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EFFECT OF COPPER CONCENTRATION ON MICROPROPAGATION AND ACCUMULATION OF SOME METALS IN THE *DENDROBIUM KINGIANUM* BIDWILL ORCHID

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Abstract

The study focused on the influence of an increased copper content in the Murashige and Skoog (1962) solid medium on the *in vitro* plant growth and development of *Dendrobium kingianum* Bidwill. Sterile explants of pseudobulbs were used for micropropagation of orchid plants on the MS regeneration medium supplemented with 0.5 mg dm^{-3} NAA and 1.0 mg dm^{-3} kinetin. Copper (as $CuSO_4 \times 5H_2O$) was added to all the combinations in concentrations of 0.025 (control), 0.625, 1.25, 2.5 and 5.0 mg dm^{-3}. The results showed that the treatments with 1.25 and 2.5 mg dm^{-3} stimulated the orchid growth and development in *in vitro* culture. After eight months of growing in *in vitro* culture, the highest number of shoots, the longest roots and the heaviest fresh weight of plantlets were obtained in these treatments. In medium with the highest copper concentration (5.0 mg dm^{-3}), a negative influence of the metal on the length of roots and fresh weight of orchids was noted. Spectrophotometric analysis (ASA) showed that the copper and iron accumulation increased in both shoots and roots with the increase in the external Cu level, whereas the zinc and calcium accumulation in these organs decreased. The copper and zinc accumulation in the roots was about 1.5-2.5 times higher than in the shoots, but the iron accumulation was about 3-3.5 times higher. The calcium accumulation in roots was only 5-12% higher than in shoots.

Keywords: biometrical features, orchids, *in vitro* culture, accumulation of Ca, Zn, Fe, Cu.

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INTRODUCTION

The genus *Dendrobium* is a member of *Orchidaceae*, which is considered to be the largest plant family. *Dendrobium* is of considerable interest owing to its broad geographic distribution and high value as a commercial commodity (Meesawat et al. 2008).

Micronutrients, such as copper, control the processes of plant growth and development (Hirayama et al. 1999, Nirwan, Kothari 2003, Yruela 2005). Being a redox-active transition metal, copper has many functions, e.g. it is a cofactor for many enzymes, it is involved in photosynthesis and respiration processes, it participates in the signalling of transcription and hormones, protein trafficking machinery, lignin formation in cell walls and in oxidative stress responses (Hirayama et al. 1999, Yruela 2005). Besides, copper is known to be a component or activator of many enzymes involved in electron transport, protein and carbohydrate biosynthesis (Purnhauser, Gyulai 1993). In plants, rapid cell division and differentiation require adequate amounts of precursors of cell wall biosynthesis. Copper plays an important role in cell wall biosynthesis, signal transduction and cell wall lignification (Nas 2004).

Many authors have observed in their studies a positive effect of higher concentrations of copper in *in vitro* cultures of various plant species. The enhancing effect of high copper concentrations on morphogenetic responses of different species has been reported by Garcia-Sogo et al. (1991) in melon (*Cucumis melo* L.), Purnhauser and Gyulai (1993) in *Triticum aestivum* L. (wheat), (X *Triticosecale Wittmack*) (triticale) and *Nicotiana tabacum* L. (tobacco), Dahleen (1995) in *Hordeum vulgare* L. (barley), Cho et al. (1998) in *Hordeum vulgare* L. (barley), Gori et al. (1998) in *Nicotiana tabacum* L. (tobacco), Saba et al. (2000) in *Lepidium sativum* L. (peppergrass), Yang et al. (1999) in *Oryza sativa* L. (rice), Sahrawat et al. (1999) in *Oryza sativa* L. (indica rice), Nirwan and Kothari (2003) in *Sorghum bicolor* L. (sorghum), Tahiliani and Kothari (2004) in *Triticum aestivum* L. (wheat), Kothari et al. (2004) in *Eleusine coracana* L. (finger millet), Kumar et al. (2003) in *Tinospora cordifolia*, Amarasinghe (2009) in *Oryza sativa* L. (indica rice), Sinha et al. (2010) in *Withania somnifera* L. (Indian ginseng) and Kowalska et al. (2012) in *Daucus carrota* L. (carrot). There have also been reports of negative effects of elevated copper concentrations on *in vitro* regeneration, e.g. Purnhauser and Gyulai (1993) in *Brassica napus* L. and *Nicotiana tabacum* L., Gori et al. (1998) in *Nicotiana tabacum* L., Kowalska et al. (2009) in *Daucus carrota* L. There are no reports on the effects of elevated copper concentrations on the accumulation of some metals in orchid organs obtained in *in vitro* conditions. Copper may antagonistically affect the uptake and accumulation of other essential metals (Hunter, Verghano 1953). Some metals, such as the micronutrients Cu, Fe and Zn, are essential to plant metabolism. Calcium is an important macronutrient and a constituent of cell walls. Because calcium is not mobile in plants, if a plant runs out of its external supply of calcium, it cannot remobilize calcium from older tissues.

