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EFFECT OF INCREASED COPPER ION CONTENT IN THE MEDIUM ON THE REGENERATION OF ANDROGENETIC EMBRYOS OF CARROT (*Daucus carota* L.)

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Abstract

The study was conducted to determine the effect of elevated concentrations of copper in the medium on the regeneration of androgenetic embryos of the carrot cultivar 'Kazan F_1' obtained in anther cultures and to determine the level of soluble phenols produced in the regenerates under copper stress. Green embryos were laid out on 4 regeneration media based on B5 medium (Gamborg et al. 1968) without hormones, containing 0.1 – control, 1, 10, and 100 µM $CuSO_4 \times 5H_2O$. The plant material was passaged 3 times, after 4, 9 and 15 weeks. During these passages the emerging structures were examined; they were classified in terms of growth and development *in vitro*, weighed and counted. The levels of soluble phenols in the freeze-dried regenerates were determined.

The elevated concentrations of copper in the regeneration media affected positively the formation of complete plants (rooted rosettes) and secondary embryos during the first 4 weeks of culture. After a longer regeneration time (9, 15 weeks), the elevated concentrations of copper caused negative effects: deformation of rosettes. After 15 weeks, the number of rooted rosettes decreased. The 9-week culture subjected to copper stress brought about an increase in the amounts of soluble phenols. The highest values were recorded in the rosettes treated with 10 µM $CuSO_4$. Prolonged exposure to media containing elevated concentrations of $CuSO_4$ caused a reduction in the accumulation of phenolic compounds in the rosettes.

Key words: $CuSO_4$, deformation/deformities, phenols, *in vitro*, rosettes, stress

INTRODUCTION

Copper belongs to a group of heavy metals; it occurs naturally in trace amounts in the soil. As a result of the expansive nature of human activity, the natural environment becomes polluted and contaminated with heavy metals, including copper. The accumulation of copper in the soil is caused by over-fertilization of fields, the use of herbicides and pesticides, landfill disposal of municipal solid waste and sewage, and industrial pollution (Gruca-Królikowska and Wacławek, 2006).

Copper is a micronutrient essential for normal growth and development of plants. It takes part in many physiological processes; it acts as an important cofactor for many enzymes in the processes of respiration, photosynthesis and the transport of electrons (Yruela, 2005).

An excess of copper and copper deficiency both lead to various disorders in plants, such as growth inhibition, decrease in biomass, and ultimately their death (Zenk, 1996). At high concentrations, copper is highly toxic to all living organisms. Copper deficiency in higher plants manifests itself in the activation of morphological changes in the leaves and roots. Symptoms of deficiency appear in the early stages of plant development and are characterized by leaf deformity, chlorosis and even necrosis (Marschner, 1995).

As a redox metal, copper generates reactive oxygen species by the Fenton reaction, which can result in oxidative stress leading to the peroxidation of the lipids in cell membranes and damage to them, and can ultimately lead to cell death (Kováčik et al. 2008). For this reason, plants have developed a precise genetically-controlled mechanism for regulating copper levels within the cell (Lenartowicz, 2002). They produce various compounds, such as phenolic acids, flavonoids, polyamines and proline, which are known as antioxidants and anticarcinogens (Sroka and Cisowski, 2003; Górecka et al. 2007; Janas et al. 2009).

Phenols play an important role in increasing the resistance to adverse environmental factors such as UV radiation, extreme temperatures, and also heavy metals. The antioxidant activity of phenolic compounds consists in, for example, their high capacity for chelating metal ions. Phenolic compounds can inactivate metal ions and in addition inhibit the formation of reactive oxygen species by the Fenton reaction (Rice-Evans et al. 1997). Phenolic antioxidants inhibit the peroxidation of lipids (Millić et al. 1998). Since the presence of heavy metals can affect the accumulation of phenols in tissue, they become suitable candidates to act as biomarkers. These compounds can thus be used as indicators of early environmental stress before any morphological and structural damage appears (Białońska et al. 2007).

Many authors have observed in their studies a positive effect of higher concentrations of copper in *in vitro* cultures of various plant species. Elevated concentrations of copper have been found to increase the effectiveness of the formation of embryos and regenerates, and improve their quality. Such observations have been reported by Purnhauser and Gyulai (1993) in *Triticum aestivum* L. (wheat), (X *Triticosecale* Wittmack) (triticale) and *Nicotiana tabacum* L. (tobacco); Tahiliani and Kothari (2004) in *Triticum aestivum* L. (wheat); Dahleen (1995) in *Hordeum vulgare* L. (barley); Sahrawat and Chand (1999) in *Oryza sativa* L. (rice); Nirwan and Kothari (2003) in *Sorghum bicolor* L. (sorghum); Kothari et al. (2004) in *Eleusine coracana* L. (finger millet); Gori et al. (1998) in *Nicotiana tabacum* L. (tobacco); Saba et al. (1999) in *Lepidium* (peppergrass); Kumar et al. (2003) in *Tinospora cordifolia* and Sinha et al. (2010) in *Withania somnifera* L. (Indian ginseng).

