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Aspects of *in situ*, *in vitro* germination and mycorrhizal partners of *Liparis loeselii*

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ABSTRACT Our present work investigated asymbiotic *in vitro* and symbiotic *in situ* germination of the rare and protected terrestrial orchid, *Liparis loeselii* (L.) Rich. as one of the possibilities for its conservation. Asymbiotic germination array was tested on four different asymbiotic media (different macroelement and organic nitrogen levels) in dark and light. Additionally the effect of cold-treatment was also investigated. *In situ* germination rate was moderate, protocorms was observed only in the close surroundings of adult *L. loeselii* individuals. The fungal partners of *L. loeselii* were identified by sequence analysis of the internal transcribed spacer (ITS) of nuclear ribosomal DNA (nrDNA). The 'root segment technique' was effective for isolation of only one species, *Tulasnella* (*Epulorhiza*), whereas using 'in situ bating' yielded isolates of both *Tulasnella* and *Ceratobasidium* (*Rhizoctonia*) species.
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KEY WORDS

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germination
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ITS sequence

In most European countries, the populations of fen orchid *Liparis loeselii* L. Rich have dramatically decreased in the past few decades, whereas special efforts of many conservation project have been made for its maintenance and propagation (Ramsay 1994; Vejsadová 2001). Although asymbiotic growing of the species has been studied yet and seedlings have usually been transferred successfully to *ex vitro* conditions, the asymbiotically grown seedlings rarely survive the re-establishment into natural habitats (Takács and Bratek 1999). To gain a deeper insight into autecology and habitat requirements of fen orchid, it is crucial to identify mycorrhizal fungal partners in order to increase the planting survival of symbiotically grown seedlings. Nevertheless, asymbiotic germination can provide both information on ecological requirements of the species and artificial multiplication by producing numerous seedlings. The Hungarian occurrences of *L .loeselii* are unique in Europe, since they live on floating mats (Balogh et al. 1980), in contrary to the rest of Europe, where the species lives on plain fens and temporary marshes. Botanical and mycological investigations may also enhance our knowledge on these unique habitats. DNA-based methods can greatly facilitate the identification of the mycorrhizal fungi, since morphological identification of the fungus partner is generally much more complicated.

Materials and Methods

Asymbiotic germination

The seeds were collected from Lake Velence, where the highest Hungarian population of *L. loeselii* is located (Balogh et al. 2002). Orchid seeds were surface sterilized for 10 min in chloride of lime, washed twice in sterile water and sown on agar in Petri dishes. The germination was tested on four asymbiotic medium: 'MS' (Murashige and Skoog 1962), 'MS1/2' (modified 'MS' containing half amount of macro elements), 'Debergh' (Van Weas and Debergh 1986) and 'Fast' (Fast 1974). Organic nitrogen resources were included in the latter two media. Cultures were maintained at 25°C under diurnal conditions or in darkness. Some dishes of the latter group were exposed to cold (4-6°C) pre-treatment for one month.

In situ baiting

Seed packets were constructed from 40×60 mm rectangles of heat-sealed nylon mesh and about 300 seeds of *L. loeselii* were placed in each. The pore size of the mesh (85 μm) was chosen to retain the seeds without impeding fungal growth. The mesh was folded once and clipped into plastic glassless slide mounts (Rasmussen and Whigham 1993). Seed baits were positioned vertically in top-peat (approx. depth 5 cm) on the floating mat of Lake Velence. Each packet was deployed in the reed and willow habitat complex at five localities. Three of the five plots contained adult *L. loeselii* plant(s). Seed baits were buried at two occasions: a) in July 2003 and b) in October 2003 and were removed at three times: a) in October 2003 and b) in June and October 2004.

