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MICROPROPAGATION OF *CHAMAEDAPHNE CALYCULATA* (L.) MOENCH BY DIRECT SHOOT ORGANOGENESIS

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K e y w o r d s: *Chamaedaphne calyculata* (L.) Moench (leather leaf), the heath family (*Ericaceae*), micropropagation, plant species conservation.

A b s t r a c t

The objective of this experiment was to investigate the effect of various concentrations of sucrose, 6-(γ,γ-dimethylallylamino)-purine (2iP) and pH values of Lloyd's and McCown's medium (1981, WPM) on the induction of lateral shoot growth in *Chamaedaphne calyculata* (L.) Moench. The explants were 2–3 cm nodal sections without the apex, with preserved leaves, from plants grown *in vitro*. The highest regenerative capacity was observed in culture media without cytokinin, with 58 mM sucrose content and pH 5.0. The lowest capacity for shoot organogenesis was reported in media with pH 5.6 with a higher sucrose content (88 mM) and 25 μM of 2iP. 80% of rooted explants were successfully transferred to *ex vitro* conditions. The survival rate of plantlets reached around 60% after three months of greenhouse cultivation.

MIKROROZMNAŻANIE *CHAMAEDAPHNE CALYCULATA* (L.) MOENCH METODĄ BEZPOŚREDNIEJ ORGANOGENEZY PĘDOWEJ

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S ł o w a k l u c z o w e: *Chamaedaphne calyculata* (L.) Moench (chamedafne północna), mikrorozmnażanie, ochrona gatunkowa, wrzosowate.

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Abstrakt

Badania podjęte w tej pracy miały na celu zbadanie wpływu różnych stężeń sacharozy, 6-(γ,γ-dimetyloallioamino)-puryny (2iP) oraz odczynu pH pożywki Lloyda i McCowna (1981,WPM) na efektywność procesu indukcji rozwoju pędów bocznych u *Chamaedaphne calyculata* (L.) Moench. Eksplantatami były fragmenty węzłowe pozbawione wierzchołka wzrostu pędu o długości od 2 do 3 cm, z zachowanymi liśćmi, pochodzące z roślin hodowlanych w kulturach *in vitro*. Najwyższy stopień regeneracji stwierdzono na podłożu bez dodatku cytokininy, zawierającym sacharozę w stężeniu 58 mM, o pH 5,0. Najsłabszą organogenezę pędową obserwowano na pożywkach o wyższym stężeniu sacharozy (88 mM), z 2iP o stężeniu 25 μM oraz o pH 5,6. 80% ukorzenionych eksplantatów pomyślnie przeniesiono do warunków *ex vitro*. Przeżywalność roślinek w warunkach szklarniowych po 3 miesiącach wynosiła około 60%.

Introduction

Chamaedaphne calyculata (L.) Moench is one of the rarest species of the family *Ericaceae* in Poland. It is a relic postglacial species characteristic of the boreal climate zone. The global distribution of the species covers the boreal and subarctic zones of Europe, Asia and North America. In Europe and Asia, it is widespread in Siberia and Scandinavia (Kloss 1999, Kruszelnicki 2001). Poland marks the south-western boundary of the species' European range, and the plant is mostly encountered in lakelands and the Masovian-Podlachian Lowland (Kloss 1999) in marshy forests and peatlands. In Europe, the species inhabits raised bogs of the *Oxycocco-Empetrion hermaphroditi* association in the subarctic and boreal zone (Kruszelnicki 2001). In Poland, the plant grows on raised bogs with acidic soils (pH below 5) within the *Sphagnetum magellanici* association and, less frequently, in *Vaccinio uliginosi-Pinetum* marshy coniferous forests (Kloss 1999, Kruszelnicki 2001). There are nine (10) leather leaf localities in Poland (out of the 13 localities known historically) which are seriously threatened by human activity, mainly peatland drainage (Kruszelnicki 2001). *Ch. calyculata* is a wildlife native species in Poland, and it remains under full legal protection.

Biotechnology offers advanced plant conservation methods which are increasingly often applied in the protection of biological diversity. In Poland, such methods are deployed to protect species of the genera *Drosera*, *Gentiana, Epipactis, Gladiolus, Asplenium* and *Polysticum*. It has been found that the best effects are produced by combining *in vitro* culture methods, which support intensive propagation of plant material, with cryopreservation techniques that allow for long-term material storage (Mikuła and Rybczyński 2006, Rybczyński and Mikuła 2007). Under laboratory conditions, plants can be propagated on a large scale and various species cultures can be preserved for many years, thus enabling the creation of tissue banks of selected plant species.

