DOKTORAL (PhD) THESIS

Habitat studies and laboratory analyses aimed at promoting the active protection of Hungarian marsh orchids, with special regard to the species fen orchid (*Liparis loeselii*) and bog orchid (*Hammarbya paludosa*)

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Introduction

Orchids are not only beautiful, with many unusual characters, but are also very rare and include a large number of endangered species. They have some of the smallest seeds in the plant kingdom, and although the plants themselves are rare, they may produce thousands or tens of thousands of seeds. However, the tiny seeds contain no reserve nutrients, so they are unable to germinate unaided. This means that the plants require the help of another organism, a specific fungal species, right from the start of their development. The symbiosis developing between the two organisms is essential if the orchid is to survive.

Although orchid species can be found in almost all types of habitats, fens nevertheless have outstanding importance for orchids. Many orchid species only grow in fens or other aqueous habitats, sometimes growing in masses on patches of near-natural habitat, but fens and swamps are extremely vulnerable habitats. Some 97% of the fens once found in Hungary have disappeared, mainly due to deliberate habitat development, having been drained and ploughed up for agricultural purposes or used for peat production. The aqueous habitats still in existence continue to be endangered, as they are low-lying and thus tend to act as collecting areas for the chemicals and mineral fertilisers applied in large quantities by farmers. This leads to weed infestation and to a reduction in plant species diversity. Due to the drastic decline in the number of fens and to their degradation, they are now protected as habitats, together with all the organisms they harbour.

Among the orchids growing on fen habitats, some can be found in large numbers, but others are extremely rare and thus warrant special attention. The fen orchid (*Liparis loeselii*) and the bog orchid (*Hammarbya paludosa*) are two of the rarest orchids in Hungary. When studying these species it is not sufficient to collect habitat and phenological data because, like other orchids, these species only occur in nature in association with their symbiotic fungi. In addition to the taxonomic classification of these fungi, knowledge on their distribution is also required if their host organisms, the orchids, are to be effectively protected. Active species protection thus requires the joint investigation, protection and preservation of the host plant and the fungus.

Aims

Research on orchid mycorrhizas is a dynamically developing branch of mycorrhizal research. An increasing depth of knowledge is being acquired on the life cycles of individual orchid species and on the taxonomic status of their fungal partners, and also on the specificity of the plant–fungus relationship. For certain rare species, however, there are still gaps in this basic knowledge, as in the case of the fen orchid (*Liparis loeselii*) and the bog orchid (*Hammarbya paludosa*). Even fewer data are available on the habitat requirements of the potential orchid-symbiotic fungi.

The main aims of the present work were thus as follows:

1. Estimation of the natural germination ratio of the fen orchid by means of *in situ* germination.
2. Detection of the symbiotic fungi of the fen orchid and bog orchid species from protocorms germinated *in situ*.
3. Detection of fungal groups potentially capable of forming orchid mycorrhizas in aqueous habitats in Hungary, and the investigation of their habitat requirements.

The methods used to achieve these aims will be detailed in the following section.

Materials and methods

The bog orchid was germinated *in situ* in Bábtava, Gelénes, the only known habitat in Hungary, in a single vegetation period.

The seeds of the fen orchid were germinated under both *in situ* and *ex situ* conditions. For the *in situ* studies, the seeds were placed in two habitats currently harbouring populations of the species (Pákozd, Dunaharaszti), in a habitat where populations were detected from 2003 to 2009 (Kistómalom), in an uncertain habitat, where no orchids of this species have been detected since 2005 (Vaja) and in the habitat of the bog orchid population. As regards the time between seed placement and retrieval, two types of experiment were carried out. In all the habitats listed, seeds were placed in spring and retrieved in autumn. In the case of Lake Velence (Pákozd) seeds were also placed in autumn, followed by retrieval in early summer and autumn the following year and two years later in autumn. For the *in situ* seed placements the seeds were placed in a piece of cheese-cloth (mesh size 85–100 μm) folded in two (Rasmussen and Whigham 1993). The *ex situ* germination of fen orchid was carried out in the Botanical Garden of Eötvös Loránd University, on a piece of peat originating from Lake Velence.

Nine orchid species were used to investigate the fungi forming orchid mycorrhizas in aqueous habitats. The fen orchid and bog orchid species that were germinated *in situ* specialise in extremely wet habitats (floating mat fens and bogs), while the remainder of the orchid taxa included in the experiment have a wider range of habitats. Three of the orchids were examined both on floating mat fens and on terrestrial habitats: the lax-flowered marsh orchid (*Orchis laxiflora* ssp. *palustris*), the early marsh orchid (*Dactylorhiza incarnata*) and the marsh helleborine (*Epipactis palustris*). The other four species were only found on terrestrial habitats: the fragrant orchid (*Gymnadenia conopsea*), the Crimean orchid (*Ophrys oestrifera*), the early spider orchid (*O. sphegodes*) and the military orchid (*Orchis militaris*). The habitats investigated were classified on the basis of their wetness (floating mat fen, terrestrial fen, swamp, steppe) and by using the vegetation as an indication of the soil water content (WB index; Borhidi 1995).

