

On the origins of extreme mycorrhizal specificity in the Monotropeoideae (Ericaceae): performance trade-offs during seed germination and seedling development

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Abstract

Fungal-induced seed germination is a phenomenon characteristic of mycorrhizal plants that produce dust-like seeds with only minimal nutritional reserves. In such systems, fungi trigger germination and/or subsidize development. We studied mycorrhizal germination in relation to mycorrhizal specificity in the Monotropeoideae, a lineage of dust-seeded non-photosynthetic plants that are dependent upon ectomycorrhizal fungi of forest trees. A total of 1695 seed packets, each containing two to five compartments with seeds from different sources, were buried for up to 2 years near known ectomycorrhizal fungi in six different native forest locations. Upon harvest, seedlings were analysed by cultivation-independent molecular methods to identify their mycorrhizal fungi. We report that (i) germination is only induced by the same fungus that associates with mature plants or by closely related congeners; (ii) seedlings associated with the latter fungi develop less than those associated with maternal fungal species in most settings; and (iii) exceptions to this pattern occur in allopatric settings, where novel plant–fungal associations can result in the greatest seedling development. We interpret these results as evidence of performance trade-offs between breadth of host range and rate of development. We propose that in conjunction with host-derived germination cues, performance trade-offs can explain the extreme mycorrhizal specificity observed at maturity. The allopatric exceptions support the idea that performance trade-offs may be based on a coevolutionary arms race and that host range can be broadened most readily when naïve fungal hosts are encountered in novel settings.

Keywords: ecology, evolution, *Monotropa uniflora*, *Pterospora*, *Rhizopogon*, *Russula*, symbiosis

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Introduction

Some plants obtain carbohydrates from mycorrhizal fungi instead of providing them (Leake 1994). These ‘myco-heterotrophic’ plants offer unique insight into the functional and evolutionary diversity of mycorrhizal systems. For instance, nonphotosynthetic mycorrhizal plants are the only obvious examples of interspecific plant-to-plant net carbon transfer via fungi, a process that has been demonstrated from *Picea* and *Pinus* to *Monotropa* (Ericaceae), from *Salix* and *Betula* to *Corallorhiza* (Orchidaceae), and from *Betula* to *Cryptothallus* (Aneuraceae) (Björkman 1960; McKendrick *et al.* 2000a; Bidartondo *et al.* 2003). Nonphotosynthetic

mycorrhizal plants are known from 87 genera and 11 families, some of which are entirely composed of nonphotosynthetic species. Those that have been studied in detail so far have turned out to be exploiters (*sensu* Bronstein 2001) of mycorrhizal mutualisms, or ‘epiparasites’, as hypothesized by Romell (1939) for *Monotropa* (Duddridge & Read 1982; Warcup 1985; Zelmer & Currah 1995; Cullings *et al.* 1996; Taylor & Bruns 1997; Bidartondo & Bruns 2001; Bidartondo *et al.* 2002, 2003). One of the most pervasive features of myco-heterotrophic plants is prolific microspermy, or the production of thousands of dust-like seeds, whose germination should occur only when mycorrhization is imminent (Bernard 1904; Francke 1934; Olson 1980; Leake 1994). For example, a *Pterospora* inflorescence produces over 200 000 seeds, less than 0.5 mm in diameter (Bakshi 1958; Wallace 1975) that germinate when exposed to *Rhizopogon* (Bruns & Read 2000).

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Myco-heterotrophic plants can cheat both from a physiological perspective (by indirectly obtaining carbon fixed by neighbouring photosynthetic plants via shared mycorrhizal fungi) and from an evolutionary perspective (by subverting the mycorrhizal symbiosis). Systems where the cheater is a third species, such as a myco-heterotrophic epiparasitic plant, in addition to the mutualists, have rarely been examined (Janzen 1975; Inouye 1983; Gaume *et al.* 1998; Irwin & Brody 1998). Anticheating behaviors can stabilize mutualisms (Risch & Rickson 1981; Pellmyr & Huth 1994), but their evolution may be prevented in the case of autotroph/fungus/myco-heterotroph systems by an 'unholy alliance' in which a photosynthetic plant is the 'victim'. This is because the photosynthetic plant does not interact directly with the myco-heterotroph that uses some of its fixed carbon. Instead, the plants' interaction is completely mediated by the fungus which actively establishes and maintains the physiological continuity of the tripartite symbiosis. Our focus is on the fungus/myco-heterotroph part of the system.

Studies of the evolutionary ecology of myco-heterotrophic plants have emphasized the role of specificity between myco-heterotrophic plant and mycorrhizal fungus. Contrary to early predictions (Molina *et al.* 1992), but in tune with parasite systems where specificity is a hallmark (Price 1980; Thompson 1994), extreme mycorrhizal specialization is widespread in dicot, monocot, and liverwort myco-heterotrophs (Cullings *et al.* 1996; Taylor & Bruns 1997; Kretzer *et al.* 2000; Bidartondo & Bruns 2001; Bidartondo *et al.* 2002, 2003). In contrast, photosynthetic mycorrhizal plants are generalists; individuals can simultaneously form mycorrhizas with a vast assemblage of fungi (Trappe 1977; Molina & Trappe 1982; Smith & Read 1997). One of the most extreme examples of ecological and evolutionary mycorrhizal narrowing is the Monotropoideae (Ericaceae); each monotrope lineage is specific to only one of five distant clades of obligately ectomycorrhizal basidiomycetes (i.e. *Rhizopogon*, *Tricholoma*, *Gautieria*, *Russulaceae*, and *Hydnellum* Bidartondo & Bruns 2001, 2002). In the process of reversing the direction of carbon flow in their mycorrhizas, the monotropes became dependent on mycorrhizal fungi that represent a minuscule subset of their arbutoid ancestors' associates (Cullings *et al.* 1996). Do mycorrhizal seed germination and seedling development ecologically recapitulate this evolutionary narrowing process? In other words, do seeds and seedlings have a broader range of fungal associates than mature plants?

