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Seed longevity in terrestrial orchids – Potential for persistent in situ seed banks

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ABSTRACT

Terrestrial orchids typically produce numerous small seeds that contain very small nutrient reserves. The seeds are structurally adapted for wind dispersal but little is known about their fate after dispersal. Some studies of seed viability in situ indicate survival for up to two years in temperate orchid species. Seeds stored in the laboratory may last much longer. We investigated seed viability of seven North American orchid species with seed packets buried in a range of soil and wood substrates within their natural habitats. In *Goodyera pubescens* most seeds germinated within one year. Four other species continued to germinate sparsely during the observation period, but after almost seven years many seeds were still viable. In one species, *Liparis liliifolia*, seeds that had been in situ for four years had germination rates as high as 68% when sown in vitro with a compatible fungus. The remaining two species did not germinate during the observation period but the seeds were judged to be intact and tested positively for viability after four years in the ground. These observations are interpreted as different species-specific strategies for in situ germination and their seed bank potential is discussed.

1. Introduction

Orchid seeds are minute, contain very small nutrient reserves, and the seedlings require a fungal symbiont to become established. When the seeds are shed, the embryos are morphologically immature and morphophysiological dormancy is likely to be common in orchids (Baskin and Baskin, 1998). Little is known about the fate of orchid seeds after dispersal. Do they become part of a seed bank, a common feature in many plant communities (Leck et al., 1989)? Does germination follow a predictable pattern over time (e.g., Grime, 1981; Baskin and Baskin, 1998)? Because of their small size, many seeds could be dispersed to sites unsuitable for germination or destroyed by soil processing animals or parasitic fungi (Rasmussen, 1995). On the other hand, orchid seeds stored under low humidity and low temperatures ex situ have remained viable for decades (Seaton and Pritchard, 2003; Ramsey and Dixon, 2003) and they are known to be highly resistant to chemical surface treatments (sulfuric acid and hypochlorites) used for sterile in vitro germination (Malmgren, 1996; Hicks, 1999).

The introduction of a method for sowing orchid seeds in situ and retrieving them later for germination assessment (Rasmussen and Whigham, 1993) has provided information on seed longevity in a few species. With the seed packet technique, the seeds are subjected to the physical and chemical conditions of the substrate, and contact with small soil organisms is possible but the sseeds are protected from larger

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animals such as millipedes and earthworms. In a study of the North American species *Spiranthes lacera*, Zelmer and Currah (1997) found that most seeds had germinated within a year. Also in the European species *Dactylorhiza maculata* and *Epipactis helleborine*, the seeds proved to be relatively short-lived (van der Kinderen, 1995). Seeds of *Caladenia arenicola* and *Pterostylis sanguinea* had either germinated or decomposed within a year in the dry Mediterranean climate of Western Australia (Batty et al., 2000). Many seeds of *Corallorhiza trifida* germinated within the first year but some seeds remained intact and appeared viable even after 31 months of burial (McKendrick et al., 2000b).

Whigham et al. (2002) reported on in situ studies of seeds of five orchid species that occur in deciduous forests in eastern North America. After one year in the ground, only one species (*Goodyera pubescens*) showed a high germination percentage and few viable seeds of this species remained. All seed packets of the other four species contained viable seeds but the small number of protocorms observed suggested a high degree of spatial variation in germination within and among the study plots. In the present paper we present additional results from that experiment and from a second field sowing experiment. Our objective is to provide evidence that seeds of some orchids persist well beyond one or two years, thus having the potential to form a persistent seed bank. We discuss the types of seed banks that were formed and their implications for conservation and restoration of threatened and endangered terrestrial orchids. The results of these studies are also interpreted in the context of orchid–fungal interactions.

