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DACTYLORHIZA FUCHSII (DRUCE) SOÓ AS A MODEL OBJECT IN *IN VITRO* CULTURE STUDY FOR DEVELOPMENT OF TERRESTRIAL ORCHIDS

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The study of orchid germination and development without fungus *in vitro* elucidates the environmental factors affecting these processes *in situ*. The *in vitro* method allows us to observe the initial stages of morphogenesis of terrestrial orchids. After the germination and *in vitro* cultivation, the next complicated problem is the acclimatization of newly obtained regenerants for further growth and development *ex vitro* in natural or half-natural conditions in botanic gardens. *Dactylorhiza fuchsii* (Druce) Soó, included in the Red Data Books of the Baltic region and listed in the Annex B of the EC Habitats Directive, was chosen as a model object for this study.

Keywords: *Dactylorhiza fuchsii*, *in vitro,* mycorrhiza, nutrient elements

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Introduction

Recalcitrant terrestrial orchids collected from the wild or *ex situ* collections are difficult to grow in culture. While there is wide general knowledge about *in vitro* cultivation of plants, it is far more limited for particular threatened species needing conservation. Various methods of sterilization and other treatments to establish clean material for culture initiation have been reported (Sarasan et al. 2006). Cultivation of terrestrial orchids is complicated and needs a thorough investigation. *Ex situ* cultivation methods must emulate those provided by mycorrhizal fungi in natural conditions, and what is the most essential during germination and early stages of development – the protocorm stage - is the formation of underground organs and sprouting. Further problems appear in acclimatization process *ex vitro*, and colonization with adequate symbionts.

In many semi-natural and natural ecosystems, mycorrhizal fungi are the most abundant and functionally important group of soil microorganisms. They are almost wholly dependent on their host plants to supply them with photosynthate in return for which they enable the plant to access greater quantities of nutrients. In some situations, plant species richness is related to mycorrhizal species richness (Johnson et al. 2005). A total of 3,617 species from 263 families of land plants were covered in this survey of mycorrhizal status. For land plants as a whole, 80% of the recorded species and 92% of the families are mycorrhizal. In angiosperms, the most species-rich clade of land plants and

the dominant group in most terrestrial plant communities, 85% and 94 % of species and families are mycorrhizal (Wang et al. 2006). The roots of most plants, not only orchids, form symbiotic associations with mycorrhizal fungi. Four major groups of plant species in this system are (1) mycorrhiza-independent or facultative mycotrophic, (2) dependent on arbuscular mycorrhizae (AM) (3) dependent on ericoid mycorrhiza (ERM) or ectomycorrhizae (EM), and (4) colonized by dark-septate (DS) endophytes. The authors hypothesized that availability of mycorrhizal propagules was related to the success of mycorrhiza-dependent plants in colonizing new substrates in naturally evolved ecosystems (Cázares et al. 2005). The symbiotic association with orchids ordinarily is related separately to orchid mycorrhiza (OM). Fungi associated with OM have traditionally been mostly regarded as saprotrophs, dead organic material thus being the energy source for the symbiosis (Rasmussen 2002).

The orchid family is the only large group of higher plants that makes consistent use of an alternative nutritional system. Orchids are to a great extent dependent on symbiotic fungi. Orchid mycorrhiza differs from other major types of mycorrhiza in that the fungus supplies the plant with energy. During some of its life stages the orchid can rely entirely on mycotrophy for nutrition, while during other stages the plant makes use of both mycotrophy and phototrophic nutrition, either alternately or the one supplementing the other (Rasmussen, 1995). Terrestrial orchids are often considered as being more dependent on their endotrophic fungi than epiphytic species, since terrestrial seedlings remain underground and are mycotrophic for months or even years, whereas epiphytic seedlings, which have immediate access to light, can begin to photosynthesize at an early stage (Rasmussen, 1995). Therefore terrestrial orchids are much more problematic to germinate and cultivate *in vitro* than epiphytic ones.