There have also been reports of negative effects of elevated copper concentrations on *in vitro* regeneration: Purnhauser and Gyulai (1993) in *Brassica napus* L. and *Nicotiana tabacum* (L)., Gori et al. (1998) in *Nicotiana tabacum* (L.).

We are not aware of any reports on the effects of elevated copper concentrations on *in vitro* regeneration of carrot. The aim of this study was therefore to determine the effect of increased concentrations of copper (as sulphate) on the regeneration of androgenetic embryos of carrot cv. 'Kazan F_1' and the levels of soluble phenols in the regenerated plant material.

MATERIALS AND METHODS

The starting material consisted of embryos of the carrot cultivar 'Kazan F_1', made to turn green under continuous light and originating from anther cultures carried out according to the procedure developed by Górecka et al. (2005). They were transferred into test tubes onto 4 regeneration media: B5 medium by Gamborg et al. (1968) without amino acids and growth regulators, with 20 g sucrose, with 0.1 μM $CuSO_4 \times 5H_2O$ (control), and its modifications with the copper content increased 10, 100, and 1000 times relative to the control. The doses of copper in the different media were as follows: control – 0.1 μM, 1 μM, 10 μM, 100 μM $CuSO_4 \times 5H_2O$. The pH of the media was adjusted to 5.8.

There were 20 carrot embryos laid out on each medium (1 embryo per 1 test tube). The test tubes were placed in a breeding room at a temperature of +20°C, with a photoperiod of 16 h illumination at about 30 $\mu mol \times m^{-2} \cdot sec^{-1}$ and 8 h darkness. Three passages of the obtained material were carried out, after 4, 9 and 15 weeks of culture. The experiment was conducted in 10 replicates (1 replicate = 1 test tube). The following categories of the obtained structures were distinguished: green incipient rosettes, normal rosettes without roots, normal rooted rosettes, unrooted rosettes with a callus heel, rooted rosettes with callus separating the root from the shoot, and secondary embryos. The obtained plant material was counted and weighed.

Some of the multiplied plant material for use in further studies was transferred into 20 test tubes with a fresh medium of the same composition.

It was a univariate experiment. The results were analyzed statistically. Mean values were assessed with an analysis of variance using the Newmann-Keuls test at a significance level of $a \leq 0.05$ (5%).

Determination of soluble phenols

After counting and weighing, the obtained plant material was collected and frozen at a temperature of –80°C, and then subjected to the process of freeze drying. To determine the levels of soluble phenols, a spectrophotometric method was used, developed by Johanson and Shaal (1957) and modified by Singelton and Rossi (1965).

The freeze-dried carrot samples with elevated levels of copper and the control were placed, about 30 mg in each case, in test tubes containing 3 ml of 96% ethanol. The samples, tightly closed, were immersed for 2 minutes in a boiling water bath. After cooling, the tissue was homogenized in a chilled porcelain mortar. The homogenate was then centrifuged for 10 min. at 4000× *g* using a centrifuge (3K30, *Sigma*), and after centrifugation the resultant supernatant liquid was placed at +4°C in the dark. After 24 hours, the extract was supplemented with 80% ethanol to the original volume (3 ml) and treated as the source of soluble phenols. The test samples containing 0.5 ml of extract, 3.65 ml of distilled water and 0.1 ml of the Folin-Ciocalteu reagent were mixed thoroughly; after 3 minutes, 1 ml of 10% Na_2CO_3 was added; the samples were mixed again and left in the dark for 60 minutes. Because the extracts were coloured (the tissue contained chlorophyll), a blank sample was prepared for each of them, which instead of the Folin-Ciocalteu reagent contained an extra 0.1 ml of distilled water. After 1 hour, absorbance was measured at a wavelength of l = 660 nm with a spectrophotometer (U-2001, *Hitachi*), and the total phenolic content was read off the standard curve prepared on the basis of known concentrations of gallic acid.

