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DACTYLORHIZA BALTICA IN VITRO AND *IN VIVO*

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For elaboration of species protection strategy and to ensure their reintroduction possibility if necessary, it is important to establish them in cultivation *ex situ* with reasonable representation of its genetic diversity. The terrestrial orchid *Dactylorhiza baltica* (Klinge) N.I. Orlova, widespread in Baltic region, has decorative inflorescence and can be used for ornamental purpose. Therefore it is chosen as a model object for *in vivo* and *in vitro* study. Our aim was an all-embracing study of *D. baltica* including observations in natural habitats (1), elaboration of *in vitro* culture methodology (2) and a subsequent acclimatization *ex vitro* and colonization with symbionts (3). The final target is to create the complete understanding of biology of Latvian wild orchids in all the three steps as base for the creation of protection strategy. A method to obtain qualitative regenerants of *D. baltica* and to maintain them in vitro as to be suitable for transplanting *ex vitro* is proposed. The most successful for acclimatization of *D. baltica* was the test with transplantation of regenerants *ex vitro* after cold treatment at 5 °C in the dark for 5 months, further growing them in a soil of garden compost mixed with a soil stratum from beneath bog moss (1:5). This paper reports the first results (2009-2010).

Key words: *Dactylorhiza baltica*, ecology, *in vitro*, *ex vitro* acclimatization, mycorrhiza.

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INTRODUCTION

Every country has three forms of wealth: material, cultural, and biological; the fauna and flora are parts of a country's heritage, the product of millions of years of evolution centred on that time and place and hence as much a reason for national concern as the peculiarities of language and culture (Wilson 1992). We must act to those great values with piety and must to leave all of them for next generations for centuries. Semi-natural grasslands have a high nature protection priority in Europe because of high diversity and occurrence of rare species, and because of aesthetic and cultural values (Znamenskiy et al. 2006). In Latvia, grassland biotopes are of special concern in biodiversity conservation, because more than 520 species of

flowering plants and pteridophytes occur mainly there, i.e. 1/3 of native flora and 40% of species listed in the Red Data Book of Latvia (approx. 128 species). Plant conservation strategy must consist of several components. For example, plant conservation in Opole province (Poland) encompasses a species-centred protection, ecosystem conservation, *ex situ* conservation, restoration and translocation, reintroduction, and monitoring (Novak et al. 2004). The conservation in Latvia involves both *in situ* protection, with a wide net of protected areas of various human activity restriction levels, and *ex situ* approach, provided in expositions of National Botanic Garden and *in vitro* in its Department of Plant Diversity In Vitro-Conservation. Our study is aimed to investigate necessary conditions for successful cultivation of Latvian rare and endangered orchids *in vitro* and further in *ex vitro* conditions (Jakobsone et al. 2010). An understanding of the ecology of orchid sites is essential to ensure their survival. Only when we have a complete understanding of orchids at habitat, ecological and population levels can we implement appropriate conservation strategies (Dixon et al. 2003).

The Orchidaceae is one of the most threatened in Latvia (26 species, i.e. 80 %, are included in Red Data Book of Latvia 2003) for various reasons. Their long lasting cultivation in laboratory conditions is complicated. In comparison, tropical orchid species are easier to maintain in asymbiotic conditions (Leroux et al. 1997). Seed germination observations *in vitro* reveal plant responses to various environmental conditions and provide essential information of early plant growth and development in natural habitats. *In vitro* asymbiotic seed culture may represent an efficient means to assess early seedling growth and development responses to different environmental conditions in orchids (Dutra et al. 2008). Terrestrial orchids typically produce numerous small seeds without endosperm. In the embryos cells are arranged along a longitudinal axis according to size; these cells contain protein and lipid reserve material (Dutra et al. 2008). Little is known about fate after seed dispersal. Some *in situ* studies indicate seed viability lasting for two years in temperate orchid species (Whighama et al. 2006). As the seeds of orchids are minute and contain few stored food reserves, colonization by a compatible fungus is essential for germination and/or early seedling development in or on the substrate (Smith et al. 1997). In the interaction, fungal hyphae grow into orchid tissues and form elaborate coiled structures known as pelotons within cortical cells. The laboratory approach in cultivation of terrestrial orchids must act as a substitute for symbionts.

One of the typical *genus* in Orchidaceae in Latvian moist grasslands, coastal grasslands, fens and shrubs is *Dactylorhiza*. It is represented by 6 species in Latvia, i.e. *D. incarnata* (L.) Soó*, D. cruenta* (O. F. Müll.) Soó*, D. russowii* (Klinge) Holub*, D. maculata* (L.) Soó*, D. fuchsii* (Druce) Soó and *D. baltica* (Klinge) N. I. Orlova (Cepurīte 2005). The number of *D. baltica* localities is not critically low at present, but the rate of their decline due to various reasons causes trouble. Therefore this species corresponds to the Fourth Threat Category of the Red Data Book of Latvia which includes "undetermined species, insufficiently studied; probably endangered, yet due to the lack of information it is possible to give an exact estimation of their present status; a deeper study is needed" (Andrusaitis et al. 2003: 18).

