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# INFLUENCE OF COBALT CONCENTRATION ON THE GROWTH AND DEVELOPMENT OF *DENDROBIUM KINGIANUM* BIDWILL ORCHID IN AN *IN VITRO* CULTURE

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**Abstract**

The study investigated the influence of increased cobalt content in MURASHIGE and SKOOG (1962) solid medium on the growth and development of *Dendrobium kingianum* Bidwill orchid plants. Explants of shoots were used for micropropagation of the orchid plants on MS regeneration medium supplemented with 0.5 mg $dm^{-3}$ NAA and 1.0 mg $dm^{-3}$ kinetin. Cobalt (as $CoCl_2 \cdot 6H_2O$) was added to all treatments in concentrations of 0.025 (control), 0.625, 1.25 and 2.5 mg $dm^{-3}$. The results obtained after eight months showed that treatments with the cobalt chloride in concentrations 0.25-1.25 mg $dm^{-3}$ did not influence the number of shoots and roots, and the length of shoots of the orchids. The treatment with the cobalt chloride in concentration 0.625 mg $dm^{-3}$ positively influenced on the length of roots and increment of the fresh weight of plantlets. However, in media with the highest cobalt concentration (2.5 mg $dm^{-3}$ $CoCl_2 \cdot 6H_2O$), a negative influence of the metal on the number of shoots of the orchids was noted.

Spectrophotometric analysis (ASA) showed that cobalt accumulation increased in both the shoots and the roots with the increase in the external Co level, whereas iron accumulation in these organs decreased. Cobalt and iron accumulation in the roots was 3-4 times higher than in the shoots.

**Keywords:** biometrical features, cobalt chloride, orchids, tissue culture.

## WPŁYW STĘŻENIA KOBALTU NA WZROST I ROZWÓJ STORCZYKA *DENDROBIUM KINGIANUM* BIDWILL W KULTURZE *IN VITRO*

**Abstrakt**

Celem badań była ocena wpływu zwiększonego stężenia kobaltu w pożywce MURASHIGE i SKOOGA (1962) na wzrost i rozwój roślin storczyka *Dendrobium kingianum* Bidwill. Do mikrorozmnażania roślin storczyka wykorzystano jego pędy, które umieszczono na stałej pożywce

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regeneracyjnej MS z dodatkiem 0,5 mg $dm^{-3}$ NAA i 1,0 mg $dm^{-3}$ kinetyny. Kobalt (w formie $CoCl_2 \cdot 6H_2O$) dodano do pożywek w stężeniach: 0,025 (kontrola), 0,625, 1,25 i 2,5 mg $dm^{-3}$. Po 8 miesiącach nie wykazano wpływu chloru kobaltu w stężeniu 0,25-1,25 mg $dm^{-3}$ na liczbę uzyskanych pędów i korzeni oraz na długość pędów. Odnotowano natomiast dodatni wpływ chlorku kobaltu w stężeniu 0,625 mg $dm^{-3}$ na długość korzeni oraz przyrost świeżej masy. Na pożywce o największym stężeniu kobaltu (2,5 mg $CoCl_2 \cdot 6H_2O$ $dm^{-3}$) stwierdzono istotnie mniej pędów niż w pożywce kontrolnej.

Analiza (ASA) zawartości kobaltu i żelaza w suchej masie pędów i korzeni storczyka wykazała, że wraz ze wzrostem stężenia kobaltu w pożywce MS następował znaczny wzrost akumulacji kobaltu oraz spadek akumulacji żelaza w tych organach. Zawartość kobaltu i żelaza była 3-4-krotnie większa w korzeniach niż w pędach.