Specialization to a fungal lineage appears to be a fixed trait that is phylogenetically correlated within each lineage of the Monotropoideae (Bidartondo & Bruns 2001). For instance *Sarcodes sanguinea* and *Pterospora andromedea* are sister taxa specialized on three closely related fungi: *Rhizopogon ellena*, *Rhizopogon salebrosus* and *Rhizopogon arctostaphyli*. The geographical range of *S. sanguinea* is contained within that of *P. andromedea* where they often

grow a few meters from each other, and yet, 93 *Sarcodes* and 77 *Pterospora* mature plants examined showed zero overlap in fungal symbionts. *S. sanguinea* always associated with *R. ellena*, while *P. andromedea* always associated with either *R. salebrosus* or *R. arctostaphyli*. Furthermore, there is some molecular genetic evidence that *P. andromedea* contains two cryptic lineages that associate with one or the other *Rhizopogon* (Bidartondo & Bruns 2002). However, *S. sanguinea* and *P. andromedea* seeds germinate *in vitro* with any of these three *Rhizopogon* species suggesting that specificity is broader in seeds than in mature plants (Bruns & Read 2000). Thus, the degree of mycorrhizal specificity at germination observed *in vitro* may be necessary, but not sufficient, to explain the extreme mycorrhizal specificity of mature plants.

Perhaps extreme adult specialization arises *in situ* after germination as a result of trade-offs in performance; less efficient associations do not lead to flowering unless the mycorrhizal fungus can be replaced. Assuming that populations of *S. sanguinea* and *P. andromedea* are stable, there is a dramatic decline from the thousands of dust seeds that can germinate in response to any of three *Rhizopogon* species, to the relatively small number of emerging inflorescences that are exclusively species-specific. This implies that the strongest selection pressure in the life history of myco-heterotrophs acts on their underground stages (Rasmussen & Whigham 1993) and that the interaction of mycorrhizal germination with mycorrhizal specificity is where a cost to specialization is likely to manifest itself. If true, this would be a fundamental modification of Hadley's (1970) 'non-specificity' symbiotic germination model. His model, which was developed from *in vitro* assays with orchid seeds and fungi, had no role for phylogenetic constraints.

Methodology is a major and often unrecognized confounding issue when interpreting mycorrhizal specificity. First, morphological identification of fungi is usually difficult and unreliable (Currah 1991; Andersen 1996; Rasmussen & Whigham 1998). Second, although only cultivation-dependent approaches can satisfy Koch's postulates, these have been superseded by DNA sequence-based methods (Fredricks & Relman 1996; Post & Ehrlich 2000). Exclusive reliance on pure isolates can exclude symbiont lineages that are obligately mycorrhizal or induce artefacts if cultivation is asymbiotic. Even for nonbiotrophic cultivable fungi, the need for aseptic nutritive media is known to induce biases in mycorrhizal specificity (Masuhara & Katsuya 1994). *In situ* seed burial techniques should circumvent these problems and they have been used to describe the ontogeny of dust-seeded mycorrhizal plants (Francke 1934; Rasmussen & Whigham 1993; McKendrick *et al.* 2000b; Batty *et al.* 2001; Leake *et al.* 2004). Here we use *in situ* burial of seed packets near known fungi in conjunction with direct PCR-based identification of fungi to test for performance trade-offs between mycorrhizal germination and mycorrhizal specificity.

Materials and methods

Seed packets

Seeds were harvested from mature capsules that were releasing seeds. In the case of *Pityopus* and *Pleuricospora*, which produce fleshy scented fruits that are rarely found mature in the field (likely caused by intense herbivory, Luoma 1987), we collected fruits and dried them at room temperature. Seeds or fruits were stored for up to 2 months after collection, and in most cases for less than 1 week, prior to seed packet construction and burial. The seeds were sieved until debris had been removed. Seed packets were constructed using 50- μ m mesh screen cloth (PGC Scientifics) by folding and sealing 1 \times 1 cm pockets (2–5) using an impulse heat sealer (PGC Scientifics). We used the multiple pockets within single seed packets to separate seeds from different plants; this enabled us to expose seeds from either different species, or from plants of one species that had different mycorrhizal fungi, to the same fungal species in nearly identical environments. One end of each packet was folded over and sealed to allow threading nylon cord through it. Approximately 200 seeds were placed in each pocket using a metal dish (1 mm in height and 3 mm in diameter) held by a fine negative tweezer. For relatively larger-seeded species (*Pityopus*, *Pleuricospora* and *Sarcodes*) we placed approximately 75 seeds in each pocket. A total of 1695 packets with 5891 pockets and over 1 million seeds were buried. In order to minimize disturbance during burial we used a trowel (5 \times 15 cm blade) pushed into the soil, which we rocked forward a few centimeters to create a crevice into which a seed packet was slid down into the soil using a metal hook, then the trowel and hook were pulled out and the soil pushed back by hand. The loose end of the cord was then attached to a flag or metal stake with a numbered metal tag. To differentiate seed packets and packets with different seed sources we used the relative position of the pocket in the seed packet, the colour of the cord and the number of knots tied on the cord.