2. Study area

The two field sowing experiments described below were conducted at the Smithsonian Environmental Research Center (SERC) in Maryland, USA. Both were established in the natural habitats of the study species. The sites for the first field sowing were all located in mature hardwood forests where the dominant trees were species of *Quercus* and *Carya* as well as *Liriodendron tulipifera* L., *Liquidambar styraciflua* L., *Fagus grandifolia* Ehrh., and *Acer rubrum* L. (Parker and Tibbs, 2004). Dominant understory trees were *Cornus florida* L. and *Carpinus caroliniana* Walter. Orchid species included in the first field sowing were: *Aplectrum hyemale* (Muhl ex Willd.) Nutt, *Corallorhiza odontorhiza* (Willd.) Nutt., *Goodyera pubescens* (Willd.) R. Br., *Liparis liliifolia* (L.) Rich ex Lindl. and *Tipularia discolor* (Pursh) Nutt. In conjunction with this first field sowing we carried out an in vitro germination study of *Liparis* seeds that had been buried for four years. The second field sowing involved *Galearis spectabilis* (L.) Raf. and *Platanthera lacera* (Michx) G. Don. Nomenclature for non-orchids follows Radford et al. (1968), and for orchids we used the World Checklist of Monocots (2004) as listed by The Board of Trustees of the Royal Botanic Gardens, Kew. Published on the Internet; http://www.kew.org/monocotChecklist/home.do.

3. Methods

3.1. *First field sowing: Aplectrum, Corallorhiza, Goodyera, Liparis and Tipularia*

In autumn 1997, locally collected seeds of these species were briefly dried in the laboratory and seeds from numerous capsules mixed. A sample containing approximately 50–300 seeds was put into each seed packet (270 seed packets per species) constructed of 50 μm pore size plankton netting fitted in plastic frames (Gepe glassless slide mounts). The packets were then placed vertically into linear plastic trays that are designed for slide projectors (Fig. 1). The trays were buried in eight different types of substrates at each of the three forest sites (Whigham et al., 2002). The schedule for examination of seed packets is shown in Table 1. Further details of the experiment and results after the first year for *Goodyera* can be found in Whigham et al. (2002). Because most seeds of *Goodyera* had germinated or deteriorated after one year, no further observations of that species were made.

Seed packets were periodically returned to the laboratory for examination and viability testing (Table 1). Seed packets not processed immediately were stored at 6 C until recorded. A visual inspection of each seed packet included examination for the presence of germinating seeds and protocorms. Germination was indicated if the embryo had emerged from the testa. Intact seeds were evaluated for viability based on visual conditions of the seed coats and embryos. After four years (Table 1) we continued to make visual observations of seed packets but we also conducted viability tests on subsets of seed packets (Table 1) using the Triphenyltetrazolium chloride (TTC) method (Rasmussen, 1995; Baskin and Baskin, 1998; Hicks, 1999; Ramsey and Dixon, 2003).

We also determined the germination rates of *Liparis* seeds after they had been in the field for four years (Table 1). The

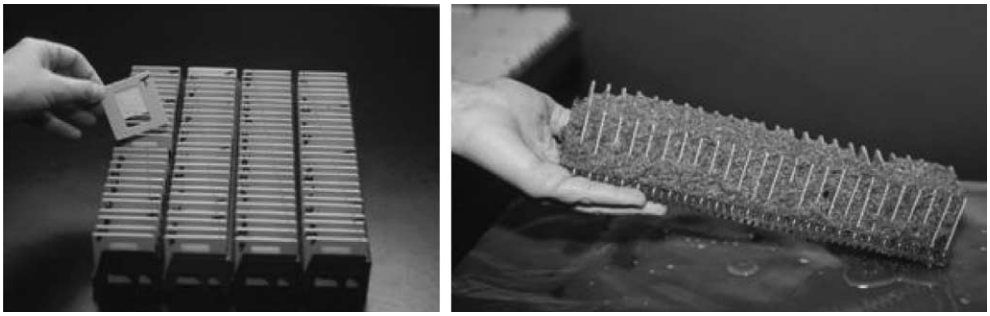

Fig. 1 – Photos of slide trays containing seed packets (left) and a slide tray that has been filled with ground wood and seed packets (right).

