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BRIEF COMMUNICATION

Effects of sugars and growth regulators on *in vitro* growth of *Dactylorhiza* species

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Abstract

The influence of sugars and growth regulators on shoot and root growth of *Dactylorhiza* species was studied under *in vitro* conditions. The seedling development was stimulated with the application of glucose and sucrose at concentration of 10 g dm^{-3} each. The improvement of shoot growth rate and shoot length was enhanced by cytokinins N^6-(2-isopentenyl)adenine or N^6-benzyladenine and their combination with auxin indolebutyric acid (IBA). The root growth rate and root length of seedlings increased in the presence of IBA and α-naphthaleneacetic acid. Individual *Dactylorhiza* species showed statistically significant differences in shoot and root development depending on sugar and growth regulator combinations.

Additional key words: auxins, cytokinins, glucose, *in vitro* cultivation, sucrose, terrestrial orchids.

Rather limited amount of data published on the development of terrestrial orchids in early stages in the life seriously hampers efforts to protect their populations. This is mainly because the seeds are so minute and much of the process occurs out of sight underground (Scott and Carey 2002). Propagation under *in vitro* conditions can not only provide amounts of genetic material for repatriations but also shed light on the intimate life stages of these rare plants.

Germination of orchid seeds is enhanced by mycorrhizal fungi (Arditti *et al.* 1990, Masuhara and Katsuya 1994, Zettler and Hofer 1998), which are, for *in vitro* cultivations, substituted by suitable sugars. While polysaccharides like starch or cellulose are suitable for germination initiation in the presence of mycorrhiza (Smith and Read 1997), for asymbiotic *in vitro* propagation, mono- or disaccharides (sucrose, glucose or fructose) are preferable (Harvais 1973). The presence of glucose in the cultivation medium was shown to stimulate seedling growth rate (Harvais and Hadley 1967) but the effect of a combination of mono- and disaccharides is unknown.

Growth regulators have a low importance for germination, but are important for the subsequent development of protocorms (Stewart 1989, De Pauw *et al.* 1995). Their influence often depends on the concentration and the orchid species (Van Waes and Debergh 1986), *e.g.* IAA inhibited germination of *Dactylorhiza purpurella* while kinetin inhibited formation of protocorm rhizoids (Hadley 1970).

The objective of this work was to determine the influence of sugars and growth regulators on shoot and root growth rate and length of *Dactylorhiza* seedlings.

We used mature seeds of *Dactylorhiza majalis* (Reichenb.) Hunt et Summerh., *Dactylorhiza incarnata* (L.) Soó ssp. *incarnata*, *Dactylorhiza incarnata* (L.) Soó ssp. *ochroleuca, Dactylorhiza maculata* (L.) Soó ssp. *maculata.* All these taxa are weakly or medium mycotrophic (Michl 1981). Mature capsules were collected during July in Southern Bohemia. The seeds

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Abbreviations: BA - N^6-benzyladenine; IAA - indole-3-acetic acid; IBA - indolebutyric acid; 2iP - N^6-(2-isopentenyl)adenine; KIN - kinetin (N^6-furfuryladenine); NAA - α-naphthaleneacetic acid.
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were stored at 4 - 5 °C until sowing.

The seeds were transferred from 5 to 20 °C one day before sowing. They were surface sterilized with 7.2 % $Ca(OCl)_2$ for 30 min and 70 % ethanol for 3 min and three times washed in sterilized distilled water for 20 min. The modified nutrient medium (Michl 1981) with pH 5.4 was used for germination and cultivation. The seeds were incubated at 22 ± 2 °C in complete darkness. After germination, all protocorms were cultured at day/night temperature of 23/20 °C, 16-h photoperiod and irradiance of 55 μmol m^{-2} s^{-1}.

After 5 months, formed protocorms were placed into the cultivation medium with sugars and growth regulators tested. The growth rate of shoots and roots was determined after 17 months from sowing. In *D. maculata* ssp. *maculata* and *D. incarnata* ssp. *ochroleuca*, the number of bulbs per seedling, the shoot and root length were evaluated after 12 months.

