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Morphogenesis of wild orchid *Dactylorhiza fuchsii* in tissue culture

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Abstract

Dactylorhiza fuchsii which is included in the Red Data Book of the Baltic Region and listed in Annex B of the EC Habitats Directive was chosen as a model to study the initial development stages of asymbiotic development in tissue culture. Half-mature seeds were removed from sterilized seed capsules and sown onto filter-paper bridges in culture tubes with initial liquid medium and kept in the dark at 23 °C. Germination started after two months of incubation. Protocorms formed were transplanted into fresh medium. Initiation of roots and tubers was observed after 3 to 6 months of germination. Plantlets with ~ 2 cm long shoots and well-developed roots were transferred into a light chamber. New plants of *D. fuchsii* with two leaves, tubers and roots appropriate for transplanting into soil were obtained in the next spring, e.g. about 8 to 9 months after the start of germination. The use of *in vitro* methods significantly reduced the relatively long period of time necessary for development of orchid plants in natural conditions.

Key words: asymbiotic development, *Dactylorhiza fuchsii,* morphogenesis, tissue culture.

Introduction

There are 33 species of wild terrestrial orchids growing in Latvia. All of these species are protected by the European Council Directive 92/43/EEC of May 21, 1992 *On the Conservation of Natural Habitats and of Wild Fauna and Flora* (Habitats Directive). In total 26 species of the family Orchidaceae are included in the Red Data Book of Latvia (2003). On them, one species is designated as extinct in the wild, five species as endangered, four species as vulnerable decreasing in number, seven species as rare, and nine species as commercially endangered. Both *in situ* and *ex situ* approaches are important for the protection of rare and endangered orchid species. Tissue culture collections are among the most important measures in *ex situ* conservation of terrestrial orchids (Jakobsone et al. 2007). Knowledge on physiological and morphological aspects of germination and development of particular orchid species is of critical importance for establishment of the collections.

Orchids have very small seeds completely lacking endosperm resembling spores of ferns. This makes it difficult to observe their further development after they are released from the seed capsule into the soil. Large seed production seems to be a common trait of plants that have a saprophytic-mycotrophic or parasitic seedling stage and therefore are extremely specific in requirements for the germination site (Rauh et al. 1975). Several

species of *Dactylorhiza* and *Orchis* can germinate in water and remain alive for some days or weeks without receiving any external nutrients (Rasmussen 1995). During the period after germination the seedlings can subsist on their own reserves while waiting for a compatible infection (Vermeulen 1947). However, the experiments of Knudson (1946) showed that asymbiotic germination of orchid seeds can be achieved using a medium with sugars as a source of reduced carbon and appropriate minerals.

The period of time necessary for development of individual plants before flowering of different terrestrial orchids is extremely long. On average period of a 12 years is necessary for development of *Cypripedium* species from seed germination until flowering (Curtis 1943; Kull 1999). The use of *in vitro* culture technique in orchid propagation allows to obtain flowering plants within 4 to 5 years of culture or even after 2 to 3 years (Stoutamire 1974; Rasmussen 1995).

