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ORIGINAL PAPER

EFFECT OF MANGANESE ON THE *IN VITRO* DEVELOPMENT AND ACCUMULATION OF IRON AND MAGNESIUM IN *DENDROBIUM KINGIANUM* BIDWILL

Roman Prażak[1], Jolanta Molas[2]

[1]Department of Genetics and Horticultural Plant Breeding
[2]Department of Plant Physiology
University of Life Sciences in Lublin

Abstract

The objective of the study was to determine the effect of increasing manganese concentrations, 2, 4, 8 and 16 times higher than the standard content in Murashige and Skoog (1962) medium, on morphological traits and on the accumulation of iron and magnesium in the roots and shoots of *Dendrobium kingianum* Bidwill. For micropropagation of orchid plants we used shoots placed on solid MS medium supplemented with 1.0 mg dm^{-3} kinetin and 0.5 mg dm^{-3} NAA, as well as different concentrations of manganese (as $MnSO_4 \cdot 4H_2O$): 22.3 (control), 44.6, 89.2, 178.4 and 356.8 mg dm^{-3}. After 18 months of *in vitro* culture, the increased concentrations of manganese, from 44.6 to 356.8 mg dm^{-3} in the MS medium, had no stimulatory effect on orchid development in the *in vitro* culture. The greatest number and length of shoots and roots and the greatest fresh weight of plantlets were noted in the control. At the high manganese concentrations of 178.4 and 356.8 mg dm^{-3} in the MS medium, the number and length of these organs and the fresh weight of entire plantlets were statistically significantly lower than in the control. Spectrophotometric analysis (ASA) showed that as the manganese concentrations in the medium increased, there was an increase in the manganese content and a decrease in the iron and magnesium content in the orchid shoots and roots. Moreover, the iron accumulation in roots was 2-3 times greater than in shoots, while the magnesium accumulation was only 15-30% greater. In contrast to these metals, the accumulation of manganese was 2-3 times greater in the orchid shoots than in the roots.

Keywords: orchid, morphological traits, manganese sulphate, accumulation of Fe, Mg and Mn.

dr hab. Roman Prażak, Department of Genetics and Horticultural Plant Breeding, University of Life Sciences in Lublin, Akademicka 15, 20-950 Lublin, Poland, e-mail: roman.prazak@up.lublin.pl

INTRODUCTION

In vitro cultures are used in orchid breeding for vegetative propagation of new genotypes. *In vitro* techniques accelerate the process of vegetative propagation of orchids (Meesawat et al. 2008, Vijayakumar et al. 2012, Abubacker et al. 2013, David, Băla 2013, Salam et al. 2013). The efficiency of *in vitro* culture depends on the composition of the medium used and on the genotype analysed. Various metals may have a beneficial effect on the development of orchids in an *in vitro* culture (Prażak 2001*a*, 2014, Prażak, Molas 2015**,** Kothari et al. 2004, Aziz et al. 2010). Micronutrients, such as manganese (Mn), control some of these processes. Manganese is essential for all stages of plant development. It is involved in photosynthesis, respiration, and lignin and amino acid biosynthesis, in addition to performing a key function in the activation of several enzymes, including decarboxylating malate dehydrogenase, malic enzyme, isocitrate dehydrogenase, or nitrate reductase (Mukhopadhay, Sharma 1991). Two Mn-containing enzymes have been identified in plants: Mn SOD and a 33-kD protein which is part of the oxygen-evolving water-oxidizing complex in PSII (Amesz 1993). On the other hand, Mn can be detrimental when available in excess in the environment. Symptoms of toxicity of excess metals, such as chlorosis, necrosis or inhibition of the growth and development, are mainly associated with interactions between these metals and other elements having an important physiological role in the plant (Badora, Kozłowska-Strawska 2011). Symptoms of Mn toxicity are quite diverse among plant species and include loss of apical dominance and enhanced formation of auxiliary shoots (Kang, Fox 1980), marginal chlorosis and necrosis of leaves in *Brassica napus* L., *Medicago sativa* L., *Lactuca sativa* L., and *Nicotiana tabacum* (Foy et al. 1978, Petolino, Collins 1985), and brown root discoloration in rapeseed (Moroni et al. 2003), soybean (Heenan, Campbell 1981) and wheat (Moroni et al. 1991). Manganese may antagonistically affect the uptake and accumulation of other metals essential to plant metabolism, such as Fe and Mg (Fageria 2002, Moosavi, Ronaghi 2011, Kleiber et al. 2014).

