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The effect of high concentration of selected calcium salts on development of microcuttings of rhododendron R. 'Catawbiense Grandiflorum' in *in vitro* cultures

Abstract: High content of calcium compounds in soil limits cultivation possibilities of the majority of cultivars of rhododendron. In the research presented an attempt was made to determine the influence of a high level of calcium salts $CaCl_2$, $CaSO_4$, $Ca(NO_3)_2$ and $CaCO_3$, pH and an increased level of auxins in a medium, on the development of microcuttings of *R.* 'Catawbiense Grandiflorum'. On the basis of the results of the research it is justifiable to state that it was the anions of some salts used that had an adverse effect on the development of rhododendron cultures, not calcium cations. Significant differences were noted in the uptake of calcium, magnesium and sodium ions by the microcuttings depending on salts used in the media. The pH value of the medium higher than optimum significantly affected the degree of chlorosis of the microcuttings analyzed. Supplementing the media with additional auxin – IBA was stimulating for the plant growth especially in the medium containing $CaCO_3$.

Additional key words: pH, IBA, magnesium, sodium

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Introduction

In natural conditions rhododendrons – *Rhododendron (Ericaceae)* grow on acid soils. Depending on the species and cultivars the optimal value of pH of the soil ranges between 3.8–6.0. The excess of calcium carbonate – $CaCO_3$ in the soil has adverse effects on the development of rhododendrons (Chaanin and Peril 1992, Czekalski 1991) and constitutes the main factor limiting the possibility of cultivating these plants. Depending on temperature, water content and concentration of CO_2 in soil, $CaCO_3$ decomposes into calcium ions – Ca^{2+} and hydrogen carbonate ions – HCO_3^-. Being strongly alkalic the HCO_3^- ions have very toxic influence on the root system of rhododendrons (Chaanin and Peril 1992). The alkalization of rhizosphere inhibits the growth of roots, which makes it more difficult to absorb nutrients. In consequence, the growth of the plant and the shoots is inhibited and may lead to the atrophy of the plant. The majority of soils in Poland are sandy and sandy clay soils with high content of calcium carbonate, which makes them unsuitable for cultivation of many cultivars of rhododendron. Finding a suitable rootstock, tolerant to the adverse conditions of the environment, would be a good solution. Research was started in 1980s in Germany to obtain rhododendrons tolerant to pH higher than optimum, which could serve as rootstock for most cultivars (Preil 1990, Preil and Ebbinghaus 1998). As a result of conducted experiments clones were selected which had increased tolerance of the adverse conditions in the soil. These plants require however proper cultivation as they are less tolerant to adverse climatic conditions

(high temperatures, low moisture) than rootstocks of *R. catawbiense, R* 'Cunningham's White' and *R. ponticum* (Maethe 1997) used in nursery production.

In the Institute of Dendrology of Polish Academy of Science (PAN) research is currently being conducted on the influence of high concentration of calcium compounds and pH on the development of selected taxons of rhododendron.

The aim of this research is learning about the sensitivity of microcuttings of *R.* 'Catawbiense Grandiflorum' to stress factors in *in vitro* conditions. The results of this research constitute the starting point for further research on the increase of tolerance of rhododendrons to high concentration of calcium salts in soil.

Materials and methods

Plant material and the conditions of plant growth

The research was conducted in *in vitro* conditions on microcuttings obtained from vegetative buds of *R.* 'Catawbiense Grandiflorum'. The buds were soaked for 2 hours in fungicide – Benlate 0.1%. They were then disinfected in 1.5% chloramine T or in 5% calcium hypochlorite (5–15 min.). After rinsing the explants several times in sterile water they were placed in modified Anderson's medium (1984) with increased amount of microelements and supplemented with casein enzymatic hydrolysates, in concentration of 500 mg/l (Bojarczuk 1995).

Microcuttings grew in glass containers of cubic capacity of 350 ml, at the temperature of 21–23°C, in the light of glow-mercury discharge lamps at 7.5 W m^{-2} intensity level for 16 hours a day. Cuttings of even growth parameters were chosen for the experiments (3 leaves per cutting). Growth regulators were added to the media: 2iP – N^6-(2-Isopentyl)adenine, IAA – Indole-3-acetic acid and IBA – Indole-3-butyric acid. The reaction of the medium was established depending on the variant of experiment on the pH levels of 5.0 and 8.0. The media were autoclaved for 20 min. at the temperature of 121°C.