Plants and fungi

We buried seed packets at six sites that either contain monotrope species or are known to contain host fungi. At all sites but one, seed packets were buried near (< 25 cm) a total of 178 mature Monotropeae whose mycorrhizal fungi had been previously identified or, in a few cases, were identified after harvesting seed packets. The Monotropeae form a dense aggregation (typically 5–20 cm in diameter) of mycorrhizal roots colonized by a single fungal lineage from which inflorescences (10–200 cm in height) are formed adventitiously. For an analysis of the spatial distribution of monotropoid roots and ectomycorrhizas near *Sarcodes*, see Bidartondo *et al.* (2000). Packets containing

Monotropa uniflora seeds were buried along transects at a site in Mount Tamalpais, Marin County, California, which is roughly 500 km south from the nearest *M. uniflora* populations, but which we knew to harbor its primary western North American host fungus (*Russula brevipes*). The sites, plants and fungi are listed in Table 1 and they are described in the following three paragraphs. The specific plant/fungal combinations tested are diagrammed in Table 2.

At various areas near Dinkey Creek, Fresno County, California, we buried packets with seeds collected from *Pterospora andromedea* plants associated with *Rhizopogon salebrosus*, *P. andromedea* associated with *R. arctostaphyli*, *Sarcodes sanguinea* associated with *Rhizopogon ellenae*, and *Pleuricospora fimbriolata* associated with *Gautieria monticola*. We have not detected other monotropoid mycorrhizal combinations in this area, which is where we have sampled Monotropeae most intensively since 1996.

We buried *M. uniflora* seed packets at three locations, one in Maryland and two in California. Seed source is important in this experiment because the North American lineage of *M. uniflora* is an example of a geographical mosaic of mycorrhizal associations. In seven locations in Oregon and California, sampled plants were associated with members of the *R. brevipes* species group and in one location in California with *Russula vesca*. Plants from one location in Vermont were associated with *R. brevipes*, *Lactarius theiogalus*, or two other *Russula* lineages, plants at a location in Maryland were associated with one of two *Russula* lineages, a location in Virginia had a *Russula*, a location in Nova Scotia a *Russula*, a Michigan location a *Lactarius*, and a location in Costa Rica two *Russula* lineages (Bidartondo & Bruns 2001; Bidartondo, unpublished). *Russula* and *Lactarius* are sister genera. The Maryland location (Smithsonian Environmental Research Center) is within the eastern North American range of the plant. The northernmost California location (Simpson Timber Company) is at the southern edge of the plant's geographical range in the western coast of North America. The central California location (Mount Tamalpais) is outside the geographical range of the plant. The seed packets buried in Maryland and at Mount Tamalpais were identical. They contained seeds collected from *M. uniflora* mycorrhizal with (i) *Russula brevipes* in northern California, (ii) *Russula nitida* in Maryland, and (iii) *Russula decolorans* in Maryland. The latter two fungi are the only ones we have detected at the Maryland location. At the Simpson Timber Company forests, we buried seed packets containing seeds collected from *M. uniflora* mycorrhizal with *Russula brevipes* and *Russula vesca*. At Mount Tamalpais, where there are no *M. uniflora* plants, we had mapped the locations of individual *R. brevipes* sporocarps in 2000 at a site where they were abundant.

At Perkins Creek and Eel Cr. creeks, Oregon, two of the most diverse Monotropeae sites known, we buried locally collected seeds of three monotropes (*Allotropia virgata*, *Monotropa hypopithys* and *Pityopus californicus*) in all

Table 1 Details of the locations where packets containing seeds of Monotropoideae plants were buried and harvested between 2001 and 2004. The column that lists Monotropoideae occurring *in situ* shows in parentheses the number of plants near which seed packets were buried and the mycorrhizal fungi detected at that location. The column that lists Monotropoideae seeds buried shows in bold those taxa that achieved the highest germination and development, the taxa followed by an asterisk showed the lowest germination, and the rest showed no germination. The mycorrhizal fungi of the plants that were used as seed sources are shown in parentheses

Location	Forest type(s)	Monotropoideae <i>in situ</i> (number used in this study) (mycorrhizal fungi)	Monotropoideae seeds buried (mycorrhizal fungi of sources)	Burial (days)	Seed packets
Dinkey Creek Sierra National Forest Fresno County California	Sierran mixed conifer, <i>Abies magnifica</i>	<i>Pterospora andromedeae</i> (36) (RH _a , RH _s) <i>Sarcodes sanguinea</i> (16) (RHe) <i>Pleuroscopora fimbriolata</i> (6) (Gm)	<i>Pterospora andromedeae</i> (RH _a , RH _s) <i>Sarcodes sanguinea</i> * (RHe) <i>Pleuroscopora fimbriolata</i> (Gm)	262/305/633	353
Bolinas Ridge Mount Tamalpais Marin County California	<i>Pseudotsuga menziesii</i>	None	<i>Monotropa uniflora</i> (Rb, Rd, Rn)	262/527/556	200
Simpson Timber Company Del Norte County California	Coastal redwood	<i>Monotropa uniflora</i> (23) (Rb, Rv) <i>Pleuroscopora fimbriolata</i> (Gm)	<i>Monotropa uniflora</i> (Rb, Rv)	236/557	320
Smithsonian Environmental Research Center Anne Arundel County Maryland	Eastern deciduous	<i>Monotropa uniflora</i> (29) (Rd, Rn) <i>Monotropa hypopithys</i> (9) (Ts)	<i>Monotropa uniflora</i> (Rb, Rd, Rn) <i>Monotropa hypopithys</i> (Tp, Ts)	552	318
Perkins Creek Eugene District Bureau of Land Management Lane County Oregon	<i>Pseudotsuga menziesii</i> <i>Tsuga heterophylla</i>	<i>Allotropia virgata</i> (9) (Tm) <i>Monotropa hypopithys</i> (9) (Tf, Tp) <i>Monotropa uniflora</i> (6) (Rb) <i>Pityopus californicus</i> (6) (Tmy) <i>Pleuroscopora fimbriolata</i> (Gm) <i>Hemitomes congestum</i> (Hsp)	<i>Allotropia virgata</i> (Tm) <i>Monotropa hypopithys</i> * (Tf, Tp) <i>Monotropa uniflora</i> (Rb) <i>Pityopus californicus</i> * (Tmy) <i>Pterospora andromedeae</i> (RH _s)	324/681	304
Eel Creek Siuslaw National Forest Coos County Oregon	<i>Pinus contorta</i>	<i>Allotropia virgata</i> (8) (Tm) <i>Monotropa hypopithys</i> (10) (Tl) <i>Pityopus californicus</i> (8) (Tfo) <i>Pterospora andromedeae</i> (3) (RH _s) <i>Hemitomes congestum</i> (Hsp)	<i>Allotropia virgata</i> (Tm) <i>Monotropa hypopithys</i> (Tf, Tl, Tp) <i>Pityopus californicus</i> (Tfo) <i>Pterospora andromedeae</i> * (RH _s)	252/603	200

