Growth regulator effect on *in vitro* regeneration of rhododendron cultivars

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ABSTRACT: In *Rhododendron* L. cv. Azuro, Bohumil Kavka, Catharine van Toll, Grandiflorum, Mars, Nova Zembla, Ortrud, Ovation, Prof. Scholz, Purple Splendour, Rebe and Van Werden Poelman, the effect of growth regulators on organogenesis induction of shoot-tip meristems was tested. All cultivars significantly showed the highest shoot regeneration on MS medium containing 6 mg/dm^3 isopentenyladenine (2iP). For most rhododendrons, the highest shoot multiplication was found on a medium with 8–10 mg/dm^3 2iP in combination with 1 mg/dm^3 indoleacetic acid (IAA). Shoots rooted successfully in the substrate with high level of peat without growth regulators. However, the commercial preparation Racine significantly increased rooting in cv. Grandiflorum, Nova Zembla and Rebe compared with 0.03% indolebutyric acid (IBA).

Keywords: *Rhododendron* L.; growth regulators; shoot regeneration; multiplication; shoot rooting

Genus *Rhododendron* L. (family *Ericaceae*) includes a number of decorative species and cultivars that are often utilized in garden and park architecture designs. In this way, the esthetic value of *Rhododendron* is important in places of public relaxation and in the environment in general, both within and outside busy urban agglomerations.

In vitro propagation from meristematic tissues is a suitable method for production of rhododendrons and azaleas; their propagation by conventional methods (seeds, cuttings, etc.) is either difficult or ineffective. The first features of initial explant growth are extended leaf primordia, followed by bud primordium growth, formation of new primordia, and multiplication of buds and shoots. Shoots were efficiently induced from stamens of rhododendron flower buds (Pavingerová et al. 2000). The development of axillary meristems is dependent on the presence of growth regulators in culture medium (Iapichino et al. 1992) but genotype response to cytokinins of many cultivars has not been explained. A positive effect of cytokinin isopentenyladenine (2iP) on shoot initiation and multiplication was found e.g. in cv. Praecox and Scintillation (Norton, Norton 1985). In *R. catawbiense* cv. Grandiflorum, the formation of buds and somatic embryos on leaf explants was induced in the presence of thidiazuron TDZ (Vejsadová, Derid 1999; Vejsadová, Preťová 2000, 2003). The objective of this study was to find the effect of growth regulators on organogenesis induction of vegetative bud meristematic tissues. In twelve rhododendron cultivars, the shoot regeneration, multiplication and rooting of shoots were determined.

MATERIAL AND METHODS

Surface sterilization of explants

Dormant terminal and axillary buds of rhododendrons, cv. Azuro, Bohumil Kavka, Catharine van Toll, Grandiflorum, Mars, Nova Zembla, Ortrud, Ovation, Prof. Scholz, Purple Splendour, Rebe and Van Werden Poelman were regularly collected from January to February 2003–2005. Meristem tips, 1–2 mm in length, were used as explants. Explants were surface sterilized for 20 min in 1.5% sodium hypochlorite NaOCl (30% commercial bleach SAVO) and washed three times with sterile distilled water.

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Culture medium

Modified MS medium for shoot induction and multiplication was used (Murashige, Skoog 1962). In the experiments, three concentrations 6, 8, 10 mg/dm^3 isopentenyladenine (2iP) were used in the induction and multiplication medium, either separately or in combination with indoleacetic acid (IAA) in the concentration 1 mg/dm^3 (Table 1).

Cultivation of explants

Explants were cultivated in Erlenmeyer flasks for a week in a thermostat at 22 ± 2°C and subsequently cultured in a controlled environment room under a 16 h photoperiod (temperature day/night 22/18°C, light intensity 55 µmol m^2/s). Subcultures were performed at a 4–6 week interval. The cultures were assessed after 8 and 24 weeks. For each treatment, 40 Erlenmeyer flasks, containing 1–3 explants, were used. Each experiment was repeated three times. A two-way ANOVA was used to find the individual and interactive effect between the factor and rhododendron cultivar. The comparative Duncan's multiple range test (P = 0.05) was used to determine differences between treatments.