**Słowa kluczowe**: chlorek kobaltu, cechy biometryczne, storczyki, kultury tkankowe.

# INTRODUCTION

Many morphological factors control the processes of plant growth and development in *in vitro* culture (Prażak 2001*a*, Soontornchainaksaeng et al. 2001, Puchooa 2004, De Faria et al. 2004, Alvarez-Pardo et al. 2006, Aziz et al. 2010). One of the most important factors of physical environment in plant tissue culture is ethylene ($C_2H_4$), a gaseous plant hormone that plays an important role in plant growth and development (Yang, Hoffman 1984, Trujillo-Moya, Gisbert 2012.). The same research was done in the past using ethylene inhibitors, that is, for example, cobalt chloride ($CoCl_2$), for promoting shoot organogenesis in several plant species which has been reviewed by Samimy (1978), Mohiuddin et al. (1995) and Kumar et al. (1998). Cobalt chloride may extend the life of cut roses (Venkatarayappa et al. 1980). Cobalt, a component of vitamin $B_{12}$, has been regarded as an essential element for animals and microorganisms but has not been recognized as such for plants. Its only physiological role is in the fixation of molecular nitrogen in root nodules of leguminous plants which possess vitamin $B_{12}$ and cobamide coenzymes and in the non-legumes *Alnus glutinosa* and *Casuarina cunninghamiana* (Bond, Hewitt 1962). Cobalt, in the form of fertilizer and pre-seeding and pre-sowing chemicals, has increased yield in many plants (Palit, Sharma 1994). Gad and Kandil (2010) reported that cobalt addition enhanced all parameters of tomato growth and yield with all sources of phosphorus fertilizers especially mono super phosphate.

The main purpose of this study was to investigate the influence of cobalt in concentrations 25, 50 and 100 times higher, than the standard content in MS medium on the growth and development of the orchid *Dendrobium kingianum* Bidwill.

## MATERIAL AND METHODS

*Dendrobium kingianum* Bidwill are extremely variable, lithophytic plants which often grow in large masses in Australia (New South Wales and Queensland). *Dendrobium kingianum* Bidwill plants were collected from a greenhouse. To develop an aseptic culture, orchid shoots (pseudobulbs 15-30 mm long with two terminal leaves) were surface sterilized with 0.1% $HgCl_2$ solution for 3 minutes, washed several times with distilled water and transferred to an initial MURASHIGE and SKOOG (1962) medium (MS) supplemented with IAA (indolyl-3-acetic acid) at 0.5 mg $dm^{-3}$ and BA (6-benzylaminopurine) at 1.0 mg $dm^{-3}$.

The pH of the medium was adjusted to 5.2 before gelled with agar. All cultures were incubated at 22-24°C with 16 h light (at an irradiance of 54 μmol $m^{-2}$ $s^{-1}$)/ 8 h dark cycle. After two months, all newly formed about 20 mm long shoots were separated, weighed (about 0.150 g/1 shoot) and individually transferred (5 shoots per vessel, and 25 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 2001b). Cobalt (as $CoCl_2 \cdot 6H_2O$) was added to all treatments in concentrations 25, 50 and 100 times higher (0.625, 1.25, 2.5 mg $dm^{-3}$) than the standard content in MS medium (0.025 mg $dm^{-3}$). All media contained 3% sucrose and 0,8% Difco bacto-agar. 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

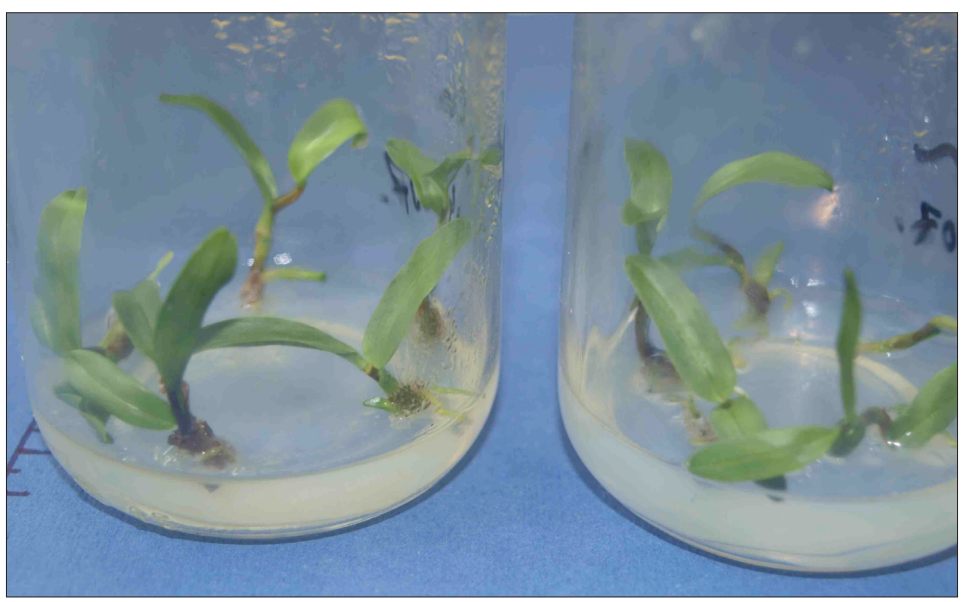

Fig. 1. *Dendrobium kingianum* Bidwill shoot explants in glass culture vessels

the fresh weight were analysed after four and eight months of growth (after 2 and 4 passages) in 22-25 plantlets from each treatment. The orchids had strong roots hence there was no risk to tear them off while measuring. The experiment was repeated twice (2 x 8 months).