Table 1 – Schedule for sampling and processing seed packets for four of the five species included in the first sowing experiment

	Time after sowing (m)	*Aplectrum hyemale*	*Corallorhiza odontorhiza*	*Liparis liliifolia*	*Tipularia discolor*
November 1997	0	Initial sowing ($N = 270$)	Initial sowing ($N = 270$)	Initial sowing ($N = 270$)	Initial sowing ($N = 270$)
May 1998	6	Visual examination ($N = 10$)	Visual examination ($N = 10$)	Visual examination ($N = 10$)	Visual examination ($N = 10$)
November 1998	12	Visual examination ($N = 15$)	Visual examination ($N = 15$)	Visual examination ($N = 15$)	Visual examination ($N = 15$)
March 1999	15	Visual examination ($N = 90$)	Visual examination and TTC test ($N = 15$)	Visual examination ($N = 85$)	Visual examination and TTC test ($N = 15$)
November 2001	48	Visual examination ($N = 125$) TTC test ($N = 15$) Returned to field ($N = 75$)	Visual examination ($N = 130$) TTC test ($N = 15$) Discontinued	Visual examination ($N = 90$) TTC test ($N = 11$) in vitro study ($N = 10$)	Visual examination ($N = 130$) TTC test ($N = 14$) Returned to field ($N = 75$)
November 2002	60	Visual examination and TTC test ($N = 15$)		Visual examination and TTC test ($N = 6$) Returned to field[a] ($N = 60$)	Visual examination and TTC test ($N = 15$)
April 2004	77	Visual examination and TTC test ($N = 15$)		Visual examination and TTC test ($N = 15$)	Visual examination and TTC test ($N = 15$)

Data for *Goodyera pubescens* are not included in the table because results for that species have been reported elsewhere (Whigham et al., 2002). N = number of seed packets.
a Stored in refrigerator for 12 months before returning to the field.

seeds were stored in a refrigerator at 6 C for another 12 months and then tested for germination by sowing them in Petri dishes on water agar that contained 2 g/L finely ground freshly collected *Liriodendron tulipifera* wood and inoculated with fungus #120, a local isolate known to support germination in vitro (Rasmussen and Whigham, 1998a; Whigham et al., 1999, 2002). Plates without the fungus served as controls.

3.2. *Second field sowing: Galearis and Platanthera*

In 2001, 12 seed packets of *Galearis* were buried in soil within a patch of the same species in a forested site that we had been monitoring for several years. At the same time, five seed packets of *Platanthera* were placed in a small population that we had been monitoring for approximately 15 years in a successional forest. Seed packets of the two species were examined in the field periodically and half were retrieved after 35 months and examined for germinated seeds and protocorms and we conducted TTC viability tests.

4. Results

4.1. *First field sowing*

After six months in the field, the seeds of all species appeared to be healthy based on visual inspection, but none of them had embryos that had increased in size. After one year, we found small numbers of germinated seeds and a few protocorms and, as already described, most *Goodyera* seeds had either germinated or completely deteriorated (Whigham et al., 2002). After two years, the seed packets of the remaining species still contained viable seeds based on visual observations but we found few germinated seeds or protocorms in the seed packets and there was a high degree of variability within and between sites. Germinated seeds of *Aplectrum* were found in seed packets at two sites but only in a single seed packet at each site. In one site almost all of the seeds in the seed packet had germinated. In the seed packet at the other site, most seeds had germinated and it contained two protocorms. A single seed packet contained five *Corallorhiza* protocorms. A few *Liparis* seeds with enlarged embryos were observed at all sites. A single *Liparis* protocorm was found at one site, and a seed packet at a second site contained numerous protocorms. Germinated seeds of *Tipularia* were found in one seed packet at each site and two protocorms were found in a seed packet at one site.