The initiation and beginning of *in vitro* culture for *Cypripedium acaule* was studied in detail by Leroux *et al.* (1997). The development of protocorm was achieved using *in vitro* germination of seeds on a culture medium containing sugar, but without a symbiont. Inside the seed, the embryo of *C. acaule* consists of about a hundred cells. The embryo cells contain protein and lipid reserve material. The meristematic dome at the anterior pole of protocorm gives rise to a scale and the apex of the seedling, which and the scale leaf develops synchronously.

The information obtained from symbiotic and asymbiotic *in vitro* cultures can be generalized to apply to the process that occurs in the nature.

It is assumed that many compounds necessary for germination *in vivo* are supplied by the fungi to the embryos; it has also been suggested that direct absorption from the soil could be a significant factor. Both soluble carbohydrates and nitrogen compounds are usually in short supply in the soil and are readily absorbed by the microorganisms in the soil and recycled (Campbell 1977, cited by Rasmussen 1995). Embryos slowly take up nutrients from the substrate *in vitro.* How can the orchid embryo, with its small and mainly non-absorbent surface, effectively interact with soil microorganisms? We understand this to mean that the large external network of hyphae to which the colonized seedling is connected, provides and ensures extensive interface with the soil. These deposits were depleted of symbiotic fungi when first exposed but colonized by them over time after exposure.

Not all microfungi are nonmalignant. Parasitic microfungi *Spermosporina gymnadeniae* (Hyphomycetes) on *Gymnadenia sp.* (Orchidaceae) collected in Tatra Mts, Poland, was described in 2003 (Woůczańska et al. 2008). The fungus discovered in new localities in Poland and Slovakia was recorded on *Gymnadenia conopsea* s.l. and *Dactylorhiza fuchsii*, which are new hosts of this parasite - on leaves of *Dactylorhiza fuchsii*; the other species of *Dactylorhiza* were similarly affected. The relations between orchids and fungi must be taken into consideration for successful cultivation of orchids in aseptic conditions.

Plant conservation programs consist of several components. For example, plant conservation in Opole Province (Poland) encompasses a species-centered protection, ecosystem conservation, *ex situ* conservation, restorations and translocations, reintroductions, and monitoring (Novak et al. 2004).

Cryopreservation is widely used as a conservation method *in vitro*. The efficiency of cryopreservation of seeds of five rare and endangered species of temperate orchids belonging to *Platanthera* and *Dactylorhiza* genera followed by their asymbiotic culture *in vitro*, as well as of *in vitro* cultured *D. fuchsii* protocorms (specific stage of orchid embryo development after release from the seed coat) was investigated by Nikishina *et al.* (2007). Germination rates of seeds after their exposure to liquid nitrogen were species-dependant and were either higher or lower than in the unfrozen control. After vitrification, 9% of *D. fuchsii* protocorms with a larger diameter of 1200 μm survived cryopreservation; however, their growth was retarded for three months when compared to control protocorms. We can say that this method needs very expensive laboratory charges and could be ineffective for terrestrial orchids.

Conservation in Latvia involves both *in situ* protection with a wide net of protected areas of various human activity restriction level and an *ex situ* approach conducted outdoors in the National Botanic Gardens and in the Tissue Culture Department. Our study investigates the necessary factors for successful cultivation of Latvian rare and endangered orchids *in vitro* and further in *ex vitro* conditions.

Material and methods

Dactylorhyza fuchsii (Druce) Soó was chosen as a model object for this study. The seeds were collected in the National Botanic Gardens of Latvia (NBG) from plants growing in semi-natural conditions in meadows of rare and endangered plants. Capsules were dipped into 96° ethanol and excised sterile seeds were sowed onto filter paper bridges in culture tubes closed with foil. A modified Knudson nutrient formula (Knudson 1946) supplemented with organic stuffs was used as a liquid initial medium for germination. Sterile cultivation protocol of protocorms and plantlets was elaborated in the Tissue Culture Department of NBG (Jakobsone, *in print*).