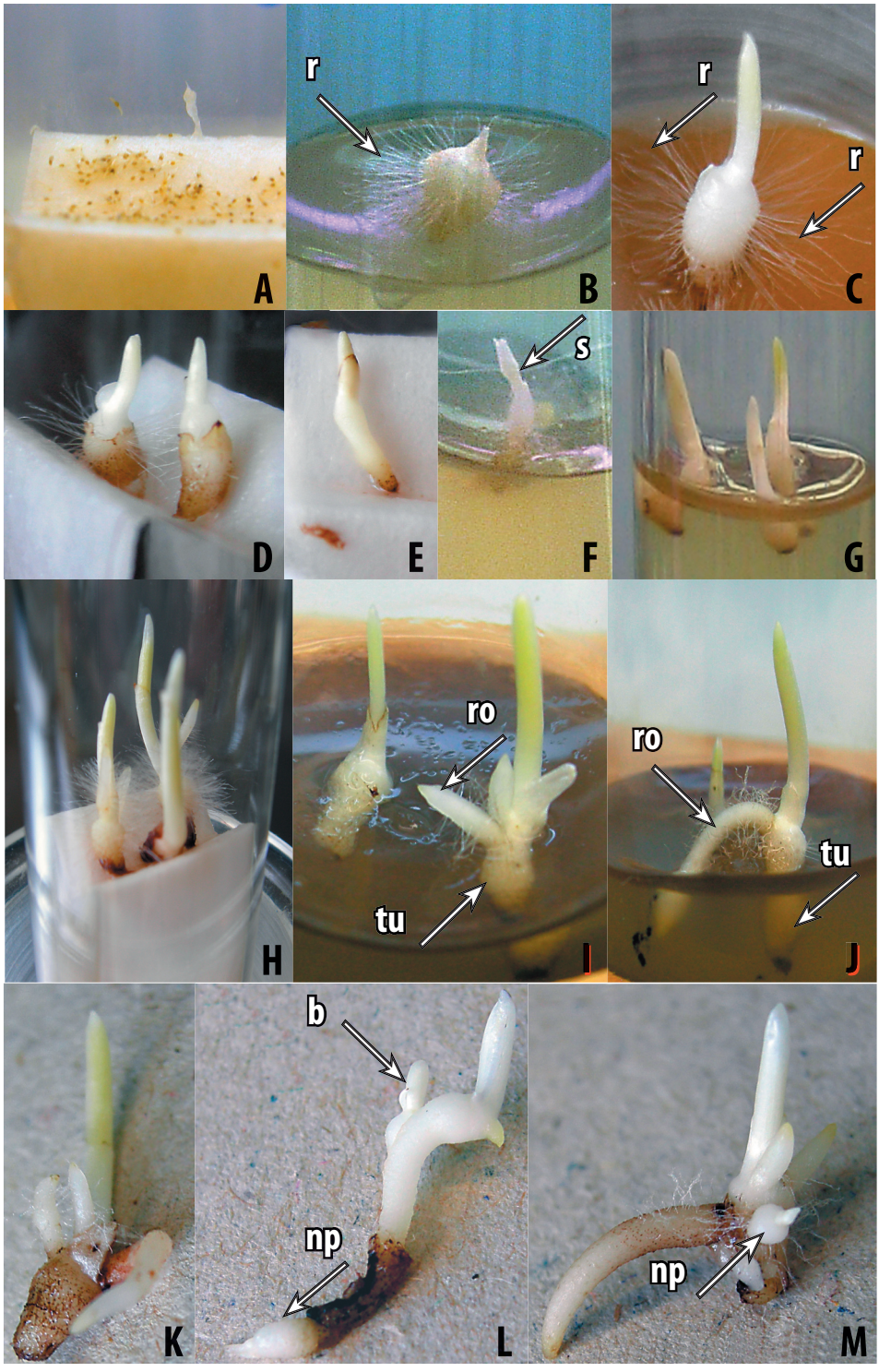
Species of the genus *Dactylorhiza* Necker ex Nevski (Orchidaceae) are terrestrial orchids from the Northern Hemisphere. They grow on a wide range of relatively open habitats from dune slacks to alpine meadows (Pillon et al. 2006). *D. fuchsii* is found in both open woodland and a variety of grasslands (Cepurīte 2005). It is a relatively shade tolerant species (McKendrick 1996). *D. fuchsii* is included in Red Data Book of the Baltic Region and listed in the Annex B of the EC Habitats Directive. The aim of the present study was to follow the first development stages of *D. fuchsii* plants from germination until formation of new regenerant. The use of tissue culture methods allowed nondestructive monitoring of developmental processes of plantlets.

Materials and methods

Seed capsules for initialization of tissue culture of *Dactylorhiza fuchsii* (Druce) Soó were collected from naturally pollinated plants growing in the National Botanical Garden of Latvia, Salaspils in a semi-natural meadow. Seed capsules were surface sterilized by a rapid dip into 96 % ethanol with subsequent passing of the capsule through a flame. The capsules containing light brown incompletely developed seeds were used for experiments. Capsules were split with a sterilized scalpel and the seeds were sowed aseptically onto filter paper bridges in culture tubes (Ø 16 mm) and closed with foil. The liquid initial media were based on a modified formula of Knudson (1946) and ½ Murashige and Skoog (1962) medium with variation of different organic compounds without addition of growth regulators.

The obtained small protocorms were transplanted onto fresh new medium for further development. Subsequent transplantation procedures were performed according to growth rate and stage of development, usually after each 1 to 2 months. During the initial stages of development cultures were incubated in the dark at room temperature. Plantlets with ~ 2 cm long shoots and well-developed roots were transferred into a light chamber