RESULTS

In the first passage, 4 weeks after setting up the cultures, there was a positive effect of the increased concentrations of copper on the effectiveness of the regeneration of androgenetic embryos. Normal, rooted rosettes, i.e. complete plants, formed in greater numbers on the media with increased copper content than on the control medium (Fig. 4). The highest number of them per 1 embryo, 7.4, weighing 0.190 grams, was obtained on the medium with a 100 times higher concentration of $CuSO_4$ (Figs 1a, 2a). About half as many rosettes formed on the control medium. The highest number of green incipient rosettes from one embryo, 1.3, was also obtained on the medium with a 100 times higher copper content (Fig. 1a). Their weight was 0.013 grams (Fig. 2a). In the initial phase of the development of androgenetic embryos on the regeneration media, yellow crumbling structures, so-called secondary embryos, were formed, which in later stages turned into rosettes. The increased concentrations of $CuSO_4$ also contributed to the formation of secondary embryos. In the first passage, after 4 weeks of culture, the largest number of these structures from 1 test tube (13.2), weighing 0.023 g, was found on the medium with 1 μM $CuSO_4$, fewer (5.1) from the tube on the medium with 10 μM $CuSO_4$, whereas on the control medium there were only 2.4 such embryos per 1 test tube.

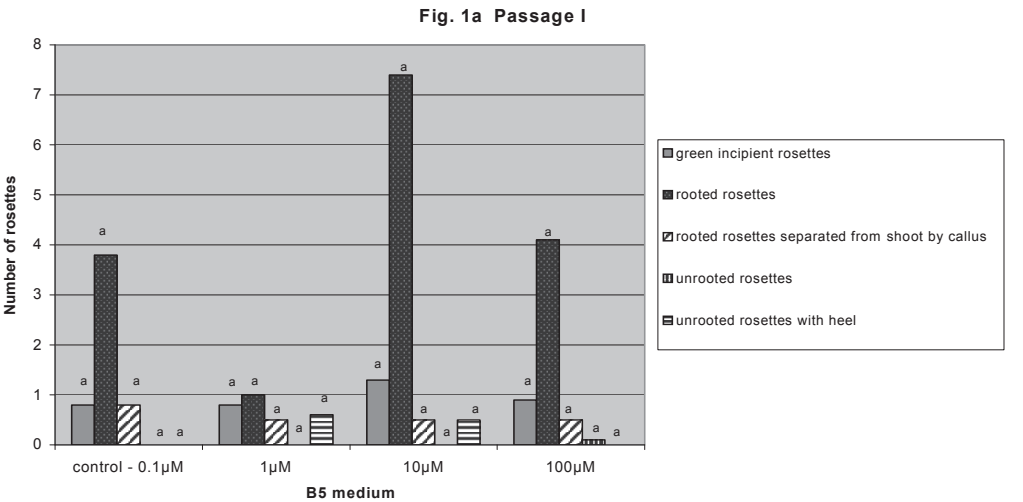
In the second passage, the 100 times higher copper content in the medium continued to positively influence the formation of complete plants. From 1 test tube, 3.7 rooted rosettes were obtained, weighing 0.229 g, while in the control 2.6, weighing 0.236 g (Figs 1b, 2b). With the increase in copper concentration, the number and weight of the resulting green incipient rosettes decreased. There was also an adverse effect of the elevated concentrations of copper on the formation of unrooted rosettes with callus at the base. About 3 times more of them were found on the medium with 1 and 10 μM $CuSO_4$ than on the control medium. Although rooted rosettes with the root separated from the shoot by callus were obtained on each of the analyzed media, the highest number of them (1.8), weighing 0.416 g, was recorded on the medium with 1 μM $CuSO_4$ (Figs 1b, 2b).

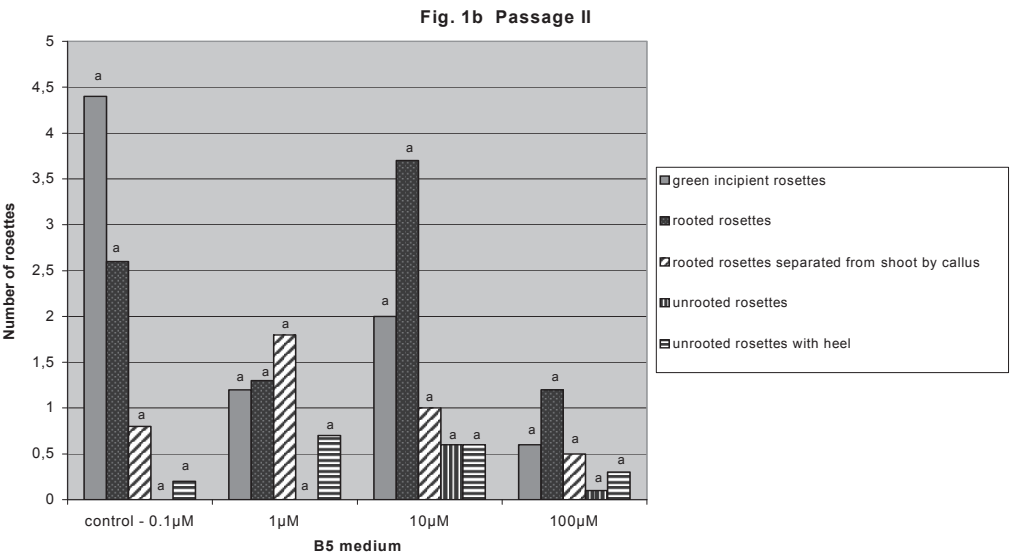
In the third passage, i.e. 15 weeks after the androgenetic embryos of carrot had been laid out on the different media, the increased levels of copper began to show a negative effect on the number of complete plants. The highest number of them from 1 test tube (7.3), weighing 0.152 g, was obtained on the control medium (Figs 1c, 2c). On the medium containing 10 μM $CuSO_4$, 5.5 rooted rosettes were obtained per 1 test tube, weighing 0.172 g. However, incipient rosettes were most numerous on the medium with 1 μM $CuSO_4$ – 4.2 against 2.9 in the control combination. The prolonged action of the media with higher Cu concentrations caused an increase in the number of unrooted rosettes with a callus heel. The highest number of them was found on the medium with 1 μM $CuSO_4$ – 2.9, weighing 0.158 g (Figs 1c, 2c).