The regenerants were replanted in soil with addition of substrate from natural meadows containing symbionts. The first transplantation in boxes was carried out in May and plants were placed in a room with white luminiscent lamps. They were replanted outside in August in earthed pots which were covered with leaves for the winter. The material for preparation i.e. roots was sampled after a year, in the next spring. Samples from nature at the Orchid path in Engure were gathered to compare their colonization with symbionts to plants acquired *in vitro*.

Roots were fixed in an FAA [formaldehyde /glacial acetic acid /95% ethanol /distilled water (10:5:50:35, v/v/v/v)] solution. Following fixation, tissues were dehydrated in an ethanol-tert-butyl alcohol series, and embedded in Histowax. Cross sections were prepared using a rotary microtome (the LEICA RM 2145), deparaffinized in a xylol-ethanol series (Ruzin 1999), stained with Astra Blue – Safranin (Braune et al. 1999) and Toluidine Blue (Norris et al. 1994), dehydrated in an ethanol-xylol series and mounted on glass slides in Canada balsam (Ruzin 1999). The method used for clearing and staining of roots and determination of fungal colonisation followed that described by Hayman (1970). Roots were washed under running tap water, cleared in 10% (w/v) KOH 1h, 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)] then washed under running tap water once more.

Sections and root squashes (total roots) were examined and photographed using a Leica DMLS light microscope equipped with the digital camera Canon S70.

The soil for investigation was sampled in NBG,

in Engure Orchid path and nutrient elements were examined in sterile culture media. The agrochemical analyses were done in the Institute of Biology of LU (by agreement with the Plant Mineral Nutrition Lab).

Results and discussion

Orchids sowed in August began germination in November, although individual seeds had produced protocorms already in October. Initial development forming protocorms 0.1 – 0.6 mm in size, specific just for Orchidaceae and sprouting and multiplication (characteristic for *in vitro* cultures) occurred during this stage (Fig.1A). The small protocorms protect against drying out in nature. Leeson *et al.* (1991) founded some protocorms of *Dactylorhyza fuchsii* in moss and grass tufts. This is an exception in observations of orchids because the minute seeds and underground seedlings are extremely difficult to observe in nature.

The protocorms have developed rhizoids typical for orchids and replace the function of roots. The protocorm stage is defined (Rasmussen 1995) as a stage from germination until production of sprouts. The further development of *D. fuchsii* is described in detail in our previous publication (Jakobsone, *in print*). These results show that new protocorms can be produced on the original one, which further develops as a tuber. The branching of tubers with initiation of additional sprouts forming tufts for initial plant has been occasionally observed *in vitro* like in nature. The shoot growth *in vitro* had occurred well during the first year (Fig. 1B), but further the dormancy period (2 ?C, dark; Fig. 1C) is advisable because it suggests avoiding necrosis of underground organs.

The first transplantation of newly obtained *D. fuchsii* regenerants in soil can be performed in the next spring i.e. six months after germination (Fig. 1D, 2). After a year, the roots of these over-wintered plants were prepared to fix the contamination with symbiotic microorganisms. Our results showed the presence of pelotons in cortex of roots (Fig. 3 A and B). These data were compared with contamination levels in plants growing in NBG outdoors (Fig. 3 C and D). The hyphae penetrate the epidermis and enter the cells of the cortical parenchyma, forming characteristic and complex hyphal coils (Fig. 4 and 5). The results of Rasmussen (1995) demonstrated that the mycotrophic tissue is found mainly in the cortex of root. A sterile cell sheath was maintained around conducting elements and epidermal cells were generally free from fungi except where the hyphae passed through them in connection with entry and exit of hyphae. It will be necessary to register the step-by-step contamination process after transplantation *ex vitro* and to determine the level of contamination in our further studies. In study with *Caladenia formosa* G.W. Carr pelotons with fine loose hyphae and monilioid cells obtained from leafing to flowering stages appear to be best for *ex situ* symbiotic seed colonization in the vulnerable (Huynh et al. 2004). New methods may now be used to store orchid-associated fungi and to store and germinate seed, leading to more efficient culture of orchid species. However, many orchid mycorrhizas must be synthesized before conservation of these associations can be attempted in the field. Further gene expression studies of orchid mycorrhizas are needed to better understand the establishment and maintenance of the interaction (Dearnaley 2007).

