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Orchid population biology: conservation and challenges.
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The underground phase: a special challenge in studies of terrestrial orchid populations

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Orchid seeds are minute and the first seedling stages of terrestrial species are underground, so there is little knowledge about the biology of the early life history and the size of seed/seedling populations relative to the number of plants that eventually emerge above ground. A recently developed field sowing technique makes it possible to explore a variety of topics such as the length of time that seeds spend as part of the soil seed bank, the phenology of seed germination, substrate requirements for germination, dependence of germination on fungal availability, substrate requirements and growth rate of seedlings, seedling phenology and development, and seedling-fungus specificity patterns in the soil. Several of these applications are relevant for conservation issues. Studies of *Goodyera pubescens*, *Corallorhiza odontorhiza* and *Liparis lilifolia* show considerable variation in germination strategies, patterns of seedling development and establishment, substrate requirements, and levels of specificity with the fungi.

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ADDITIONAL KEY WORDS:—*Corallorhiza* – demography – germination – *Goodyera* – life history – *Liparis* – mycotrophy – Orchidaceae – seed – seedling (protocorm).

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INTRODUCTION

Orchid seedlings are fully mycotrophic, and those of terrestrial orchids develop underground for extended periods of time. The phase lasting from the time when the mature seed is dispersed until a seedling appears above ground is a considerable part of life history. However, the small size of seeds makes it difficult to follow the fate of both them and the underground seedlings, which means that this phase is largely neglected in life history studies. None of the life history investigations presented in a previous volume on the ecology of terrestrial orchids adequately addressed the fate of seeds and underground seedlings (Wells & Willems, 1991).

The first results of experimental sowings in the field have shown that seeds may remain dormant in the soil for a considerable time. *Goodyera pubescens* and *Corallorhiza odontorhiza* required 24–30 weeks in the soil before germinating (Rasmussen & Whigham, 1993), and rupture of testa was first observed in *Epipactis helleborine* after 8 months in the soil (Van der Kinderen, 1995b); infection and protocorm development in *Epipactis* occurred after about a year. While *Dactylorhiza maculata* is reported to germinate within 3.5 months, *Microtis parviflora* had germinated after 12 weeks in the soil and *Spiranthes sinensis* var. *amoena* germinated after only 8 weeks (Van der Kinderen, 1995a; Perkins, Masuhara & McGee, 1995; Masuhara & Katsuya, 1994). Season of germination in these studies varied from early spring (*Microtis*), and early summer (*Goodyera*, *Corallorhiza*, *Spiranthes*), to autumn (*Epipactis*) and early winter (*Dactylorhiza*). In growth chambers in a natural substrate and with a constant temperature and humidity, but varying photoperiods to simulate summer and winter periods, Johnson (1994) obtained germination in *Bletilla striata* in early December, about 6 weeks after sowing.

Estimates of the length of time spent as purely heterotrophic seedlings (i.e. until the first foliage leaf is produced) vary considerably, the usual range being 2–4 years (including the amount of time between seed dispersal and germination; Wooster, 1935; Willems, 1982; references in Rasmussen, 1995), with a minimum of 3–4 months after germination (*Liparis loeselii*, Mrkvicka, 1990). Individual variation within the population is probably great, because the germination and growth rates of seedlings depend on slightly differing individual requirements and conditions. One example is the requirement for a cold period in seedlings of *Dactylorhiza majalis*, the length of which appears to be inversely related to the size of the seedling (Jørgensen in Rasmussen, 1995). This means that individuals below a critical size would require more than one winter for the first leaf or leaves to sprout.

Understanding the dynamics of the underground phase is important for understanding changes in the overall population structure of terrestrial orchids. The uncertain duration of the underground phase means that individuals appearing above ground for the first time ('seedlings' in most contexts) cannot be assigned with certainty to any particular previous flowering/fruiting season and, indeed, may not be a genuine cohort but an unevenly aged group. Provided that the population is fairly stable there is a dramatic decline in numbers from the thousands of seeds produced to the relatively small number of emerging seedlings, which makes it obvious that a strong selection pressure acts on the underground stages. It is important to know when and how this depletion occurs and what factors control it.

Three approaches can be used in combination to gain insight into the underground phase of terrestrial orchids.

(1) Field observation of naturally dispersed seeds is virtually precluded because of

TABLE 1. Species of terrestrial orchids that germinate in water or on water agar (W). In some germination percentages rise when either soluble carbohydrates, macroelements (S+M) or organic nitrogen sources (OrN) are included in the substrate

	W	S+M	OrN	
Goodyera pubescens	+++			Orig.
Spiranthes spp.	+	+	++	Stoutamire, 1964, 1974
Platanthera hyperborea	+		++	Harvais, 1974
P. obtusata	+		++	Harvais, 1974
Dactylorhiza purpurella	+			Harvais & Hadley, 1967
D. spp.	++	++	+++	Downie 1941; Van Waes & Debergh, 1986
Coeloglossum viride	+			Downie, 1941
Gymnadenia conopsea	+			Downie, 1941
Orchis spp.	+	+	++	Eiberg, 1969; Vermeulen, 1947; Van Waes & Debergh, 1986
Anacamptis pyramidalis	(+)	+	++	Van Waes & Debergh, 1986

TABLE 2. Species of terrestrial orchids that do not germinate in water (W), but germinate if either soluble carbohydrates, macroelements (S+M), an organic nitrogen sources (OrN), or other additives are included in the substrate. Fungus/potato (F/P); yeast + amino acids (Y+A).

