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Preservation of the rare terrestrial orchids *in vitro*

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Abstract

The aim of this study was to establish viable tissue culture of Estonian terrestrial orchids for *in vitro* conservation and for final reindroduction of propagated plants to their areas. In total 28 meristems of *Dactylorhiza baltica* (Klinge) Orlova were excised from protocorm-like bodies and planted to modified Murashige and Skoog medium supplemented with 0.5 mg l^{-1} naphthaleneacetic acid and 2.0 mg l^{-1} N^6-Δ^2-isopentenyl adenine. After four weeks of culture, 60 % of excised apical meristems of *D. baltica* had started to grow while none of the explants originating from the lateral meristems expressed any tissue development. Semi-ripened seeds of *D. ruthei* (M. Schulze ex Ruthe) Soo and *D. praetermissa* (Druce) Soo. were sown to modified Heller, Lindemann, Norstog or Murashige and Skoog (control) medium. The current studies showed that high concentrations of microelements are necessary for germination of orchid seeds. Depending on species, germination of orchid seeds started two months after initiation. Preliminary results have been obtained regarding in vitro culture of *D. baltica*, *D. ruthei* and *D. praetermissa*.

Key words: orchids, preservation, regeneration, seeds, tissue culture.

Introduction

In vitro germination of seeds of north temperate terrestrial orchids species has been more difficult than with tropical epiphytic orchids. Terrestrial species have more stringent requirements for germination but little is known about their specific requirements (De Pauw et al. 1995). In Estonia there are 36 species of terrestrial orchids, only three of them have been taken under protection. Declining numbers of plants in the populations are caused by climatic changes, human activities, increased overgrowth of growing areas with bushes etc.

The present investigation examined the specific requirements for the germination and protocorm growth of *Dactylorhiza ruthei*, *D. praetermissa* and of meristem initiation of *D. baltica*.

Materials and methods

The protocorm-like bodies of *Dactylorhiza baltica* (Klinge) Orlova and semi-ripened seeds of *D. ruthei* (M. Schulze ex Ruthe) Soo and *D. praetermissa* (Druce) Soo were used as explants. The seed capsules of *D. ruthei* and *D. praetermissa* were collected seven and ten weeks after pollination. The protocorm-like bodies and semi-ripened capsules

were dipped in 75 % ethyl alcohol and rinsed with distilled autoclaved water. This was followed by sterilization in sodium hypochlorite solution for 20 min. In total 28 meristems of *D. baltica* were excised from buds from protocorm-like bodies and planted to modified Murashige and Skoog medium supplemented with 0.5 mg l^{-1} 1-naphthaleneacetic acid (NAA) and 2.0 mg l^{-1} N^6-Δ^2-isopentenyl adenine (2iP). The size of meristems was measured every tenth day.

Semi-ripened seeds were sown to modified Heller (1953), Lindemann (1970), Norstog (1973) or Murashige and Skoog (1962) (control) medium. Seeds from each individual capsule were assigned to all treatments: approximately 100 seeds per plate and two replications of each treatment per capsule. The size of seeds was measured after sowing every ten days. Germination was considered to have occurred when the embryo emerged from testa.

Results

After four weeks of culture, 60 % of excised apical meristems of *D. baltica* had started to grow while none of the explants orginated from the lateral meristems expressed any tissue development (Table 1). Protocorm-like bodies of *D. baltica* formed after four weeks on meristems taken from the apical section of growth buds form the protocorm-like bodies. Root formation of *D. baltica* occurred two months after planting of meristems to media.

The composition of culture medium and the stage of ripeness of seeds had a strong influence on the development of orchids seeds *in vitro*. Depending on species, germination was initiated approximately two months after inoculation in most media variants (Table 2, 3). After four and six months in culture, a higher percentage of the final germination occurred on modified Norstog (1973) medium compared to the control Murashige and Skoog (1962) medium. The germination percentage of seeds of *D. ruthei* (M. Schulze ex Ruthe) Soo was 14 % higher on modified Norstog (1973) than control Murashige-Skoog (1962) medium *D. praetermissa* (Druce) Soo.

Discussion

The present investigation examined specific requirements for *D. baltica* meristem initiation. Protocorm-like bodies of *D. baltica* formed after four weeks on in vitro culture. Explants of *Diuris longifolia* R.Br. formed protocorm-like bodies after forty nine days on the in vitro culture (Collins et al 1992). No tissue development was expressed on meristems of *D. baltica* which orginated from the lateral buds.

Research on *in vitro* germination of terrestrial orchid seeds is often a long, slow process due to the considerable periods of time required for germination to occur (De

Table 1. Regeneration of *Dactylorhiza baltica* meristems depending on position of buds on protocorm-like bodies

Position of buds	Number of excised explants	Number of live explants		
		4 weeeks	6 weeks	8 weeks
Apical	14	8	8	8
Lateral	14	0	0	0

Table 2. Time-course of germination percentage of *Dactylorhiza ruthei* seeds on different cultivation media

Medium	Time of cultivation (months)			
	2	4	6	8
Heller	0	5	7	8
Lindemann	0	6	9	10
Norstog	0	10	20	25
Murashige and Skoog (control)	0	6	10	11

Table 3. Time-course of germination percentage of *Dactylorhiza praetermissa* seeds on different cultivation media

Medium	Time of cultivation (months)			
	2	4	6	8
Heller	0	0	4	8
Lindemann	0	2	5	5
Norstog	0	7	15	20
Murashige and Skoog (control)	0	2	6	9

Pauw et al. 1993). *D. ruthei* and *D. praetermissa* seeds started to germinate after four mounth of culture. Germination of *D. ruthei* and *D. praetermissa* seeds depended on the concentration of $MnSO_4$ in the media. Stancato (1996) showed that micronutrients play a fundamental role in the growth of seedlings of Laelia cinnabarina Batem. Seeds of *Dactylorhiza maculata* started to germinate after 3.5 months of incubation in soil (Kinderen 1995). Our study showed that a high precentage of seeds germinate within three months of culture, and after this time, the increase in germination is very small.

Modified Norstog (1973) medium is characterised by a high concentration $MnSO_4$ (4 g l^{-1}) compared to other media tested. It can be assumed that $MnSO_4$ is an important component in germination of orchids seeds and that it significantly enhances the development of protocorm-like bodies.

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