Fungal lineage abbreviations: Gm, *Gautieria monticola*; Hsp, *Hydnellum* sp.; RH_a, *Rhizopogon arctostaphyli*; Rb, *Russula brevipes*; Rd, *Russula decolorans*; Rn, *Russula nitida*; RH_s, *Rhizopogon salebrosus*; Rv, *Russula vesca*; Tf, *Tricholoma flavovirens*; Tfo, *Tricholoma focale*; Tl, *Tricholoma luteo-maculosum*; Tm, *Tricholoma magnivelare*; Tmy, *Tricholoma myomyces*; Tp, *Tricholoma portentosum*; Ts, *Tricholoma sejunctum*; RHe, *Rhizopogon ellenae*.

possible combinations between maternal fungi and fungi forming mycorrhizas with *in situ* mature plants. These monotropes use mutually exclusive sets of *Tricholoma* (Bidartondo & Bruns 2002). [Note: *Monotropa* is polyphyletic; *Monotropa uniflora* is sister to *Monotropastrum*, and *Monotropa hypopithys* is sister to *Pityopus* (Bidartondo & Bruns 2001).] At Perkins Creek we also buried seeds collected from *Pterospora andromedeae* associated with *Rhizopogon salebrosus* from Dinkey Creek, California.

Analysis

Seed packets were harvested by removing the flag, or metal stake, and pulling the cord with its attached seed packet out of the soil. The seed packets were then sealed in plastic bags and stored on ice until examined within 2 or 3 days after harvest. For examination, packets were rinsed in running water, opened with a scalpel under a dissecting

microscope (40× magnification), and each seed or seedling was categorized as ungerminated, seed coat cracked, embryo/hypocotyl emerging, mycorrhizal, or branched. Many germinated but nonmycorrhizal seeds were also examined with a microscope (100–400× magnification). Mycorrhizal *M. uniflora* seedlings were routinely examined in this manner because one of *M. uniflora*'s dominant mycorrhizal fungi, *R. brevipes*, forms characteristic flask-shaped cystidia with an apical knob in the outer surface of the mycorrhizal mantle. All seedlings were collected by using fine tweezers, flash-frozen in liquid N₂, lyophilized, and weighed using a MX5 microbalance (accuracy ± 1 µg) (Mettler-Toledo). In many cases, rhizomorphs (linear hyphal aggregations) were also collected from inside seed packets. Fungi were identified from rhizomorphs and from at least one mycorrhizal seedling or one fragment of a mycorrhizal seedling from each pocket. We used fungal-specific polymerase chain reaction (PCR) strategies and protocols for

Table 2 Experimental design. Monotropeoideae seeds were collected from plants with known mycorrhizal fungi at five sites (horizontal) and buried near plants with known mycorrhizal fungi at six sites (vertical) in the 96 combinations indicated by grey cells. Mount Tamalpais, California, was the only site without any Monotropeoideae plants, but it is known to harbor *Russula brevipes* an associate of *Monotropa uniflora*. Germination was observed in the 26 combinations indicated by a check mark (✓)

		Seed source (plant/mycorrhizal fungus)																			
		Dinkey Creek, CA				SERC, MD			Del Norte County, CA		Perkins Creek, OR				Eel Creek, Oregon						
Seeds buried at↓	Seeds buried near↓	<i>Pa/RHa</i>	<i>Pa/RHs</i>	<i>Ss/RHe</i>	<i>Pf/Gm</i>	<i>Mu/Rd</i>	<i>Mu/Rn</i>	<i>Mh/Ts</i>	<i>Mu/Rb</i>	<i>Mu/Rv</i>	<i>Ave/Tm</i>	<i>Mh/Tf</i>	<i>Mh/Tp</i>	<i>Pc/Tmy</i>	<i>Mu/Rb</i>	<i>Ave/Tm</i>	<i>Mh/Tl</i>	<i>Pc/Tfo</i>	<i>Pa/RHs</i>		
Dinkey	<i>Pa/RHa</i>	✓	✓	✓																	
	<i>Pa/RHs</i>	✓	✓	✓																	
	<i>Ss/RHe</i>	✓	✓	✓																	
	<i>Pf/Gm</i>																				
SERC	<i>Mu/Rn</i>					✓	✓								✓						
	<i>Mu/Rd</i>					✓	✓														
	<i>Mh/Ts</i>																				
Del Norte	<i>Mu/Rb</i>								✓	✓											
	<i>Mu/Rv</i>								✓	✓											
Perkins	<i>Ave/Tm</i>																				
	<i>Mh/Tf</i>											✓									
	<i>Mh/Tp</i>												✓								
	<i>Pc/Tmy</i>													✓							
	<i>Mu/Rb</i>																				
Eel	<i>Mh/Tl</i>																				
	<i>Ave/Tm</i>																				
	<i>Pc/Tfo</i>																				
	<i>Pa/RHs</i>		✓																✓		
Tamalpais	<i>Rb</i>					✓	✓								✓						