The 100 times increase in Cu concentration in the medium in the 4th week of culture did not have a significant effect on the amount of soluble phenols in tissues and decreased it only slightly (Fig. 3). In the combinations with 1 and 100 μM $CuSO_4$, too small amounts of plant material were collected to enable their analysis.

After 9 weeks, the lowest levels of soluble phenols were found in the rosettes growing on the medium supplemented with 1 μM $CuSO_4$. In all the other variants, the amount of these compounds increased, and in the case of the concentration of 10 μM $CuSO_4$ it reached a value twice as high as that after the first passage.

The levels of soluble phenols decreased markedly in all the analyzed variants after 15 weeks (third passage), but the rosettes treated with 10 μM $CuSO_4$ still accumulated the highest amount of these compounds (Fig. 3).





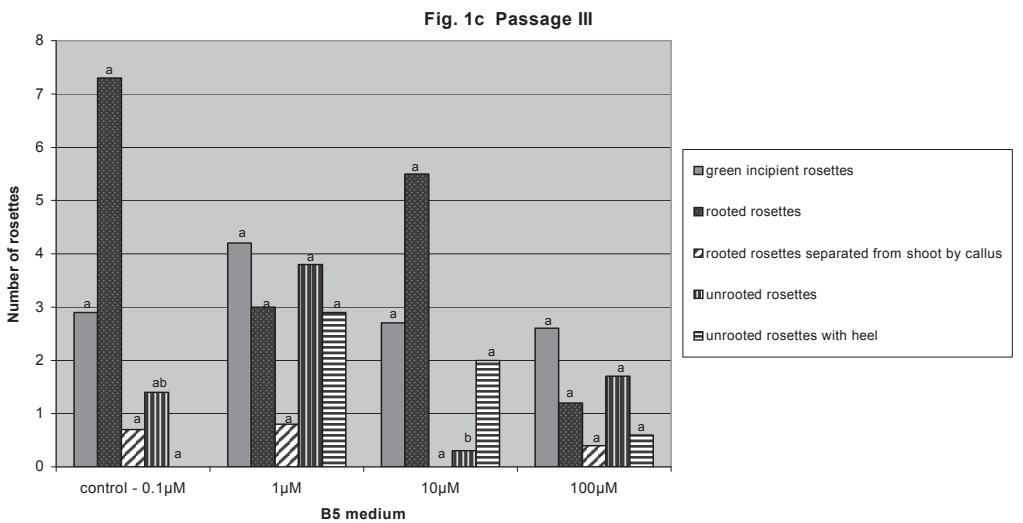
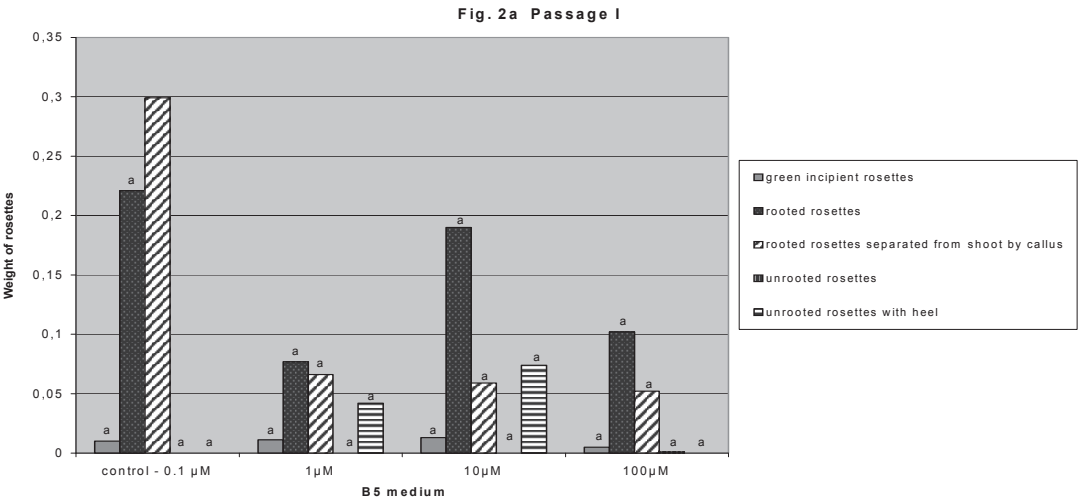
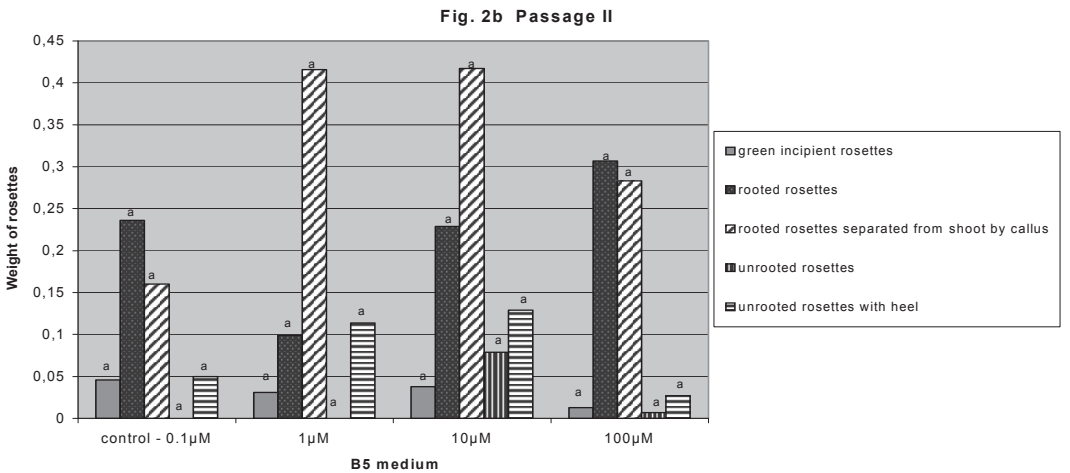


Fig. 1. Effect of elevated copper sulfate content in the medium on the number of rosettes regenerated on androgenetic embryos of carrot cv. 'Kazan F_1'
Fig. 1a – Passage I; Fig. 1b – Passage II; Fig. 1c – Passage III
It was a univariate experiment. Mean values were analyzed using the Newman-Keuls test at a significance level of $\alpha \leq 0.05$ (5%).





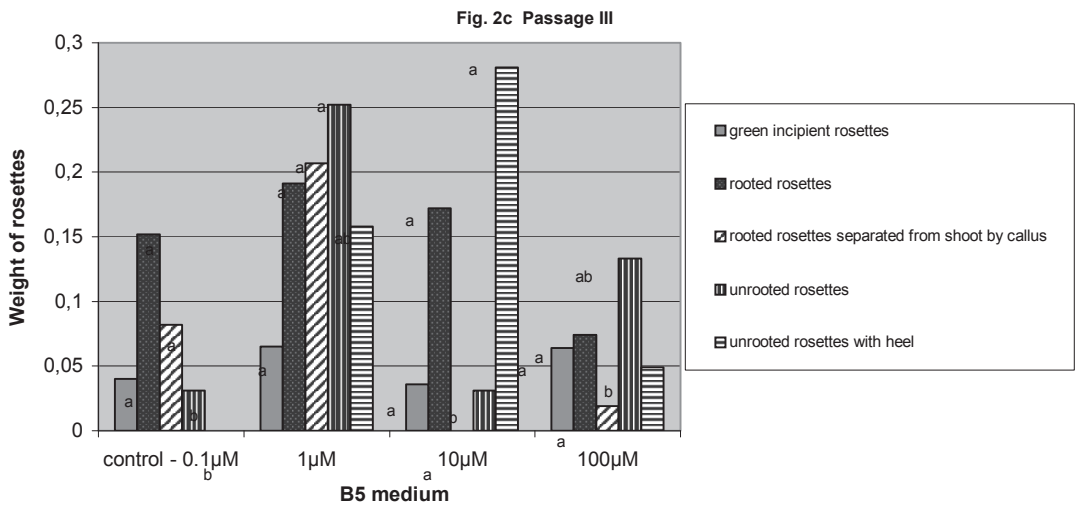


Fig. 2. Effect of elevated copper sulfate content in the medium on the weight of rosettes regenerated on androgenetic embryos of carrot cv. 'Kazan F_1'

Fig. 2a – Passage I; Fig. 2b – Passage II; Fig. 2c – Passage III

It was a univariate experiment. Mean values were analyzed using the Newman-Keuls test at a significance level of $\alpha \leq 0.05$ (5%).