This paper investigates the micropropagation of *Chamaedaphne calyculata* (L.) Moench by direct organogenesis. The development of a highly effective micropropagation method supporting the production of a large number of seedlings over a short period of time would significantly contribute to species preservation in the natural habitat. Such a method would also enable the colonization of well-preserved raised bogs that offer an ideal habitat for this endangered species.

Materials and Methods

Plant material

Secondary explants were 2–3 cm shoot sections with 4–8 nodes, without the apex and with preserved leaves, isolated from stabilized *in vitro* cultures of *Chamaedaphne calyculata* (L.) Moench shoots on the WPM medium (LLOYD and MCCOWN 1981). Input material for maternal cultures comprised sections of *Ch. calyculata* shoots grown naturally. They were sampled from two selected individuals of a single population at a locality in the Masurian Landscape Park, municipality of Piecki.

Medium composition for inducing the formation of lateral shoots

Each secondary explant was transferred to one of twelve induction media (Table 1). All growth media contained salts, vitamins and amino acids, according to the methodology proposed by LLOYD and MCCOWN (1981, WPM), as well as 8 g l^{-1} of agar. Medium variants differed with regard to their sucrose concentrations (58 and 88 mM), 2iP concentrations (0, 10 and 25 μM) and pH (5.0 and 5.6).

Explants were cultured at a temperature of 20°C (+/– 2°C) under a fluorescent light (OSRAM L36W/77 Fluora, Flora type) photoperiod (16 h light and 8 h dark). The cultures were passaged every six weeks.

The experiment was performed in a completely randomized block design. Every experimental treatment was represented by at least five replications. Every replication consisted of 10 test tubes (50 ml each) with 10 ml of the medium where individual explants were placed vertically (the bottom internode without the lateral bud was inserted into the medium). A total of 50 explants were cultured in each medium type throughout the experiment.

The formation of lateral shoots and aseptic culture conditions were monitored every 5–7 weeks. The development of lateral shoots was observed

Table 1
Differences in the composition of Lloyd and McCown's medium variants (WPM, 1981) used in the experiment. The underlined growth media were also applied in the rooting process

Medium	Sucrose [mM]	2iP [μM]	pH
111	58	0	5.0
112	58	0	5.6
121	58	10	5.0
122	58	10	5.6
131	58	25	5.0
132	58	25	5.6
211	88	0	5.0
212	88	0	5.6
221	88	10	5.0
222	88	10	5.6
231	88	25	5.0
232	88	25	5.6

based on the following scale (RPB): 1° – swollen bud; 2° – shoot with folded leaves in a bud, 3° – shoot shorter than 3 cm with unfolded leaves, 4° – shoot longer than 3 cm with unfolded leaves.

Rooting and acclimatization in *ex vitro* conditions

To induce rooting, explants were cultured in each of the four WPM variants which did not contain phytohormones and differed in their sucrose content and pH (Table 1).

After 18 weeks (three growth cycles), plants with branching roots, where the length of the main root exceeded 1.5 cm, were transferred to *ex vitro* conditions. During the first six weeks, the plantlets were grown on perlite saturated with a 50% solution of WPM salts (LLOYD and MCCOWN 1981). After the successive six weeks, the plantlets were transferred to a 3:1 mix of acidic peat and perlite, and they were watered with a 50% solution of WPM salts (LLOYD and MCCOWN 1981). After the following six weeks, the 50% WMP salt solution was replaced with deionized water (pH 5.0–5.2). Prior to transfer, the plantlets were rinsed in distilled water to remove medium residues. The *ex vitro* culture was carried out in conditions identical to the *in vitro* culture. The vessels containing the plantlets were covered to maintain high levels of air humidity.

Statistical analysis

The obtained data were processed by an analysis of variance and Duncan's test at a significance level of $\alpha=0.05$ using Microsoft Excel 2007 and STATISTICA 8.0 software.

Results and Discussion

The highest rate of lateral shoot induction was observed in medium 111 without 2iP with sucrose content of 58 mM and pH 5.0. The data presented in Table 2 suggest that higher sucrose concentrations, the presence of 2iP and higher pH values had a detrimental effect on the induction and growth of lateral shoots in *Ch. calyculata*. The noted differences were statistically significant.