Plant lineage abbreviations: *Av*, *Allotropia virgata*; *Mh*, *Monotropa hypopithys*; *Mu*, *Monotropa uniflora*; *Pc*, *Pityopus californicus*; *Pa*, *Pterospira andromeda*; *Ss*, *Sarcodes sanguinea*. Fungal lineage abbreviations: *Gm*, *Gautieria monticola*; *RHa*, *Rhizopogon arctostaphyli*; *Rb*, *Russula brevipes*; *Rd*, *Russula decolorans*; *Rn*, *Russula nitida*; *RHs*, *Rhizopogon salebrosus*; *Rv*, *Russula vesca*; *Tf*, *Tricholoma flavovirens*; *Tfo*, *Tricholoma focale*; *Tl*, *Tricholoma luteo-maculosum*; *Tm*, *Tricholoma magnivelare*; *Tmy*, *Tricholoma myomyces*; *Tp*, *Tricholoma portentosum*; *Ts*, *Tricholoma sejunctum*; *RHe*, *Rhizopogon elleneae*.

the nuclear ribosomal internal transcribed spacer described in Gardes & Bruns (1993) but using a silica emulsion (Q-Biogene) for DNA binding and purification. All positive PCR products were directly sequenced bidirectionally using BigDye version 3.1 chemistry (Applied Biosystems). All unique fungal DNA sequences not previously reported for the monotropoid symbiosis have been deposited in GenBank (Accession nos AY878655–AY878661).

When germination was obtained, we performed multi-factor mixed model nested ANOVAs using JMP 4.0.2 (SAS Institute) with detected fungus (A), maternal seed source (C), and seed packet block nested within detected fungus [B(A)] as main effects, and an A*C interaction term. We did not include a B(A)*C interaction term because we did not replicate seed sources within packets. If the ANOVA and interaction term were significant ($P = 0.01$), we carried out one contrast between $x,x/y,y$ (same detected fungus as the mycorrhizal fungus of the maternal seed source) and $x,y/y,x$ (different detected fungus from the mycorrhizal fungus of the maternal seed source) testing whether their least square means were significantly different. In the case of *P. andromedea* we performed one ANOVA for the arcsine-transformed proportion of seeds for each developmental stage within seed pockets. In the case of *M. uniflora*, we tested the cube-root-transformed cumulative weight of seedlings within seed pockets. The transformations were used to normalize the data. For ANOVA, the data sets were balanced by removing randomly selected blocks, except for *M. uniflora* from Mount Tamalpais where we instead relabelled all non-*R. brevipes* fungi as 'other'.

Results

We detected a single fungal lineage in each seedling examined and all seedlings formed mycorrhizas exclusively with the same fungal genus as their maternal plants, that is the fungal genus that their plant lineage was known to specialize towards. However, the inside and outside of all seed packets had been densely colonized by various other fungi, particularly ectomycorrhizal fungi, both as rhizomorphs and hyphae. For example, at Mount Tamalpais, approximately one-third of seed packets contained *Rhizopogon vinicolor*, and a smaller number contained *Tomentella*, *Cortinari*, *Phomopsis*, and *Verticillium* spp. At Eel Creek, roots of *Pinus contorta* were able to cross the 50- μ m opening and form ectomycorrhizas with *Cenococcum* sp. Remarkably, the vast majority of ungerminated seeds appeared intact even 2 years after burial and this was most pronounced at the driest locations (Dinkey Creek, Perkins Creek, Eel Creek). Seedlings were often densely aggregated within seed packets, to the point of being difficult to extricate when they were highly branched (Fig. 1.4, 1.8 and 1.9). In fact, they appear identical to the dense root masses of mature plants. Thus, our counts of mycorrhizal branched

seedlings are likely to be conservative estimates. For *M. uniflora*, we do not consider these estimates to be accurate and we analyzed instead the cumulative biomass (dry weight) per seed packet. At three sites, we examined the correlation at harvest between blooming (presence or absence of inflorescences) of the plant near which seed packets were buried and germination (presence or absence of germinated seeds) in those packets. The correlations were 0.11 for *P. andromedea* at Dinkey Creek, 0.37 for *M. uniflora* in Maryland, and 0.60 for *M. uniflora* at Del Norte County.

At Dinkey Creek, *Pleuricospora* seeds did not germinate and *Sarcodes* seeds germinated at the lowest rate (< 1%). In this area, we had obtained greater *Sarcodes* germination but no mycorrhization in an earlier pilot experiment during 1999–2000 when seed packets (100 seed packets, 50 seeds per packet, 250- μ m mesh) were buried in transects more than 5 m from mature *Sarcodes* or near individual mature *Sarcodes*. *Pterospora* seeds germinated at a relatively high level; 10% of seed packets buried had seeds with cracked seed coats, approximately 5% had an emergent embryo/hypocotyl, and approximately 1% became mycorrhizal (Fig. 1). Because *Rhizopogon* produces distinctive and abundant large rhizomorphs, we were able to verify which fungi were present in the pockets at the time of harvest even when there were no colonized seeds by collecting rhizomorphs for DNA sequence analysis. In all seed packets with germinated seeds, if adjacent pockets contained *Rhizopogon* rhizomorphs, these rhizomorphs were only either *Rhizopogon ellena*, *Rhizopogon salebrosus* or *Rhizopogon arctostaphyli*. Furthermore, in this study, and a related pilot study, the *Rhizopogon* species detected inside seed packets corresponded, with only one exception, to the same *Rhizopogon* forming mycorrhizas with the Monotropoideae plants that the packets were buried near. Rhizomorphs of other fungi were also commonly found inside seed packets, but they had not triggered any seed germination. All three *Rhizopogon* spp. known to form mycorrhizas with monotropes led to cracking of the seed coat, as previously observed *in vitro* (Bruns & Read 2000), with *R. arctostaphyli* appearing to outperform the other fungi. At embryo/hypocotyl emergence, *Sarcodes*' associate (*R. ellena*) was ineffective. When the fungus detected was the same as the mycorrhizal fungus of the maternal seed source, embryo/hypocotyl emergence was greatest ($P = 0.0001$, $n = 34$). At mycorrhization, only the mature *Pterospora* maternal combinations were observed in seedlings.