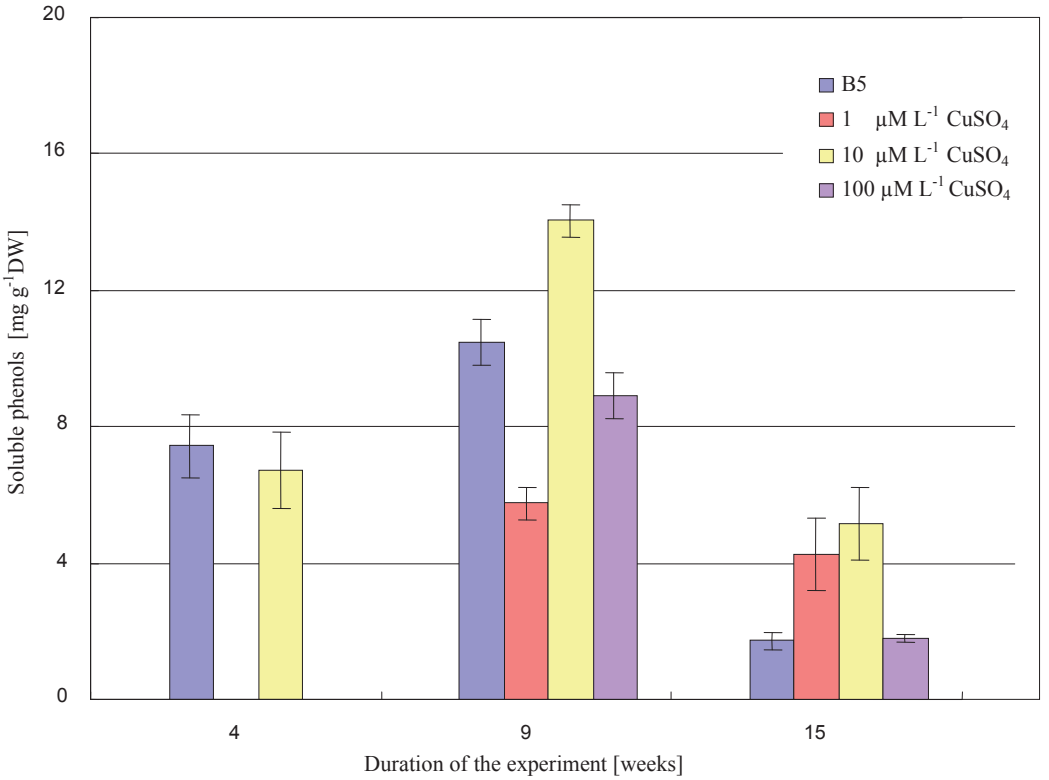
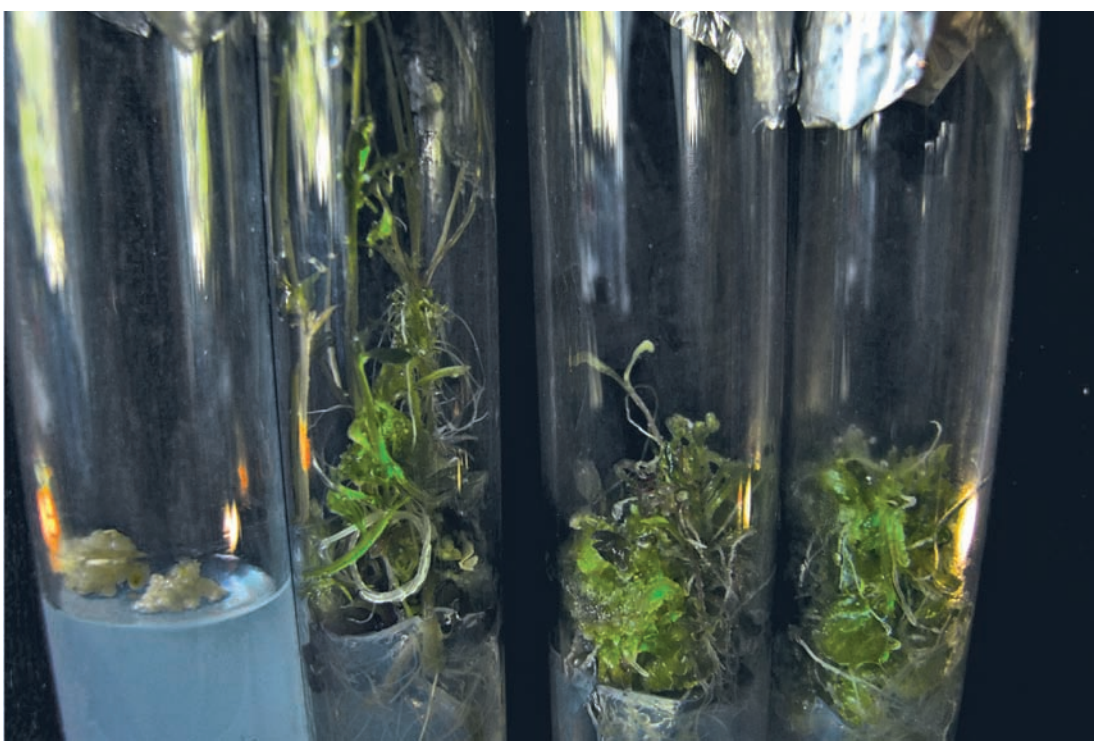