The aim of this paper was to study the effects of elevated levels of copper (25, 50, 100 and 200 times higher) in the MS culture medium on micropropagation of *Dendrobium kingianum* Bidwill ex Lindl. and copper, iron, zinc and calcium accumulation in its organs.

MATERIAL AND METHODS

Cultures of *Dendrobium kingianum* Bidwill were initiated from pseudobulbs about 30 mm long with two terminal leaves, collected from a plant growth chamber at the Subdepartment of Plant Biology, Faculty of Bioeconomy, University of Life Sciences in Lublin. The pseudobulbs were rinsed in 10% liquid dishwasher detergent, surface sterilized in 70% ethanol for 30 s, and then rinsed with sterile water three to four times. The explants were then sterilized in 0.1% $HgCl_2$ solution for 3 minutes, rinsed five times in sterile water, blotted dry with sterilized filter paper, and put on the MS (Murashige, Skoog 1962) basal medium containing 3% sucrose and 0.8% Difco bacto-agar, and supplemented with IAA (indolyl-3-acetic acid) at 0.5 mg dm^{-3} and BA (6-benzyl-aminopuryne) at 1.0 mg dm^{-3}. The explants were grown at 23±2°C with a photon fluence of 54 μmol m^{-2} s^{-1} and 16-h photoperiod. After two months, newly formed shoots about 20 mm long were separated, weighed and individually transferred (3 shoots per vessel, and 30 per treatment) to an MS multiplication medium (Figure 1) supplemented with NAA (1-naphthalene acetic acid) at 0.5 mg dm^{-3} and kinetin (6-furfurylaminopurine) at 1.0 mg dm^{-3} (Prażak 2001, 2014). Copper (as $CuSO_4 \times 5H_2O$) was added to all treatments in concentrations 25, 50, 100 and 200 times higher (0.625, 1.25, 2.5 and 5.0 mg dm^{-3}) than the standard content in the MS medium (0.025 mg dm^{-3}). Test glass culture vessels (100 ml) with Magenta B-caps as closures were dispensed with 20 ml medium respectively. The number and length of shoots and roots and the fresh weight were analysed after four and eight

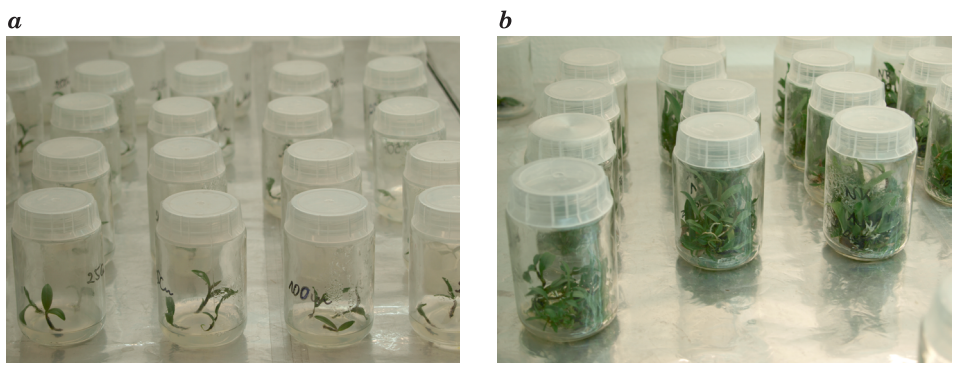

Fig. 1. *Dendrobium kingianum* Bidwill in the MS medium supplemented with different concentrations of copper: shoot explants in *in vitro* culture (*a*), plantlets after 8 months of *in vitro* culture (*b*)

months of growth (after 2 and 4 passages) in 20-24 plantlets from each treatment. This experiment was repeated twice (2 x 8 months).