Monotropa uniflora achieved the greatest levels of germination and development that we have observed (approximately 15%) and in all cases seedlings were associated with *Russula* species. To our knowledge, seedlings of this plant had never been observed before. Furthermore, *M. uniflora* was the only species where we observed the production of adventitious inflorescence buds breaking through the mycorrhizal mantle (Fig. 1). Because members of the Russulaceae produce only very thin hyaline hyphae and in

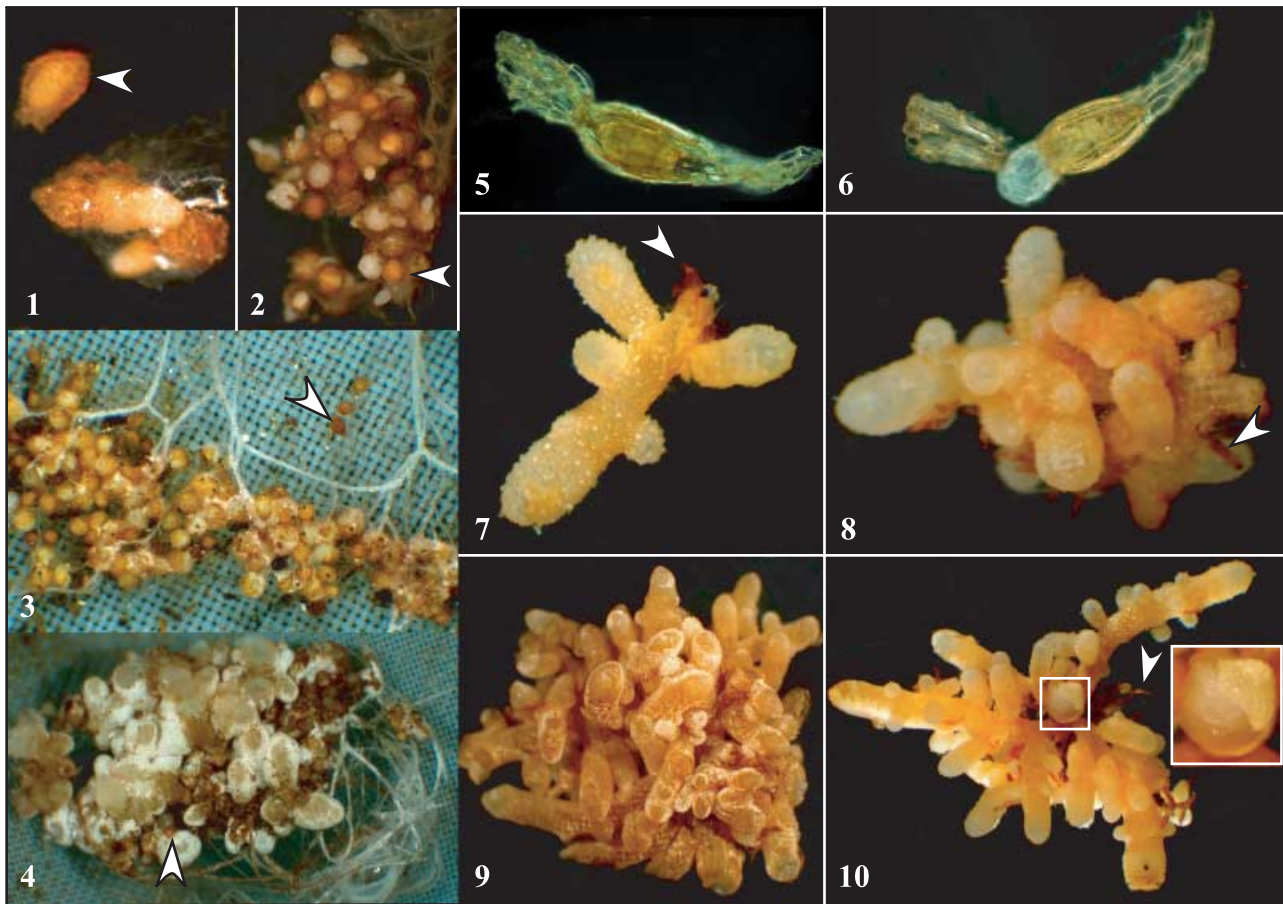


Fig. 1 Seeds and mycorrhizal seedlings of three Monotropoideae and five Basidiomycete lineages. 1. *Pityopus californicus* colonized by *Tricholoma myomyces*. 2. *Pterospora andromedea* with seed coats cracked and/or embryo/hypocotyl emerged surrounded by white hyphae and rhizomorphs of *Rhizopogon salebrosus*. 3. *P. andromedea* colonized by *R. salebrosus*, shown within seed packet. 4. *P. andromedea* branched and mycorrhizal with *Rhizopogon salebrosus*, shown within seed packet. 5. Imbibed *Monotropa uniflora* seed. 6. *M. uniflora* seed with cracked seed coat and emerged embryo/hypocotyl. 7. *M. uniflora* mycorrhizal with *R. decolorans*. 8. *M. uniflora* mycorrhizal with *R. nitida*. 9. *M. uniflora* mycorrhizal with *R. brevipes*. 10. *M. uniflora* mycorrhizal with *R. decolorans*, the inset shows an adventitious inflorescence bud that has broken through the mycorrhizal mantle. White arrows indicate ungerminated seeds: *Pityopus* is 0.25 mm in diameter (photograph 1), *Pterospora* is 0.5 mm in diameter (photographs 2–4), and *M. uniflora* is 1 mm long (photographs 5–10).