Transfer of shoots to *ex vitro* conditions

Microcuttings (with about 4 leaves) from 12 month-old *in vitro* regenerants (without acclimatization) were soaked for 15 min in tap water to be subsequently dipped for a short time in one of the following growth stimulators: 0.03% indolebutyric acid (IBA) and 0.03% commercial preparation Racine containing 2.5% naphthylacetic acid (NAA), and subsequently grown in the substrate in pots. The mixture of 10% of perlite: 90% of pure peat was used as the elementary substrate. Controls were cultured without any growth regulator. Microcuttings were cultivated at a day/night temperature of 22/18°C and under 16 h photoperiod. Polyethylene covers and regular irrigation were needed to maintain a relative humidity of 80–95%. Rooting of the shoots was evaluated after 18 weeks.

Table 1. Composition of the modified MS medium

Medium compounds	(mg/dm^3)
NH_4NO_3	250
KNO_3	285
$CaCl_2 \cdot 6\ H_2O$	220
Na_2EDTA	52.3
$FeSO_2 \cdot 7\ H_2O$	19.5
H_3BO_3	3.1
$Na_2MoO_4 \cdot 2\ H_2O$	0.12
$CoCl_2 \cdot 6\ H_2O$	0.012
KH_2PO_4	85
$NaH_2PO_4 \cdot 2\ H_2O$	50
$MgSO_4 \cdot 7\ H_2O$	185
$MnSO_4 \cdot 4\ H_2O$	11.1
$ZnSO_4 \cdot 7\ H_2O$	4.3
$CuSO_4 \cdot 5\ H_2O$	0.012
KJ	0.04
Myo-Inositol	100
Thiamine	0.4
Nicotinic acid	0.4
Pyridoxine	0.2
Glycine	4
Adenine sulphate	40
Isopentenyladenine (2iP)	6, 8, 10
Indoleacetic acid (IAA)	1
Polyvinylpyrrolidone (PVP)	2,000
Sucrose (%)	3
Agar Sigma (%)	0.75
pH	5.3

RESULTS AND DISCUSSION

Successful establishment of *in vitro* cultures is substantially influenced by multiple factors, in particular by the composition of induction medium. The nutrient medium according to Anderson (1984) is frequently used for regeneration of rhododendrons (Iapichino, Chen 1995; Mertens et al. 1996; Pavingerová et al. 2000). Nevertheless, some authors prefer modified MS medium, e.g. Preil and Engelhardt (1977) reported a high regeneration frequency in *R. simsii* Helmut Vogel on MS medium at a 1/10 dilution of macro/microelements. MS medium with optimum pH 5.3 was selected on the basis of our previous pilot experiments; more significant results were achieved with modified MS salt concentration as compared to Anderson's medium. Almost all rhododendron cultivars need specific culture conditions, in particular addition of suitable growth regulators. In the experiments, three concentrations of cytokinin 2iP were used in the induction and multiplication medium, either separately or in combination with auxin IAA. After 8 weeks of culture, all cultivars exhibited shoot induction except for explants cultivated on the medium without growth regulators showing progressive necrosis (Table 2). Induction of shoots was not stimulated by increas-

Table 2. Effect of growth regulators on the shoot regeneration in rhododendron cultivars after 8 weeks of culture – shoot regeneration (%)