Likewise, after four years, we found protocorms of all four remaining species (Table 2). They were more abundant than after the first observations, but again there was a high degree of spatial variability within and between study sites. *Corallorhiza* protocorms ($N = 542$) were present in 42.9% (58 of 135) of the seed packets examined. They were found at all sites and substrate types. In contrast, relatively few protocorms of *Tipularia* ($N = 11$), *Aplectrum* ($N = 120$), and *Liparis* ($N = 91$) were present, and they were found in a small number of seed packets ($N = 4$, 7 and 3, respectively), substrates ($N = 2$, 2, and 1, respectively), and sites (2, 2, and 1, respectively). For all of the species, the majority of seed packets contained viable seeds based on visual inspection (Table 2). TTC tests indicated

Table 2 – Summary data for seed packets for four species (Aplectrum hyemale, Corallorhiza odontorhiza, Liparis liliifolia, Tipularia discolor) used in the first seed sowing study

	# of Protocorms per seed packet (mean/maximum/minimum)	# Seed packets with protocorms (maximum = 135)	# Seed packets with viable seeds (maximum = 135)	# Sites with protocorms (maximum = 3)	# Substrates with protocorms (maximum = 8)
Aplectrum	1.0/88/0	7	119	2	3
Corallorhiza	3.9/140/0	58	94	3	8
Liparis	0.8/91/0	3	78	1	1
Tipularia	0.1/6/0	4	119	2	2

Seed packets were placed into the field in 1997 and retrieved four years later (2001).

that the mean (±1 Ste) seed viability for *Aplectrum* was 42.9 ± 7.8% after four years and 74.9 ± 2.4% after five years. Viability for the same periods of time was 62.3 ± 6.9% and 48.8 ± 8.9% for *Liparis* and 30.5 ± 8.1% and 31.7 ± 3.7% for *Tipularia*. We did not test seed viability in *Corallorhiza* with the TTC test because most of the seeds had germinated and the few ungerminated seeds that remained did not stain or stained so lightly so that it was not possible to make accurate counts.

After six years and five months, visual assessment and TTC tests indicated that all *Aplectrum* and *Tipularia* seed packets that had been returned to the field sites after a brief inspection in year four, contained viable seeds. All *Liparis* seed packets contained viable seeds at two sites, but only 20% of the seed packets contained viable seeds according to the TTC test at the third site. In this last sampling we found no protocorms of any species in the seed packets.

The mean germination of *Liparis* seeds in the laboratory study with fungus #120 was 68.7 ± 10.4% and seeds failed to germinate on the asymbiotic control plates.

4.2. *Second field sowing*

Seed packets of *Galearis* and *Platanthera* retrieved after 35 months in the field contained no protocorms but all of them contained viable seeds based on TTC testing (data not shown).

5. Discussion

After one year there were germinated seeds and protocorms of *Goodyera* at all sites and in all eight substrates and few viable seeds remained in the seed packets. In contrast, the other four species in the first field sowing were capable of forming a seed bank that would last from 4–5 (*Corallorhiza*) to almost 7 years (*Aplectrum*, *Liparis*, *Tipularia*). Judging from TTC viability testing, *Platanthera* and *Galearis* also could form seed banks that last for at least three years, as shown in the 2nd sowing experiment.

Previous studies have indicated that seeds of terrestrial orchids are relatively short-lived (van der Kinderen, 1995; Zelmer and Currah, 1997; Batty et al., 2000; McKendrick et al., 2000b). These studies, however, were not intended to be long-term and seed longevity may have been underestimated. McKendrick et al. (2000b), for example, found that some seeds of *Corallorhiza trifida* were still intact when they ended their study after 31 months.