D. baltica occurs in European moderate zone (Tutin et al. 1980). It is widespread in Baltic region, occurring as scattered individuals, rarely/sometimes growing in dense groups (Kuusk et al. 2003), and it is believed (http://www.latvijasdaba.lv) that through its area it is most abundant just in Latvia. The area of *D. baltica* is disjoined, being reported also for West Asia (Cepurīte 2005). Status of *D. baltica* in Latvia has been considered unclear (Andrusaitis et al. 2003) until recently. Although the data are gradually accumulating, increasing the list of biotopes where this species occurs– moderate moist, swampy, alluvial lands and seaside grasslands, low swamps, bushes, slope of the ditch, rarely forests (Galenieks 1953, Feodorov 1974, Baroniņa 1994, Andrusaitis et al. 2003,

Cepurīte 2005), only in the last ten years has added thorough knowledge of its ecology, as well as the reasons limiting its distribution. It was very important for species protection suggestions development, reintroductions emergency case and species like potential decorative plant selection to get decorative forms with adequacy diverse environment conditions. The terrestrial orchid *D. baltica* is characteristic directly in Baltic region, has quite decorative inflorescence and so can be selected as a new ornamental cultivar. Therefore is preferable to choose it as model object for our study *in vivo* and *in vitro*.

Our objective was to start detailed study of *D. baltica* including observations in their natural habitat (1), *in vitro* culture methodology (2) and subsequent acclimatization *ex vitro* and colonization with symbionts (3). The future aim will be to create the complete understanding about wild Latvian orchids in all three numbered objectives as base for creating of protection strategy.

MATERIAL AND METHODS

Observations *in situ*. The field studies were carried out in five localities of Latvia: *D. baltica* was observed in protected areas near the Lake Engure (Engure-lagoon), at the Lake Pape (Brušvīti), at the Lake Tosmāre, Lake Kaņieris and in semi-natural grasslands in NBG. The type of communities as well as the accompanying species was inspected in these locations.

NBG (total area 129 ha) is located 18 km to southeast of Riga (56º52' N, 24º21' E). The territory is plain with poor relief – 14-26 s.m. There is both closed and opened drainage system. In ditches, completely drying out in summer, there are calcareous springs of underground waters. The dendrological plantations are intermixed with open grassland areas where a wide range of species, typical for forests, forest margins and meadows are represented. In 2008, the inventory of habitats in NBG has been undertaken. The aim of this activity was to locate suitable and potentially suitable biotopes in NBG to work out either their appropriate conservation measures or to enrich their biodiversity, respectively. Methodology elaborated by Bakker (2005), Gazenbeek (2008) and Jermacāne et al. (2002) was applied. Data about biotope management history, current management, possible future threats for the biotope existence were assessed; all species found, especially indicator species, were registered and their cover proportion (%) or number of individuals was filed in a biotope survey form (Strode & Roze 2011).

The transect method was used in biotope inspection. Taxa were named according to "Flora of Latvian vascular plants. List of taxa" (Gavrilova, Šulcs 1999) and biotope classification followed "Habitats of Latvia. Classifier" (Auniņš 2010, Kabucis 2001).

Sterilization of seeds. The fully ripe seeds were collected in the National Botanic Garden of Latvia (NBG; 56° 52.072 N, 024°21.751 E) from plants growing in semi-natural conditions in meadow of rare and endangered plants, in November 2007. The capsules were wiped with 60 % ethanol and excised seeds wrapped into filter-paper discs and closed with staple. They further were sterilized in ACE (commercial chlorine reagent): sterile distilled water = 1 : 1 for 10, 15 and 20 minutes and after then rinsed with sterile distilled water three times for 5 minutes, then delivered in distillate for one hour. The wrappings were carefully opened after rinse and seeds with filter paper placed into jars with 50 ml half-solid initial medium for germination and closed with polypropylene caps.

Cultivation. Sterile cultivation was carried out following the protocol elaborated in Department of Plant Eco-physiology, National Botanic Garden NBG, similar to that for *D. fuchsii* (Jakobsone 2008, Jakobsone et al. 2010) and other species (Belogrudova & Jakobsone 2010). The sprouts, which germinated in 2007-2008, were sampled for *in vitro* studies in 2009.

Cultivation experiments. To create the gene

bank of orchids, two necessary factors in preliminary studies were clarified: the cold treatment bringing physiological rest (1) and initiation of development of new shoots to keep the microculture in rejuvenescence (2). The experiment with addition of various stuffs to basic culture medium for solving the problem (2) was carried out:

A. Yeast extract (YE) in five concentrations - 0; 0.5; 1; 1.5 and 2 $g.L^{-1}$ without (A-1) or in composition with BAP, 0.2 $mg.L^{-1}$ to each concentration (A-2).
B. 6-benzylaminopurine (BAP) in four concentrations – 0; 0.1; 0.2 and 0.3 $mg.L^{-1}$ without (B-1) or in composition with IAA, 0.05 $mg.L^{-1}$ (B-2).
C. 6-(3-hydroxybenylamino) purine (Meta-Topolin, MT) in concentrations 0.1; 0.2 and 0.3 $mg.L^{-1}$.

The duration of this experiment was 1.5 months. The results were evaluated by eight parameters:
1. number of tubers per plant,
2. number of roots per plant,
3. summary root length per plant (mm),
4. number of shoots per plant, by proliferation,
5. number of shoots per plant, generating on roots,
6. browning of medium, by five-point scale,
7. level of necrosis (% in total of a plant),
8. summary vitality, by five-point scale.

Statistical analysis was used Kruskal Wallis Test with the program SPSS 17. As the data of our experiment do not correspond with standard SPSS 17 programme division, there were used non-parametric methods. First, by Kruskal-Wallis test there were compared all sample groups, of which there were selected those sample groups, which showed essential difference in particular parameters. There selected sample groups were further compared by Mann-Whitney U-test. For the final result there was done Pearson correlation assessment to check how the sample groups differ in particular parameter – correlate positively or negatively.