Fig. 1. Morphogenesis of *Dactylorhiza fuchsii in vitro* in the dark at 23 °C. A, imbibed seeds; B, formation of protocorms with rhizoids (r); C - E, polarization and elongation of protocorms; F, sprouting (s); G, development of tuber at the basal part of the protocorm and shoot formation at the apical part; H - J, elongation of shoots and initiation of rooting (ro – root, tu – tuber); K, rooting; L, initiation of a new orchid plantlet (np) on the elongated tuber of the stock plant, branching of tuber (b); M, initiation of new orchid plantlet (np) on the tuber of the stock plant.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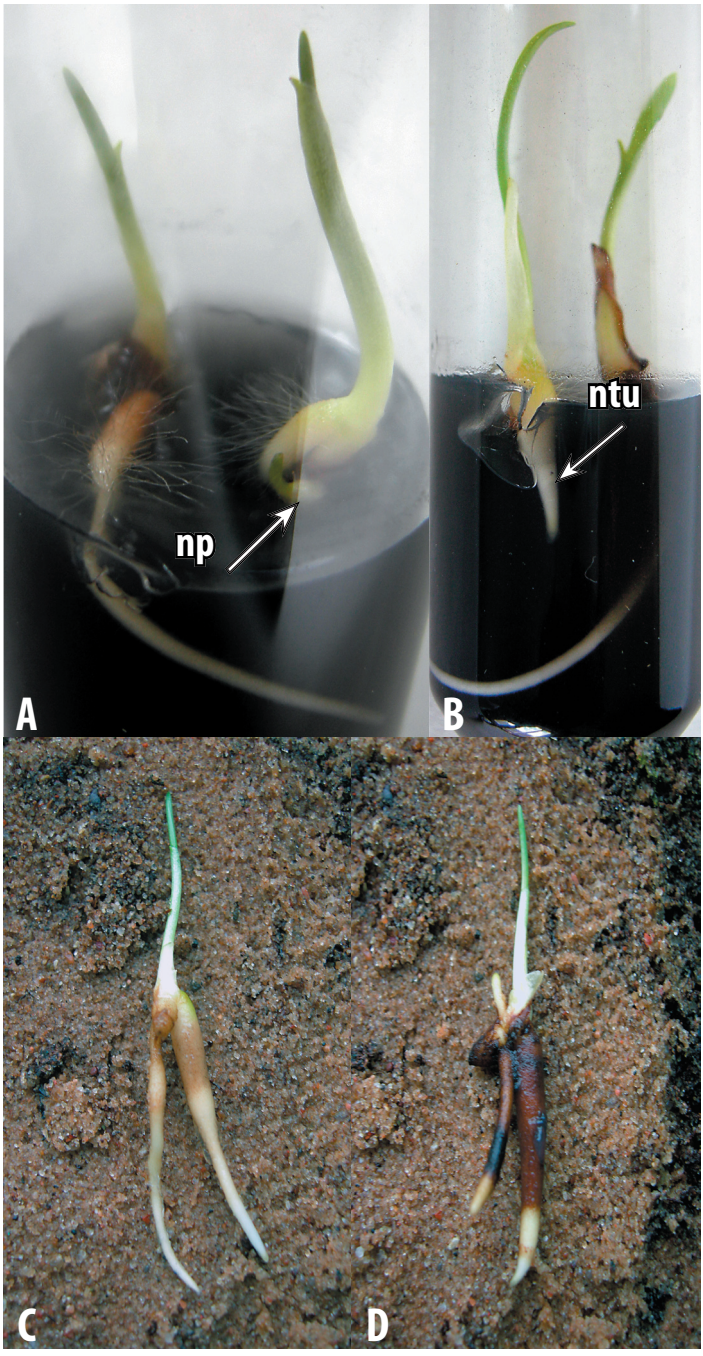

Fig. 2. Development of regenerants of *Dactylorhiza fuchsii* under light conditions. A, intensive growth of underground organs and initiation of secondary plantlets (np); B, unfolding of first leaves and start of development of true secondary tubers (ntu); C - D, obtained *in vitro* plants before transplanting in soil.

illuminated by white fluorescent lamps and a 16-h photoperiod. Photosynthetic photon flux density was 40 mmol m^{-2} s^{-1}. Temperature during incubation was 23 ± 2 °C. All stages of development were documented by photography.

Results and discussion

The development of most species of *Dactylorhiza* from germination to shoot and tuber formation is about four years (Rasmussen 1995). *D. fuchsii* belongs to a group of orchids with sympodial growth of attenuate root tubers (Tatarenko, Kondo 2003). In natural conditions a new shoot apex is formed in late autumn, remains in undifferentiated state for more than 18 months, and appears above ground during the 4th year after formation (Tatarenko, Kondo 2003). Thus, the estimated life span for underground shoots of *D. fuchsii* is more than 30 months, which is relatively long period. The present experiments confirmed that in conditions of tissue culture the whole development period of *D. fuchsii* from seed germination until formation of first leaves is about 8 months.

Seed germination started relatively slowly within 7 weeks of culture (Fig. 1 A). The seeds sown on August 19 started to germinate in October. The germination of ungerminated seeds continued for a prolonged period of time lasting more than 2.5 years. The first protocorms (about 1 mm in diameter) appeared in November. The presence of numerous rhizoids was evident (Fig. 1B). Rhizoid formation usually occurs in parallel with development of an apical bud. Polarization and subsequent elongation of protocorms started in December (Fig. 1 C - E).

