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ESTABLISHMENT OF *IN VITRO* CULTURE COLLECTION OF ENDANGERED EUROPEAN ORCHIDS

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SUMMARY

In order to establish *in vitro* culture collection of five endangered European orchid species – *Cypripedium calceolus* L., *Dactylorhiza majalis* (Rchb.) Hunt et Summerh., *Epipactis atrorubens* (Hoffm.ex Bernh.) Besser, *Epipactis palustris* (Will.) Cr. and *Orchis morio* L., asymbiotic seed germination was performed. To estimate the method yielding the highest percentage of germination, mature and immature seeds were used (green pod technique).

INTRODUCTION

Orchids belong to endangered species threatened with extinction all over the world. The presence of 46 species of orchids belonging to 22 genera was indicated in Poland. Out of that number two are already extinct in Poland: *Anacamptis pyramidalis* and *Orchis tridentata* (Szlachetko 2001). If we consider only the region of Western Pomerania, then 11 species are already extinct and 16 are in direct danger of extinction (Szlachetko 1995). Therefore all species of *Orchidaceae* family come under full preservation in Poland (Dz. U. Nr 27, poz. 134) (Żukowski and Jackowiak 1995).

Despite their legal protection orchids are still decreasing in number. In natural conditions the life cycle of orchids is very long, it takes them approximately 5–10 years to bloom and produce seeds. Such low proliferation rate makes it very difficult for wild orchids to re-establish their position in natural habitats. That is the reason why a more efficient approach to conservation of orchids is needed. Biotechnology and modern technologies such as asymbiotic germination and micropropagation give the opportunity for increasing reproduction rate and reintroduction of orchids to natural habitats. The conditions of asymbiotic germination of some European species of orchids have already been studied in several research centers, in Poland at the Botanical Garden of the University of Wrocław (Arczewska 1993, 1998, Arczewska and Kukułczanka 1991). Spectacular results concerning asymbiotic germination and reintroduction of rare and endangered orchids were obtained at the Royal Botanic Gardens, Kew (Ramsay and Stewart 1998). The aim of this project was to compare the efficiency of asymbiotic germination of immature and mature seeds.

MATERIALS AND METHODS

Plant material – Experiments were carried out on the following species: *Cypripedium calceolus* L., *Dactylorhiza majalis* (Rchb.) Hunt et Summerh., *Epipactis atrorubens* (Hoffm.ex Bernh.) Besser, *Epipactis palustris* (Will.) Cr. and *Orchis morio* L. The seeds were excised from capsules produced by natural pollination. The capsules were collected from plants growing in the botanical garden in Gołubie (Pomerania) and from natural habitats in Mierzeja Wiślana. Asymbiotic germination of immature and mature seeds was performed in order to estimate the best conditions for seed germination and to investigate the most suitable moment in seed development to obtain the highest percentage of their germination. The method based on sowing immature seeds is called "green pod technique". Immature seeds were collected at different time intervals post pollination – from approximately 20 days from flowering in the case of E. atrorubens to 50, 60 and 80 days in the case of *C. calceolus*.

Sterilization – unripe, intact capsules were surface-sterilized in 5% calcium hypochlorite for 15 min. Afterwards they were dipped in

70% ethanol and then the solvent was burnt off and seeds were scooped for sowing on solid media. Mature seeds were sterilized for 15 min. in 1,5% calcium hypochlorite solution in packages made of filter paper.

Culture media -After sterilization the seeds were sown on eight different solid media: 1) C1 cypr. (http://www.orchidseed.com/), 2) Fast (Fast 1981), 3) $^1/_2$ MS (Murashige and Skoog 1962), 4)1/5 MS Wetsteyn modification (http://www.orchidseed.com/), 5) PB2 – Tsuchiya medium modified by Kukułczanka and Sarosiek (1971) supplemented with Heller's microelements (Kukułczanka and Paluch 1971) 6) RM (Reinert and Mohr 1967), 7) T 849 (http://www.phytotechlab.com) modified by the addition of casein hydrolysate, 8) v/w (Vacin and Went 1949) modified by the addition of ferrous sulphate instead of ferric tetrate and 9) W (van Waes 1986). Some of the media were supplemented with 2–10% coconut water (c) and/or 0.01–0.2% activated charcoal (CA).

Culture conditions – Cultures were maintained at 23°C either under a 16/8 hour photoperiod, illumination of 25 µmol • m^{-2} • s^{-1}, or in continuous dark. Germination was considered to have occurred when protocorms were observed.