Transplantation *ex vitro*. The first transplantation in boxes was carried out in May 2009 and plants were placed in a room under white luminescent lamps. Six types of substrates were tested, all with pH = 6.8:
1. gravel : meadow soil : pine bark mulch (1:1:0,5);
2. substrate No. 1 covered with forest soil of partly-decayed leaves;
3. substrate No. 1 covered with mixture of meadow soil and pine bark mulch above (1:1:1);
4. garden compost : soil stratum from just beneath of bog moss (1:3);
5. garden compost : soil stratum from just beneath of bog moss (1:5);
6. garden compost : soil stratum from just beneath of bog moss (1:5).

For No. 1-5, *in vitro*-plants were transplanted *ex vitro* from an illumination room, at 23 – 25 °C. For No. 6, the regenerants were transplanted *ex vitro* after cold treatment, at 5 °C in the dark for 5 months.

After a month the boxes were brought outdoors and covered with a textile used for shading in horticulture, also avoiding excessive evaporation. No additional fertilizer was used. The plantlets were estimated in September 2009 by morphology parameters:
1. number of roots per plant,
2. number of droppers (tuberidious roots) per plant,
3. number of tubers per plant (g),
4. fresh mass per plant.

Ten examples of roots from all variants were sampled to check the contamination with symbionts.

The new plantlets were replanted at NBG in the field for the future studies.

The acquired results were processed with PC-ORD 5.0 programme multi-dimension Klaster analysis method.

Root sampling and assessment of mycorrhizal colonization. Ten examples of roots from all

variants were taken to test their contamination with symbionts. Of each variant a root from eight individuals were collected 5 months after beginning of experiment. The method used for cleaning and staining of roots and determination of fungal colonization followed that described by Hayman (1970). Roots were washed under running tap water, heated for 1 h in 10% KOH, washed under running tap water again, stained with 0.05% (w/v) Trypan Blue in lactoglycerol [lactic acid, glycerol and water (v/v/v 1:1:1)] and washed under running tap water. Root pieces were examined and photographed using a Leica DMLS light microscope equipped with a digital camera Leica DFC490.

Mycorrhizal colonization was estimated after Trouvelot et al. (1986) testing one root per plant. Intensity of mycorrhizal colonization in the root system (M%) was calculated with the computer program Mycocalc (Trouvelot et al. 1986). M% was calculated accordingly to the formula M% = (95 × n5 + 70 × n4 + 30 × n3 + 5 × n2 + n1)/(total nb), where n5 = number of fragments with a degree of colonization rated as "5", n4 = number of fragments with a degree of colonization rated as "4", etc.; nb = number of eyeshots. Frequency of mycorrhiza in the root system F% = (nb of fragments myco/total nb)*100 was calculated accordingly Trouvelot et al. (1986) (http://www2.dijon.inra.fr/mychintec/Protocole/protoframe.html).

RESULTS AND DISCUSSION

The grassland biotopes inspected in NBG had natural structure and they had indicator species typical for non-fertilized grasslands. Total number of species in them exceeded 30. *Dactylorhiza baltica* was found as an indicator species of non-fertilized grassland biotopes in NBG (Strode, Roze 2011), where it occurred in the following habitats:

38. Mesophyle grasslands:
35.12. Agrostis-Festuca grasslands, fallow lands
Anthoxanthum odoratum – Briza media grasslands
Anthoxanthum odoratum – Agrostis tenuis grasslands.

38.2. Lowland hay meadows, natural or semi-natural grasslands
Festuca pratensis grasslands
Helictrotrichon pubescens grasslands

These grasslands have relatively fertile soil with an average to rich moisture conditions through all vegetation period or the most of it (Kabucis 2001).

The other four inspected areas were in Kurzeme-region, where *D. baltica* was represented by scattered individuals, but was fairly abundant in grasslands of Brušvīti (at Lake Pape).

D. baltica was present in the following habitats:
1. at Lake Engure in ecotone zone between forest and biotope 54.223, Northern brown bog-rush fens with *Primula farinosa*, *Pinguicula vulgaris, Parnassia palustris*, *Phragmites australis, Potentilla erecta, etc*.
2. at Lake Kaņieris in ecotone zone between bushes and biotope 7210*, Calcareous fens with *Cladium mariscus* and species of the *Caricion davalliane*.
3. at Lake Tosmāre in ecotone zone - some individuals between bushes and biotope 7210*, Calcareous fens with *Cladium mariscus* and species of the *Caricion davalliane* with *Peucedanum palustre*, *Angelica sylvestris*, *Stachys sylvatica*, *Lysimachia vulgaris*, *Filipendula ulmaria*, *Ranunculus acris, Thalictrum flavum*, *Tusillago farfara*.
4. in Brušvīti grasslands (at Lake Pape) in ecotone zone between bushes and *Anthoxanthum odoratum – Agrostis tenuis* grasslands.

This paper presents the very first results obtained in 2009 - 2010. The future studies will be focused on the assessment of role of the above listed species in a plant community, including the orchids, as well as detailed investigation of their biology. *D. baltica* is a highly decorative plant with some infra-specific variation and could be used for a breeding programme.