The presence of a seed bank is an important component of many plant communities (Leck et al., 1989) and seed banks have been described as being either short-term (seeds lasting for 1–5 years) or long-term (seeds lasting >5 years) persistent (Bakker et al., 1996 as cited in Baskin and Baskin (1998)). In deciduous forests, however, few herbaceous species are known to persist in the seed bank for more than a year. Species that have long-lived seed banks in temperate deciduous forests are typically good colonizers of disturbed sites and they are often associated with early successional habitats (Pickett and McDonnell, 1989). The majority of the orchid species that we have studied are probably good colonizers because they are among the few herbs of mature deciduous forests that also occur in 70-year old successional forests at SERC. *Liparis* occurs mostly as scattered individuals or in small groups in mature and successional forests, and the largest population at SERC occurs in a successional stand (Whigham and O'Neill, 1991). *Tipularia* is the most widespread orchid in SERC forests (Whigham and O'Neill, 1991; Rasmussen and Whigham, 1998b), and it occurs in both successional and mature forests. Established plants of *Aplectrum* also occur in mature and older successional forests but for years we have looked in vain for spontaneous seedlings. *Platanthera lacera* occurs only at one site at SERC, a young successional forest that developed on a site that had been abandoned after it had been used as part of a managed experimental garden for almost 10 years. We are certain that there were no orchids at the site during the time that it was used as an experimental garden. The most likely scenario is that seeds were blown to the site after the experimental garden had been abandoned, but the source population must be far away as we are unaware of any other populations of the species on the 1200 hectare SERC property. Orchid seeds are known to disperse for great distances (Arditti and Ghani, 2000, Table 5), however.

Corallorhiza, Galearis, and *Goodyera* do not fit the colonizer model based on their distribution at SERC as they as primarily occur in mature forests. *Corallorhiza* and *Galearis*, however, are seed bank species, according to our results. *Corallorhiza* primarily occurs at one site, but scattered individuals can be found elsewhere in mature forests at SERC that have not been disturbed for more than 150 years. *C. odontorhiza*, as generally within this genus, is an achlorophyllous species that produces no leaves. A related species, *C. trifida* relies on recruitment from buried seeds (McKendrick et al., 2000a,b), but natural propagation of *C. odontorhiza* may also occur by fragmentation of the underground coralloid rhizome and by the formation of bud-like structures at the tips of branches (J. O'Neill and D. Whigham, personal observations).

Galearis spectabilis is a common species in mature forests at SERC. It interacts with a fungus that is probably a species of *Ceratobasidium* (M.K. McCormick, unpublished DNA analysis) and the slow growth of the fungus in vitro indicates that it may be at least facultatively ectomycorrhizal. We have monitored individual *Galearis* plants in permanent plots for more than a decade. Most individual plants (D.F. Whigham, personal observations) appeared above ground for only 1–2 years, although some individuals persisted longer but none of them ever flowered. While some orchids are known to persist underground for varying periods of time (e.g., Rasmussen, 1995; Shefferson et al., 2001; Shefferson, 2002; Willems, 2002), we have found no evidence that this occurs in *Galearis*. as excavations at the site of plants that produced leaves one year but not the next confirmed that the plants had died. In addition, no plants in or near the permanent plots have produced seeds, yet new individuals appear each year in one or more of the permanent plots. The source of the new individuals is unknown but we believe that it is most likely the emergence of plants from a persistent seed bank.

Goodyera is the only species we studied that does not produce a seed bank, nor is it often found in successional forests – a pattern similar to many herbs of deciduous forests (Pickett and McDonnell, 1989). Also like many woodland herbs (Whigham, 2004), it is a clonal species with long-lived ramets; thus, there may be less selection for long-lived seeds. Two other clonal orchids that we have worked with that have rhizomes are *Tipularia* and *Aplectrum* and both can produce new ramets. The rhizomes of both species typically only branch after flowering or disturbance (Whigham and O'Neill, 1991) and branches eventually become separate ramets as the older parts of the rhizome disintegrate.

It is difficult to interpret our data in the context of seed bank types as described by Thompson and Grime (1979), Grime (1981) and Baskin and Baskin (1998). *Goodyera* has a variant of a Type I seed bank (Thompson and Grime, 1979) since seeds germinated within the first year and do not persist, but the other species do not seem to follow any recognized seed bank types. The only general germination pattern we found was the presence of decreasing numbers of protocorms in seed packets over time. Even this pattern, however, is uncertain since the number of protocorms was always small and spatial variability was high among and within the study sites and substrates (Table 2).