Media variants used

As basal medium [BM] modified Anderson's medium was used (Table 1).

Experiment 1. The influence of pH value on the development of microcuttings.

441 mg/l $CaCl_2 \cdot 2H_2O$ and growth regulators (2iP 0.25 mg/l and IAA 0.1 mg/l) were added to the [BM] medium. The reaction of the medium was established depending on variant: K_1 (control) – pH 5.0 and K_2 – pH 8.0.

Experiment 2. The influence of calcium salts and IBA on the development of microcuttings.

Table 1. Basal medium [BM] contents

Compounds	Concentration [mg/l]
NH_4NO_3	400
KNO_3	490
KH_2PO_3	300
H_3BO_3	9.3
$Na_2MoO_4 \cdot 2H_2O$	0.375
$CoCl_2 \cdot 6H_2O$	0.037
$MgSO_4 \cdot 7H_2O$	835
$MnSO_4 \cdot H_2O$	25
$ZnSO_4 \cdot 7H_2O$	15
$CuSO_4 \cdot 5H_2O$	0.037
Na_2EDTA	75
$FeSO_4 \cdot 7H_2O$	56
adenine hemisulfate salt	80
myo-inositol	100
thiamine	0.4 ml
saccharose	30 g
agar	10 g

Depending on the variant of medium, different calcium salts were added to the [BM] medium (Table 2).

Growth regulators were added to the media: in culture I (2iP 0.25 mg/l, IAA 0.1 mg/l, IBA 2 mg/l), in culture II and III (2iP 0.25 mg/l, IAA 0.1 mg/l). pH level was established at 5.0.

Table 2. Calcium salts used in media

Medium variant	Calcium salts concentration [mg/l]	Ca^{2+} concentration [mg/l]
K [control]	441 $CaCl_2 \cdot 2H_2O$	120
Cl	1764 $CaCl_2 \cdot 2H_2O$	480
N	2832 $Ca(NO_3)_2 \cdot 4H_2O$	480
S	2060 $CaSO_4 \cdot 2H_2O$	480
C	1200 $CaCO_3$	480

Evaluation of the development of cultures

After 44-day of culture, depending on the variant of experiment the following factors were determined: the shoot length, the number of leaves per cutting, the number of shoots with leaves 3 cm, the degree of callus development in 0–3 scale: 0 – no callus; 1 – weak (callus diameter <0.1 cm); 2 – medium (callus diameter 0.1–0.3 cm), 3 – large (callus diameter >0.3 cm); the degree of chlorosis and browning of cultures in 0–3 scale: 0 – no chlorosis or browning, 1 – weak (1–20% chlorosis or browning), 2 – medium (21–60% chlorosis or browning), 3 – large (61–100% chlorosis or browning). At the end of every culture the pH value of media was measured.

Quantitative determination of chemical elements

In the microcuttings from experiment 2 in the non IBA variant, the content of Ca, Mg, Na was determined. The plant material was dried at 65°C until solid mass was obtained. Next the material was ground and mineralized in the mixture of concentrated acids HNO_3 and $HClO_4$ at the ratio of 3:1. After mineralization the content of Ca, Na and Mg was determined through atom absorption spectrometry (AAS). Chemical analyses were conducted in the Department of Fertilization of Horticultural Plants of the Academy of Agriculture in Poznań.

Statistical analysis of the results

60 microcuttings grown in 12 glass containers (1 replication = 5 microcuttings per glass container) were used for each medium variant. The results were analyzed statistically using one-way ANOVA. For comparing means the Tukey's test of significant difference was applied (P = 0.05). Analyzing the degree of callus development and the degree of chlorosis and browning Bliss transformation was applied (Snedecor and Cochran 1976).