Table 1. Resulting parameters of *Dactylorhiza baltica* microplants after *in vitro* cultivation experiment

Variants	Number of roots per plant	Length of all roots per plant (mm)	Number of tubers per plant	Number of new plants, by proliferation	Number of new plants, on roots	Browning of culture medium, by 5-point scale	Necrosis (% in total of a plant)	Vitality 5-point scale
A-1 YE 0 g.L^{-1}, control	3.3	23	1.0	0.5	0.1	0.5	3.3	4.4
YE 0.5 g.L^{-1}	2.7	21	0.7	0	0.2	0.1	3.5	4
YE 1 g.L^{-1}	2.2	25	1.1	0.2	0.2	0.2	0	**4.5**
YE 1.5 g.L^{-1}	2.8	21	0.8	0.3	0.2	0.3	3.6	3.7
YE 2 g.L^{-1}	2.4	23	1.1	0.2	0	0.3	1	3.3
A-2 YE 0 g.L^{-1} + BAP 0.2 mg.L^{-1}	1.4	22	1.0	0.1	0.1	1.3	18	3.1
YE 0.5 g.L^{-1} + BAP 0.2 mg.L^{-1}	2.3	22	1.2	0	0.4	0.6	3.5	4.3
YE 1 g.L^{-1} + BAP 0.2 mg.L^{-1}	2.0	26	0.9	0	0.3	1.5	13	3.5
YE 1.5 g.L^{-1} + BAP 0.2 mg.L^{-1}	1.6	27	1.0	0	0.1	1.3	21	3.1
YE 2 g.L^{-1} + BAP 0.2 mg.L^{-1}	2.0	27	0.8	0	0	1.5	18	3
B-1 BAP 0 mg.L^{-1}	2.4	31	0.7	0	0	2.4	13	3.6
BAP 0.1 mg.L^{-1}	2.1	26	0.7	0	0.1	2.6	9	3.4
BAP 0.2 mg.L^{-1}	2.2	22	0.4	0	0.1	2.6	14	3.3
BAP 0.3 mg.L^{-1}	2.4	24	0.2	0.3	0	2.1	8	3,3
B-2 BAP 0.1 mg.L^{-1} + IAA 0.05 mg.L^{-1}	1.7	31	0.7	0.1	0	2.4	11	3.6
BAP 0.2 mg.L^{-1} + IAA 0.05 mg.L^{-1}	2	29	0.6	0.1	0.4	1.8	13	3.3
BAP 0.3 mg.L^{-1} + IAA 0.05 mg.L^{-1}	2.2	28	0.2	0	0.1	2.1	11	3.2
C MT 0.1 mg.L^{-1}	1.1	37	0.2	0	0.7	2.5	25	2.5
MT 0.2 mg.L^{-1}	2.5	22	0.4	0	0.3	2.8	29	2.1
MT 0.3 mg.L^{-1}	2.1	24	0.7	0	0.1	2.9	19	2.9

Table 2. Statistical analysis of influence of BAP and Yeast exstract on plantlet's quality *in vitro* using Kruskal Wallis Test with the program SPSS 17

	Tubers	Number of roots	Vitality	Proliferation rate	Newly developed shoots	Necroses	Length of roots	Previous medium
Chi-Square	12,429	9,215	14,379	10,186	20,169	16,481	7,425	4,449
Asymp. Sig.	0,190	0,418	0,109	0,336	**0,017***	0,057	0,593	0,879

Table 3. Statistical analysis of influence of BAP, MT and IAA on plantlet's quality *in vitro* using Kruskal Wallis Test with the program SPSS 17

	Tubers	Number of roots	Vitality	Proliferation rate	Newly developed shoots	Necroses	Length of roots	Previous medium
Chi-Square	6,861	6,072	20,496	14,427	8,339	17,233	3,154	18,300
Asymp. Sig.	0,652	0,733	**0,015***	0,108	0,500	**0,045***	0,958	**0,032***

***In vitro* cultivation experiments.** The germination and re-growing of plantlets in sterile culture with subsequent transplanting *ex vitro* into the suitable soil mixture was an important constituent of this work with wild orchids. The other aim was to keep the sterile regenerants in microculture conditions for a longer time to create the gene bank. It means that we must get the development of new microshoots, by proliferation or in any other way. The next problem was that the prolonged cultivation (in prevalence in the light) stimulated browning of the culture medium what caused necrosis of plant.

The results of *in vitro* cultivation experiments are summarized in Table 1**.** We observed the propagation in two ways – by proliferation (Fig.1A) and by initiation of new microshoots on roots (Fig. 1B). The proliferation was obtained only in particular cases and did not strongly depend on application of cytokinines. No repeated applications were attempted in this study. The initiation of new microshoots on the roots we considered as one of the natural ways to form plant groups from one initial plant like *in situ*. For *D. fuchsii* we obtained the third way of propagation – by branching of tuber *in vitro* (Jakobsone 2009). Comparison of experimental results showed that morphological parameters were satisfactory nearly in all variants. The highest propagation (the initiation of new 0.7 plantlet on roots /per plant) was observed in the variant with MT 0.1 mg.L^{-1}. Nevertheless, the most important parameters what show the quality of microplants in general were the vitality and the percentage of necrosis of whole plant. Unfortunately the cytokinine MT application