After eight months all plants from the different treatments were separated into roots and shoots. Then both 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 plants material was digested using a diacid ($HNO_3$-$HClO_4$) mixture. After dilution of the digests, they were processed for cobalt (Co) and iron (Fe) analysis using the ASA method.

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

## RESULTS

In this study, the formation of multiple shoots was successfully induced from single shoot explants of *Dendrobium kingianum* Bidwill. The results obtained after four months showed that the 25-, 50- and 100-fold increases in cobalt content in MS solid medium did not influence the number and length of shoots and roots, and fresh weight of the plantlets (Table 1). In the

Table 1

The influence of increased cobalt (as $CoCl_2 \cdot 6H_2O$) content in 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 $CoCl_2 \cdot 6H_2O$ (mg $dm^{-3}$) | | | | $LSD_{p=0.05}$ |
|---|---|---|---|---|---|---|
| | | 0.025 (control)** | 0.625 (25 · 0.025) | 1.25 (50 · 0.025) | 2.5 (100 · 0.025) | |
| Number of shoots | 4 | 4.02 | 4.09 | 3.92 | 3.50 | n.s. |
| | 8 | 12.22 | 11.56 | 8.89 | 7.91* | 3.92 |
| Shoot length (mm) | 4 | 38.50 | 37.87 | 37.50 | 38.35 | n.s. |
| | 8 | 44.00 | 46.11 | 38.56 | 41.46 | n.s. |
| Number of roots | 4 | 11.01 | 10.91 | 8.50 | 8.22 | n.s. |
| | 8 | 19.38 | 21.44 | 14.67 | 14.36 | n.s. |
| Root length (mm) | 4 | 15.30 | 16.46 | 16.83 | 18.46 | n.s. |
| | 8 | 34.60 | 49.78* | 35.33 | 34.91 | 11.33 |
| Fresh weight of plant Increment (g) | 4 | 0.632 (0.482) | 0.791 (0.641) | 0.553 (0.423) | 0.540 (0.390) | n.s. n.s. |
| | 8 | 2.018 (1.868) | 3.233* (3.083*) | 1.692 (1.542) | 1.654 (1.504) | 1.024 (1.012) |

* - result significantly different in relation to the control at $p$=0.05
** - standard content of Co in MS medium
n.s. - not significant in relation to the control at $p$=0.05

Fig. 2. *Dendrobium kingianum* Bidwill plantles after 8 months growing in an *in vitro* culture

series with cobalt concentration 25 times higher than the standard content in MS medium means of 4.09 shoots and 0.791 g fresh weight per 1 explant were obtained. They were higher than the means in the control treatment, but the differences were not statistically significant (Table 1). The increment of orchid plant fresh weight after four months of *in vitro* culture was 0.641 g in the case of the cobalt concentration increased 25-fold and 0.482 g for the control. However, the 50-, 100-fold increases in cobalt content in MS medium led to a lower mean number of shoots and roots and lower fresh weight of orchid plants than in the control (Table 1).

The results after eight months of *Dendrobium kingianum* Bidwill *in vitro* culture (Figure 2) show that the 25-fold increase in cobalt content in MS solid medium positively influenced root length and plantlets fresh weight (Table 1). In the series with the 25-fold increase in cobalt concentration, significantly longer roots per 1 explant (49.78 mm) were obtained than in the control treatment (34.60 mm). In the same series, significantly higher fresh weight of plant per 1 explant (3.233 g) was noted in comparison with in the control (2.018 g) – Table 1.