Fig. 3. Changes in the levels of soluble phenols in the rosettes of the carrot cultivar 'Kazan F_1' treated with various concentrations of $CuSO_4 \times 5H_2O$ for 4, 9 and 15 weeks.
Control plants were grown on B_5 medium with 0.1µM $CuSO_4$ (G a m b o r g et al. 1968).



Control – 0,1µM 1 µM 10 µM 100 µM

Fig. 4. Regeneration of androgenetic embryos of carrot, cv. Kazan F_1, after for 4 weeks on medium with various concentrations of $CuSO_4 \times 5H_2O$

DISCUSSION

The beneficial effects of elevated concentrations of copper in *in vitro* cultures have been emphasized by a number of authors. At higher concentrations of $CuSO_4$, Purnhauser and Gyulai (1993) observed significant growth of shoots regenerated from androgenic callus in *Triticum aestivum* L. (wheat) and X *Triticosecale* Wittmack (triticale). They also showed that the increased copper content in the medium for regeneration also had an effect on increasing the plant survival rate at the adaptation stage. Tahiliani and Kothari (2004), to improve regeneration from wheat callus, used culture media with increased $CuSO_4$ content in two lines, C-306 and R-3777. As little as 0.5 μM $CuSO_4$ had an effect on improving the induction of callus from immature embryos and the regeneration of plants. Based on his research, Dahleen (1995) concluded that the concentration of $CuSO_4$ in the standard MS medium is not optimal for callus cultures of *Hordeum vulgare* L. (barley) and that regeneration can be improved by using higher concentrations of copper. In rice, *Oryza sativa* L., increased concentrations of copper played an important role in improving its regeneration. The concentrations of 10 and 50 μM $CuSO_4$ in MS medium had a beneficial effect on the regeneration of plants from immature embryos in two Indian rice cultivars (Sahrawat and Chand, 1999). Wojnarowiez et al. (2002) claim that the concentration of copper in the medium is an important factor affecting *in vitro* androgenesis in cereals. In liquid media for isolated microspore cultures of barley, increased copper content can initiate the development of green plants in frequently occurring albino lines, thereby strengthening the viability of the plant material obtained in the process of regeneration. Joshi and Kothari (2007) reported that elevated concentrations of copper in the medium had a positive effect on the induction of shoot buds and their further development from cotyledon explants of *Capsicum annuum* (L.). On a medium with 3 μM $CuSO_4$, 41 shoots were obtained from 1 explant, and 17 of them were more than 2 cm in length.

In the study presented here, the increased concentrations of copper stimulated the formation of normal rooted rosettes during the first 4 weeks of culture.

There have also been reports in various species of adverse effects of elevated concentrations of copper in the induction of embryos and their regeneration. Researchers have also shown differences in the response of the tested species to the applied concentrations of $CuSO_4$. Purnhauser and Gyulai (1993) reported that $CuSO_4$ (0.1-100 μM) had no significant effect on the morphogenesis of callus cultures of *Brassica napus* (L.). Higher concentrations of copper did not improve the regeneration of shoots, and the highest concentration (100 μM) completely inhibited their regeneration and resulted in reducing the number of roots. Gori et al. (1998) observed in the tobacco variety Bel W3 that 50 μM $CuSO_4$ considerably inhibited the growth of callus and the regeneration of shoots after one month of culture. In the presence of 100 μM and 150 μM $CuSO_4$, the fresh matter content decreased substantially. 200 μM $CuSO_4$ almost completely inhibited the growth of callus. Copper concentrations higher than in the standard medium in longer-lasting cultures had a negative effect on the regeneration of androgenetic embryos of carrot cv. 'Feria F_1' (Kowalska et al. 2009).