Carbohydrates added to in vitro culture media (mainly disaccharides – sucrose, maltose, and monosaccharides – glucose, fructose, galactose and maltose) are the main source of carbon for the cultured plant material. They also stabilize the substrate's osmotic equilibrium, thus enhancing nutrient uptake and the growth of explant cells (STEFANIAK 2004, DEBNATH 2005). Research results show that the sugar which is most often applied in the micropropagation of plants of the family *Ericaceae* is sucrose at a concentration level of 88 mM, although lower doses of 44–58 mM are recommended by some authors (e.g. KYTE and KLEYN 2003, CAO et al. 2003, LITWIŃCZUK and WADAS 2008). In this study, culture media were enriched with 58 mM sucrose (this concentration level is generally applied to the media proposed by LLOYD and MCCOWN 1981, as well as ZIMMERMAN and BROOM 1980) and 88 mM sucrose (most popular concentration for ANDERSON'S medium, 1975). After 18 weeks (three growth cycles), higher sucrose concentrations limited the number of induced lateral shoots and impaired their growth (Table 2). The above could be attributed to the fact that the presence of sugar in culture media affects the substrate's osmotic pressure. The sucrose content of 88 mM could impair nutrient uptake in comparison with substrates characterized by lower sugar concentrations, thus inhibiting the growth of lateral shoots. The fact that infection rates were twice lower in media with lower sucrose concentrations (30% and 60%, respectively; data not presented) additionally contributes to the above hypothesis. The obtained results showed statistically significant differences in the number of explants induced on culture media with varied sucrose concentrations.

Axillary buds are usually dormant under *in vivo* conditions, but their development can be induced when the bud is separated from the shoot apex

Table 2
Percentage share of lateral buds and stems at various development stages (on the RPB scale) in variants of the WPM medium after three growth cycles (18 weeks). Data presented in columns were analyzed

Medium	Number of explants/ number of explants with inducted lateral buds [%]	Percentage of inducted lateral buds/explant	Percentage of lateral buds at various development stages on the RPB scale/ explant			
			1^0	2^0	3^0	4^0
111	50/36 [72%]	39.68^{a}	25.61^{a}	15.85^{a}	18.29^{a}	40.24^{a}
112	50/24 [49%]	25.37^{b}	5.88^{b}	9.80^{b}	54.90^{b}	29.41^{b}
121	50/17 [35%]	27.89^{b}	11.32^{c}	11.32^{a}	41.51^{c}	32.07^{b}
122	50/15 [30%]	20.30^{b}	15.00^{c}	17.50^{a}	50.00^{b}	17.50^{c}
131	50/13 [27%]	26.46^{b}	18.64^{c}	11.86^{a}	52.54^{b}	16.95^{c}
132	50/12 [25%]	16.37^{c}	16.22^{c}	18.92^{a}	62.16	$2.70^{d,e}$
211	50/31 [62%]	22.54^{b}	14.58^{c}	12.50^{a}	56.25^{b}	16.67^{c}
212	50/20 [40%]	15.24^{c}	31.25^{a}	25.00^{c}	34.37^{d}	9.37^{d}
221	50/15 [30%]	20.19^{b}	16.28^{c}	20.93^{c}	$46.51^{b,c}$	$11.63^{c,d}$
222	50/11 [23%]	9.09^{d}	31.58^{a}	26.32^{c}	42.10^{c}	0.00^{e}
231	50/11 [23%]	14.56^{c}	40.00^{d}	30.00^{c}	30.00^{d}	0.00^{e}
232	50/10 [20%]	10.55^{c}	17.39^{c}	26.09^{c}	$47.83^{b,c}$	8.69^{d}

(elimination of apical dominance). For this reason, culture media that do not contain phytohormones are suitable for the propagation of plants via shoot apices and axillary buds. Nevertheless, the addition of phytohormones, in particular cytokinins, speeds up the elimination of apical dominance, it stimulates shoot branching and increases the propagation rate (e.g. KIRKORIAN 1995, MOK et al. 2000, CZERPAK and PIOTROWSKA 2003, STEFANIAK 2004, SACHS 2005). The most popular cytokinins applied in *in vitro* propagation of ericaceous plants are 2iP and zeatin, used in combination or separately. The selection of optimal concentrations for each species is a vital consideration because the above compounds may exhibit phytotoxic effects (LITWIŃCZUK 2007). According to reference data, the preferred 2iP concentrations for plants of the family *Ericaceae* range from 1 to 73.82 μM, and the range of 10–30 μM is most highly recommended (e.g. MCCOWN 2000, TOMSONE and GERTNERE 2003, LITWIŃCZUK 2007). In view of the above, we decided to test 2iP concentrations of 10 and 25 μM in our experiment. The reported results (Table 2) indicate that although 2iP is popularly used in the micropropagation of other species of the family *Ericaceae*, it does not effectively stimulate direct shoot organogenesis in *Ch. calyculata*.