There are no reports on the effects of elevated manganese concentrations on micronutrient accumulation in orchid organs obtained in *in vitro* conditions.

The aim of this paper was to study the effects of elevated levels of manganese (2, 4, 8 and 16 times the standard content) in the MS culture medium on the growth and development of *Dendrobium kingianum* Bidwill and on the iron and magnesium accumulation in its organs.

MATERIAL AND METHODS

The plant material for the project consisted of young pseudobulbs of *Dendrobium kingianum* Bidwill, about 3 cm long, obtained from mature plants growing in a growth chamber. Pseudobulb explants were washed with sterile

distilled water and immersed in 70% alcohol for 30 s and in 0.1% mercuric chloride ($HgCl_2$) for 6 minutes. After this procedure the pseudobulbs were cleansed 3 times with sterile distilled water, blotted dry with sterilized filter paper, and placed on MS medium (Murashige, Skoog 1962). The *in vitro* orchid culture was conducted in 100 ml glass jars sealed with Magenta B-caps. MS medium was poured into the jars in the amount of 20 ml. The MS medium, containing 3% sucrose and 0.8% Difco Bacto Agar, was supplemented with 1.0 mg dm^{-3} kinetin (6-furfurylaminopurine) and 0.5 mg dm^{-3} NAA (1-naphthalene acetic acid) (Prażak 2001*b*). The pH of the medium was adjusted to 5.2 before being gelled with agar. After two months, new shoots grown on the explants, about 20 mm in length, were separated, weighed and transferred to jars (3 shoots per jar, 30 per treatment) on MS medium with the same composition of plant hormones as before. Manganese was added to all treatments in concentrations 5.5 (control), 10.8, 21.8, 43.6 and 87.2 mg dm^{-3} (as $MnSO_4 \cdot 4H_2O$: 22.3, 44.6, 89.2, 178.4 and 356.8 mg dm^{-3}). The orchid explants were grown at 23±2°C with a photon fluence of 54 mmol m^{-2} s^{-1} and a 16 h photoperiod. The number and length of shoots and roots and the fresh weight of the plantlets were analysed after 12 and 18 months of growth (after 6 and 9 passages) in 24-26 plantlets from each treatment. This experiment was repeated twice.

Next, 17-22 of the plantlets obtained from each combination were washed in distilled water and separated into shoots and roots. The separated plant parts were dried and ground. The ground material was digested using a diacid (HNO_3-$HClO_4$) mixture. After dilution of the digests, they were processed for iron (Fe), magnesium (Mg) and manganese (Mn) analysis by the ASA method.

The remaining plantlets were washed and planted in pots filled with wet pieces of bark, and then transferred to a growth chamber with a temperature of 22-24°C, a photon fluence of 200 mmol m^{-2} s^{-1} and a 16 h photoperiod.

Statistical analysis of the results was performed using one-way analysis of variance (ANOVA) in Statistica 10.0 PL. Significance of differences was verified using the Tukey's test at a significance level of $\alpha = 0.05$.

RESULTS

The results obtained after 12 months show that the treatments with manganese sulphate at concentrations 2, 4, 8 and 16 times higher (44.6, 89.2, 178.4 and 356.8 mg dm^{-3}) than the standard content in MS medium (22.3 mg dm^{-3}) had no stimulatory effect on the number and length of shoots and roots or on the fresh weight of *D. kingianum* Bidwill (Table 1). As the manganese concentration in the medium increased, the values for these morphological traits decreased. The most shoots (21.3 shoots/explant) were noted in the plantlets grown at a concentration of 22.3 mg $MnSO_4 \cdot 4H_2O$ dm^{-3} (con-