Results and discussion

The role of calcium in plant metabolism is not known sufficiently. One of the most widely accepted theories assumes that calcium plays crucial role in the mechanism of auxin activity, and that it functions as a secondary and tertiary transmitter of information (Tretyn 1997). Calcium functions also as a neutralizer of organic acids. It activates enzymes and affects the absorption of water and nutrients while maintaining proper Ca^{2+}/K^+ ion ratio (Jankiewicz 1984).

The influence of high concentration of calcium on rhododendrons depends on taxon and chemical form in which this element is provided (Bojarczuk 2000). In the case of easily soluble salts, the whole pool of ions Ca^+ and anions bound with them is transferred to the solution and is readily available to plants. High degree of chlorosis of microcuttings grown on medium containing calcium chloride (Cl variant) might have been the effect, even though the pH value was only slightly lower than pH of control medium (K variant), (Table 3). Most probably the excess of chlorine was the cause of chlorosis of the blade. Higher concentration of $CaCl_2$ (1764 mg/l $CaCl_2 \cdot 2H_2O$) weakened also the growth of plants (Table 3). Ballinger (1962) suggested that toxic concentration of Cl^- ions in the leaves of Highbush Blueberry *Vaccinium corymbosum* (*Ericaceae*), grown on medium containing NaCl stems from the fact that this species lacks the mechanism of removal of excess chloride ions from its tissues. Mularitharan et al. (1990) confirmed these observations and indicated a significantly higher toxicity of NaCl for Highbush Blueberry as compared to Na_2SO_4. Ziska et al. (1990) stated that the increase in concentration of Cl^- anions in the leaves of *Prunus saliciana* is one of the main causes of the decrease of assimilated CO_2 which negatively influences metabolic processes connected with photosynthesis.

Calcium nitrate (N variant) did not have negative impact on the development of microcuttings as compared to control variant (Table 3). Higher pH value of medium in this variant as compared to control resulted from physiology of NO_3^- anion uptake by the plants. On the one hand nitrates are absorbed through H^+ ion symport (McClure et al. 1990, Kłobus 1995), which results in decrease of hydrogen ion concentration and increase of the pH value of the medium. On the other hand NO_3^- anions are absorbed in larger quantities than Ca^{2+} cations which bind with HCO_3^- or OH^- ions separated by the plant and additionally alkalize the environment (Szweykowska 1997).

Small quantities of calcium carbonate stimulate the growth of seedlings and rooting of cuttings of rhododendron (Czekalski 1991). The fact that microcuttings grown on medium containing $CaCO_3$ (C variant) were characterized by good growth (Table 3) may result from very weak solubility of this compound. In effect, only part of calcium contained in the medium may have assimilable form. In the course of Ca^{2+} ion absorption by the plant, their new pool may gradually be released into solution. As a result most probably the concentration of HCO_3^- ions in medium did not increase abruptly. These ions might have influenced the change of acidity of medium without causing major chlorotic changes in leaves (Table 3). The increase in concentration of HCO_3^- anions in medium causes decrease in absorption of iron or slowing down of transport of iron from roots to shoots which may be manifested by internerve chlorosis (Marschner 1986, Boxma 1972, Kolesh et al. 1984). Drehmel and Preil (1992) indicated noxious influence of HCO_3^- ions on the degree of rooting and the length of the root system of selected rhododendron cultivars.

Calcium sulfate (S variant) because of weaker solubility and acidifying SO_4^{2-} group did not cause the decrease in the quality of the analyzed cultures, i.e. the degree of leaf chlorosis (Table 3). The reaction of the medium in S variant was higher than the reaction of control, however it remained within the scope of advocated optimum for rhododendrons. Earlier experiments confirm insignificant noxiousness of plaster for cuttings of *R.* 'Cunningham's White' (Drehmel and Preil 1992) and Highbush Blueberry (Borkowska 1996). Rennberg (1984) states that plants can control the amount of SO_4^{2-} anions absorbed from the medium (contrarily to Cl^- ions). Therefore, their concentration in tissues does not exceed optimal values.