	W	S	S+M	OrN	Other	
Cypripedium reginae	0		+	++	+++ (Kinetin)	Harvais, 1973
Epipactis atrorubens				+	+++ (BA)	Van Waes & Debergh, 1986
E. helleborine				+	++ (BA)	Van Waes & Debergh, 1986
Listera ovata	0		+	++	+ (Potato)	Downie 1941, 1949b; Eiberg, 1969; Van Waes & Debergh, 1986
Googyera repens	0		+		++ (Potato)	Downie, 1940, 1941; Hadley, 1982
G. oblongifolia	0	+				Harvais, 1974
Platanthera bifolia	0		+	++	++ (Potato)	Downie, 1941; Van Waes & Debergh, 1986
Corallorhiza trifida	0	0	0		++ (F/P)	Downie, 1949a
Galeola septentrionalis	(+)				+++ (Y/A)	Nakamura, 1962

their small size. However, excavated seedlings are occasionally observed in the field and most of our present knowledge of the underground phases is based on such field observations. Practical difficulties in finding underground seedling stages limit the usefulness of this approach and it is impossible to age (and sometimes even identify) underground seedlings. When found in large numbers (e.g. Leeson, Haynes & Wells, 1991), seedlings show wide individual size variation and no clear segregation into cohorts. Large individual differences in time of germination or seedling growth rates could account for this lack of pattern.

(2) Germination *in vitro* and culture of seedlings on an artificial substrate is another widely used approach. With due caution, valuable information can be gained about the requirements of the underground phases, especially if the ingredients used in the growth medium are tested one at a time and with water agar as control ('minimal' media). Many orchids germinate *in vitro* in pure water and without the aid of fungi, indeed behave like any other plant seeds (Table 1; Rasmussen, 1995). However, the germination percentage of the seed batch often increases compared with the water agar control if the substrate is provided with sucrose or other simple organic compounds, such as amino acids. This reflects a certain seed polymorphism. In another group of species, germination does not occur in water but rises to detectable levels with the substrate is enriched (Table 2).

Results from *in vitro* studies need to be treated with caution since conditions offered *in vitro* are very different from conditions in the soil. There are no competing organisms, usually a richer substrate, and the symbiont can be a carefully selected fungus. On the other hand, substrate compositon may be suboptimal and environmental stimuli that trigger development in nature may be lacking. Hence, development of seedlings grown *in vitro* can either be slower or faster than in the natural habitat. Asymbiotic culture and growth regulators in the substrate may furthermore change the natural course of organogenesis.

(3) A third approach is controlled sowing of seeds in retrievable seed packets in the field (Rasmussen & Whigham, 1993; Masuhara & Katsuya, 1994; Van der Kinderen, 1995a). Germination percentage can be recorded at regular intervals of time and seedling sizes analysed to estimate seedling growth rates of mortality. With frequent retrievals the phenology of germination and seedling growth can be analysed. By manipulation of soil components and physical factors, or by inoculation with micro-organisms, experimental studies on edaphic, climatic, or biotic requirements for germination and seedling development are possible Mycorrhizal status of seedlings can be assessed, infection sites located, and endophytes isolated and identified to determine whether the association varies with time or place. One draw-back to this method is the limited time during which seedling growth can be contained unharmed within the confines of the seed packets.

In this paper we present results from the combined use of the second and third approaches to study seed germination and seedling biology of three species of terrestrial orchids growing in deciduous forests in Maryland, USA.

MATERIAL AND METHODS

Field study sites

All field studies were conducted in deciduous forests at the Smithsonian Environmental Research Center (SERC). The forests have been classified as being part of the Tulip Poplar Association (Brush, Lenk & Smith, 1980). As suggested, *Liriodendron tulipifera* is a dominant species but many other tree species are common (e.g. *Liquidambar styraciflua*, *Acer rubrum*, *Quercus alba*, *Q. velutina*, *Q. falcata*, *Carya tomentosa*, *C. glabra*, *Fagus grandifolia*) along with several shrub species (e.g. *Cornus florida*, *Carpinus caroliniana*).

Habitats for terrestrial orchids at SERC include mature and successional forest, i.e. *c.* 50 years since abandonment (Whigham & O'Neill, 1988, 1991). In successional forest with *Liriodendron tulipifera* and *Liquidambar styraciflua* as dominant species, *Liparis lilifolia* and *Tipularia discolor* are most commonly found. *Goodyera pubescens* is mostly restricted to the dry, mature forest dominated by *Quercus prinus*, *Q. alba*, *Q. falcata* and *Nyssa sylvatica* with an understory containing ericaceous plants such as *Kalmia latifolia* and *Vaccinium* spp. On more mesic sites with mature forest, *Corallorhiza odontorhiza* is found together with *Galearis spectabilis*, *Tipularia discolor* and *Aplectrum hyemale*. *G. pubescens* may also occur in such habitats but usually in areas where litter accumulation is less due to slope position.