The pH of the substrate significantly affects ion bioavailability (e.g. WILLIAMS 1993), and it may exert a powerful influence on organogenesis *in vitro* (e.g. ANTHONY et al. 2004). According to most researchers, pH 4.6 to 5.8 is the

optimum range for ericaceous plants (e.g. LITWIŃCZUK 2007). In this experiment, culture media had the pH of 5.0 and 5.6. In general, a higher rate of lateral shoot induction was observed in media with a lower pH (5.0), and statistically significant differences were reported in the number of explants induced in media with different pH values (Table 2). Nutrient availability is lower in media with a lower pH, nevertheless, *Ch. calyculata* is naturally habituated for peatlands with a low pH, and the obtained results should not be surprising.

Effective rooting of regenerated shoots and high plant survival rates in soil are the critical final stages of micropropagation. The shoots of ericaceous plants grown *in vitro* on media enriched with auxins (indole-3-butanoic acid, IBA) and the shoots grown *ex vitro* in a controlled environment chamber without the addition of phytohormones develop roots relatively easily. The effectiveness of rooting is often similar in both environments, therefore, the *in vitro* rooting phase may be omitted, and the shoots produced by tissue cultures may be rooted directly in the greenhouse (ALMEIDA et al. 2005, CANTOS et al. 2007, MEINERS et al. 2007). In several referenced studies, shoots developed weak root systems on agar media, and more satisfactory results were reported *in vitro* (e.g. ORLIKOWSKA 1986, LYRENE and PERRY 1988). The existing body of research on *Ch. calyculata* describes only the rooting of shoot cuttings from mature plants growing in the Drawa National Park (MALINOWSKA et al. 2004). Our previous experiments have demonstrated that *Ch. calyculata* roots equally well in media without phytohormones and media enriched with auxins. The shoots grown *ex vitro* develop significantly weaker root systems (data not presented). For this reason, in this experiment, the rooting process was carried out *in vitro* on media not containing phytohormones. Adventitious root formation was observed during six weeks of culture in 75% shoots. The roots of plantlets grown on media with a higher sucrose content (211 and 212) were notably shorter and less branched than the roots of plants cultured in media with lower sucrose concentrations (111 and 112) – Figure 1. No differences were reported in the root systems of explants cultured on media with various pH (Figure 1).

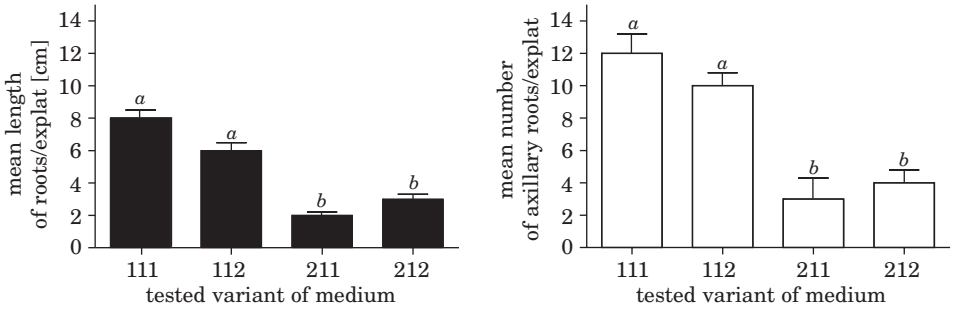

Fig. 1. *In vitro* rooting and root development in explants grown on different media after 18 weeks

80% of rooted shoots were successfully transferred to *ex vitro* conditions, and the remaining 20% died in the first two weeks of acclimatization. The survival rates of plantlets grown *in vitro* reached around 60% after three months.

Conclusions

Increasing 2iP concentrations in the culture medium had an inhibitory effect on the induction of lateral shoots and their growth. Higher sucrose concentrations reduced the efficiency of *Ch. calyculata* micropropagation. The rate of lateral shoot induction was higher in media with lower pH values (5.0). The applied growing regime supported the growth of *Ch. calyculata* under *in vitro* conditions.

The results of this study indicate that *Ch. calyculata* micropropagation by direct organogenesis yields the best results on a WPM medium (LLOYD and MCCOWN 1981) not enriched with 2iP, with a sucrose content of 58 mM and pH 5.0.

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