, które zakwalifikowano do odpowiednich kategorii pod względem wzrostu, rozwoju i przebiegu regeneracji w kulturze in vitro.

Pożywka B5 do regeneracji zarodków androgenetycznych marchwi zawierająca 1000 μM.L-1 CuSO4 była silnie toksyczna dla ich wzrostu i rozwoju. Wyższe niż w standardowej pożywce stężenia CuSO4: 1, 10, 100 μM.L-1, w drugim i trzecim pasażu, po 9 i 15 tygodniach negatywnie wpłynęły na regenerację z zarodków androgenetycznych marchwi. Wywoływały deformację liści i tworzenie się nieprawidłowej piętki kalusowej pomiędzy częścią nadziemną a korzeniem.