Seeds were distributed in packets of plankton netting (Nutex, 35 μm mesh size) mounted in 24 × 36 glassless slide frames (Gebe). These packets were tied to flags

TABLE 3. Substrate recipes

Ground recipe (per litre demineralized water):	
Ca $(NO_3)_2 \cdot 4H_2O$	200 mg
KCl	100 mg
KH_2PO_4	200 mg
$MgSO_4 \cdot 7H_2O$	100 mg
yeast extract (Difco)	100 mg
sucrose	2 g
12 g agar	
(pH adjusted before autoclaving to 5.8).	

H_1 : ground recipe plus included 3 g of oat.
H_2 : as H_1 but with 2 g glucose in place of sucrose.
W_1 : ground recipe plus 3 g dried and granulated (<2 mm), decayed wood, and 2 g glucose in place of sucrose.
W_2 : as W_1 but with no soluble carbohydrates.
W_6 : as W_1 but Ca $(NO_3)_2$ was replaced by 27 mg urea.
CL : ground recipe plus 3 g of cellulose.
LR : ground recipe plus 3 g of dried, powdered *Liriodendron tulipifera* leaf litter.

and buried about 2 cm deep in 45 × 55 cm plots distributed at random in selected areas of the forest. At consecutive times one packet was retrieved and recorded destructively from each plot (Rasmussen & Whigham, 1993).

Fungi

Pelotons were extracted from root and seedling tissues, transferred through six rinses with sterile water before being placed singly on 60 mm agar plates (Rasmussen, 1995, for details of isolation technique). Of the substrates used for isolation and maintenance, H_1, H_2 (collectively called 'oat'), W_1, W_2, W_6 ('wood'), CL and LR were all based on a ground recipe with various modifications, mainly with respect to carbohydrate sources (Table 3). Classical fungal media were also used, i.e. MA: 20 g malt, 12 g agar; MP: 20 g malt, 1 g peptone, 12 g agar; DP: 5 g glucose, 1 g peptone, 12 g agar; PDA: 10 g potato flour, 10 g glucose, 12 g agar.

The following fungi, isolated locally in the forest at SERC, were used in the experiments *in vitro*.

M5-1 was isolated from the first rhizome of a young seedling of *Goodyera pubescens* from mature, mesic forest in November 1991. The mycelium was a typical *Rhizoctonia* with monilioid cells, aggregating to show signs of loose sclerotia, and forming hyphal coils in pure culture. It could be grown on a range of substrates, e.g. oat, DP, MA, and W_2.

M7-1 was isolated from a young root on a seedling of *Tipularia discolor*, on which the first leaf was developed and a corm beginning to form. The seedling was found in November 1991 in mature mesic forest on a fallen tree log too decayed for identification. The mycelium formed many monilioid cells in pure culture. Best growth was obtained on W_6, but it also grew slowly on other wood media, oat, CL, MA, MP, LR, and PDA.

M9-2 was isolated from the rhizome of a flowering individual of *Corallorhiza odontorhiza*, in mature mesic forest in December 1991. This fungus formed clamp connections and thus cannot be referred to *Rhizoctonia* (Rasmussen & Whigham,

1993). It was difficult to grow in pure culture, and we only had limited success on DP and PDA.

M23-2 was isolated from a second population of *Corrallorhiza odontorhiza*, also from a rhizome of a flowering individual, growing in fairly dry, mature forest February 1992. This mycelium resembled M9-2 in all respects, but grew slightly better on a broader range of substrates.

M25-1 was isolated from a root of an adult plant of *Aplectrum hyemale* in mesic mature forest in February 1992. This was a typical *Rhizoctonia*, forming monilioid cells and hyphal coils in pure culture and growing rapidly on all substrates tested.

M26-1 and M26-4 were probably identical isolates, originating from a tuberous root of *Galearis spectabilis*, collected in mature mesic forest in February 1992. These isolates had typical features of *Rhizoctonia*, such as short lateral hyphal branches and sclerotia-like aggregates on the mycelium in pure culture (H_1). They were also grown on DP, PDA and W_1.

M29-2 was an isolate from a root of a first-year seedling of *Tipularia discolor* with remaining protocorm, growing on a fallen log of *Quercus alba*, in late February 1992. The isolate was a typical *Rhizoctonia*, with monilioid cells and pelotons in pure culture, which was grown on H_2, CL, DP and PDA.

M31 was isolated from the rhizomatic base of the pseudobulb on a mature plant of *Liparis lilifolia*, collected in successional forest in March 1992. The isolate had the characteristics of *Rhizoctonia* sp. and was grown on H_1 and PDA.

M43 was an isolate from material as above, but collected in a separate population, in a successional forest area in spring 1992. This mycelium was similar to M31 in morphological features and culture.