Increased amount of auxin positively influenced the growth of plants. It is indicated by higher degree of callus development and the number of shoots with leaves 3 cm (Table 4), especially in C variant with calcium carbonate (Table 3). Because of weak solubility of calcium carbonate the concentration of Ca^{2+} and HCO_3^- ions could be low, which coupled with increased dose of IBA, stimulated the development of microcuttings better than other combinations. It is believed that auxin increases the permeability of cell membranes for calcium ions, which influence the activity of growth hormones – auxins and cytokinins (Bethke et al. 1995). Strong growth of cultures causes however faster dwindling of nutrients from the medium, which may in consequence lead to the decrease in their quality (Bojarczuk 2000). In the research presented, higher degree of chlorosis was found in plants grown on medium including increased doses of auxin (Table 4).

High pH value, achieved through increased concentration of OH^- ions (K_2 variant), significantly influenced the degree of chlorosis of microcuttings, despite re-acidification of the medium after 44-day of culture (Table 5). Rhododendrons grow well on acid and strongly acid soils, probably because they require considerable amounts of iron, which is more easily available at lower pH and low alkali content in the sorption complex (Czekalski 1991, 1998). Holt et al.

Table 3. Influence of different calcium salts and additional auxin (IBA 2 mg/l) on the development of microcuttings of *R.*'Catawbiense Grandiflorum'. pH at the beginning of the culture for all variants had the value of 5.0

Medium variant	Number of leaves per cutting	Degree of callus development in 0-3 scale	Number of shoots with leaves 3cm	Degree of chlorosis in 0–3 scale	pH at the end of culture
K [control]	4.5 ab	1.2 a	0.25 a	1.6 ab	4.2
Cl [$CaCl_2$]	4.3 a	1.2 a	0.04 a	2 a	4.1
N [$Ca(NO_3)_2$]	4.8 abc	1.1 a	0.5 ab	1.65 ab	4.4
S [$CaSO_4$]	5.7 c	1.3 a	0.3 a	1.5 b	5.1
C [$CaCO_3$]	5.6 bc	1.9 b	0.9 b	1.5 b	5.6

Means in a column which are followed by a common letter are not significantly different at P=0.05

Table 4. Influence of additional auxin – IBA on the development of microcuttings of *R.*'Catawbiense Grandiflorum'

IBA concentration [mg/l]	Number of leaves per cutting	Degree of callus development in 0–3 scale	Number of shoots with leaves 3 cm	Degree of chlorosis in 0–3 scale
0	5.3 b	1.2 a	0.2 a	1.5 b
2	4.7 a	1.4 b	0.6 b	1.8 a

Table 5. Influence of pH of the medium on the development of microcuttings of *R.*'Catawbiense Grandiflorum'

Medium variant	Shoot length [cm]	Degree of callus development in 0–3 scale	Degree of chlorosis in 0–3 scale	Degree of browning in 0–3 scale	pH at the beginning of culture	pH at the end of culture
K_1 [control]	1.2 a	1.6 a	0.7 b	0.4 a	5.0	4.5
K_2	1.0 a	1.2 a	1.8 a	0.5 a	8.0	5.3

Means in a column which are followed by a common letter are not significantly different at P=0.05

Table 6. Influence of different calcium salts on the content of selected elements in microcuttings of *R.*'Catawbiense Grandiflorum'

Medium variant	Content of elements in microcuttings [% in d.w.]		
	Ca^{2+}	Mg^{2+}	Na^+
K [control]	0.81 b	0.28 b	0.2 d
Cl [$CaCl_2$]	1.64 d	0.28 b	0.22 e
N [$Ca(NO_3)_2$]	1.54 c	0.27 a	0.19 c
S [$CaSO_4$]	1.5 c	0.28 b	0.15 a
C [$CaCO_3$]	0.71 a	0.32 c	0.18 b

Means in a column which are followed by a common letter are not significantly different at P=0.05

(1998) observed higher percentage of rooted cuttings of selected rhododendron cultivars in soil of pH 4.5 as compared to soil of pH 7.5.

Presence of different calcium salts in medium significantly influenced the content of Ca^{2+}, Mg^{2+} and Na^{+} in microcuttings (Table 6). Increasing the concentration of calcium compounds in medium (Table 2) caused the increase of Ca^{2+} ion content in plants grown on Cl, S and N variants as compared to control K (Table 6). Because of very weak solubility of $CaCO_3$, high concentration of this compound in medium did not influence the increase of the number of calcium ions in the plant material analyzed. Lower content of Ca^{2+} and higher content of Mg^{2+} in microcuttings grown on C variant as compared to the remaining variants containing high concentration of calcium salts, might be the result of antagonism obtaining between magnesium and calcium ions.