17-19 plantlets from each treatment were divided into roots and shoots. Then both the shoot and root samples were washed in distilled water and placed in a forced air oven to dry at 70°C for 72 hrs. The dried plant material was digested in a diacid (HNO_3-$HClO_4$) mixture. After dilution, the digests were processed for copper (Cu), zinc (Zn), iron (Fe) and calcium (Ca) analysis using the ASA method.

The remaining plantlets were washed gently with tap water to remove traces of agar and nutrients, after which they were transplanted into plastic pots containing wet pieces of bark. The pots and plantlets were covered with a plastic bag for 2 weeks and were transferred into a plant growth chamber at 22-24°C, with a photon fluence of 200 μmol m^{-2} s^{-1} and a 16-h photoperiod.

Statistical analysis of the results was performed using analysis of variance on mean values, applying the Tukey's test for assessment of differences, at the significance level $\alpha = 0.05$.

RESULTS

The results obtained after four months showed that the treatments with copper sulphate in concentrations 0.625-2.5 mg dm^{-3} did not influence essentially the number of shoots and the number and length of roots of *Dendrobium kingianum* Bidwill (Table 1). However, mean values of morphological

Table 1

The influence of an increased copper (as $CuSO_4 \times 5H_2O$) content in the MS medium on biometrical features of *Dendrobium kingianum* Bidwill after 4 and 8 months *in vitro* culture (mean value of feature/1 explant)

Biometrical feature	Months	Concentration of $CuSO_4 \times 5\ H_2O$ (mg dm^{-3})					$LSD_{p=0.05}$
		0.025 (control) **	0.625 (25 x 0.025)	1.25 (50 x 0.025)	2.5 (100 x 0.025)	5.0 (200 x 0.025)	
Number of shoots	4	7.1	8.4	8.8	9.7	3.5	n.s.
	8	9.3	10.6	12.9*	13.9*	5.8	2.93
Shoot length (mm)	4	35.70	43.30*	38.40	40.59	37.06	6.422
	8	38.62	47.56*	45.50	41.05	38.57	7.598
Number of roots	4	10.8	13.2	11.8	11.6	6.5	n.s.
	8	14.2	15.7	16.1	16.6	11.2	n.s.
Root length (mm)	4	25.10	33.90	31.70	30.22	7.81*	6.941
	8	33.90	45.10*	42.90*	40.98*	17.50*	6.924
Fresh weight of plant (increment) (g)	4	0.92	1.22	1.39*	1.41*	0.29*	0.465
		(0.77)	(1.07)	(1.24*)	(1.26*)	(0.14*)	(0.459)
	8	1.59	2.05	2.27*	2.33*	0.66*	0.623
		(1.44)	(1.90)	(2.02*)	(2.18*)	(0.51*)	(0.617)

* – result significantly different from the control at $p = 0.05$
** – standard content of Cu in MS medium
n.s. – not significant versus the control at $p = 0.05$

traits showed an increasing trend following the increasing Cu concentration in the growth medium. The highest number of shoots (9.7) was noted in the 2.5 mg $CuSO_4 \times 5H_2O$ dm^{-3} treatment. The significantly longest shoots (43.30 mm) versus the control (35.70 mm) were noted in the 0.625 mg $CuSO_4 \times 5H_2O$ dm^{-3} treatment. The highest fresh weights (1.39 and 1.41 g) and fresh weight increment (1.24 and 1.26 g) of orchid plantlets were recorded in the treatments 1.25 and 2.5 mg $CuSO_4 \times 5H_2O$ dm^{-3}. These mean values significantly differ from the control treatment. The 5.0 mg $CuSO_4 \times 5H_2O$ dm^{-3} treatment led to a lower number of shoots and roots, root length (7.81 mm – significantly different from the control) and significantly lower fresh weight and fresh weight increment of orchid plantlets than in the control (0.025 mg $CuSO_4 \times 5H_2O$ dm^{-3} - standard content in MS medium).