Cultivar/ regulator (mg/dm^3)	Azuro	Bohumil Kavka	Catharine van Toll	Grandiflorum	Mars	Nova Zembla
None*	–	–	–	–	–	–
2iP 6	39.1 ± 6.9 g	6.8 ± 3.1 b	12.4 ± 2.8 c	10.9 ± 2.3 c	10.6 ± 1.9 c	9.7 ± 2.9 c
8	35.4 ± 8.9 f	4.6 ± 2.3 a	9.2 ± 3.4 b	7.5 ± 2.9 b	8.7 ± 3.5 b	6.8 ± 3.3 ab
10	34.8 ± 8.9 f	3.9 ± 2.1 a	7.6 ± 3.5 b	6.5 ± 3.0 b	7.4 ± 3.2 b	6.4 ± 3.0 ab
2iP 6+ IAA 1	38.9 ± 7.1 g	6.4 ± 2.0 b	12.1 ± 2.4 c	9.3 ± 1.8 c	10.2 ± 1.5 c	7.8 ± 3.2 b
2iP 8 + IAA 1	36.2 ± 9.0 f	5.2 ± 2.6 a	10.5 ± 2.9 c	7.6 ± 2.9 b	9.1 ± 4.1 b	7.6 ± 3.4 b
2iP 10 + IAA 1	35.5 ± 8.9 f	4.2 ± 2.3 a	8.3 ± 3.5 b	7.0 ± 2.9 b	8.3 ± 3.5 b	6.1 ± 3.5 b
	Ortrud	Ovation	Prof. Scholz	Purple Splendour	Rebe	Van Werden Poelman
None*	–	–	–	–	–	–
2iP 6	9.2 ± 2.1 c	8.9 ± 2.0 c	22.6 ± 4.0 e	27.7 ± 4.3 f	46.3 ± 7.5 h	41.2 ± 7.1 g
8	6.4 ± 3.0 b	5.6 ± 2.8 a	15.4 ± 4.9 d	21.6 ± 4.5 e	40.9 ± 8.1 g	35.7 ± 8.9 f
10	5.8 ± 2.8 ab	4.8 ± 2.3 a	12.7 ± 5.5 c	18.4 ± 5.4 d	36.7 ± 9.1 f	29.8 ± 4.8 e
2iP 6 + IAA 1	8.7 ± 3.3 b	8.5 ± 2.5 b	18.0 ± 5.4 d	21.3 ± 4.5 e	47.5 ± 7.6 h	42.9 ± 7.0 g
2iP 8 + IAA 1	7.2 ± 3.1 b	8.8 ± 2.5 b	15.8 ± 4.9 d	22.3 ± 4.5 e	42.8 ± 8.3 g	36.3 ± 8.9 f
2iP 10 + IAA 1	6.2 ± 3.2 b	5.2 ± 2.1 a	13.0 ± 5.5 c	18.9 ± 5.4 d	37.5 ± 9.1 f	32.9 ± 8.2 e

*necrosis. The values are mean of 40 replicates, ± SE. Values followed by the same letter are not significantly different according to the Duncan's test (P = 0.05)

Table 3. Effect of growth regulators on the shoot number in rhododendron cultivars after 24 weeks of culture – shoot number (>10 mm)/explant