RESULTS

In the case of three out of five species studied: *C. calceolus*, *D. majalis* and *E. palustris*, the highest level of germination was observed when immature seeds were used. The seeds harvested about 50 days from flowering germinated with the result of 10% on media T 849 and 1/5 MS in the case of *C. calceolus* (Fig. 1) and 30% in the case of *D. majalis* on Fast medium supplemented with activated charcoal 0.2% (Fig. 2). The seeds of *E. palustris* harvested 35 days from flowering germinated with the result of 7%. Some germination occurred also with older seeds but generally they proved to be too mature, although sown from green pod, to germinate without breaking seed dormancy. Seeds younger than mentioned above did not germinate. Seedlings of *C. calceolus* and *D. majalis* after two years of *in vitro* culture are presented in Fot. 1 and 2.

In the case of *O. morio*, only mature seeds were tested. The highest, 10% germination, was obtained on modified MS medium.

Despite numerous media combinations and plant growth regulators, each medium had five variants supplemented with a combination of the following plant growth regulators: BAP,

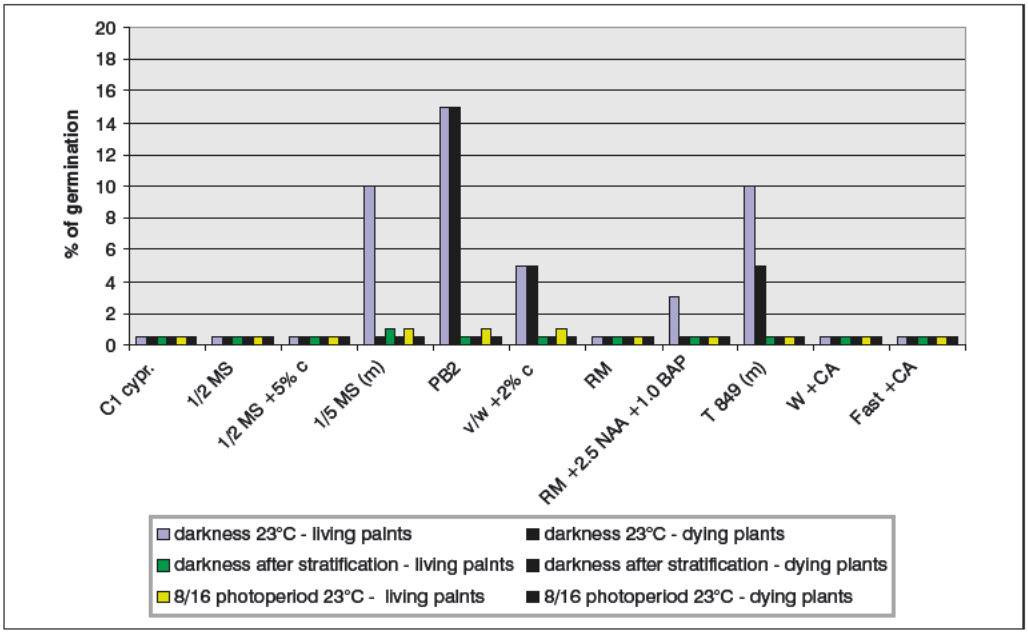


Fig. 1. *Cypripedium calceolus*, seeds harvested on 05.07.02. approximately 50 days from flowering.