Table 1

The influence of increased manganese (as $MnSO_4 \cdot 4\ H_2O$) content in MS medium on biometrical features of *Dendrobium kingianum* Bidwill after 12 and 18 months of *in vitro* culture (mean value of feature/1 explant)

Biometrical feature	Months	Concentration of $MnSO_4 \cdot 4\ H_2O$ (mg dm^{-3})					$LSD_{p=0.05}$
		22.3 (control) **	44.6 (2 x 22.3)	89.2 (4 x 22.3)	178.4 (8 x 22.3)	356.8 (16 x 22.3)	
Number of shoots	12	21.3	18.8	18.6	17.7	12.3*	8.14
	18	38.0	33.2	27.2*	26.3*	17.6*	10.10
Shoot length (mm)	12	37.50	31.52	31.14	26.03*	22.92*	10.760
	18	47.52	42.53	38.34	32.44*	29.54*	12.905
Number of roots	12	19.8	18.9	16.0	15.6	11.6*	8.02
	18	29.8	22.8	20.1*	19.3*	14.6*	7.99
Root length (mm)	12	33.80	17.98	15.27	14.53*	13.45*	19.125
	18	37.54	27.76	23.82*	18.34*	13.98*	10.145
Fresh weight of plant (increment) (g)	12	3.08 (2.99)	1.84 (1.75)	1.75 (1.66)	1.27* (1.18*)	1.05* (0.96*)	1.356 (1.343)
	18	8.45 (8.36)	4.93* (4.84*)	4.62* (4.53*)	2.72* (2.63*)	2.61* (2.52*)	3.253 (3.181)

* result significantly different in relation to the control at $p = 0.05$
** standard content of Mn in MS medium

trol). Plantlets grown at 178.4 and 356.8 mg dm^{-3} $MnSO_4 \cdot 4H_2O$ dm^{-3} had statistically significantly shorter shoots (26.03 and 22.92 mm) than the control (37.50 mm). The most roots (19.8 roots/explant) were counted in the plantlets of the control treatment. This was statistically significantly more roots than in the 356.8 mg dm^{-3} treatment. Concentrations of 178.4 and 356.8 mg dm^{-3} $MnSO_4 \cdot 4H_2O$ dm^{-3} produced significantly shorter roots than the control. The control plantlets had the statistically significantly higher fresh weight and increment of fresh weight than the 178.4 and 356.8 mg dm^{-3} $MnSO_4 \cdot 4H_2O$ dm^{-3} treatments (Table 1).

After 18 months of the *in vitro* culture of *D. kingianum* Bidwill, we again analysed the effect of manganese concentrations increased 2-, 4-, 8- and 16-fold on the number and length of shoots and roots and on the fresh weight and increment of fresh weight. In this case as well the values for these traits in the treatments with increased manganese concentrations were lower than in the control (Figure 1, Table 1). Higher manganese sulphate concentrations of 89.2, 178.4 and 356.8 mg dm^{-3} had a statistically significant negative effect on the number of shoots and roots and on the length of the roots of the *D. kingianum* Bidwill plantlets. The 178.4 and 356.8 mg dm^{-3} concentrations had an adverse effect on the length of the shoots. All increased manganese concentrations significantly reduced the fresh weight of the orchid plantlets and the in-

Fig. 1. *Dendrobium kingianum* Bidwill plantlets in *in vitro* culture

crement of fresh weight. The highest number of shoots (38.0 shoots/explant), shoot length (47.52 mm), number of roots (29.8 roots/explant), root length (37.54 mm), fresh weight (8.45 g/explant) and increment of fresh weight (8.36 g/explant) were achieved in the control treatment.