Fungal strains were isolated from the protocorms and roots of the orchids using the root segment technique (Bernard 1904) and the peloton extraction technique. The isolated fungal strains were examined and grouped using a molecular taxonomic method. For some groups of

fungi, the sequences of the ribosomal ITS region can be used for identification even at the species level (Frøslev et al. 2007), and allowed the fungal strains used here to be classified in clades. The DNA sequences were aligned using the ClustalW program (Thompson et al. 1994) and with the MEGA 4 program package (Tamura et al. 2007), which uses the same algorithm. Phylogenetic analysis was also carried out with the MEGA 4 program package.

Results and discussion

New results presented in the thesis:

- The mycorrhizal fungal partner of the bog orchid (*Hammarbya paludosa*) was successfully isolated and identified from protocorms germinated *in situ*. Judging from its ITS sequence, the symbiont belongs to the *Tulasnella* genus of fungi.
- The *in situ* germination method proved to be applicable even on floating mat fens. The orchid seeds germinated both on the reed-sedge peat of the two floating mat fen habitats of the fen orchid in Pákozd (Lake Velence) and Dunaharaszti (Soroksár branch of the Danube), and on the peat moss of the Bábtava habitat of the bog orchid.
- It was successfully demonstrated that the seeds of fen orchid have an extremely low germination ratio (0.1–0.5%) in the wild. This provided extremely important information on a vital phase in the life cycle of the species that had not previously been investigated (Wheeler et al. 1998, Rolfsmeier 2007).
- The fact that a substantially higher ratio of fen orchid seeds were found to germinate in the close vicinity of adult plants of the species suggested the enhanced presence of the mycorrhizal fungus. Further studies will be needed to decide whether the more frequent occurrence of the fungus resulted in the settlement of the orchids, or whether the development of successful symbiosis resulted in the multiplication of the fungus in the vicinity of the orchids.
- A *Tulasnella* (anamorph: *Epulorhiza* group I) taxon was found to be dominant on floating mat fens, while members of the *Epulorhiza* group II, shown by ITS sequence similarity to be genetically distant from the former, were dominant on drier habitats. This is in agreement with the findings of Bonnardeaux et al. (2007), who suggested that the spread of the *Disa bracteata* orchid species could be attributed to

the stress tolerance of its mycorrhizal partner, a fungus of the *Epulorhiza* type, similar to the group I taxon identified in the present work.

- On terrestrial habitats the fungal groups forming orchid mycorrhizas exhibited greater diversity than on the floating mat fens. The apparent fungal partner species specificity of the fen orchid, a floating mat fen specialist in Hungary, could be due to the fact that the number of potential fungal partners declines in extremely wet habitats, so the possibility that other fungal species could be satisfactory symbionts for fen orchid cannot be excluded.
- The fact that the fen orchid is extremely rare in Hungary and is only found on floating mat fens cannot be explained by the exclusive occurrence of its symbiotic fungal partners on floating mat fens, since these species were also detected in terrestrial habitats. It can thus be concluded that there are (micro)climatic reasons for the fact that the fen orchid is only found on floating mat fens in Hungary.

With respect to the three aims designated in the section entitled "Aims", the following results were obtained:

1. No field knowledge was previously available on germination, the first phase in the life cycle of fen orchids. The *in situ* germination method elaborated for orchids was successfully employed to observe the germination processes taking place in the natural habitats of fen orchids and to determine the magnitude of germination.
2. Studies on the mycorrhizal partners of rare orchids raise nature protection problems, since conventional fungus isolation techniques destroy the orchid plants. By contrast, the *in situ* examination of seed germination does not endanger the natural orchid population. The application of this method allowed the mycorrhizal fungus partners of these rare orchids to be identified at the only location in Hungary where a few tens of bog orchids are to be found and at two fen orchid habitats.
3. Aqueous habitats are rapidly being lost not only in Hungary, but throughout Europe. These habitats harbour many valuable plant and animal associations, and also provide a home for many very precious orchids. The habitat specificity of orchid species has long been studied, and a large body of knowledge has been compiled. Few data are available, however, on the habitat preferences and variability of the fungus species living in symbiosis with orchids. In the course of the present work, the complex orchid-fungus associations characteristic of floating mat fens, peat bogs, terrestrial fens and swamps were examined, together with those found in neighbouring, drier

steppe habitats. The results indicated that the fungal flora of each type of habitat was quite distinct, with certain fungus groups becoming dominant in some habitats (*Tulasnella* spp. – floating mat fens, *Epulorhiza* group II – dry habitats).

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