Seeds and in vitro *experiments*

For experiments with minimal media, the basic formulation was the ground recipe (Table 3, Fig. 1) and varied thus:

'-salts' – no inorganic salts; '$\frac{1}{2}$salt' – half amount of all inorganic salts; '-sucr' – no sucrose; '$\frac{1}{2}$sucr' – 1 g sucrose; '-yeast' – no yeast extract; '$\frac{1}{2}$yeast' – 50 mg yeast extract; 'WA' – 12 g agar and water only.

Goodyera pubescens

The seeds were a mixture of collections from several SERC populations, as well as other localities in Maryland and Virginia. They were dried at room atmosphere for 1.5 months, then stored at 5°C until use. Prior to sowing on agar they were surface sterilized 1 h in saturated $Ca(OCl)_2$ with *c.* 0.3% Tween 80. Germination on minimal media was recorded after 7 weeks, in 10 replicate Petri dishes per substrate including WA. The length of five randomly chosen seedlings in each dish was measured 2 weeks later.

Five months after the beginning of this asymbiotic experiment the seedlings were reused for a symbiotic screening experiment. Seedlings growing on either full basic formulation, or substrates with half strength mineral salts, sucrose, or yeast extract, were transferred to H_1 agar Petri dishes so that seedlings originating on the same germination plate were spread on four new plates. These were randomly incoculated with one of eight local symbiont fungi, initially with 17 replicate dishes of each

strain (some replicates were subsequently lost to contamination). Lengths of *c.* 100 seedlings of each treatment were measured 4 weeks after inoculation.

Corallorhiza odontorhiza

Seeds were harvested from SERC plants in November 1991. Immediately after harvest they were surface sterilized for 1–8 h and sown on H_1 medium. After asymbiotic incubation at 20°C for *c.* 5 months the seeds had not germinated. The dishes were then inoculated with isolate M9-5, incubated for 6 additional weeks, and subsequently randomly distributed into four treatments: chilling for 3, 6 or 9 weeks at 5°C and a control, remaining at 20°C. After chilling the dishes were returned to 20°C until they were all recorded 3.5 months after cold stratification treatments began.

Liparis lilifolia

Seeds were collected at SERC and stored in the same manner as those of *Goodyera*, for a maximum of *c.* 35 weeks before being used in experiments.

Before sowing, the seeds were surface sterilized for 2 h in $Ca(OCl)_2$ as described above. On the series of minimal media, as described above (10 replicate Petri dishes of each substrate) no asymbiotic germination had taken place at 20°C 7 weeks after sowing; the plates were then inoculated with a fungal strain, M31, and maintained at 20°C. Germination and maximal seedling length in each dish were recorded 12 weeks after inoculation.

For symbiotic screening W_2 medium was used. Seeds were sown, and the Petri dishes either inoculated with 1 of 8 symbionts (10 replicates of each treatment) or left as asymbiotic controls (20 replicates) and incubated at 20°C. Germination percentage and maximal seedling length of each dish were recorded 6 weeks after sowing.

Field sowings

Seeds of *G. pubescens* and *C. odontorhiza* were sown in the field in seed packets and retrieved at regular intervals as previously described (Rasmussen & Whigham, 1993). Freshly harvested seeds of *Liparis lilifolia* were sown on 3 November 1993 in the same successional forest area where they had been collected. Six seed packets were placed in each of six plots in a population of adult *L. lilifolia* at two soil depths, approx. 10 and 35 mm. Starting 24 weeks after sowing, one seed packet from each plot was retrieved at 6-week intervals. Packets were fixed and stored in 70% ethanol until they were opened to record germination percentage and size distribution of seedlings.

RESULTS

Goodyera pubescens

In vitro germination of *Goodyera pubescens* on minimal media ranged from 93% on full substrate minus sucrose to 87% on water agar, and there were no significant

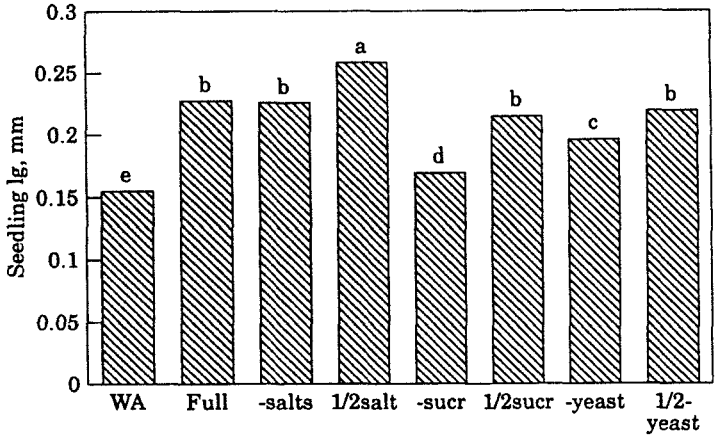

Figure 1. Asymbiotic seedlings of *Goodyera pubescens*, length 9 weeks after sowing when grown on water agar, a full medium and a series of media in which macroelements, sucrose or yeast extract had been partly or entirely removed. Each column represents mean of 50 seedlings originating from 10 replicate agar plates. Different lettering signifies differences at least at the 5% significance level (analysis of variance and pairwise comparisons by two-sample *t*-tests, a>b>c>d>e).