The observed temporal pattern of *D. majalis* and *D. incarnata* ssp. *incarnata* growth was linear, rather than exponential. We therefore first fitted the temporal dependence of the aerial plant part length and the number of roots by means of linear regression to obtain their relative growth rates (slopes of the regression lines). Further we analyzed the dependence of the relative growth rates in *D. majalis* and *D. incarnata* ssp. *incarnata* and the dependence of shoot and root length on the combinations of sugars and growth regulators. In *D. maculata* ssp. *maculata* and *D. incarnata* ssp. *ochroleuca*, the growth was expressed by seedling length and bulb number.

One-way *ANOVA* and the subsequent Duncan's multiple range test ($P < 0.05$) were used to determine differences between treatments.

In *D. incarnata* ssp. *incarnata,* the highest growth rate of shoots and roots was achieved in the presence of glucose at concentration of 40 g dm^{-3}. In *D. majalis*, a combination of glucose and sucrose enhanced growth rate of shoots (10 + 10 g dm^{-3}) and roots (20 + 20 g dm^{-3}) but a significant difference was confirmed only in the roots (Table 1). In *D. incarnata* ssp. *incarnata*, high concentration of glucose had the strongest positive effect on shoot and root growth rate, which was counteracted by promotion of rapid necrosis of seedlings. A medium concentration of sucrose (20 g dm^{-3}) is therefore preferable, as it still yielded a relatively high growth rate of shoots and roots and a low percentage of necrosis. In *D. majalis*, a combination of sucrose and glucose at concentrations of 10 + 10 g dm^{-3} seems to be the optimal one, as it resulted in a low percentage of necrosis. Our results indicate that a higher concentration of sucrose inhibits orchid seedling growth (expressed by the growth rate of shoots and roots), which is consistent with the observations of Rasmussen (1995), Pritchard and Prendergast (1989) and Van Waes and Debergh (1986). However, the combination of glucose with sucrose had more positive effect on shoot and root growth rate compared with addition of glucose alone. Thus it appeared that the best strategy to stimulate the seedling development was the application of glucose with sucrose at concentration of 10 g dm^{-3} each.

Table 1. Effects of sugars [g dm^{-3}] and growth regulators [μM] on growth rates [mm mm^{-1} $year^{-1}$] of shoots, r_s, and roots, r_r,, in *Dactylorhiza incarnata* ssp. *incarnata* and in *D. majalis* after 17 months from the sowing date. In each column values accompanied by the same letters are not significantly different at $P < 0.05$ (Duncan's test).

Sugars/growth regulator	*D. incarnata*		*D. majalis*	
	r_s	r_r	r_s	r_r
Glucose 20	4.08^{a}	0.36^{a}	2.41^{a}	0.25^{a}
Glucose 40	4.46^{a}	0.55^{c}	2.71^{ab}	0.26^{a}
Glucose + sucrose 10 + 10	4.26^{a}	0.42^{a}	2.77^{b}	0.28^{a}
Glucose + sucrose 20 + 20	4.17^{a}	0.52^{bc}	2.65^{ab}	0.39^{b}
Sucrose 20	4.28^{a}	0.50^{b}	2.28^{a}	0.25^{a}
None	4.05^{c}	0.61^{ac}	1.83^{ab}	0.24^{ac}
IBA 4.9	3.36^{abc}	0.61^{ac}	2.52^{a}	0.31^{abc}
NAA 5.4	3.46^{abc}	0.63^{a}	1.77^{b}	0.25^{ac}
IAA 5.7	2.64^{b}	0.47^{c}	1.78^{b}	0.32^{bc}
KIN 4.7	3.16^{bc}	0.63^{ac}	2.32^{ab}	0.33^{b}
BA 4.4	4.08^{ac}	0.64^{a}	2.20^{ab}	0.25^{ac}
2iP 2.5	4.62^{a}	0.81^{b}	1.89^{b}	0.24^{c}