The analysis of the content of selected metals (Mn, Mg and Fe) in the orchid shoots and roots, performed after 18 months, showed that accumulation of manganese in the shoots and roots increased with the Mn concentration in the MS medium (Figure 2). Manganese accumulation in the shoots was greater than in the roots in all treatments. In the plantlets in the control treatment (22.3 mg dm^{-3} $MnSO_4 \cdot 4H_2O$), manganese accumulation was 109 mg kg^{-1} DW of roots and 269 mg kg^{-1} DW of shoots. Manganese content in the roots and shoots showed a significant increasing trend with increasing Mn concentration in the growth medium. Manganese concentration increased about 18 times (109 to 1,920 mg Mn kg^{-1} DW from 22.3 to 356.8 mg dm^{-3} $MnSO_4 \cdot 4H_2O$ of MS medium) in the roots and about 11 times (269 to 2,980 mg Mn kg^{-1} DW from 22.3 to 356.8 mg dm^{-3} $MnSO_4 \cdot 4H_2O$ of MS medium) in the shoots (Figure 2).

The iron (Fe) content showed a decreasing trend in the roots and shoots with an increasing Mn concentration in the growth medium (Figure 3). Iron concentrations decreased up to 35% (272 to 177 mg Fe kg^{-1} DW with Mn in

Fig. 2. *Dendrobium kingianum* Bidwill plantlets after 18 months in MS medium supplemented with $MnSO_4 \cdot 4\ H_2O$ (from the left): 22.3, 44.6, 89.2, 178.4, 356.8 mg dm^{-3}

the medium increasing from 22.3 to 356.8 mg $MnSO_4 \cdot 4H_2O$ dm^{-3} of MS medium) in the roots and 28% (110.0 to 79.4 mg Fe kg^{-1} DW with Mn in the medium increasing from 22.3 to 356.8 mg $MnSO_4 \cdot 4H_2O$ dm^{-3} of MS medium) in the shoots (Figure 3).

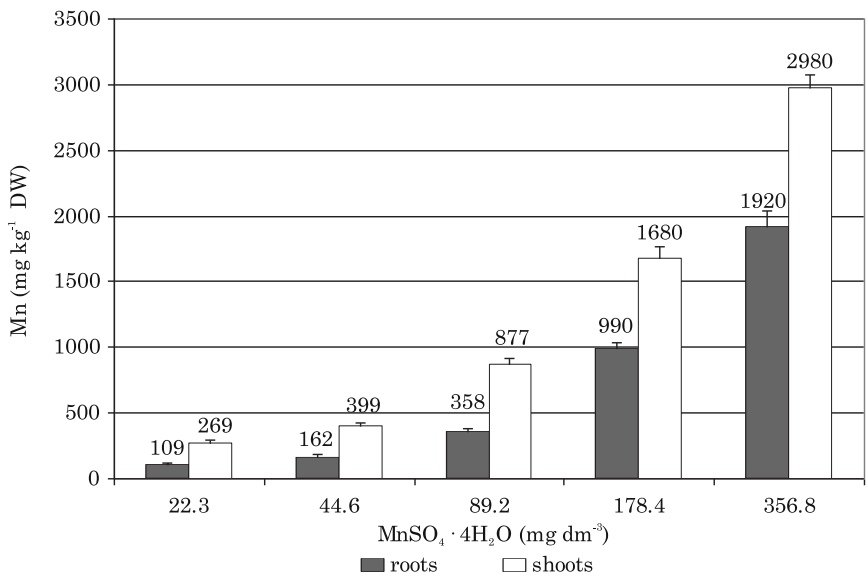

Fig. 3. Manganese content in the roots and shoots of *Dendrobium kingianum* Bidwill after 18 months of culture in MS medium supplemented with different concentrations of manganese (as $MnSO_4 \cdot 4\ H_2O$)

The magnesium (Mg) content in the roots and shoots also showed a decreasing trend with an increasing Mn concentration in the growth medium (Figure 4). Magnesium concentrations decreased up to 18% (1.58 to 1.29 g Mg kg^{-1} DW with Mn in the medium increasing from 22.3 to 356.8 mg $MnSO_4 \cdot 4H_2O$ dm^{-3} of MS medium) in the roots and 16% (1.22 to 1.03 g Mg kg^{-1} DW with Mn in the medium increasing from 22.3 to 356.8 mg $MnSO_4 \cdot 4H_2O$ dm^{-3} of MS medium) in the shoots (Figure 5).