differences between treatments (data not shown). The largest seedlings during the 9 weeks of the experiment were obtained on the substrate with half concentration of salts (Fig. 1, '$\frac{1}{2}$salt'), perhaps because the full medium provided more than required and, due to its osmotic strength, inhibited the seedlings' uptake of water. Omitting the macroelements entirely ('-salts') also had a slight negative influence on seedling growth compared with the $\frac{1}{2}$salts medium, indicating that in spite of the seeds' reserves of macroelements, deficiencies were beginning to show within the first 9 weeks. Complete omission of either sucrose ('-sucr') or yeast ('-yeast') reduced seedling sizes significantly, but the growth was still better than on water agar ('WA'), indicating that these substances were utilized and to some extent able to replace each other, although seedlings did slightly better without yeast than without sucrose. A 50% reduction in the supply of sucrose or yeast did not limit growth (within the 9 weeks of measurement) as compared with the full medium.

Goodyera pubescens germinated readily in field sowings after 6 months in the soil in all three localities tested, although the species is rare in one of them. At first, the seedlings appeared uninfected (Rasmussen & Whigham, 1993) but in the subsequent retrievals most of the seedlings had established a visible mycorrhiza. Few of the seedlings that germinated in the seed packets developed beyond the first stages: no more than 13.6% of the seedlings reached 0.3 mm or more in width during the observation period (Fig. 2), roughly corresponding to 0.7 mm in length. Most of these seedlings occurred in the two localities in which the species was numerous.

Inoculation studies *in vitro* showed that the growth rate of the seedlings was significantly dependent on the fungus (Fig. 3). With four of the local strains tested the seedlings did not exceed *c.* 0.7 mm in length after 4 weeks, with three others seedling growth was slightly, although significantly, stronger. However, with one isolate, originating from *G. pubescens* (M5-1) the seedlings grew about three times longer during the same time span.

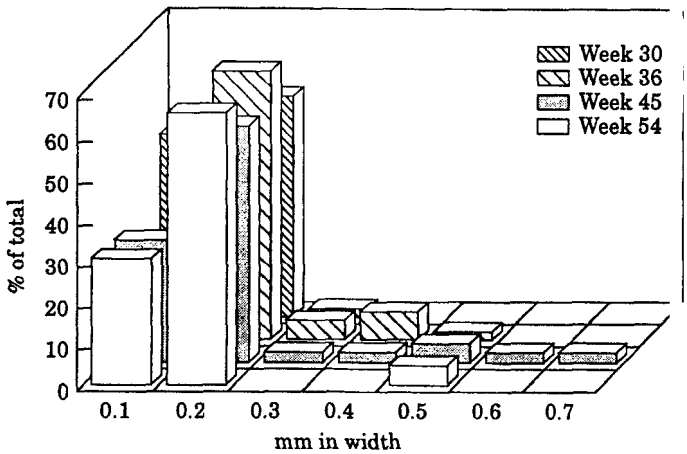

Figure 2. Size distribution of seedling populations of *Goodyera pubescens* as found at subsequent retrievals of field sown seed packets. Size represented as maximal width of the turnip-shaped seedlings.

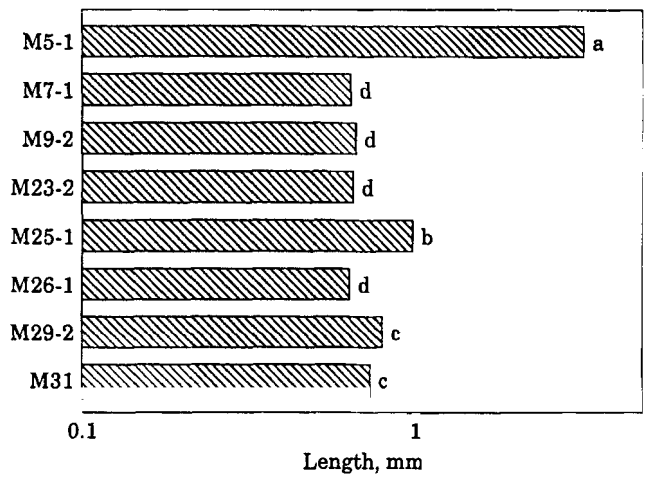

Figure 3. Seedlings of *Goodyera pubescens* produced asymbiotically and inoculated with a range of orchid endophytes (see materials and methods for details of fungi), on substrate H_1. Bars represent mean length of 100 seedlings. Different lettering signifies differences at least at the 5% significance level.

Corallorhiza odontorhiza

This species germinated *in vitro* only after cold stratification and with inoculation with an isolate originating from the rhizome of an adult *C. odontorhiza*, M9-5 (Fig. 4). We were not able to repeat the result in a later experiment when seeds and fungus culture had been stored for a longer time. The data suggested an increase in germination with increasing length of cold stratification, but none of the treatments were significantly different from each other due to the large variation within treatments. A range of other orchid symbionts were tested with cold stratification, but none effected germination.