In treatment with the highest cobalt concentration (2.5 mg $dm^{-3}$ $CoCl_2 \cdot 6H_2O$) a significantly negative influence of the metal on the number of shoots of *Dendrobium kingianum* Bidwill was noted. The effect of Co toxicity was more severe on shoots than on roots. The percentage decrease in number of shoots relative to the control was greatest (35%) at the highest Co level (2.5 mg $dm^{-3}$ $CoCl_2 \cdot 6H_2O$), followed by a 27% at 1.25 mg $CoCl_2 \cdot 6H_2O$ $dm^{-3}$ of MS medium, and 5% at 0.625 mg $dm^{-3}$ $CoCl_2 \cdot 6H_2O$

of MS medium. The reduction in the number of roots was 27% at 2.5 mg $CoCl_2 \cdot 6H_2O$ $dm^{-3}$ of MS medium, and 26% at 1.25 mg $CoCl_2 \cdot 6H_2O$ $dm^{-3}$ of MS medium.

The significant increment of orchid plant fresh weight after eight months of *in vitro* culture was 3.083 g in the case of the cobalt concentration increased 25-fold and 1.868 g for the control (Table 1). In media with cobalt content increased 50 and 100 times a negative influence of Co on the number and length of shoots and roots, and on the weight of plant fresh mass were noted. The treatment with a 100-fold increase cobalt led to a significantly smaller number of shoots and roots than in the control (Table 1).

Cobalt accumulation in both shoots and roots increased with the increase in external Co level. Cobalt accumulation in the roots was higher than in the shoots in all treatments (Figure 3). The maximum Co contents was observed in both the roots (3978.71 µg $kg^{-1}$ DW) and the shoots (1126.6 µg $kg^{-1}$ DW) at the highest level of Co (2.5 mg $dm^{-3}$ $CoCl_2 \cdot 6H_2O$), followed by 1675.38 and 430.07 µg Co $kg^{-1}$ DW of roots and shoots, respectively, at 1.25 mg $CoCl_2 \cdot 6H_2O$ $dm^{-3}$ of MS medium, and 999.89 and 239.91 µg Co $kg^{-1}$ DW of roots and shoots, respectively, at 0.625 mg $CoCl_2 \cdot 6H_2O$ $dm^{-3}$ of MS medium.

Iron content in the roots and shoots showed a decreasing trend with increasing Co concentration in the growth medium (Figure 2). Iron concentration decreased by 35% (266 to 173 mg Fe $kg^{-1}$ DW from 0.025 to 2.5 mg $CoCl_2 \cdot 6H_2O$ $dm^{-3}$ of MS medium) in the roots and by 18% (67.4 to 55.3 mg Fe $kg^{-1}$ DW from 0.025 to 2.5 mg $CoCl_2 \cdot 6H_2O$ $dm^{-3}$ of MS medium) in the shoots (Figure 4).

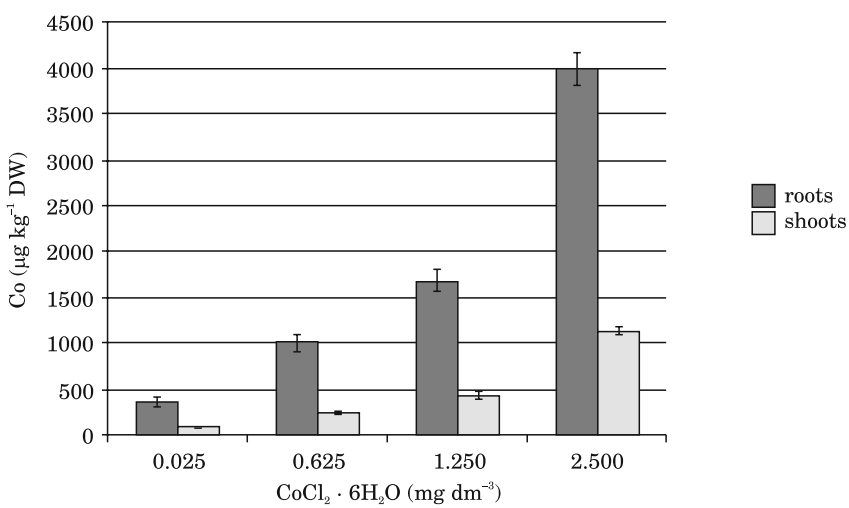


Fig. 3. Cobalt content in the roots and shoots of *Dendrobium kingianum* Bidwill after 8 months growing in MS medium supplemented with different concentrations of cobalt (as $CoCl_2 \cdot 6\ H_2O$)