Higher content of calcium in plants (Table 6) and good growth in medium containing $CaSO_4$ (Table 3) may indicate its lack of noxiousness for the microcuttings analyzed. Decrease of quality of plants in Cl and N variants in comparison to microcuttings from S variant, was most probably caused by excess of Cl^- and NO_3^- ions (Drehmel and Preil 1992, Bergmann 1983). Mordhorst et al. (1990) compared the calcium content in internerve spaces of rhododendron leaves in leaves with and without the symptoms of chlorosis. It was noted that significantly higher concentration of calcium in leaves without chlorosis might indicate that the ions of this element are not noxious. Research conducted on plum trees (Hartmann 1979), soya (Coulombe et al. 1984) and grapevine (Mengel et al. 1984) indicated lower content of calcium in chlorotic leaves as compared to leaves without symptoms of chlorosis. Tod (1959) on the basis of chemical analyses conducted for two species of rhododendron stated that calcium content in the tissues of chlorotic and non-chlorotic leaves was similar.

The weakest growth of microcuttings of *R.* 'Catawbiense Grandiflorum' was noted in the variant of increased level of calcium chloride in comparison to control. Simultaneously, these microcuttings showed highest calcium content (Table 6). Higher calcium content in leaves might have stabilizing properties for stress factors (Christiansen and Foy 1979; Chuntanaparb and Cummings 1980; Polito 1986). Detoxicating properties towards other elements e.g. heavy metals are also ascribed to calcium ions (Bergmann 1983; Marschner 1986). Mordhorst et al. (1990) stated that because of higher level of calcium ions in non-chlorotic leaves, as compared to calcium ion content in chlorotic leaves, calcium content in tissues cannot constitute a criterion in the evaluation of the degree of tolerance of rhododendrons to high levels of calcium salts in medium. This also refers to plants from *in vitro* cultures. The results of the research presented confirm this view.

In natural conditions high value of pH in soil (caused by HCO_3^- ions) is one of the main factors limiting the possibility of cultivating the majority of species and cultivars of rhododendron in soils with high content of calcium carbonate. Because of its low degree of solubility the application of $CaCO_3$ as the selecting factor in *in vitro* cultures in order to obtain plants with higher tolerance to increased concentration of calcium carbonate in the ground seems to be inefficient. In research conducted on *in vitro* cultures soluble carbonate salts should be used as selecting factor (thanks to carbonate salts it is possible to considerably increase the concentration of HCO_3^- ions in the medium). Research methods can also be introduced which allow more efficient obtaining of specimens with higher tolerance to adverse soil conditions. Further research is planned to use seeds of rhododendrons and mutagenic factors, which will allow for significant broadening of genetic diversity of material analyzed and will increase the probability of obtaining plants tolerant to adverse environmental conditions.

Conclusions

1. The weakest development of microcuttings was obtained in the variant of increased level of $CaCl_2$ as compared to control. These microcuttings showed highest calcium content in comparison to the plants treated with remaining salts.
2. Weakly soluble salts – calcium sulfate and calcium carbonate influenced the development of microcuttings of *R.* 'Catawbiense Grandiflorum' more positively in comparison to control than easily soluble calcium nitrate and calcium chloride.
3. Adding $CaSO_4$ and $CaCO_3$ to medium increased its reaction (pH above 5.0 at the end of every culture) as compared to control (pH 4.2).
4. Increased amount of auxin in medium was stimulating for growth and quality of plants.
5. High pH value of medium caused by the increase of OH^- ion concentration increased the degree of chlorosis of analyzed microcuttings. This fact may indicate the key role of pH in rhododendron cultivation.
6. Application of different calcium salts influenced significantly the assimilation of Ca^{2+}, Mg^{2+}, and Na^{+} ions by the microcuttings.
7. The influence of calcium compounds on the development of analyzed microcuttings did not depend on the amount of assimilated Ca^{2+} ions but on the type of anion present in given salt.

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