After eight months (Figure 1), the number of multiple shoots and the increment of fresh weight increased essentially with the copper sulphate supplementation at concentrations 25-, 50- and 100-fold higher than the standard content in the MS medium (Table 1). The treatments with 1.25 mg $CuSO_4 \times 5H_2O$ dm^{-3} and 2.5 mg $CuSO_4 \times 5H_2O$ dm^{-3} were found to have a significant effect on the *Dendrobium kingianum* Bidwill plantlets' number of shoots and length of roots, and fresh weight as well as fresh weight increment. The elevated levels of copper were very effective in stimulating shoot formation. The highest number of shoots (12.9 and 13.9 shoots/explant), and the highest fresh weight (2.27 and 2.33 mg/explant) and fresh weight increment (2.02 and 2.18 mg/explant) were achieved while using copper sulphate at these concentrations. The 5.0 mg $CuSO_4 \times 5H_2O$ dm^{-3} treatment led to a lower mean number and length of shoots and roots and lower fresh weight and its increment of orchid plantlets than in the control (0.025 mg $CuSO_4 \times 5H_2O$ dm^{-3}), but the differences were not significant except the length of roots.

The copper accumulation in both shoots and roots increased with the increase in the external Cu level (Figure 2). The accumulation of copper in roots was higher than in shoots in all the treatments (Figure 2). The maximum Cu content was observed in both roots (73.6 mg kg^{-1} DW) and shoots (28.7 mg kg^{-1} DW) at the highest level of Cu (5.0 mg dm^{-3} $CuSO_4 \times 5H_2O$), followed by 33.4 and 15.0 mg Cu kg^{-1} DW of roots and shoots, respectively, at 2.5 mg $CuSO_4 \times 5H_2O$ dm^{-3} of the MS medium, and 14.6 and 8.19 mg Cu kg^{-1} DW of roots and shoots, respectively, at 1.25 mg $CuSO_4 \times 5H_2O$ dm^{-3} of the MS medium, and 7.8 and 4.45 mg Cu kg^{-1} DW of roots and shoots, respectively, at 0.625 mg $CuSO_4 \times 5H_2O$ dm^{-3} of the MS medium.

The iron (Fe) content in roots tended to increase with the increasing Cu concentration in the growth medium, while just slightly rising in shoots (Figure 3). The iron concentration increased by up to 72% (228 to 391 mg Fe kg^{-1} DW from 0.025 to 5.0 mg $CuSO_4 \times 5H_2O$ dm^{-3} of MS medium) in roots and 40% (76.5 to 107.0 mg Fe kg^{-1} DW from 0.025 to 5.0 mg $CuSO_4 \times 5H_2O$ dm^{-3} of MS medium) in shoots (Figure 3).

The zinc (Zn) content in roots and shoots showed a decreasing trend with the increasing Cu concentration in the growth medium (Figure 4). The zinc

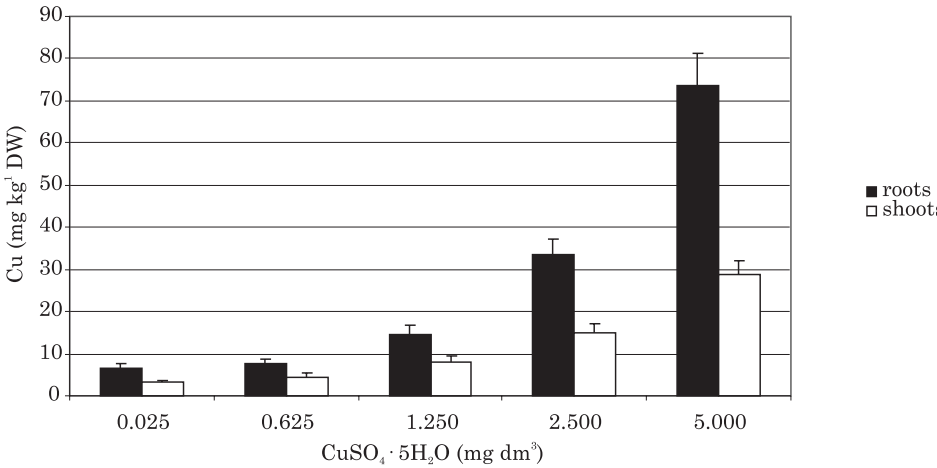

Fig. 2. Copper content in roots and shoots of *Dendrobium kingianum* Bidwill after 8 months growing in the MS medium supplemented with different concentrations of copper (as $CuSO_4 \times 5\ H_2O$)