The endophytic fungi associated with orchid protocorms are presumably obtaining carbon from the breakdown of cellulosic materials in the soil. It is likely therefore that orchid mycorrhizal fungi express cellulytic enzymes in soil, at least in the early stages of orchid development. Both groups (ericoid mycorrhizal (ERM) fungi) can degrade cellulose, cellobiose and carboxymethylcellulose. This suggests the production of cellobiohydrolases, endoglucanases and glucosidases enabling the complete hydrolysis of cellulose to glucose. OM fungi indicate utilisation of arabinose, cellobiose, cellulose, glucose, galactose, maltose, mannose, pectin, starch, tannic acid and xylan (Midgley et al. 2006). Sterile media for cultivation of plants *in vitro* contain sugars, commonly sucrose or glucose because the photosynthesis comes in

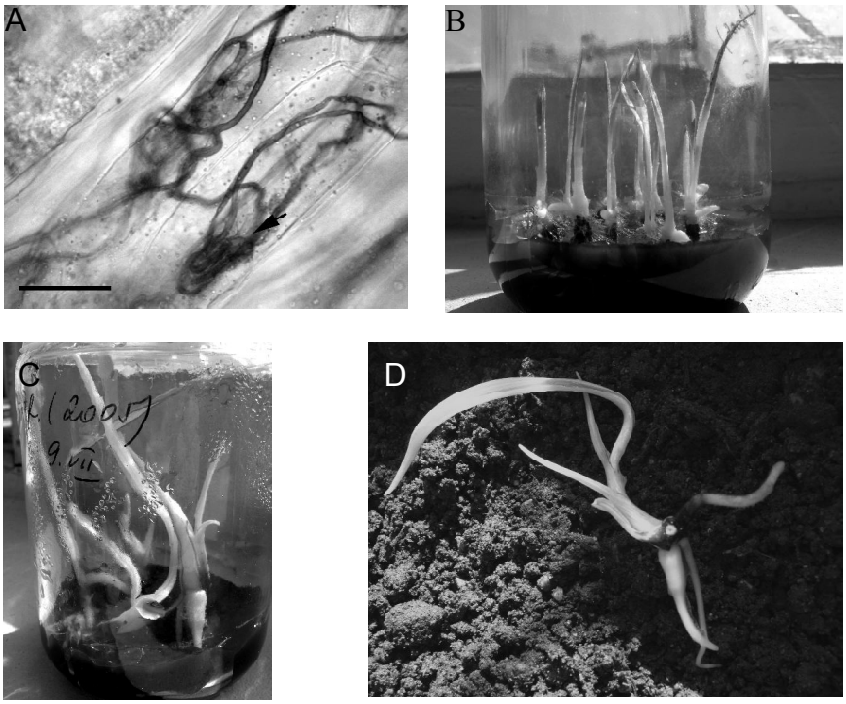

Figure 1. Development of *Dactylorhiza fuchsii* (Druce) Soó in vitro conditions: A – beginning of morphogenesis after germination with development of protocorms, characteristic for orchids; B – initialization of rooting; C – sterile plantlets after exposition at 2 °C in the dark, simulating the natural dormancy period; D – plantlet before transplanting ex vitro (ro – root; tu – tuber).