In seed packets in the field, this species germinated after 7 months in a locality

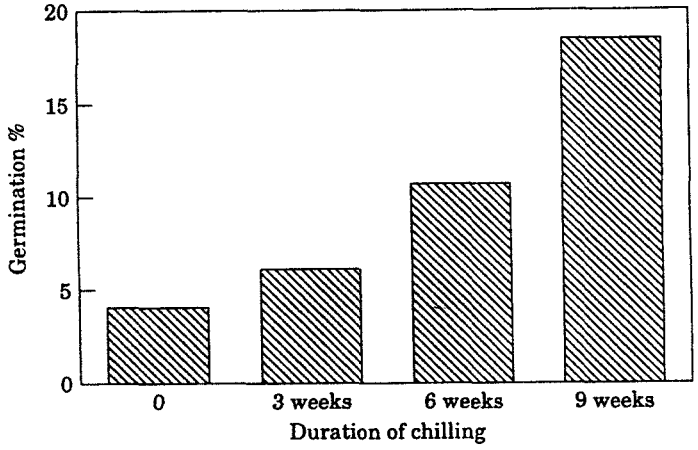

Figure 4. Germination in *Corallorhiza odontorhiza*. Surface sterilization for 1–8 hours, incubation at 20°C for 5 months, then inoculation with isolate from *C. odontorhiza* (M9-5), incubation for 6 weeks, then cold treated at 5°C for 0, 3, 6 and 9 weeks, respectively, and returned to 20°C. Germination recorded 9.5 months after sowing. No treatments differ significantly.

TABLE 4. Germination percentages in *Corallorhiza odontorhiza* in successive retrievals of seed packets from 3 plots, *c.* 45 x 55 cm large, all placed within a population of *C. odontorhiza*, 30–54 weeks after sowing in November

Plot no	w30	w36	w45	w54
1	7.69	66.67	23.08	81.25
2	6.45	34.78	21.43	3.28
3	48.78	8.46	61.27	89.09

where the adults are numerous, and with germination percentages ranging from 3 to 89% (Table 4). In another locality tested, in which adults were not found, there was no germination. Variation within germinating plots was considerable, suggesting a high degree of spatial variation in the distribution of compatible symbionts or other environmental factors essential for germination. Germination percentage did not tend to increase with time.

The field seedlings were all visibly infected with mycorrhiza immediately after germination (Rasmussen & Whigham, 1993). Analysis of the seedling sizes in subsequent retrievals of seed packets suggests that a large percentage of the seedlings (*c.* 50%) increased in size over the course of the experiment (Fig. 5).

Liparis lilifolia

Asymbiotic germination *in vitro* of *Liparis lilifolia* was unsuccessful even on a complete nutrient substrate. However, the seeds germinated when inoculated with a symbiont isolated from adult *L. lilifolia* (31). Symbiotic germination occurred at varying levels on all minimal media, even sporadically on water agar (Fig. 6).

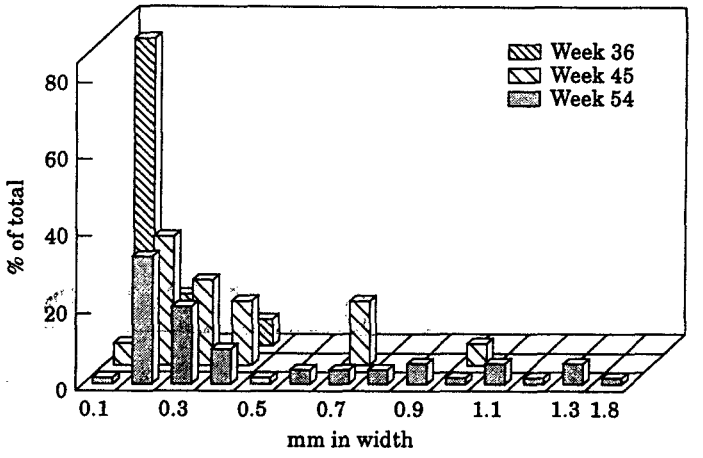

Figure 5. Size distribution of seedling populations of *Corallorhiza odontorhiza* as found at subsequent retrievals of field sown packets. Size represented as maximal width of the turnip-shaped or elongated seedlings.

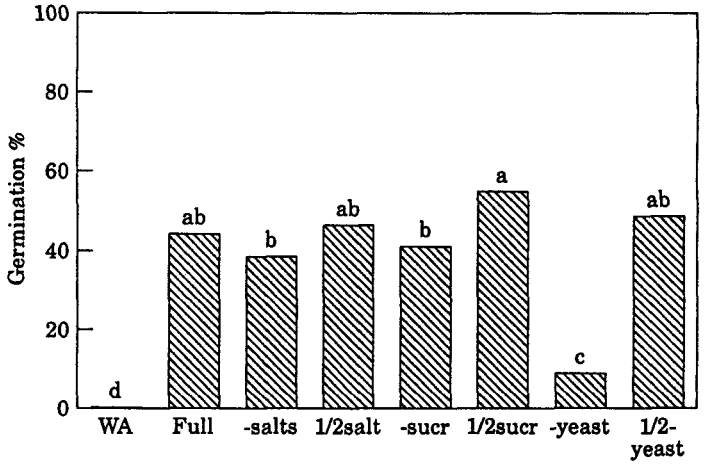

Figure 6. Symbiotic germination of *Liparis lilifolia*, recorded 19 weeks after sowing and 12 weeks after inoculation with an endophyte isolated from *L. lilifolia* (M31). Sown on water agar, a full medium and a series of media in which macroelements, sucrose or yeast extracts had been partly or entirely removed. Each column represents mean of 10 replicate agar plates. Different lettering signifies differences at least at the 5% significance level.