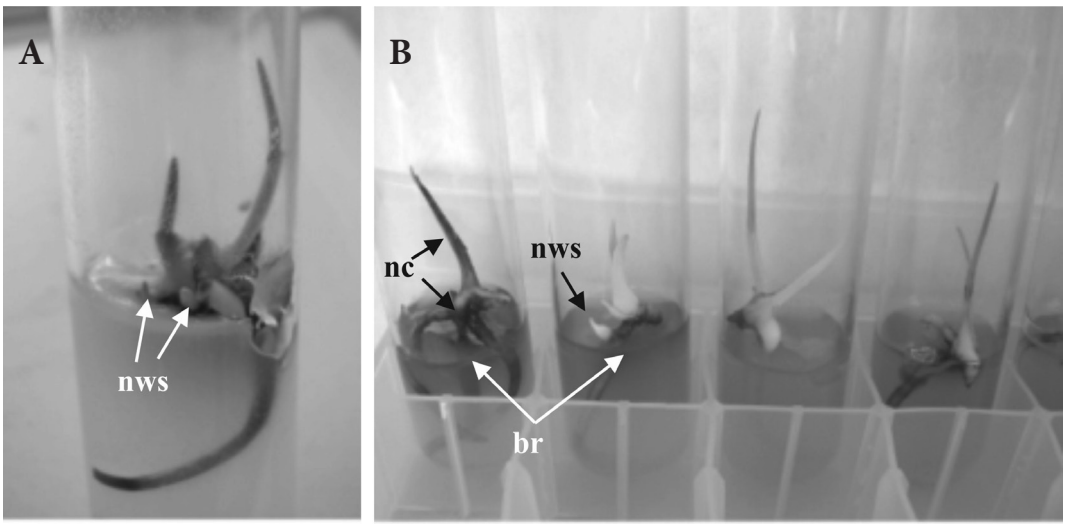

Fig. 1. Microcultivation of *Dactylorhiza baltica* (Klinge) N.I. Orlova: A – proliferation; B – the variant BAP 0.2 mg.L-1 + IAA 0.05 mg.L-1 (nws – development of new shoot; br – browning of culture medium; nc – necrosis).

also caused the browning of culture medium and therefore the highest level of plant necrosis (19 – 29 %) respectively. Some multiplication (0.4 plantlets on roots /per plant) was obtained in variant with application of YE 0.5 $g.L^{-1}$ + BAP 0.2 $mg.L^{-1}$. The vitality rate was 4.3 in this case, i.e. the same as in control (Table 1).

The sample groups differ essentially if *Asymp. Sig.* = 0.00 < 0.05, therefore Van Waes and co-authors' (1986) basic medium modification experimental variants with BAP and YE were essentially different in new sprout formation on roots (variants A1 and A2; table 1) (0.017) (Table 2). In the series of experiments on Fast (1974) basic medium modification with addition of phyto hormone BAP, MT and IES the variants did essentially differ in vitality (0.015), previous medium (0.032) and necroses (variants B1, B2, C; table 1) (0.045) (Table 3). Experimental variants were additionally assessed by making comparison of two sample groups with Mann-Whitney U-test which showed that in these parameters variants mutually did not differ essentially. Basing on the results of Mann-Whitney test, there was also done Pearson correlation test to find out which parameters mutually correlate positively and which - negatively. The assessment of experimental work results by Kruskal-Wallis test, Mann-Whitney U-test and Pearson Correlation test allows predicting the further *in vitro* cultivation experimental work directions in more detail. (Tables 2 and 3).

Some authors (Nakamura; Arditti) had reported that YE contains vitamin B, particularly in the form of nicotinic acid, about 10 % amino acids and considerable amounts of phosphorus (*c*. 10 %) (cited by Rasmussen 1995). The amino acids contain organic nitrogen that is more suitable form for uptake in orchids than inorganic compounds. Altogether, the best result was only in one variant of this experiment – with an addition of YE 1.0 $g.L^{-1}$ in culture medium (0 % necrosis, average vitality of plants 4.5, 1.1 tubers per plant, *etc*.) (Fig. 2). As the death of plants from necrosis was established largely in many cases and was not rejected by application of AC, we started to elaborate another methods for solving this problem. The first findings of preliminary experiment were taken with maximizing Ca^{2+} in culture media using Ca gluconate monohydrate (Fig. 3). This study needs the continuation.

In summary, the study to provide prolonged microplant cultivation method without

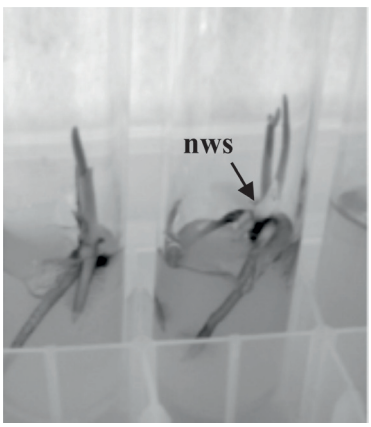

Fig. 2. Microcultivation of *Dactylorhiza baltica* (Klinge) N.I. Orlova, YE 1 g.L-1 (nws – development of new shoot).