minor amounts, we could not verify their presence within seed packets at harvest, unless they had colonized seedlings. If adjacent pockets within a single seed packet had mycorrhizal seedlings, these were invariably colonized by the same fungal lineage. Thus, we assumed that seeds in all adjacent pockets within a seed packet had been exposed to the same fungus. In Maryland, western seeds (from *Russula brevipes* mycorrhizal plants) performed worst; only three packets had very small unbranched seedlings. Local seeds performed best when colonized by the fungus detected on the roots of their maternal plants and they performed at a lower level when colonized by nonmaternal fungi ($P = 0.0001$, $n = 13$). At Mount Tamalpais, western seeds (from *R. brevipes* mycorrhizal plants) performed best when colonized by *R. brevipes* and worst when colonized by four other *Russula* species that were never detected on

mature *Monotropa uniflora* roots ($P = 0.0007$, $n = 25$). Eastern seeds (from plants associated with either eastern *Russula* species) developed to intermediate levels when paired with *Russula brevipes* and four other *Russula* species never detected on mature *M. uniflora* roots. In Del Norte County, the above pattern of maternal combinations performing best was partially reversed; seeds obtained from *M. uniflora* plants mycorrhizal with *R. brevipes* performed best when colonized by *R. vesca*, and all other pairings performed worse ($P = 0.014$, $n = 23$). In two seed packets, seedlings were colonized by one *Russula* lineage never before detected on mature *M. uniflora* roots (i.e. *Russula chloroides*).

At Perkins Cr. and Eel Cr. germination was very low (*Monotropa hypopithys*, *Pityopus californicus*, *Pterospora andromedea*) or absent (*Allotropia virgata*), but when it occurred, seedlings were always associated with the fungal genus of

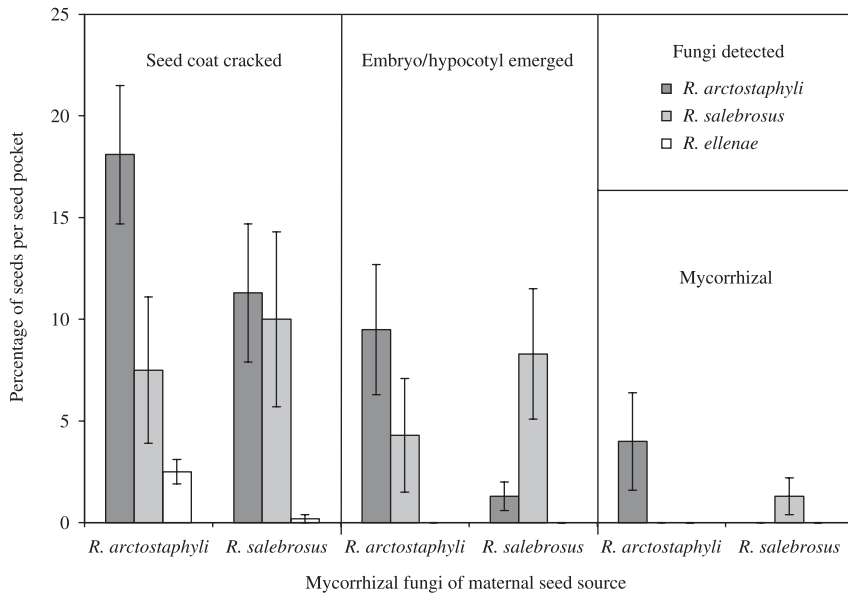


Fig. 2 *Pterospora andromedea* seed germination at Dinkey Creek, California, for three categories of development, two maternal sources of seed with different mycorrhizal fungi (*Rhizopogon arctostaphyly* or *R. salebrosus*), and three colonizing fungi.

mature plants. Oregon experienced a record 100-year drought in 2003 and an unusually low number of monotropes flowered that year at both locations. To our knowledge, seedlings of *Pityopus* had never been observed before (Fig. 1). Only seedlings of *Pityopus* (two seed packets at Perkins Creek) and *Pterospora* (two seed packets at Eel Creek) reached mycorrhization, but only when buried near conspecific plants with the same mycorrhizal fungi as their maternal plants (*Tricholoma myomyces* and *Rhizopogon salebrosus*, respectively).

Discussion

The results of this and an earlier study (Bruns & Read 2000) are consistent with a model in which extreme specificity of mature plants in the Monotropoideae is achieved by germination only being triggered by cues produced by closely related fungi, followed by narrowing during seedling development down to an even smaller subset of fungi. Thus, the cost of specialization is likely paid by seeds whose development is arrested, or retarded, if they germinate in response to the wrong fungus. However, during the seedling development stage, the situation may differ if the seeds or the fungi are not local, indicating a tightly coevolved and geographically structured interaction. In this case, some novel plant/fungal genotype combinations, albeit restricted to fungal congeners, may be favoured perhaps because the fungi have had no opportunity to evolve defences. This model predicts that host shifts should occur at higher frequency at the fringe of the geographical range and in rare cases of long-distance seed dispersal.