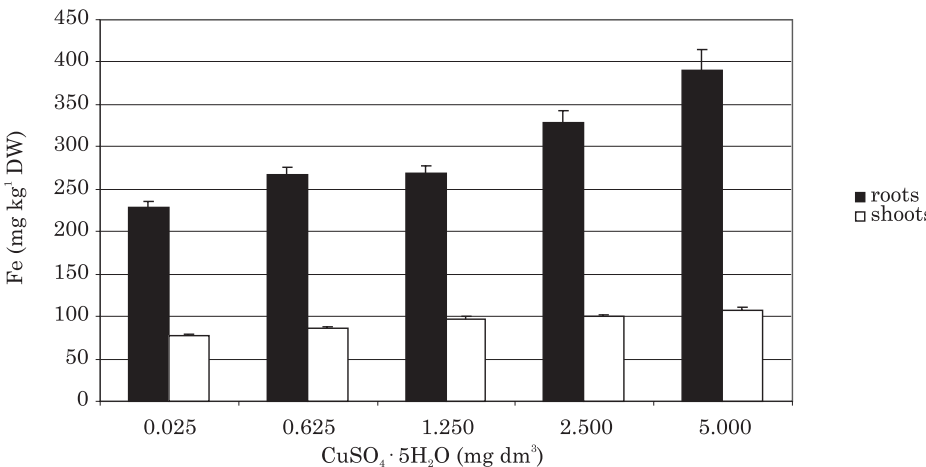

Fig. 3. Iron content in roots and shoots of *Dendrobium kingianum* Bidwill after 8 months growing in the MS-medium supplemented with different concentrations of copper (as $CuSO_4 \times 5\ H_2O$)

concentration decreased by as much as 21% (151 to 119 mg Zn kg^{-1} DW from 0.025 to 5.0 mg $CuSO_4 \times 5H_2O$ dm^{-3} of MS medium) in roots and 9% (78.9 to 71.7 mg Zn kg^{-1} DW from 0.025 to 5.0 mg $CuSO_4 \times 5H_2O$ dm^{-3} of MS medium) in shoots (Figure 4).

The calcium (Ca) content in roots and shoots showed a decreasing trend (0.025-5.0 mg $CuSO_4 \times 5H_2O$ dm^{-3}) with the increasing Cu concentration in the growth medium (Fig. 5). The calcium concentration decreased by 23% (5530 to 4260 mg Ca kg^{-1} DW) from 0.025 to 5.0 mg $CuSO_4 \times 5H_2O$ dm^{-3} of

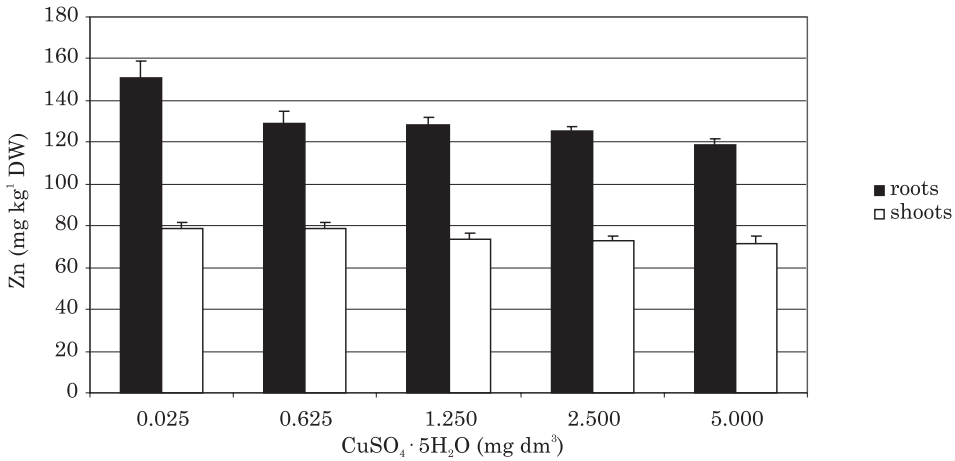

Fig. 4. Zinc content in roots and shoots of *Dendrobium kingianum* Bidwill after 8 months growing in the MS-medium supplemented with different concentrations of copper (as $CuSO_4 \times 5\ H_2O$)