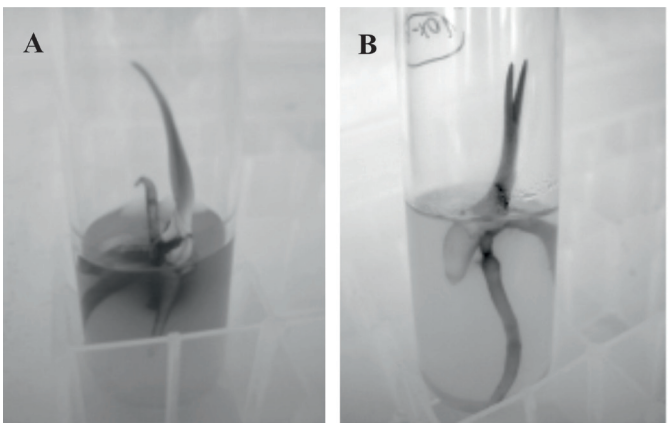

Fig. 3. *Dactylorhiza baltica* after cultivation in vitro in Norstog and co-authors (1973) modified basal medium for 1.5 months: A – Ca^{2+} dose accordingly the reception, coloring the medium induced necrosis of plants; B - 10x dose of Ca^{2+} (2.28 g/L-1) promoted rejecting of necroses.

transplantation *ex vitro* and with limited development of new microshoots (the mass multiplication we find undesired in wild species collection) is only in the beginning stage.

In vitro techniques have found increasing use in the conservation of threatened plants in recent years and this trend is likely to continue as more species face risk of extinction. While a large body of knowledge is available on the *in vitro* culture of plants, there are limited publications relating to threatened plant conservation (Sarasan et al. 2006). As the available plant material for culture initiation is scarce and in many cases associated with inherent problems such as low viability and endogenous contamination, reliable protocols on multiplication, rooting, and storage methods are very important. Future conservation biotechnology research and its applications must be aimed at conserving highly threatened, mainly endemic, plants from conservation hotspots (Sarasan et al. 2006).

The acclimatization *ex vitro*. The aim of this experiment was to compare the effect of soil mixtures on micropropagated orchids: development of regenerants and interaction with associated fungi.

All the *vitro*-plants transplanted *ex vitro* acclimatized satisfactory, although there was variation (Fig. 5). An essential distinction was observed in the variant No. 6 (garden compost: soil from a stratum from beneath bog moss, 1:5), transplanted *ex vitro* after cold treatment, at 5 °C in the dark for 5 months (Fig. 4A). These plants were vigorous with well-developed root system, leaves and particularly tubers. The other regenerants were considerably smaller; they had approximately 1-2 roots and a dropper (tuberidious root) (Fig. 4B-C) or tuber. All these plants were transplanted outside in NGB in various ecology conditions for the further study. Data analysed by Cluster method showed the similarity of the variants (Fig. 6**)**. However the parameters of the variant No. 6 were noticeably different from the others (Fig. 5.B). It may be caused from exposition five months in the dark at 5 °C. In the next year, some of the outdoors overwintered plants from variant No. 6 came into flowers (Fig. 9) and obtained seed-capsules with ripe seeds.

The investigation under microscope showed that at the end of acclimatization time fungal hyphae were observed in cortical cells of roots in all variants (Table 4). The highest intensity (M % = 11.7) of colonization was in the variant

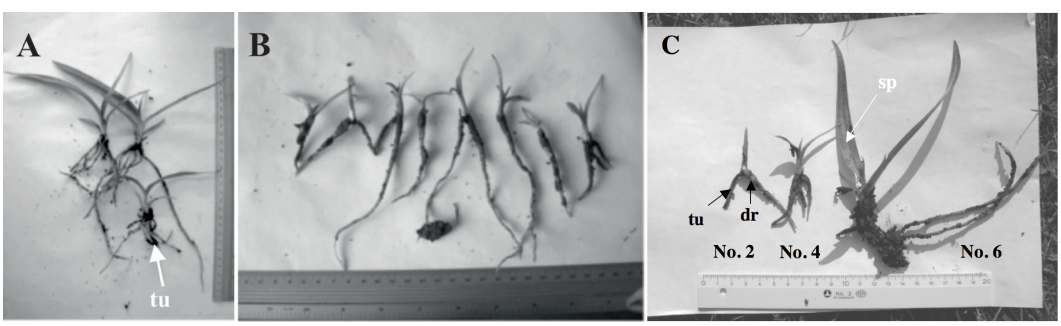

Fig.4. In vitro obtained regenerants of *Dactylorhiza baltica* (Klinge) N.I. Orlova 5 months after acclimatization ex vitro in soil mixtures: A – the variant No. 6. garden compost : soil stratum from just beneath of bog moss (1:5); B – the variant No. 2. substrate gravel: meadow soil: pine bark mulch (1:1:0,5) covered with forest soil of partly-decayed leaves; C – the comparison of variants No. 2. substrate gravel: meadow soil : pine bark mulch (1:1:0,5) covered with forest soil of partly-decayed leaves, 4. garden compost: soil stratum from just beneath of bog moss (1:3) and 6. garden compost : soil stratum from just beneath of bog moss (1:5) (dr – dropper /tuberidious root; tu – tuber; sp – spots characteristic for D. baltica).