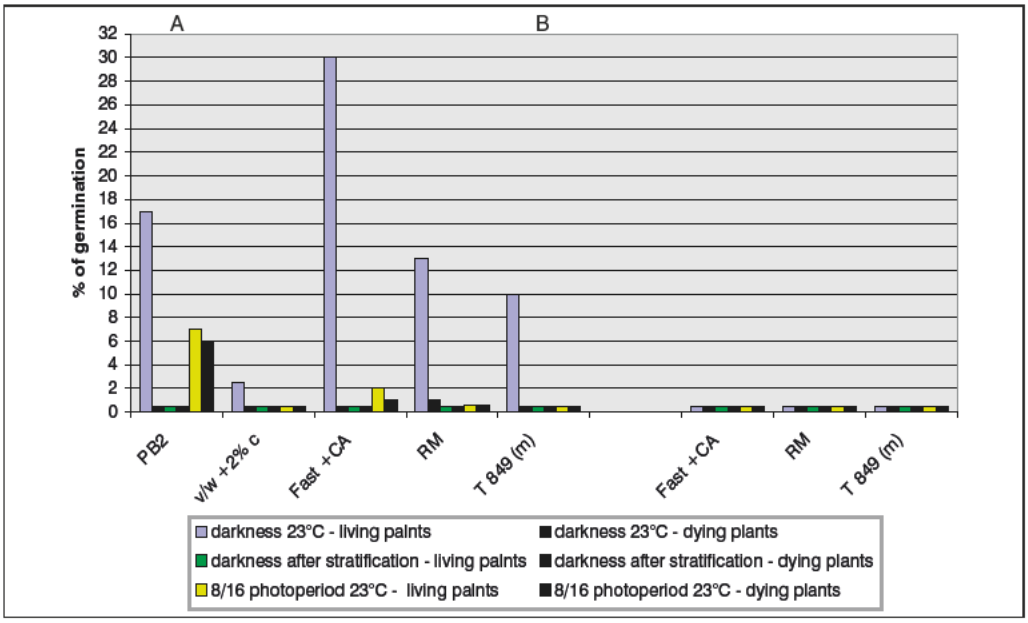
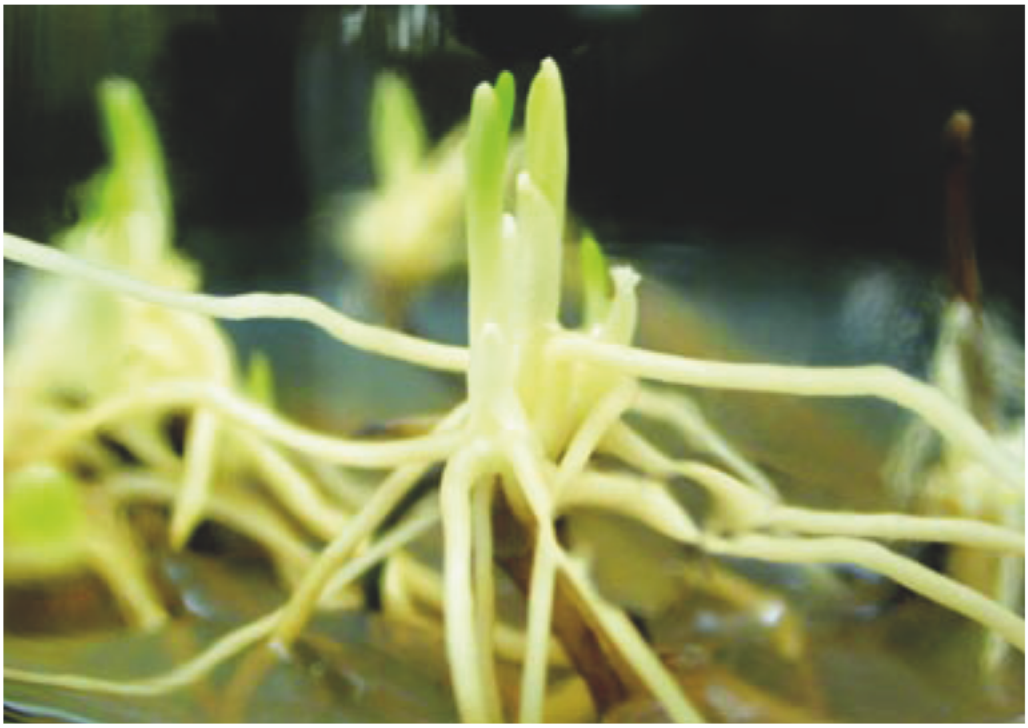
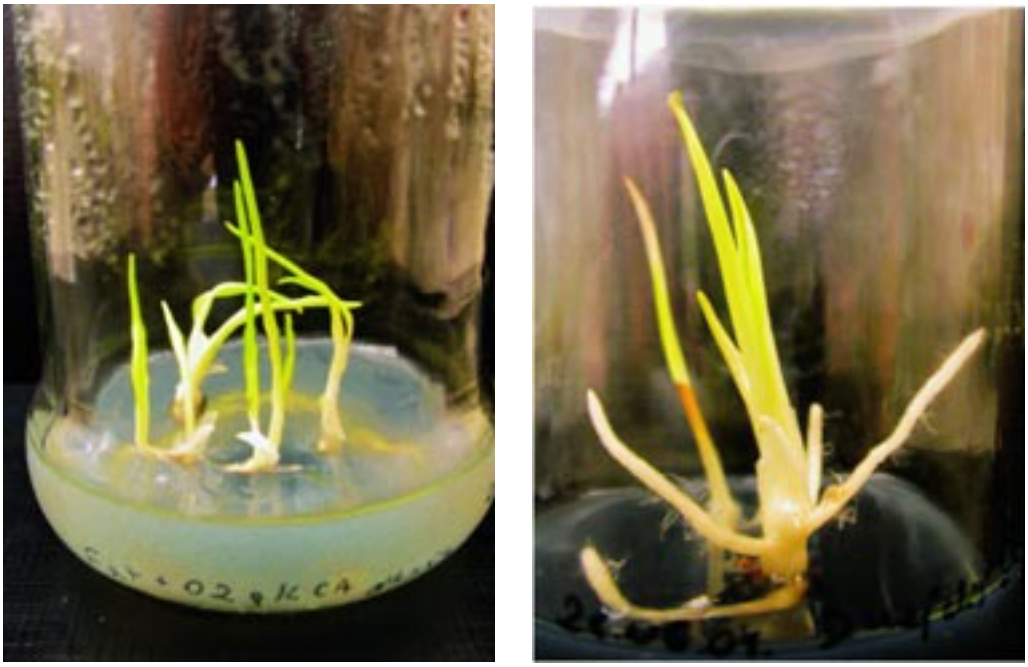