Generally, auxins stimulate root formation and cytokinins enhance development of shoots and cell division. In *D. incarnata* ssp. *incarnata*, the highest shoot and root growth rate was found on the medium supplemented with 2.5 μM of N^6-(2-isopentenyl)adenine (2iP), significant differences were evaluated in the roots. On the contrary, a positive growth effect of 4.9 μM indolebutyric acid (IBA) on shoots and 4.7 μM kinetin (KIN) on roots, has been observed in *D. majalis*. In *Dactylorhiza maculata* ssp. *maculata* and *D. incarnata* ssp. *ochroleuca*, shoot length was significantly the largest in the presence of 4.9 μM IBA in a combination with 4.4 μM N^6-benzyladenine (BA) or 4.7 μM KIN (Table 2). In the seedlings of *D. ochroleuca*, a significantly larger length of the shoots was found in the presence of 5.4 μM α-naphthaleneacetic acid (NAA) and 4.4 μM BA. Separate additions of IBA, BA or kinetin had no influence on the growth of shoots. For both species, the significantly the largest root length was achieved on the medium supplemented with NAA alone. NAA with BA or kinetin significantly increased the bulb formation, NAA stimulated the bulb number in *D. maculata* ssp. *maculata*. Indole-3-acetic acid (IAA; 5.7 μM) alone or combined with cytokinins had no effect on seedling development.

A positive effect of cytokinins (*e.g.* BA or KIN) on orchid development was reported by Rasmussen (1995). A negative effect of KIN was observed by Hadley (1970) who reported that 1 - 10 μg g^{-1} KIN with 1 μg g^{-1} IAA inhibited germination but enhanced growth rate in *Dactylorhiza purpurella*. In our experiments, a significantly higher shoot growth was observed in the presence

of combinations of IBA with BA or KIN. The growth of roots was stimulated with NAA.

In all *Dactylorhiza* species, statistically significant differences in shoot and root growth depending on sugar and growth regulator combinations were found. The results have shown a positive growth effect of glucose with sucrose and cytokinins 2iP, BA, kinetin and their combination with auxin IBA on *in vitro* seedling development.

Table 2. Effects of growth regulators on shoot and root length and number of bulbs per plant in 12-month-old *Dactylorhiza maculata* ssp. *maculata* and *D. incarnata* ssp. *ochroleuca* seedlings. In each column, values accompanied by the same letters are not significantly different at $P < 0.05$ (Duncan's test).

Growth regulators [μM]	*D. maculata* ssp. *maculata*			*D. incarnata* ssp. *ochroleuca*		
	shoot length [cm]	root length [cm]	bulb number [seedling^{-1}]	shoot length [cm]	root length [cm]	bulb number [seedling^{-1}]
None	3.90^a	7.23^a	0.00^a	5.81^a	6.12^a	0.00^a
IBA 4.9	4.35^a	7.68^a	0.00^a	6.24^a	6.57^a	0.00^a
NAA 5.4	4.33^a	10.53^b	1.80^b	6.22^a	9.41^b	0.00^a
IAA 5.7	3.01^a	6.40^a	0.00^a	5.00^a	5.37^a	0.00^a
BA 4.4	4.07^a	7.96^a	0.00^a	6.02^a	6.84^a	0.00^a
BA + IBA 4.4 + 4.9	6.85^b	7.94^a	0.00^a	8.70^b	6.81^a	0.00^a
BA + NAA 4.4 + 5.4	4.65^a	8.08^a	2.00^b	7.42^b	9.01^a	1.40^b
BA + IAA 4.4 + 5.7	3.42^a	6.57^a	0.00^a	5.31^a	5.46^a	0.00^a
KIN 4.7	3.05^a	6.80^a	0.00^a	5.02^a	5.75^a	0.00^a
KIN + IBA 4.7 + 4.9	5,72^b	7.06^a	0.00^a	7.61^b	6.11^a	0.00^a
KIN + NAA 4.7 + 5.4	3.86^a	5.79^a	1.68^b	6.00^a	4.58^a	1.75^b
KIN + IAA 4.7 + 5.7	3.50^a	7.06^a	0.00^a	5.48^a	6.15^a	0.00^a

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