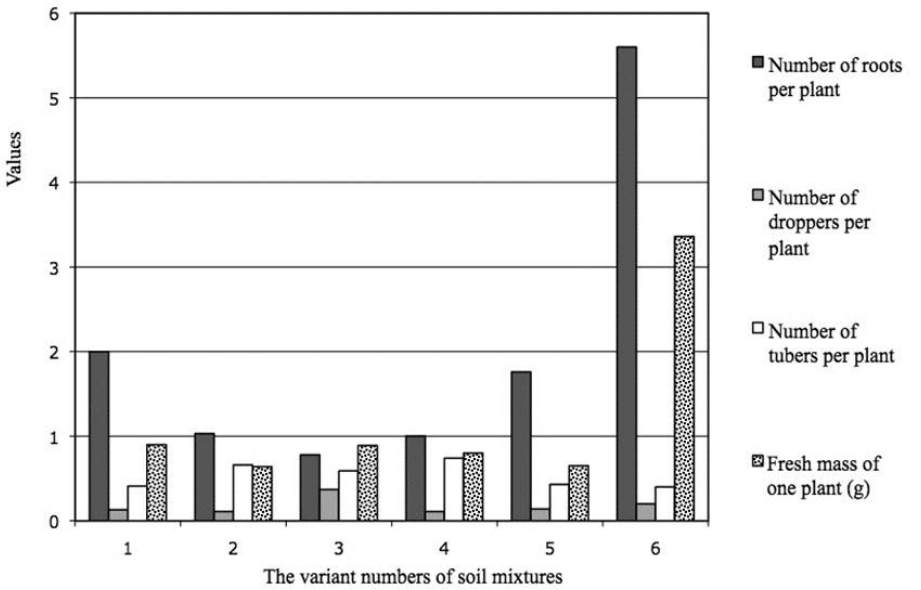

Fig. 5. The morphological parameters of *Dactylorhiza baltica* after 5 months regrowing in various soil mixtures (No. 1. gravel: meadow soil : pine bark mulch (1:1:0,5); 2. substrate No. 1 covered with forest soil of partly-decayed leaves; 3. substrate No. 1 covered with mixture of meadow soil and pine bark mulch above (1:1:1); 4. garden compost : soil stratum from just beneath of bog moss (1:3); 5. garden compost : soil stratum from just beneath of bog moss (1:5); 6. garden compost : soil stratum from just beneath of bog moss (1:5) after cold treatment).

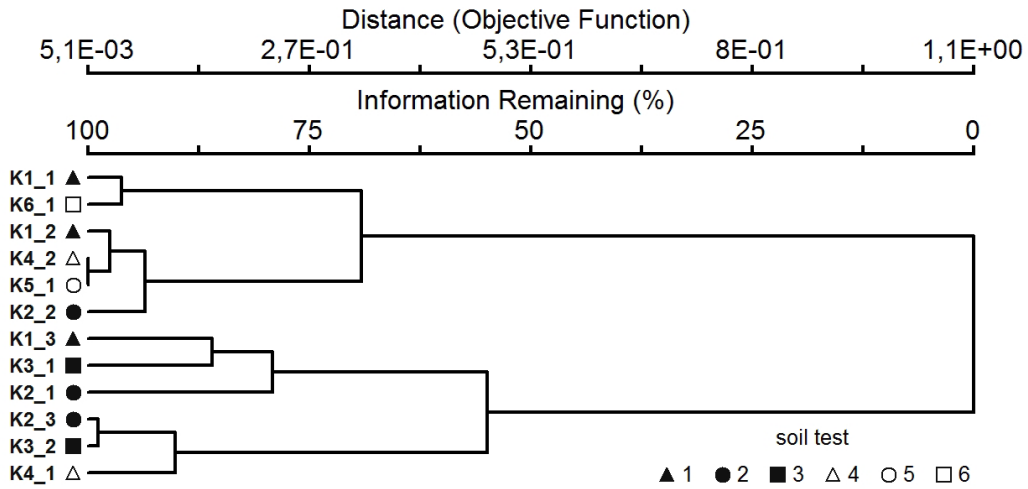

Fig. 6. Dendrogram of Cluster method with the results of acclimatization *Dactylorhiza baltica* by evaluating the six types of substrates tested.

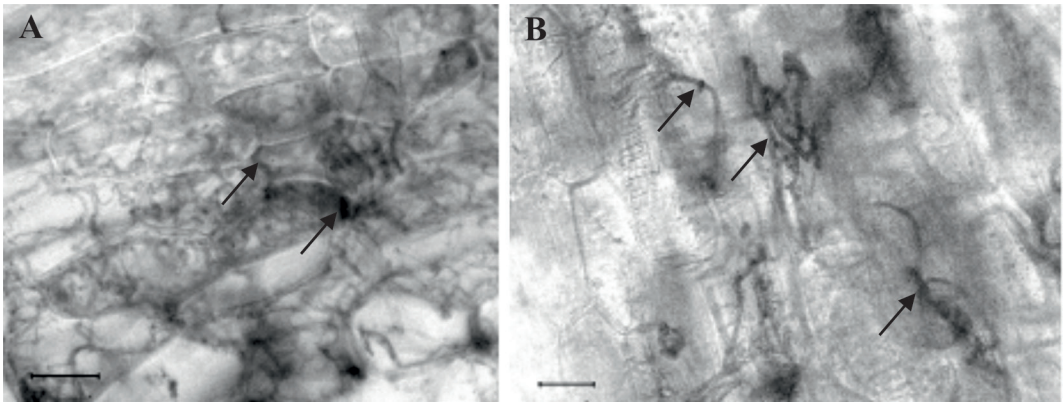

Fig. 7. The colonization of roots with symbionts in in vitro obtained plants of *Dactylorhiza baltica* 5 months after acclimatization ex vitro. A – the variant No. 2. substrate gravel: meadow soil: pine bark mulch (1:1:0,5) covered with forest soil of partly-decayed leaves and B – the variant No. 6. garden compost: soil stratum from just beneath of bog moss (1:5) after cold treatment (arrows indicates the fungal hyphae. Bar A, B 30 μm).