Omission of sucrose from the germination substrate had no apparent effect, but germination was significantly reduced when yeast extract was omitted, showing that the orchid/fungus relationship requires an external source of organic compounds other than sucrose. During the first weeks of seedling development the omission of

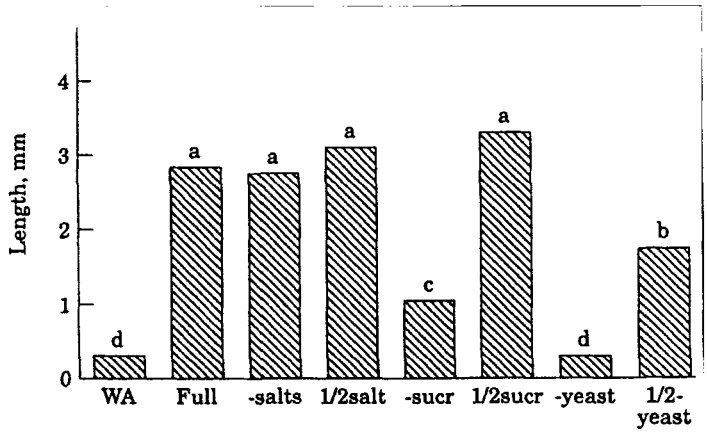

Figure 7. Seedling length of symbiotic *Liparis lilifolia* recorded 19 weeks after sowing and 12 weeks after inoculation with an endophyte isolated from *L. lilifolia* (M31). Growth on water agar, a full medium and a series of media in which macroelements, sucrose or yeast extracts had been partly or entirely removed. Each column represents mean of maximum seedling length in 10 replicate agar plates. Different lettering signifies differences at least at the 5% significance level.

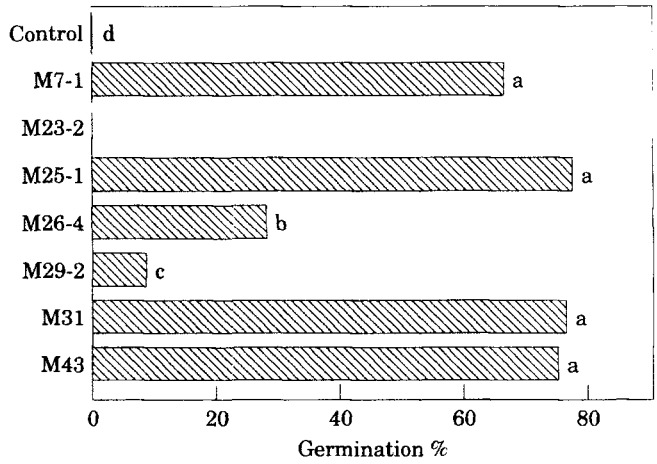

Figure 8. Symbiotic germination in *Liparis lilifolia* with a range of orchid endophytes (see materials and methods for details of fungi) on substrate W_2. Recorded 6 weeks after sowing. Bars represent means of 10 replicate dishes. Different lettering signifies differences at least at the 5% significance level.

yeast extract inhibited growth to the level of WA and more so than a removal of sucrose from the substrate (Fig. 7).

The presence of fungi affected germination percentages markedly; among a range of orchid symbionts tested *in vitro* there was compatibility with at least four isolates (Fig. 8). Amongst these, two strains originated from *L. lilifolia* (M31 and M43), and two were isolates from *Aplectrum hiemale* (M25) and *Tipularia discolor* (M7). Two of the remaining strains stimulated the germination percentage to a lower level.

Except for the isolate originating from *C. odontorhiza*, all isolates supported some

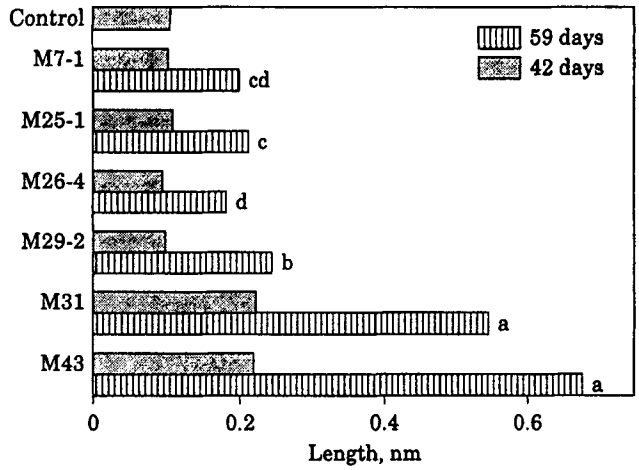

Figure 9. Growth of symbiotic seedlings of *Liparis lilifolia* with a range of orchid endophytes (see Material and methods for details of fungi) on substrate W_2. Recorded 6 weeks and $8\frac{1}{2}$ weeks after sowing. Bars represent means of maximum seedling length in each of 10 replicate dishes. Different lettering signifies differences at least at the 5% significance level on day 59.

development from day 42 to day 59. However, seedlings grown with isolates from *L. lilifolia* obtained significantly larger sizes. Neither M7 nor M25, both of which stimulated germination, enhanced seedling development more than other non-specific isolates (Fig. 9).