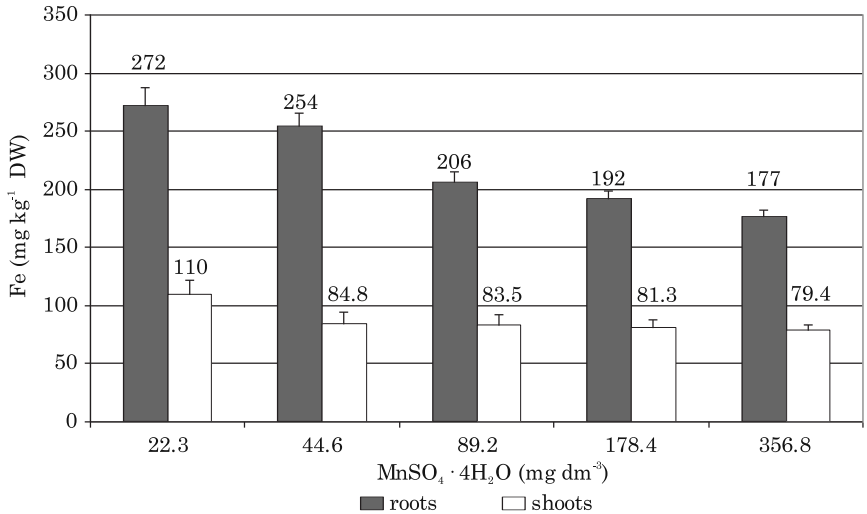

Fig. 4. Iron content in the roots and shoots of *Dendrobium kingianum* Bidwill after 18 months of culture in MS-medium supplemented with different concentrations of manganese (as $MnSO_4 \cdot 4\ H_2O$)

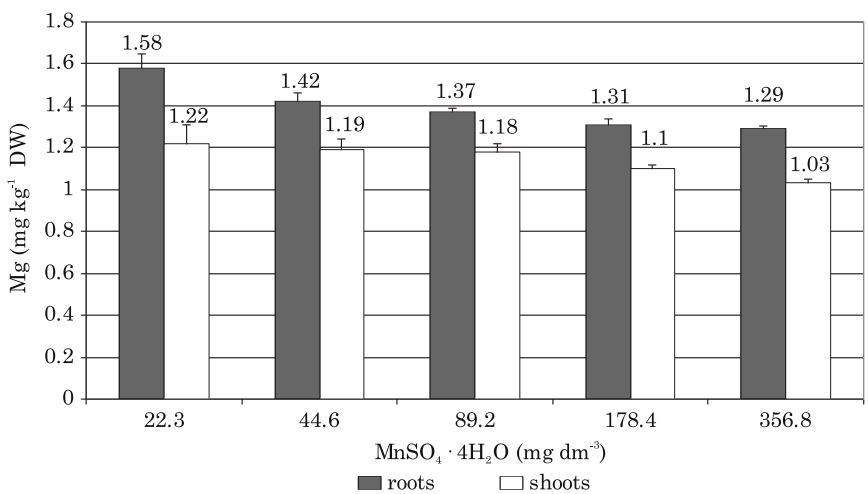

Fig. 5. Magnesium content in the roots and shoots of *Dendrobium kingianum* Bidwill after 18 months of culture in MS-medium supplemented with different concentrations of manganese (as $MnSO_4 \cdot 4\ H_2O$)

DISCUSSION

MS medium supplemented with 1.0 mg dm^{-3} kinetin and 0.5 mg dm^{-3} NAA was used for micropropagation of the orchid *D. kingianum* Bidwill.