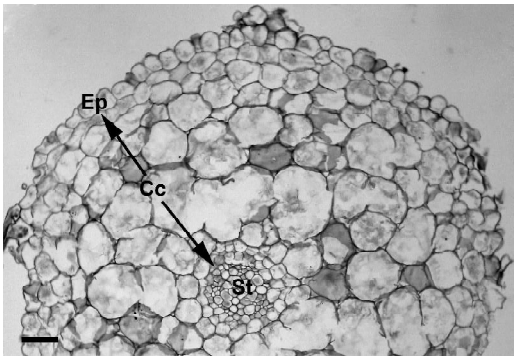

Figure 2. Cross-section of root *in vitro* (asymbiotic condition) showing epidermis (Ep), cortex (Cc), stele (St). Section was stained with Astra Blue-Safranin. Bar = 100 µm.

at low levels. In comparison, the orchids in underground stages that continue some years in natural conditions make similar nutrition by keeping glucose from combination with definite fungus.

We established the approximate nutrient content in media for *in vitro* cultivation of *D. fuchsii* and analyzed the nutrient elements in the natural substrate in Orchid's path in Engure and NBG (Table 1). The contents of some nutrient elements (Fe, B) were almost the same in *in vitro* medium, natural habitat of Orchid path in Engure and in NBG, whereas others were at variance. The widest difference was in Ca content while the acidity was similar. Evidently, Ca is largely combined in a poorly soluble compound in natural substrate and with limited availability for a plant. The same might refer to Mg, whereas S and Mn were in abundance. Despite this, we cannot adequately compare the culture media with substrate in the ground because symbionts are highly selective in taking up nutrients from soil. It can serve in another way: when we have found the optimal nutrient reception in sterile culture media, we can establish the role of fungus.

The net flux of nutrients, particularly phosphorus (P), from the soil into the plant is greater in

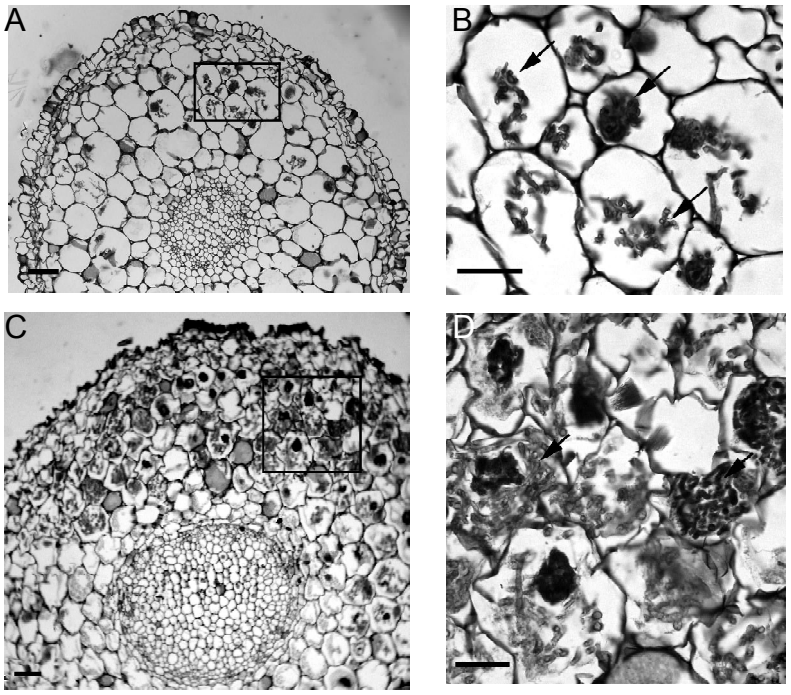

Figure 3. Cross-section of root of *D. fuchsii* growing in the soil: A – from plants one year after transplanting *ex vitro* (marked area see in fig.3B; bar = 100 μm); B - arrows indicate the hyphal coils of mycorrhizal fungi in the root cortex (section was stained with Toluidine Blue; bar = 50 μm); C – from plants in the exposition in the NBG (marked area see in fig.3D; bar = 100 μm); D – arrows indicate the hyphal coils of mycorrhizal fungi in the root cortex (section was stained with Toluidine Blue; bar = 50 μm).