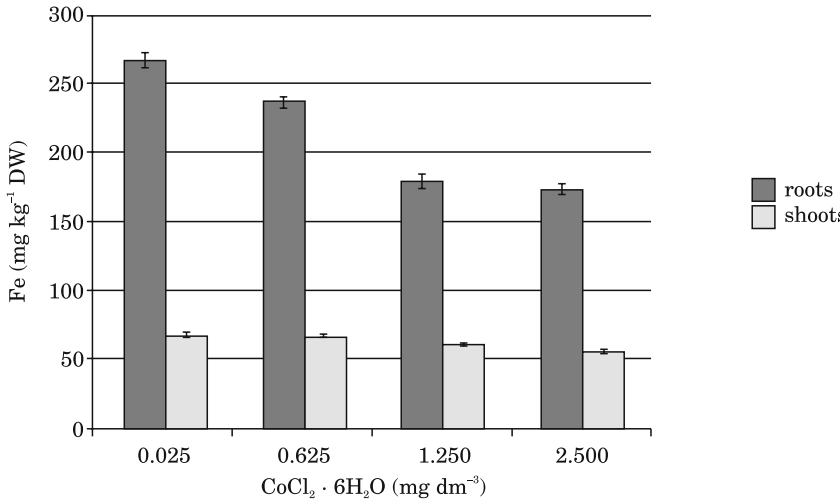


Fig. 4. Iron content in the roots and shoots of *Dendrobium kingianum* Bidwill after 8 months growing in MS-medium supplemented with different concentrations of cobalt (as $CoCl_2 \cdot 6\ H_2O$)

# DISCUSSION

The experiment showed that 25- and 50-fold increase cobalt concentration in MS medium (0.625, 1.25 mg dm$^{-3}$) did not affect the number of shoots in *Dendrobium kingianum* Bidwill orchid plants growing in *in vitro* conditions. The 25-fold increase in cobalt concentration in MS medium strongly stimulated root length and plant fresh weight increment.

Chae et al. (2012) reported that shoot organogenesis and plant regeneration in gloxinia (*Sinningia speciosa Bail)* were improved by using ethylene inhibitors such as, inter alia, cobalt chloride ($CoCl_2$). The leaf explants were cultured by them on initial shoot regeneration media (MS media with BAP at 2 mg dm$^{-3}$ + NAA at 0.1 mg dm$^{-3}$) supplemented with different concentrations of cobalt chloride ($CoCl_2$). The addition of $CoCl_2$ significantly improved the regeneration frequency giving higher shoots per explant and longer shoots. In treatment of 1 mg dm$^{-3}$ $CoCl_2$ 12% more shoots were produced by per explant compared to control. Inhibition of ethylene formation by cobalt chloride did not confirm Santana-Buzzy et al. (2006) in case of habanero peppers (*Capsicum sinense* Murray) and Chae and Park (2012) in case of purple coneflower (*Echinacea angustifolia* D.C.). Their results showed that $CoCl_2$ added to the culture medium did not help to inhibit the production of ethylene.

Amarasinghe (2009) reported that the rate of callus proliferation in the screened traditional indica rice (*Oryza sativa* L.) varieties in Sri Lanka was significantly higher in the medium supplemented with both 5 mg dm$^{-3}$ copper sulphate and 5-10 mg dm$^{-3}$ cobalt chloride together. In regeneration,

the highest number of normal plants with the least number of albino plants could be obtained in the media containing 5 mg $dm^{-3}$ copper sulphate in combination with 5 mg $dm^{-3}$ cobalt chloride. The results indicate that the *in vitro* performance in Sri Lanka traditional indica rice varieties can be improved by using the media containing both copper sulphate and cobalt chloride.

ATTA-ALY et al. (1991) found that supplementing nutrient solution with a low level of cobalt (0.25 mg $dm^{-3}$) improved growth of tomato plants and enhanced both flowering and fruiting. GAD (2005*a*) demonstrated that cobalt at 7.5 mg $dm^{-3}$ significantly increased growth parameters, fruit yield and nutrient concentration in tomatoes, as well as total soluble solids, total soluble sugars and L-Ascorbic acid, while titratable acidity decreased. On the other hand, supplementing nutrient solution with a higher level of cobalt resulted in a negative response.