Five initial stages during germination and protocorm development, universal for all terrestrial orchids, are described as follows (Dixon et al. 2003): ungerminated seed (stage 0); embryo (protocorm) ruptures testa (stage 1); protocorm appears to be larger than seed with production of rhizoids (stage 2); initial bud development on rapidly growing protocorm (stage 3); chlorophyllous leaf tissue or root initials produced (stage 4); protocorm enlargement with well developed leaf and initialization of dropper (stage 5). According to the other sources, six stages of orchid seedling development can be described (Kauth et al. 2006). Formation of leaves is related to stage 4, and is described morphologically as protocorm with developing leaves and rhizoids. In contrast to Dixon et al. (2003), in the present experiments with *D. fuchsii* the first well-developed leaf formed only on relatively highly-developed plantlets instead of protocorms (Fig. 2B). Polarization and elongation of protocorms was visible well before leaf formation (Fig. 1 E). The polarized protocorms further developed into sprouts and tubers (Fig. 1 F - G). In terrestrial orchids tubers represent dormancy survival structures that function as storage organs (Rasmussen 1995). Tubers are produced on a stalk often referred to as a dropper.

Initiation of rooting took place only after polarization and elongation of protocorm (Fig. 1 H) at the base of tubers (Fig. 1 I - K). Until this time the plantlets take up nutrient elements with the aid of rhizoids. *D. fuchsii* regenerants with only shoots and tubers did not initiate rooting in conditions of a light chamber. Consequently only the plantlets with relatively well-developed roots could be placed into the light chamber for further development. Initiation of tubers and roots were observed after 3 to 6 months from the start of germination.

Vegetative propagation was observed on *D. fuchsii* explants on medium without growth regulators in early stages of development by either of two mechanisms: as

elongation and branching of tubers (Fig. 1 L) or as direct formation of new plantlets in the form of protocorm-like bodies on the first developed tuber (Fig. 1 M). To our knowledge this is the first description of development of new orchid plantlets on tubers without a dormancy period for *Dactylorhiza* species, clearly showing intensification of development in conditions of tissue culture. In contrast, it was reported that tuberization of orchid seedlings occurs more rapidly and frequently *in situ* than under *in vitro* conditions (Debeljak et al. 2002).

Well developed plantlets of *D. fuchsii* were obtained by means of sterile culture in the next spring after sowing in August (Fig. 2 A and B). Unfolding of first leaves and start of development of true secondary tubers indicated the stage for transplanting of *D. fuchsii* plantlets to *ex vitro* conditions. The first germinated plants with two leaves, tubers and roots were appropriate for transplanting into soil after eight months (Fig. 2 C and D). Thus, the use of *in vitro* methods significantly reduced the relatively long period of time necessary for development of plants in natural conditions. Furthermore, application of *in vitro* methods gave a possibility to observe developmental stages and to record crucial morphogenic characteristics of orchids without destructive sampling.

Studies on *in vitro* propagation and subsequent *ex vitro* cultivation of rare and endangered terrestrial orchid species could be important also for species reintroduction programs. Recently asymbiotic propagation of the vulnerable Japanese orchid *Cephalanthera falcata* and successful introduction into natural habitat has been described (Yamato, Iwase 2007).

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Savvaļas orhidejas *Dactylorhiza fuchsii* morfoģenēze audu kultūrā

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Kopsavilkums

Dactylorhiza fuchsii, kura ir iekļauta Baltijas reģiona Sarkanajā grāmatā un minēta arī ES biotopu direktīvas B pielikumā, izmantoja par modeli, pētot asimbiotiskās attīstības sākuma stadijas audu kultūrā. Daļēji nobriedušas sēklas atdalīja no sterilizētas sēklu kapsulas, uzsēja uz filtrpapīra tiltiņiem kultivēšanas mēģenēs ar sākotnējo šķidro barotni un turēja tumsā 23 °C temperatūrā. Dīgšana sākās pēc divu mēnešu inkubācijas. Sakņu un gumu veidošanās bija novērojama 3 līdz 6 mēnešus no dīgšanas sākuma. Mikroaugus ar ~ 2 cm gariem dzinumiem un labi attīstītiem gumiem pārnesa tālākai audzēšanai gaismā. Jaunos *D. fuchsii* augus ar divām lapām, gumiem un saknēm, kuri bija piemēroti pārstādīšanai augsnē, ieguva nākamajā pavasarī, t.i., aptuveni 8 līdz 9 mēnešus pēc dīgšanas sākuma. *In vitro* metožu izmantošana ievērojami samazināja relatīvi ilgo laiku, kāds nepieciešams orhideju augu attīstībai dabiskos apstākļos.