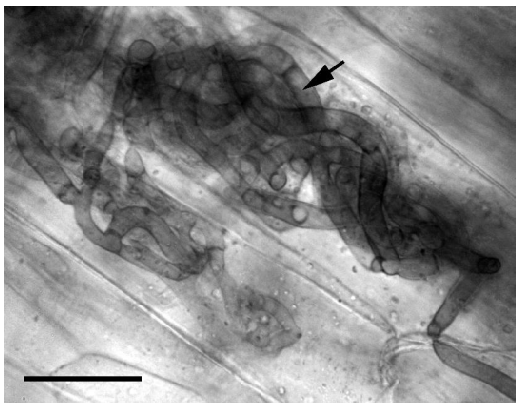

Figure 4. Root of *D. fuchsii* growing in the exposition in NBG: arrow indicates elaborate coiled structures known as pelotons of mycorrhizal fungi in the root cortex (section was stained with Trypan Blue; bar = 50 mm)

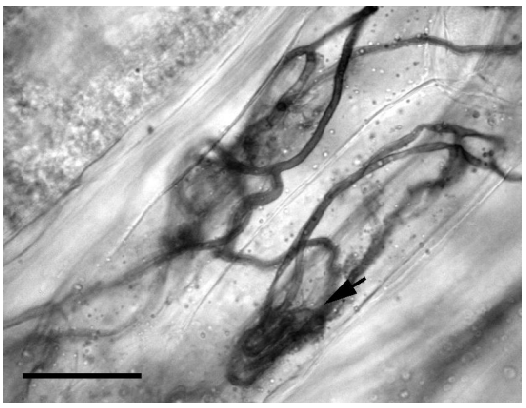

Figure 5. Root of *D. fuchsii* one year after transplanting *ex vitro*: arrow indicates some peloton-like structure as they do within the orchid cells (section was stained with Trypan Blue; bar = 50 mm).

Table 1. Content of nutrient elements for *Dactylorhiza fuchsii*

Chemical element	($mg.L^{-1}$, with extract in 1 M HCl) Content in the soil in Engure by Orchids path	Content in the soil in exposition of NBG	($mg.L^{-1}$) Content in the sterile culture medium
N	19	29	225,5
P	30	149	57
K	140	62	72
Na	4,50	11	In trace amount
Ca	3413	2275	213
Mg	413	475	24
S	20	65	365
Cl	40	-	0,15
Fe	330	695	560
Mn	29	53	117,3
Zn	3,65	5	1,95
Cu	0,35	2,20	0,01
Mo	0,04	0,04	0,1
B	0,40	0,70	1,1
C	-	-	14,2
pH_{KCl}	5,50	6,41	5,9

mycorrhizal than in comparable non-mycorrhizal plants. However, despite the widespread occurrence of mycorrhizal associations, the processes controlling the transfer of solutes between the symbionts are poorly understood. To understand the mechanisms regulating the transfer of solutes, information about conditions at the interface between plant and fungus is needed (Ayling et al. 1997). Alterations in free and conjugated polyamines and the enzymes involved in their biosynthesis, namely arginine decarboxylase, ornithine decarboxylase and S-adenosyl methionine decarboxylase have been reported to occur during cell division, growth, embryogenesis and rhizogenesis in an array of plant materials. It seems that specific polyaminoacids at specific concentration ranges are required during critical stages of growth and morphogenetic events (Kakkar et al. 2000).

We can state that tissue culture methods give the possibility to observe underground stages in every detail. To optimize the nutrient media for cultivation and establishment in soil, it is necessary to elucidate the correlations between orchids and symbionts. We have obtained the first essential results in our study and consider all of them for general direction in our future investigations.

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References

Ayling S. M., Smith S. E., Smith F. A., Kolesik P. 1997 Transport processes at the plant-fungus interface in mycorrhizal associations: physiological studies. Plant and Soil, 196: 305-310.

Cázares E., Trappe J. M., Jumpponen A. 2005 Mycorrhiza-plant colonization patterns on a subalpine glacier forefront as a model system of primary succession. Mycorrhiza 15: 405–416.