Why did many of the seed packets contain viable seeds but few germinated seeds and protocorms? Except for *Liparis* (see below), we do not know whether the seeds judged to be viable based on visual inspection or TTC tests were in a dormant state or if they are capable of germination but had not germinated because an appropriate fungus was not present. Baskin and Baskin (1998) found evidence for morphophysiological dormancy in orchid seeds but little evidence of long-term dormancy in seeds of terrestrial orchids. In view of the high seed quality (Whigham et al., 1999), the generally low incidence of in situ germination (except in *Goodyera* and *Corallorhiza*) suggests seed polymorphism, with few seeds having had their individual requirements for germination fulfilled. The importance of an appropriate mycorrhizal fungus in seed packets has been demonstrated by others (e.g., Zelmer and Currah, 1997; McKendrick et al., 2000a, 2002; Batty et al., 2001a) and our results also indicate that the presence or absence of appropriate fungi at least partially explains the spatial variation and the low germination response of orchids.

The importance of the fungal–orchid interaction is clearly demonstrated by the in vitro study of *Liparis* seed germination and observations of *Liparis* in seed packets. *Liparis* protocorms were only found in seed packets in a single substrate at one site and it occurred on two occasions. We isolated the fungus from one of the seed packet protocorms and identified it through DNA analysis as the same fungus that associates with adults of *Liparis* (McCormick et al., 2004), thus providing a strong case for a high degree of specificity. We do not know if the high germination response in vitro was also influenced by the seed pretreatment but the importance of the fungus for successful germination is clear.

This research also suggests the importance of the distribution of fungi that form mycorrhizal interactions with terrestrial orchids, especially species that do not form a seed bank. The reason why *G. pubescens* germinated so readily in all places could be that the seedlings do not require infection before the rhizoids are formed (Rasmussen and Whigham, 1993, 1998b); even though they germinate sooner in vitro when a compatible fungus is present (Whigham et al., 2002). For germinated orchid seeds to survive as seedlings, however, an appropriate fungus must be present (e.g., Zettler and Hofer, 1998; McKendrick et al., 2000b; Whigham et al., 2002). It would seem that this strategy could only be successful if there is a low specificity for fungi or if appropriate fungi are widespread. For species that form a seed bank, the temporal urgency is less, as the chances that a fungus will be present would increase over time if patches of fungi expand and appear in new sites where they would encounter seeds. The germination strategy of seed banking orchid species could either be that germination is very sparse and spread over a long time span, or that it is fungus-induced. Species like *Liparis liliifolia* apparently require that the fungi have arrived before germinating, as seen in this study. Little is known, however, about the distribution and spatial dynamics of fungi in soil systems (e.g., Batty et al., 2001a). We are currently addressing this issue in our research program.

Our results and those of others (e.g., Zelmer and Currah, 1997; McKendrick et al., 2000a,b; Batty et al., 2001a) also have a significant bearing on the conservation and restoration of terrestrial orchids. A number of terrestrial orchids are rare, threatened, or endangered (Whigham and Willems, 2003). Species may be in decline for a variety of reasons, but for orchids one critical factor undoubtedly is the decrease or loss of the fungi, required for seedling development and at other life history stages. Another important factor would be the seed bank depletion following long-term reproductive failure (Roberts, 2003). The latter could result from general problems such as grazing of inflorescences, and lack of appropriate pollinators, but especially for orchids, flowering could be prevented by habitat changes that discourage above-ground photosynthetic structures altogether and support mycotrophic, vegetative persistence (Rasmussen, 1995). Similarly, efforts to restore orchids to habitats from which they have been extirpated (e.g., Zettler et al., 2001; Ramsey and Dixon, 2003) require knowledge about the ecology of seeds and protocorms and information on the presence or absence of an appropriate

fungus. The absence of an appropriate fungus must be dealt with if restoration is to be successful. The results of these studies indicate that seed banks of terrestrial orchids may be more important than previously thought. As is already being done in Western Australia (Ramsey and Dixon, 2003; Batty et al., 2001b), we encourage others to explore this aspect of the ecology of terrestrial orchids through the use of simple and inexpensive seed packets.

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