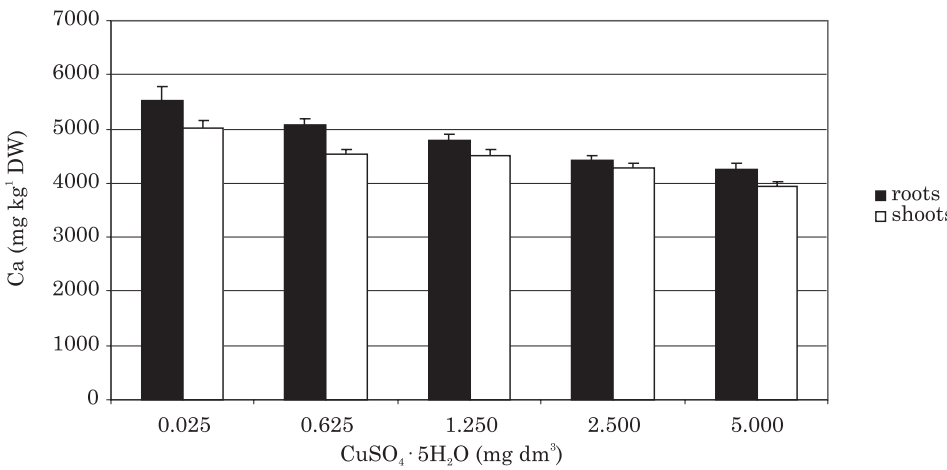

Fig. 5. Calcium content in roots and shoots of *Dendrobium kingianum* Bidwill after 8 months growing in the MS-medium supplemented with different concentrations of copper (as $CuSO_4 \times 5\ H_2O$)

MS medium) in roots and 22% (5020 to 3930 mg Ca kg^{-1} DW from 0.025 to 5.0 mg $CuSO_4 \times 5H_2O$ dm^{-3} of MS medium) in shoots (Figure 5).

DISCUSSION

The experiment showed that 25-, 50-, 100-fold increase in the copper sulphate concentration in the MS-medium had a favourable effect on the

growth and development of *Dendrobium kingianum* Bidwill orchid plants in *in vitro* conditions. The increase in copper concentration strongly stimulated the shoot and root growth and development as well as the plantlets' fresh weight increment. The experiment showed that 50- and 100-fold increase in copper sulphate concentration in the MS medium (1.25, 2.5 mg dm^{-3}) after 8-months of growth significantly affected the number of shoots and fresh weight increment in *Dendrobium kingianum* Bidwill orchid plantlets growing in *in vitro* conditions. In these treatments, the plantlets developed the biggest number of shoots. These data suggest that the higher levels of copper stimulated shoot formation in the explants, and it might be necessary to use higher levels of this medium component in order to enhance the morphogenetic potential of explants. The 200-fold increase in the copper content in the MS medium led to a lower mean number and length of shoots and roots and lower fresh weight and its increment of orchid plantlets than in the control (0.025 mg $CuSO_4 \times 5H_2O$ dm^{-3} - standard content of Cu in MS medium), but the differences were not significant.

Many authors point to a positive influence of higher copper ions concentrations on organogenesis of many plants. Nas (2004) suggested that higher levels of Cu (up to 5.1 mg $CuSO_4 \times 5H_2O$ dm^{-3}) enabled hazelnut (*Corylus* spp.) explants to divert their resources from unorganized cell division (callus production) to cell differentiation (shoot and bud formation), and it might be necessary to use higher levels of this medium component in order to enhance the morphogenetic potential of explants. These findings are supported by the results observed by Garcia-Sogo et al. (1991), who reported that the addition of $CuSO_4 \times 5H_2O$ at high concentrations (0.1 to 5.0 mg dm^{-3}) to culture medium increased the organogenic response of explants, the frequency of organogenic callus, the extension of organized growing areas in the calli and the development of organogenic structures in melon (*Cucumis melo* L.). The most favourable concentration was found to be 1.0 mg of copper sulphate per dm^3.