A prior study (Prażak 2001*b*) had shown that this composition of plant hormones in MS medium was optimal for micropropagation of this orchid. Other studies have also used a different cytokine, BAP (6-benzylaminopurine), for orchid propagation. David and Băla (2013) observed the best propagating rate of *D. nobile* Lindl. when MS medium was supplemented with BAP at 2.0 mg dm^{-3} and NAA at 1.5 mg dm^{-3}. The combination of 2.0 mg dm^{-3} BAP and 0.1 mg dm^{-3} NAA was shown to be most effective for the development of shoots of *D. candidum* Wall. ex Lindl. in a study by Shiau et al. (2005). Abubacker et al. (2013) reported that the combination of 2.0 mg dm^{-3} BAP and 1.0 mg dm^{-3} NAA yielded the highest number of shoots of *D. barbatum* Lindl. Vijayakumar et al. (2012) reported that MS medium supplemented with 1.5 mg dm^{-3} BAP favoured production of more shoots, elongation of shoots, and root formation in *D. aggregatum* Roxb.

Our experiment tested the effect of a 2-, 4-, 8- and 16-fold increase in the manganese sulphate concentration in MS medium on the micropropagation of *D. kingianum* Bidwill orchids in an *in vitro* culture. The pH of the medium was adjusted to 5.2. Mn, as an essential micronutrient, is required in low concentrations in media for *in vitro* plant cultures (Todorović et al. 2009). Absorption of manganese by plants increases in an acidic (pH < 5.5) or alkaline (pH about 8.0) environment. According to Badora et al. (2000), additional application of Mn increased grain and straw yield of the Helia and Ismena varieties of common wheat.

The experiment showed that 2-, 4-, 8- and 16-fold increases in the manganese sulphate concentration in MS medium had a negative effect on the growth and development of *Dendrobium kingianum* Bidwill orchid plants in *in vitro* conditions. The experiment showed that after 18 months the 4-, 8- and 16-fold increase in manganese sulphate concentration in MS medium (89.2, 178.4 and 356.8 mg dm^{-3}) had a significant negative effect on the number of shoots and roots, root length, fresh weight and increment of fresh weight in *Dendrobium kingianum* Bidwill orchid plantlets in *in vitro* conditions. The 2-fold increase in manganese content in MS medium also led to significantly lower fresh weight of orchid plantlets and increment of fresh weight compared with the control. Todorović et al. (2009) reported that application of Mn at a high concentration had a negative effect on the stem length of lesser centaury (*Centaurium pulchellum* (Sw.) Druce) in *in vitro* culture.

Manganese is taken up as a divalent cation and is very rapidly transported to the aerial parts of the plant, and therefore symptoms of manganese deficiency or toxicity are first visible on the aerial parts. Manganese toxicity in plants depends on transformations of its compounds in the soil and on the plant genotype. The effects of manganese on physiological processes in plants can be considered in terms of its essential role as a microelement and in terms of its toxicity. As in the case of other microelements, the boundary between deficiency and oversupply of Mn is very narrow (Amesz 1993, Todorović et al. 2009, Hong et al. 2010).

Toxic Mn effects have been described in various *in vitro* developmental processes, including callus induction and growth and shoot regeneration from callus (Petolino, Collins 1985, Clairmont et al. 1986, Santandrea et al. 1997, 1998*a*). Moreover, high Mn levels have direct cytotoxic effects, causing extensive cytoplasmic injury, mitochondrial modification, and plasma membrane ruptures in the outer root cap and meristematic cells (Santandrea et al. 1998*b*). For all these reasons, it is very important to establish the optimal Mn dose for the growth of plant species in *in vitro* culture.

Some authors point out a positive influence of higher manganese ion concentrations on *in vitro* organogenesis of plants. Shibli et al. (2007) reported that in an Mn experiment, fresh and dry weight and the length of microshoots of apple tree varieties increased with the Mn level in the medium (up to 16.2 mg dm^{-3}), while any increase beyond this concentration caused a decrease in both weight and microshoot length. In the present study we used treatments with manganese sulphate in concentrations of 22.3, 44.6, 89.2, 178.4 and 356.8 mg dm^{-3}, equivalent to 5.4, 10.8, 21.6, 43.2 and 86.4 mg Mn dm^{-3}.