Fig. 2. *Dactylorhiza majalis*, seeds harvested on 05.07.02. approximately 50 days from flowering. A – seeds with white to yellowish embryos that could be wiped off with a scalpel without cutting into the placenta, B – seeds with yellowish to light brown embryos with loose contact to the placenta.



Fot. 1. *Cypripedium calceolus* plants after 2 years of culture on modified 1/5 MS medium.



Fot. 2. *Dactylorhiza majalis* plants after 2 years of culture on medium Fast supplemented with activated charcoal a) 0.02% and b) 0.2%.

2,4-D, KIN, NAA (data not shown), asymbiotic germination of *E. atrorubens* was unsuccessful.

DISCUSSION

From the data presented, one can conclude that optimal conditions for asymbiotic germination (maturity of seeds, methods of sterilization and medium for germination) have to be developed for each species of terrestrial orchids. In most cases, when seeds form as a result of natural pollination and are harvested from natural habitants, it is very difficult to precisely determine the date of pollination. Because of this the period after flowering was used for the description of the stage of seed development.

The highest level of *D. majalis* germination was observed with the seeds harvested about 50 days from flowering (Fig. 2). However, according to embryo colour, some of the seed capsules contained seeds of slightly different stages of maturity. The majority of seeds in each capsule could be grouped into one of the following categories: 1) seeds with white to yellowish embryos that could be wiped off with a scalpel without cutting into the placenta (Fig. 2A), 2) seeds with yellowish to light brown embryos with loose contact with the placenta (Fig. 2B). The seeds from the second category did not germinate at all, most probably due to the fact that they were already entering the period of dormancy. This may explain the results of Arczewska (1993) who obtained low or even no germination when mature seeds of *C. calceolus* and *E. palustris* were used for asymbiotic germination. The data of Ramsay and Stewart (1998) indicate that the period of time when immature seeds germinate efficiently in axenic conditions is short, approximately 7–10 days and should be experimentally estimated for each species. Such results make it clear that precise evaluation of maturity of the seeds is crucial and to ensure it, further study employing hand-pollinated seeds are planned for the next summer.

The media tested in the study differed widely in the concentrations of inorganic nitrogen (and other ions) and varied in substances used to supplement organic nitrogen. A wide range

of complex additives has been supplied in various media: casein hydrolysate, coconut water, peptone and yeast extract. The most efficient media were those supplemented with peptone, yeast extract and casein hydrolysate.

Many of the substrate recipes for seed germination of terrestrial orchids have been developed. The main trend has been towards reducing the concentrations of mineral salts and increasing the amount of organic compounds, for example Wetsteyn's modification of MS medium is based on reduction in macroelements to 1/5 of the original concentration and supplementation with yeast extract. Such attempts aim at simulating the assumed contribution of the mycorrhizal fungus. The soils of typical orchid habitats are exceedingly poor in inorganic nitrogen. In a number of European species it has been observed that the germination percentage improved as the concentration of ammonium and nitrate salts decreased. The results obtained confirm the earlier data of Rasmussen (1995), which show that asymbiotic germination is often stimulated by exogenous organic nitrogen.

The results indicated that light proved to have an inhibitory effect on germination. These findings are completely in line with the results obtained by Arczewska and Kukułczanka (1991) and Arczewska (1993).

The failure with *E. atrorubens* asymbiotic germination is most probably due to the fact that seeds were harvested either too early and therefore were not capable of germination or had already achieved maturity and in order to germinate needed breaking seed dormancy.

The results obtained in this paper indicate that problems with low germination of some seeds may be overcome by precise estimation of time-interval post-pollination and by conducting asymbiotic germination on media adjusted to the specific requirements of certain orchid species.

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