Isolation of fungal symbionts of *Liparis loeselii*

In order to determine the characteristics of fungus forming pelotons in seedlings and roots of *L. loeselii*, symbionts were isolated using aseptic techniques and cultured on Potato Dextrose Agar medium (Hadley 1970). Seedlings taken from packets and roots were surface-sterilized by immersion in 0,1% $AgNO_3$ for 1-3 min. after washing by distilled water they were placed onto the agar. One of the two adult fen orchids was collected from Lake Velence (Hungary), and the

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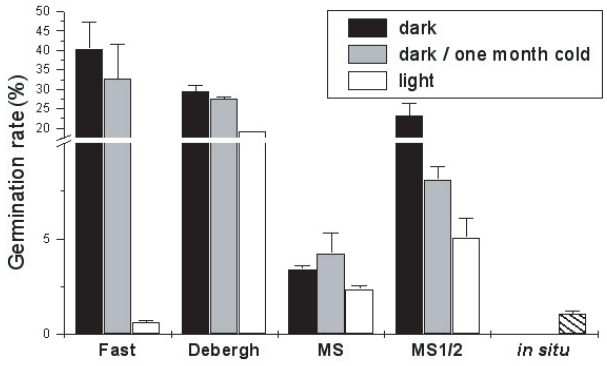

Figure 1. *In vitro* germination 4 months after sowing and *in situ* germination.

other was originated from Ceska Lípa (Czech Republic).

Molecular identification of fungi

DNA analysis was carried out on 12 fungal strains isolated from in situ germinated seedlings and 3 strains isolated from adult *L. loeselii* roots. DNA extraction, PCR and sequencing were carried out according to McKendrick et al. (2000).

Results and Discussion

The highest germinating rate allied with the fastest germination was observed on 'Fast' and 'Debergh' media, both of which contained organic nitrogen sources. Higher germination rate was detected on the 'MS1/2' medium, comparing to 'MS' (Fig. 1), may be related to the ecological requirements of the plants occurring in oligotrophic habitats. Although the cold pre-treatmented seeds began to germinate later than the others, after 120 days they showed similar germination rate. The effect of light/dark on germination of temperate terrestrial orchid is considered to be controversial (Arditti et al. 1990; Rasmussen and Rasmussen 1991). Nevertheless, we found that *L. loeselii* seeds germinated on light, but exhibited much lower level of germination than in dark (Fig. 1).

In situ germination rate was moderate (Fig. 1) presumably due to low abundance of potential symbionts in the constantly humid fen peat. Other facts also support this scenario: germination was observed 1) in separated groups of seeds in the slide mounts and 2) only in the close surroundings of adult *L. loeselii* plants. *In vitro* germination of seeds at room temperature began four-six weeks after the sowing, in contrast to in situ germination, which started germination two months later. The delay of germination might be caused by the cold microclimate of floating mat.

McCormick (2004) found that *L. liliifolia* considered to be the closest relative of *L. loeselii* was associated with a single fungal species, while we isolated likely the same and an additional species from our *L. loeselii* samples. Fungal ITS sequences resembling that of *Tulasnella* (anamorph: *Epulorhiza*) reported from *L. liliifolia*, were isolated from roots of two adult *L. loeselii* individuals (Hungary, Czech Republic) and also from 9 *L. loeselii* protocorms (Table 1). The protocorms also hosted 3 species probably the genus *Ceratobasidium* (anamorph: *Rizoctonia*) (Table 1). Considering the symbiont fungus invariability as for the photosynthetic *L. liliifolia* species, McCormick et al. (2004) rejected the widespread idea that the symbiont specificity only occurs in case of non-photosynthetic plants. Our results support the classical views, whereas the results by McCormick et al. (2004) may be attributed to the poverty in potential symbiont fungal species of the *L .liliifolia* habitats.

The infection of asymbiotically cultivated plants by isolated symbiont fungi can give a huge help to survive for the *L loeselii* individuals when re-establishing.

Acknowledgments

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Table 1. Isolated symbionts of *L. loeselii.*

Origin	Isolation source	Genus	No. of strains	Accession no.
Czech Republic	Root	Epulorhiza	2	AJ549128
Hungary	Root	Epulorhiza	1	AJ549124
Hungary	Protocorm	Epulorhiza	9	AM040890
Hungary	Protocorm	Rhizoctonia	3	AM040889

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