No. 2 (substrate No. 1 [gravel : meadow soil : pine bark mulch (1:1:0,5)] covered with forest soil of partly-decayed leaves) and with a forest soil layer of partly decomposed leaves above). The highest colonization frequency (F % = 83) was in the variant No. 6, transplanted *ex vitro* after cold treatment at 5 °C in the dark for 5 months. The roots of other plants were barely colonized with fungus (Fig. 7). In some studies was observed that many orchid mycorrhizas must be synthesized before conservation of these associations can be attempted in the field (Dearnaley 2007). Seedlings that are unable to grow under natural conditions could grow in such an artificial environment. This may be one of the reasons for the low survival rate of the planted seedlings (Yamato et al. 2008). In our experiment roots were colonized with native orchid-associated endophyte from soil in natural way. The intensity of mycorrhizal colonization in the plant roots varied depending on soil mixture. Consequently, the further studies could be carried out to find out the most suitable soil mixture for plant development associated with intensity of mycorrhizal formation, in order to ensure the optimal acclimatization - development and survival of orchid transplants without application of mineral fertilizers. In adult green individuals of *Cephalanthera damasonium*, it was shown that about 50% of carbon source was obtained from mycorrhizal fungi (Julou et al. 2005), suggesting that *C. falcata* also shows mixotrophy, obtaining carbon both from mycorrhizal fungi and via photosynthesis in the adult stage. Phosphorus and nitrogen (as glycine) transfer from fungus to plant was confirmed in radiolabelling experiments (Cameron 2006, 2007).

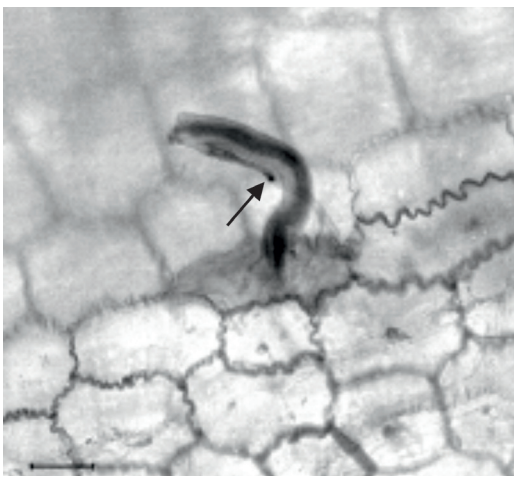

Fig. 8. The colonization of roots with symbionts in *in vitro* obtained plants of *Dactylorhiza baltica* 5 months after acclimatization *ex vitro*; the variant No. 4. garden compost: soil stratum from just beneath of bog moss (1:3) (arrows indicates the fungal hyphae in the root hair. Bar 30 μm).

Fig. 9. The flowering of *Dactylorhiza baltica* regenerants after one year acclimatization in the field of NBG (variant No. 6.: garden compost: soil stratum from just beneath of bog moss (1:5), after cold treatment).

The rapid loss of native orchid habitat throughout ecologically important areas (e.g., Florida) has prompted researchers to develop appropriate plans for the propagation and reintroduction of many native orchid species. Ideally, symbiotic orchid seed germination methods are utilized in the production of orchid seedlings to be used in plant reintroduction programs (Stevart et al. 2006). Largely because of human-induced habitat loss and theft of attractive individuals, many orchid species are in danger of extinction across the planet. Conservation measures require a full understanding of the biology of each species in question (Dearnaley 2007).

CONCLUSIONS

1. The detailed study of biology of *Dactylorhiza baltica* is important for biodiversity *ex situ*-conservation in Latvia; our data could be important in conservation strategy in region where it stretches, especially regarding reintroduction necessity.

2. D. baltica was found in the following inspected habitats:

• 38. Mesophyle grasslands: 35.12. Agrostis-Festuca grasslands, fallow lands (*Anthoxanthum odoratum – Briza media* grasslands, *Anthoxanthum odoratum – Agrostis tenuis* grasslands) and 38.2. Lowland hay grasslands, natural grasslands (*Festuca pratensis* grasslands, *Helictrotrichon pubescens* grasslands);

• in the ecotone zone between bushes and biotope 7210* Calcareous fens with *Cladium mariscus* and species of the *Caricion davalliane;*

• in the ecotone zone between forest and biotope 54.223, Northern brown bog-rush fens.

3. The methodology to keep qualitative regenerants of *D. baltica in vitro*, suitable for transplanting *ex vitro*, has been elaborated. Additional studies are needed to ensure long-term *in vitro* cultivation so as to periodically obtain limited amount of new viable microshoots, for example by branching of tubers or by root shooting.

4. The most appropriate variant for acclimatization of *D. baltica* was the test with transplantation of regenerants *ex vitro* after cold treatment at 5 °C in the dark for 5 months, re-growing in the soil mixture - garden compost : soil stratum from beneath bog moss, 1:5.

5. The colonization with symbionts took place 5 months after re-growing of regenerants in all tested soil mixtures without any artificial addition of fungus culture. The highest intensity (M % = 11.7) of colonization was in the variant No. 2 (substrate No. 1 [gravel : meadow soil : pine bark mulch (1:1:0,5)] covered with forest soil of partly-decayed leaves) and with a forest soil layer of partly decomposed leaves above). The highest colonization frequency (F % = 83) was in the variant No. 6 (garden compost: a soil stratum from beneath bog moss, 1:5), transplanted *ex vitro* after cold treatment at 5 °C

in the dark for 5 months.

6. In vitro obtained regenerants of *D. baltica* begun to flower after one-year acclimatization in the field, in some cases.

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