Our findings suggest that higher levels of Cu (mostly 0.625 mg $CuSO_4 \times 5H_2O$ dm^{-3}) stimulated the formation of longer orchid shoots and roots. Rafail et al. (2012) reported that the highest number of branches and leaves per explant for apple and pear was obtained in a treatment with 0.05 mg dm^{-3} of copper in culture medium. At the rooting stage, the elevated levels of copper were very effective in stimulating root formation in both apple and pear shoots. The highest number of roots in apple was achieved while adding 0.1-0.2 mg dm^{-3} of copper to culture medium. These data suggest that the higher levels of copper enabled shoot and root formation in the explants, and it might be necessary to use higher levels of this medium component in order to enhance the morphogenetic potential of explants. Joshi and Kothari (2007) reported that elevated concentrations of copper in medium had a positive effect on the induction of shoot buds and their further development from cotyledon explants of *Capsicum annuum* L. The highest number of shoots and longer shoots were obtained from 1 explant on medium with 4.8 mg $CuSO_4$ dm^{-3}.

Dahleen (1995) concluded that the concentration of $CuSO_4$ in the standard MS medium is not optimal for callus cultures of *Hordeum vulgare* L. and that regeneration can be improved by using higher concentrations of copper. Amarasinghe (2009) reported that the rate of callus proliferation and plant regeneration from callus in the screened traditional indica rice (*Oryza sativa* L.) varieties in Sri Lanka was significantly higher in the medium supplemented with 5 mg dm^{-3} copper sulphate. In regeneration, the highest number of normal plants with the least number of albino plants could be obtained in media containing 5 mg dm^{-3} copper sulphate in combination with 5 mg dm^{-3} cobalt chloride.

The results of the current study are partially in agreement with those published by Garcia-Sogo et al. (1991), who reported that the addition of high concentrations of $CuSO_4 \times 5H_2O$ (0.1 to 5.0 mg dm^{-3}) to culture medium increased the organogenic response of explants in melon. Only the concentration of 5.0 mg $CuSO_4 \times 5H_2O$ dm^{-3} in the MS medium negatively influenced the biometric features of *Dendrobium kingianum* Bidwill, i.e. number of shoots, number and length of roots, and fresh weight of plants.

As in many other plants, differential Cu transport may antagonistically affect the uptake and accumulation of other essential inorganic nutrients (Hunter, *Verghano* 1953). In the present study, an increase in the copper accumulation in both shoots and roots was associated with an increase in the external Cu level. An increased copper content in the MS medium led to a slight decrease in Zn and Ca accumulation and an increase in Fe accumulation in these organs. The copper and zinc accumulation in roots was about 1.6-2 times higher than in shoots, but that of iron was 3-3.7 times higher. Jankowski et al. (2014) also reported that the copper and zinc accumulation in oilseed rape roots was higher than in its straw.

Our findings further showed that the calcium accumulation in roots was only 5-12% higher than in shoots. Calcium is an essential macronutrient for plants, for example it plays an important role in intercellular signaling, stomatal regulation and cell wall stabilization (White, Broadley 2003). Zinc is an essential minor element, which plays many functional, regulatory and structural roles in many enzymes. This element is also important for auxin production, carbohydrate and protein metabolism, protection of cells against oxidative stress, photosynthetic reaction and maintenance of the membrane structure and functions (Brennan, Bolland 2006). Zn toxicity induces chlorosis in plants, either by competing with the uptake of other elements or due to reduction in the synthesis of chlorophyll because of Fe deficiency (Broadley et al. 2007, Dospatliev 2011).

CONCLUSIONS

1. The 50- and 100-fold increase in copper content in the MS medium positively influenced micropropagation (number of shoots) in *Dendrobium kingianum* Bidwill orchid plants. These higher levels of copper in the MS culture medium could be said to enhance morphogenetic response of orchid pseudobulb explants and the success of many micropropagation programs.

2. In the variant with the highest copper concentration (5.0 mg $CuSO_4 \times \times 5H_2O$ dm^{-3}), a negative influence of the metal on the length of roots and fresh biomass of *Dendrobium kingianum* Bidwill was noted.

3. The copper accumulation in both shoots and roots increased with the increase in the external Cu level, but the content of other metals was only slightly affected at higher Cu concentrations (Zn and Ca content decreased but Fe increased both in roots and in shoots).

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