Kleiber (2014) reported that excessive Mn significantly reduces lettuce yield. This effect was observed when the concentration of manganese in a nutrient solution increased from 0.5 mg dm^{-3} to 19.2 mg dm^{-3}. The significantly highest reduction in plant yield was noted for the combination in which 19.2 mg Mn dm^{-3} was applied (Kleiber 2014, Kleiber et al. 2015). This was confirmed in our experiments. After eighteen months, a significant reduction in the number and length of roots and shoots was noted in the treatments with 21.6-86.4 mg Mn dm^{-3}, and a significant reduction in fresh weight of plantlets in the treatments with 10.8-86.4 mg Mn dm^{-3}.

In our study, an increase in the manganese content in MS medium led to an increase in the Mn accumulation in the roots and shoots of *D. kingianum* Bidwill. Manganese accumulation in the shoots was about 2-3 times higher than in the roots. This distribution of manganese indicates that the aerial green parts of the plant contain the largest quantity of this element, which is linked to its concentration in the chloroplasts (Amesz 1993, Todorović et al. 2009, Hong et al. 2010). In a study by Jankowski et al. (2014), the straw of winter and spring rapeseed contained 8.3-21.4 mg Mn kg^{-1} DW. Spiak et al. (2007) reported that in field conditions 1 kg DW of winter rapeseed straw contained 10.7-14.3 mg Mn. The straw of spring rapeseed was a rich source of manganese, whose content ranged from 34.6 to 37.2 mg kg^{-1} DW.

As in many other plants, differential Mn transport may antagonistically affect the uptake and accumulation of other essential inorganic nutrients (Fageria 2002, Moosavi, Ronaghi 2011). In our study, an increase in the manganese content in MS medium led to a decrease in the Fe and Mg accumulation in the roots and shoots of *D. kingianum* Bidwill. The iron accumulation in the roots was about 2-3 times higher than in the shoots, while that of

magnesium was about 15-30% higher. Other studies have also found a negative interaction between Mn and Fe (Shibli et al. 1997, Kintzios et al. 2006, Kleiber et al. 2014). Sawwan et al. 2000, on the basis of a study on African violet, suggested that Mn interferes with Fe transport. Shibli et al. (2007) reported that the Fe concentration decreased in tissues of the MM 106 apple tree variety in *in vitro* culture as the Mn level increased in the medium.

The most common symptoms of manganese toxicity in physiologically mature plant tissues are chlorosis on the leaf edges or the tip of the leaf blade, the appearance of dark spots on the leaves, drying out of leaves and defoliation (Hong et al. 2010). According to Kitao et al. (2001), a concentration of about 10-50 mg Mn·dm^{-3} in the medium also leads to chlorosis on younger leaves of white birch (*Betula platyphylla var. japonica*). In our study, in *in vitro* conditions, treatments with manganese sulphate at concentrations of 178.4 and 356.8 mg dm^{-3}, equivalent to 43.2 and 86.4 mg Mn dm^{-3}, caused some of the leaves to yellow.

In many cases, chlorosis of the leaf blade is also observed as a result of iron deficiency caused by excess manganese (Hong et al. 2010). Magnesium is the central component of chlorophyll and an activator of an entire set of enzymes. Excess manganese in the environment leads to magnesium and iron deficiency in plants, chloroplast damage, and a reduced photosynthesis rate, thereby significantly reducing yield (Clairmont et al. 1986, Kitao et al. 2001). In our study, as the manganese concentration in the MS medium increased, we observed a lower increment of fresh weight of *D. kingianum* Bidwill.

CONCLUSIONS

1. In combinations with higher manganese concentrations (89.2, 178.4 and 356.8 mg dm^{-3}), a negative influence of the metal was noted for such morphological traits as the number of shoots and roots, root length, fresh weight, and increment of fresh weight of *Dendrobium kingianum* Bidwill.

2. Manganese accumulation in both shoots and roots increased with the increase in external Mn level. Manganese accumulation in the shoots was about 2-3 times higher than in the roots.

3. The study confirmed that manganese may antagonistically affect the uptake and accumulation of other essential metals, such as iron and magnesium. The content of Fe and Mg in the roots and shoots decreased as the external Mn level increased. Iron and magnesium accumulation in the roots was higher than in the shoots.

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