Also in our study, we found that a longer exposure of the cultures on the media with elevated concentrations of copper resulted in deformities and adversely affected the number of rooted rosettes.

Research has been conducted on the mechanism of action of heavy metals, including copper, on living organisms. It shows that heavy metals may directly affect life processes of plants, their growth, development and aging, or indirectly through the production of jasmonates and ethylene (Maksymiec, 2007).

The production of phenolic compounds can play a very important defensive role under copper stress. Similar results have been observed in suspension cultures of ginseng roots of *Panax ginseng* C.A. Mayer (Ali et al. 2006), gibbous duckweed *Lemna gibba* L. (Babu et al. 2003), in the material regenerated from embryos obtained from anther cultures of carrot *Daucus carota* L. cv. 'Narbonne F_1' (Górecka et al. 2007), and in cultures of chamomile *Matricaria chamomilla* L. (Kováčik et al. 2008).

In our experiment, in the 9th week of culture, the lowest level of soluble phenols was observed in the regenerated rosettes on the medium with 1 μM $CuSO_4$. In other variants, the amount of these compounds increased markedly, and in the case of 10 μM $CuSO_4$ it reached a value twice as high as that after the first passage.

As reported by Jahangir et al. (2008), plants under the influence of stress caused by the action of heavy metals produce secondary metabolites, whose number increases with the concentration of the metal, but only to a certain point, beyond which a decrease in these metabolites can be observed.

In the present study, after 15 weeks of culture, the amounts of soluble phenols decreased markedly in all the analyzed variants. It seems that in this case such a situation might have been expected, and the prolonged period of stress caused a fall in the levels of soluble phenols; nevertheless, in all the passages the rosettes treated with 10 μM $CuSO_4$ accumulated the largest amounts of phenolic compounds.

CONCLUSIONS

Increased concentrations of copper had a positive effect on the formation of rooted rosettes during the first 4 weeks of culture.

After a longer regeneration time (9, 15 weeks), the elevated concentrations of copper caused negative effects, such as deformations of rosettes.

A 9-week culture on media with increased concentrations of copper had an effect on increasing the levels of soluble phenols. The highest values were recorded in the rosettes treated with 10 μM $CuSO_4$.

Prolonged exposure to media containing increased concentrations of copper caused a reduction in the accumulation of phenolic compounds.

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Wpływ podwyższonej zawartości jonów miedzi w pożywce na regenerację zarodków androgenetycznych marchwi (*Daucus carota* L.)

Streszczenie:

Badania przeprowadzono w celu określenia wpływu wyższych stężeń miedzi w pożywce na proces regeneracji zarodków androgenetycznych marchwi odmiany 'Kazan F_1' uzyskanych w kulturach pylnikowych oraz określenia poziomu rozpuszczalnych fenoli powstałych w regenerantach pod wpływem stresu miedzi. Zazielenione zarodki wykładano na 4 pożywki regeneracyjne B5 (Gamborg i in. 1968) bez hormonów zawierających 0,1 – kontrola; 1; 10; i 100 µM $CuSO_4 \times 5H_2O$. Materiał roślinny przepasażowano 3-krotnie po 4, 9 i 15 tygodniach. Podczas tych pasaży obserwowano powstające struktury, które odpowiednio sklasyfikowano pod względem wzrostu i rozwoju w kulturze *in vitro*. Analizowano liczbę i masę otrzymanych struktur. W zliofilizowanych regenerantach określono poziom rozpuszczalnych fenoli.

Podwyższone stężenia miedzi w pożywkach regeneracyjnych wpływały korzystnie na powstawanie kompletnych roślin (ukorzenionych rozet) i wtórnych zarodków w trakcie pierwszych 4 tygodni kultury.

Po dłuższym czasie regeneracji (9,15 tygodni) wyższe stężenia miedzi wywoływały negatywne efekty: deformacje rozet. Po 15 tygodniach spadała liczba ukorzenionych rozet.

9-tygodniowa kultura poddana stresowi miedzi wywołała wzrost zawartości fenoli rozpuszczalnych. Najwyższe wartości odnotowano w rozetach traktowanych 10µM $CuSO_4$. Długotrwała ekspozycja na pożywkach zawierających podwyższone stężenia $CuSO_4$ powodowała obniżenie akumulacji związków fenolowych w rozetach.