Concentrations of 2.5 mg $CoCl_2 \cdot 6H_2O$ $dm^{-3}$ in MS medium the most negatively influenced the number of shoots of *Dendrobium kingianum* Bidwill. LIU et al. (1995) showed that with a higher level of cobalt in medium the growth of onion roots increased more than that of shoots. SAMIMY (1978) reported that cobalt promotes the elongation of the hypocotyl of soybean seedlings and decreases their thickness, and concluded that cobalt exerts its effects on soybean hypocotyl growth, at least in part, by inhibiting ethylene formation. JAYAKUMAR and JALEEL (2009) found that higher concentrations of Co (level of 100-200 mg $kg^{-1}$ in the soil) resulted in maximum accumulation in all parts of soybean plants, while low concentrations of cobalt (50 mg $kg^{-1}$ Co level) proved to be favourable to the overall growth of soybean plants.

As in many other plants, differential Co transport may antagonistically affect the uptake and accumulation of other essential inorganic nutrients, such as iron. High levels of many trace elements are known to commonly induce iron deficiency in plants (HUNTER, VERGHANO 1953).

In the present study, an increase in cobalt accumulation both in shoots and roots was associated with an increase in external Co level. Increased cobalt content in MS medium led to a decrease in Fe accumulation in these organs. Cobalt and iron accumulation in the roots was 3-4 times higher than in the shoots.

GAD and KANDIL (2008) reported that increasing cobalt levels in plant media from 5 up to 15 mg $dm^{-3}$ increased cobalt content in the roots of sweet potato plants as compared to the control treatment. These results clearly indicated that cobalt content increases with the concentration of added *cobalt*. Increasing cobalt concentration in the plant media was also shown to result in a progressive depression effect on iron content in the tubers of sweet potato plants. This may be explained by results obtained by BLAYLOCK et al. (1993) and GAD (2005*a* and 2006), who showed certain antagonistic relationships between cobalt and iron. They also stated that cobalt contributes to a wilted appearance and reduces net photosynthesis. Data obtained by

GAD and KANDIL (2010) showed that cobalt increased the content of micronutrients, with the exception of iron. The reduction rate of Fe indicates the competition between Fe and Co (GAD 2005*b*). ROMERA and ALCANARA (1994) reported that the presence of the ethylene inhibitor, such as, cobalt chloride in the nutrient solution inhibited the Fe- deficiency stress responses ferric-reducing capacity and subapical cucumber root swelling. LUCENA et al. (2006) and WATERS et al. (2007) showed that treatment of *Arabidopsis*, tomato, cucumber plants with 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene, induced the expression of ferric reductase (*AtFRO2*, *LeFRO1*, *CsFRO1*), iron transporter (*AtIRT1*, *LeIRT1*, *CsIRT1*), $H^+$-ATPases (*CsHA1*), and transcription factors (*LeFER*, *AtFIT*), that regulate the iron deficiency response. Conversely, the addition of ethylene inhibitors, such as $Co^{2+}$, markedly repressed the expression of these genes.

Under lower cobalt application, the improved root system has helped plants to better absorb water and other nutrients (JAYAKUMAR, JALEEL 2009). Improvement of water uptake associated with Co may be attributed to its action on inhibiting vascular blockage and closing stomata (REDDY 1988). It probably influenced a higher increment of fresh weight and root growth in orchid plantlets.

## CONCLUSIONS

1. Ethylene inhibitor cobalt chloride significantly did not promote the shoot regeneration of *Dendrobium kingianum* Bidwill. The experiment showed that 25-, 50- and 100-fold increases in cobalt concentration in MS medium (0.625, 1.25, 2.5 mg $dm^{-3}$) did not affect positively the number of shoots in *Dendrobium kingianum* Bidwill orchid plants growing in *in vitro* conditions.

2. The increase 25-fold in cobalt content (0.625 mg $dm^{-3}$) positively influenced the number and length of the roots and fresh weight of *Dendrobium kingianum* Bidwill.

3. Spectrophotometric analysis (ASA) of cobalt and iron content in the roots and shoots of orchid plants confirmed an antagonistic relationships between these metals – an increase in cobalt accumulation both in the shoots and the roots as the external cobalt level increased, and a decrease in iron in these organs.

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