Cultivar/ regulator (mg/dm^3)	Azuro	Bohumil Kavka	Catharine van Toll	Grandiflorum	Mars	Nova Zembla
None	2.3 ± 1.1 a	1.1 ± 0.7 a	1.0 ± 0.4 a	1.8 ± 0.9 a	3.2 ± 0.7 a	1.1 ± 0.8 a
2iP 6	4.1 ± 2.5 b	2.2 ± 0.8 a	1.2 ± 0.5 a	2.2 ± 0.9 a	11.5 ± 3.1 e	1.8 ± 0.8 a
8	5.8 ± 2.9 bc	3.8 ± 0.5 b	2.8 ± 0.6 a	7.3 ± 0.5 c	12.2 ± 3.1 e	2.9 ± 0.9 a
10	6.5 ± 2.2 c	3.5 ± 0.5 b	2.9 ± 0.6 a	7.5 ± 0.5 c	14.8 ± 1.8 f	3.1 ± 0.9 a
2iP 6 + IAA 1	5.7 ± 2.8 bc	2.8 ± 0.8 a	2.1 ± 0.5 a	3.2 ± 1.3 a	11.8 ± 2.9 e	2.0 ± 0.8 a
2iP 8 + IAA 1	7.9 ± 3.2 c	7.0 ± 2.7 c	3.5 ± 0.9 a	9.6 ± 1.0 de	14.5 ± 1.8 f	4.1 ± 0.6 b
2iP 10 + IAA 1	8.6 ± 2.1 d	7.2 ± 2.7 c	3.4 ± 0.9 a	9.1 ± 1.0 d	16.8 ± 2.0 f	4.5 ± 0.6 b
	Ortrud	Ovation	Prof. Scholz	Purple Splendour	Rebe	Van Werden Poelman
None	1.6 ± 1.1 a	2.6 ± 1.6 a	1.9 ± 1.2 a	1.2 ± 0.9 a	1.7 ± 1.1 a	2.0 ± 1.3 a
2iP 6	3.2 ± 1.3 b	13.1 ± 1.8 e	2.1 ± 1.5 a	3.9 ± 0.7 b	6.9 ± 0.9 c	9.3 ± 2.1 d
8	3.8 ± 1.4 b	16.4 ± 1.8 f	6.5 ± 1.1 c	4.5 ± 1.2 b	7.4 ± 0.9 cd	9.8 ± 2.1 d
10	3.5 ± 1.4 b	15.2 ± 1.7 f	5.0 ± 1.9 bc	4.8 ± 1.2 b	7.9 ± 1.0 c	9.5 ± 2.0 d
2iP 6 + IAA 1	3.9 ± 1.5 b	15.8 ± 1.7 f	3.0 ± 2.1 a	3.9 ± 0.9 b	7.2 ± 1.1 c	9.9 ± 2.1 d
2iP 8 + IAA 1	4.9 ± 2.0 bc	16.6 ± 1.8 f	8.7 ± 2.0 d	5.8 ± 2.1 bc	9.5 ± 0.8 d	11.4 ± 1.9 de
2iP 10 + IAA 1	4.2 ± 2.0 b	16.7 ± 1.8 f	6.7 ± 1.3 c	5.5 ± 2.1 bc	9.2 ± 0.7 d	9.7 ± 2.1 d

The values are mean of 40 replicates, ± SE. Values followed by the same letter are not significantly different according to the Duncan's test (P = 0.05)

Table 4. Effect of growth stimulators on root length in rhododendron cultivars after 18 weeks of *ex vitro* culture – root length (mm)

Cultivar/ stimulator	Bohumil Kavka	Grandiflorum	Mars	Nova Zembla	Prof. Scholz	Rebe
None	22.3 ± 6.3 a	24.7 ± 6.1 a	44.3 ± 6.7 c	21.9 ± 6.8 a	26.1 ± 6.9 a	46.9 ± 6.7 c
0.03% IBA	28.6 ± 8.5 a	26.3 ± 8.2 a	45.8 ± 6.7 c	22.4 ± 6.8 a	30.4 ± 7.1 ab	49.6 ± 6.9 c
0.03% Racine	32.2 ± 9.1 ab	49.3 ± 6.9 cd	47.0 ± 6.5 c	38.6 ± 5.7 b	34.1 ± 5.9 b	57.4 ± 5.4 e

IBA (indolebutyric acid), Racine (commercial formulation containing 2.5% NAA). The values are mean of 20 replicates, ± SE. Values followed by the same letter are not significantly different according to the Duncan's test ($P = 0.05$)

ing the cytokinin concentration; on the contrary, a reverse effect in most of the cultivars has been observed. The important culture medium component for stimulation of axillary buds and shoots is IAA that proved to be effective at concentrations of 1 to 6 mg/dm^3, e.g. for shoot regeneration of cv. Praecox and Scintillation (Norton, Norton 1985). These authors observed the highest shoot induction on a medium supplemented with 2iP or zeatin. In these experiments, significantly the highest regeneration of all the cultivars was achieved on the medium containing 2iP (6 mg/dm^3). The presence of 2iP (6 mg/dm^3) with IAA (1 mg/dm^3) improved regeneration in cv. Rebe and Van Werden Poelman (Table 2).