Germination in field sown seed packets of *L. lilifolia* was generally poor, the first trial being entirely negative (Rasmussen & Whigham, 1993). In a second sowing experiment, germination first occurred in July after 8 months in the soil but germination was observed only in 4 of the 24 seed packets that were subsequently retrieved, and germination was usually below 10%. Average seedling sizes increased from 0.284 mm in July (1993) to 1.08 mm in January (1994).

DISCUSSION

These case studies display a range of strategies and requirements for germination and seedling growth. In *Goodyera pubescens* germination could occur independently of external provisions and could possibly take place asymbiotically in nature, as suggested by the seedlings that were found in the field sowings. A time delay between rupture of testa and infection was also reported in *Epipactis helleborine*, in which germination *in situ* was observed 9 months after sowing and differentiated protocorms first observed in a sample taken 6 months later (van der Kinderen, 1995a). These observations indicate that seedlings are able to establish their mycorrhiza with available local fungi, expressing little specificity at this early stage. However, since the subsequent development of the seedlings of *G. pubescens* requires sucrose and organic compounds from outside, and growth rate is highly dependent on properties of the symbiont, great mortality can be expected during this phase. This expectation appears to be confirmed by the low number of field-sown seedlings that grew beyond a small size. Depletion of the seedling population would hence be strong during

early stages, probably most critical in the autumn months immediately after germination. Under semi-natural growth-chamber studies of *Bletilla striata*, a fairly unspecific germination was also observed, but these seedlings, which presumably were symbiotic, suffered high mortality in early spring about 2 months after germination, at a stage when the seedlings began to develop a green, terminal bud (Johnson, 1994).

The screening of *Corallorhiza odontorhiza* suggests that the presence of very specific fungi is required for germination, and the low overall germination *in vitro* even with a compatible strain indicates that the germination requirements of the seeds are complex. *C. odontorhiza* is apparently much more demanding at the time of germination that *G. pubescens*, and most individuals of *C. odontorhiza* are likely to be lost as ungerminated seeds in sites where the highly specific fungi are unavailable and where the seeds are not adequately subjected to low temperatures. However, it appears from the behaviour of the seedlings in the seed packets, that once established, mycorrhizal seedlings have a larger chance of successful development than those of *G. pubescens*.

The ingredient in yeast extract that proved essential for germination of *Liparis lilifolia in vitro* could be one or more amino acids or vitamins. Symbiotic germination *in vitro* was unsuccessful without it, so in nature it must be obtained through the mycelium from organic compounds in the substrate. This suggests a dependency on a certain kind of plant or animal debris, similar to the requirement that appears to exist in *Tipularia discolor* for decaying wood (Rasmussen & Whigham, in prep.). This requirement may limit the number of potential germination sites considerably and may explain the poor results of field sowings.

In vitro results with germination of *L. lilifolia* suggest that the symbiosis with fungi becomes increasingly specific as the seeds germinate and begin to grow. While induction of germination could be brought about by a relatively simple and brief stimulus, a prolonged symbiosis in the seedling requires metabolic adjustment between the partners. The eventual change towards photoautotrophy is probably also a critical stage. If germination in *L. lilifolia* is achieved with a variety of fungi with incomplete compatibility, which, based on the results *in vitro*, appears to be possible, a gradual reduction in seedling numbers will be expected during seedling development. However, information of compatibility patterns *in vitro* should be treated with caution because they may be rather different from those existing in a natural environment (e.g. Masuhara & Katsuya, 1994; Perkins *et al.*, 1995).

It is hardly surprising that the seedling recruitment in all three orchid species appears to be limited by the presence of appropriate conditions, particularly substrate requirements, for a symbiont. All species exhibit specificity patterns, but these appear to apply entirely to the seeds and germination phase in *C. odontorhiza*. In contrast, it is in the seedling stage that specificity patterns are expressed in *G. pubescens*. *L. lilifolia* occupies an intermediate position between the other two species, showing increasing levels of specificity from germination to young seedling development.

In each species and population under study there is a need to identify the bottleneck stages in the life history and environmental factors that control them. Evidently the stages as seeds and underground seedlings are critical in this respect. Considering the large surplus of seed that is being produced in most orchid populations, a slight change in the survival curve of the underground stages could dramatically affect above-ground recruitment. Hence, a better understanding of the

underground phase would provide a powerful tool with which to control the size and proliferation of orchid populations.

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