Braune W., Leman A., Taubert H. 1999 Pflanzenanatomisches Praktikum I. Spektrum Akademischer Verlag Heidelberg, Berlin. 368 S.

Dearnaley J. D. W. 2007 Further advances in orchid mycorrhizal research. Mycorrhiza 17: 475–486

Hayman D.S. 1970 Endogone spore numbers in soil and vesicular-arbuscular mycorrhiza in weat as influenced by season and soil treatment. Transactions of the British Mycological Society, 54: 53-63.

Huynh T.T., McLean C.B., Coates F., Lawrie A.C. 2004 Effect of developmental stage and peloton morphology on success in isolation of mycorrhizal fungi in Caladenia formosa (Orchidaceae). Aust J Bot 52:231–241

Jakobsone G. (in print) Morphogenesis of wild orchid Dactylorhiza fuchsii in tissue culture. Acta Univ. Latv., ser. Biology.

Johnson D., IJdo M., Genney D. R., Anderson I. C., Alexander I. J. 2005 How do plants regulate the function, community structure, and diversity of mycorrhizal fungi? J. Exper. Bot. 56: 1751–1760.

Kakkar R.K., Nagar P.K., Ahuja P.S., Rai V.K. 2000 Polyamines and plant morphogenesis. Biol.Plant. 43: 1-11.

Knudson L. 1946 A new nutrient solution for orchid seed germination. Amer.Orchid Soci.Bull. 15: 214-217.

Leroux G., Barabé D., Vieth J. 1997 Morphogenesis of the protocorm of Cypripedium acaule (Orchidaceae). Pl. Syst. Evol. 205: 53-72.

Leeson E., Haynes C, Wells T.C.E. 1991 Studies of the phenology and dry matter allocation of Dactylorhiza fuchsii. In: Population ecology of terrestrial orchids, ed. T.C.E. Wells & J.H.Willems, the Hague: SPB Academic Publishing, pp.125-138.

Midgley D. J., Jordan L. A., Saleeba J. A., McGee P. A. 2006 Utilisation of carbon substrates by orchid and ericoid mycorrhizal fungi from Australian dry sclerophyll forests Mycorrhiza 16: 175–182.

Nikishina T.V., Popova E.V., Vakhrameeva M.G., Varlygina T.I., Kolomeitseva G.L., Burov A.V., Popovich E.A., Shirokov A.I., Shumilov V.Yu., Popov A.S. 2007 Cryopreservation of Seeds and Protocorms of Rare Temperate Orchids. Russ. J. Plant Physiol. 54: 121–127.

Norris JR, Read D, Varma AK. 1994 Techniques for Mycorrhizal Research Methods in Microbiology. Academic Press Inc., San Diego, CA. 928 pp.

Novak A., Novak S. 2004 The Effectiveness of Plant Conservation: A Case Study of Opole Province, Southwest Poland. Environmental Management 34: 363–371.

Rasmussen H. 1995 Terrestrial orchids from seed to mycotrophic plant. Cambridge University Press, New York, 444 pp.

Rasmussen H. 2002 Recent developments in the study of orchid mycorrhiza. Plant and Soil 244: 149–163.

Ruzin S.E. 1999 Plant microtechnique and microscopy. Oxford University Press, New York Oxford, 322 p.

Sarasan V., Cripps R., Ramsay M. M., Atherton C., McMichen M., Prendergast G., Rowntree J. 2006 Conservation In vitro of threatened plants—Progress in the past decade. In Vitro Cell.Development.Biol.-Plant. 42: 206-214.

Wang B., Qiu Y.-L. 2006 Phylogenetic distribution and evolution of mycorrhizas in land plants. Mycorrhiza 16: 299–363.

Wołczańska A., Mulenko W., Oklejewicz K., Bacigálová K. 2008 Parasitic microfungi of the Tatra Mountains.3. Spermosporina gymnadeniae (Hyphomycetes):species new to Slovakia and new records from Poland. Biologia 63: 50—52.

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