Higher cytokinin concentrations and the presence of IAA had no effect on shoot regeneration. For rhododendron cv. Fuchsia, Hsia and Korban (1997) reported low shoot induction in the presence of 2iP, while the highest regeneration frequency was induced with thidiazuron (TDZ). Some authors (Mertens et al. 1996; Hsia, Korban 1997; Vejsadová, Preťová 2003) reported TDZ to be an essential growth regulator for the shoot induction of leaf explants. Our results indicate specificity of rhododendron cultivars to cytokinin 2iP.

According to Procházka et al. (1989) media containing cytokinin derivatives induce growth of lateral buds (and then of shoots) in the axils of leaf primordia. On the media without growth regulators, the development of axillary meristems that can proliferate into lateral branches was inhibited due to apical dominance. This assumption was tested in multiplication experiments. For most of the cultivars a significantly enhanced shoot multiplication in higher 2iP concentrations (8 to 10 mg/dm^3) combined with 1 mg/dm^3 IAA was found (Table 3). Shoot formation was reduced on the medium without growth regulators. Auxin stimulated shoot numbers except for cv. Catharine van Toll, Ovation and Van Werden Poelman. The cultivars showed statistically significant differences in shoot multiplication depending on 2iP concentration in the presence of IAA. The best regeneration of shoots was found in cv. Mars, Ovation and Van Werden Poelman.

We found that the *ex vitro* rooting of rhododendrons was superior to *in vitro* rooting and an acclimation phase had no significant influence on multiplication procedure (unpublished results). Economou and Read (1986) showed that root induction and growth depended on the number of subcultures. They found the highest root growth rates for the third and fourth subcultures, which is consistent with our results for microcuttings after the fourth passage. After 18 weeks of *ex vitro* culture, root length was significantly enhanced by the preparation Racine at a concentration of 0.03% in cv. Grandiflorum, Nova Zembla and Rebe (Table 4). Application of 0.03 % IBA had no significant effect on root length in any of the cultivars. *In vitro* shoots of cv. Mars and Rebe (that showed good regeneration ability and growth in general) rooted successfully without growth stimulators.

In rhododendrons tested, growth regulators 2iP and IAA significantly enhanced organogenesis induction of vegetative bud meristematic tissues in response to cultivar-specificity. The lowest regeneration frequency was determined in cv. Bohumil Kavka, Nova Zembla and Ovation explants. The lowest shoot formation was found in cv. Catharine van Toll, Nova Zembla and Ortrud (1–5 shoots per explant). The highest *in vitro* explant responsibility was recorded for cv. Rebe, Azuro and Van Werden Poelman.

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Vliv růstových regulátorů na *in vitro* regeneraci kultivarů rododendronu

ABSTRAKT: U kultivarů rodu *Rhododendron* L. (Azuro, Bohumil Kavka, Catharine van Toll, Grandiflorum, Mars, Nova Zembla, Ortrud, Ovation, Prof. Scholz, Purple Splendour, Rebe a Van Werden Poelman) byl testován účinek růstových regulátorů na indukci organogeneze z meristémů vzrostného vrcholu. Všechny kultivary vykazovaly nejvyšší regeneraci výhonů na MS médiu s obsahem 6 mg/dm^3 izopentenyladeninu (2iP). U většiny rododendronů byla zjištěna nejvyšší multiplikace výhonů na médiu s 8–10 mg/dm^3 2iP v kombinaci s 1 mg/dm^3 kyseliny indolyloctové (IAA). Výhony úspěšně zakořenily v substrátu s vysokým obsahem rašeliny bez růstových regulátorů, ale u cv. Grandiflorum, Nova Zembla a Rebe komerční přípravek Racine průkazně zvýšil zakořeňování ve srovnání s 0,03% kyselinou indolylmáselnou (IBA).

Klíčová slova: *Rhododendron* L.; růstové regulátory; *in vitro* regenerace; multiplikace; zakořeňování

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