The phylogenetic loyalty of monotrope seeds and seedlings that we have observed closely mirrors the phylogenetic conservatism of monotrope lineages that we reported ear-

lier from analyses of the roots of mature plants (Bidartondo & Bruns 2001, 2002). We know from *in vitro* studies that germination of *Sarcodes* and *Pterospora* seeds is triggered by diffusible or volatile chemical cues produced by the closely related *Rhizopogon* species that colonize mature plants (Bruns & Read 2000). The pattern we found with seed packets *in situ* within a broader set of monotropes and fungi indicates that germination is similarly cued only by fungi closely related to those associated with mature plants. These observations suggest that jumps to distantly related fungi are effectively constrained during germination by the absence of recognizable fungal cues. Nonetheless, shifts among closely related fungal hosts are not constrained during germination and early development; several associations between plants and fungi that we never observed in mature plants were recorded from seedlings (Figs 3 and 5). However, there must be secondary constraints involving physiological interactions between a seedling and its fungal host as evidenced by differences in performance observed among various plant–fungal genotype combinations (Figs 3, 4 and 5). This observation finally provides a mechanism to explain situations where plant genotypic diversification at local or regional scales is correlated with mycorrhizal divergence among closely related fungi, a phenomenon reported for both monotrope and orchid myco-heterotrophs (Bidartondo & Bruns 2002; Taylor *et al.* 2003a, b). For instance, *Pterospora andromedea* seeds can germinate in response to *Rhizopogon arctostaphyly*, *Rhizopogon salebrosus* and *Rhizopogon ellenae*, all of which are members of *Rhizopogon* section *Amylopogon*. However, seeds obtained from *Pterospora andromedea* plants mycorrhizal with *R. arctostaphyly* only manage to become mycorrhizal with *R. arctostaphyly*. Similarly, seeds obtained from plants mycorrhizal with *R. salebrosus* only manage to become mycorrhizal with *R. salebrosus* (Fig. 2). These two

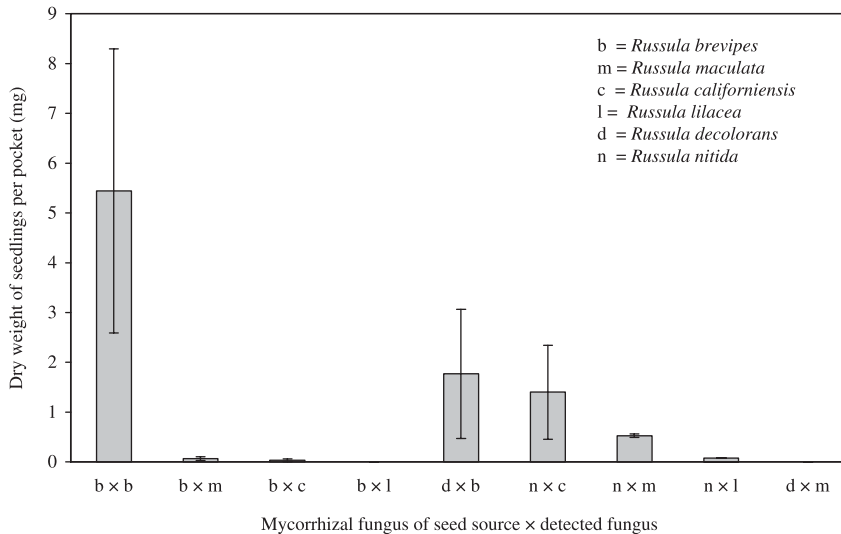


Fig. 3 *Monotropa uniflora* seed germination at Mount Tamalpais, California. This location is outside the geographical range of *M. uniflora*. The first four bars correspond to seeds from Oregon and the last five bars correspond to seeds from Maryland.

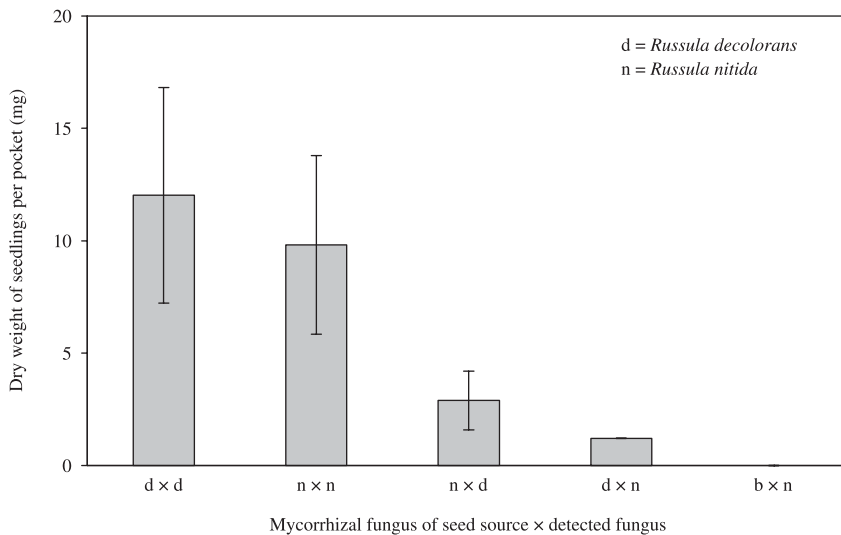


Fig. 4 *Monotropa uniflora* seed germination at the Smithsonian Environmental Research Center, Maryland. The first four bars correspond to seeds from Maryland and the last bar corresponds to seeds from Oregon.

Rhizopogon lineages are sympatric sister taxa and they are the exclusive associates of mature *Pterospora andromeda* plants. *P. andromeda* populations contain some plastid haplotypes that are significantly correlated with *R. arctostaphyli* and others with *R. salebrosus* (Bidartondo & Bruns 2002). Although we do not have genotype information for *Monotropa uniflora*, the seedling development data from Maryland agrees with our observations on *P. andromeda*. In the Maryland sites that we sampled, blooming *M. uniflora* plants associate sympatrically with *Russula decolorans* or *Russula nitida*. Seeds obtained from *M. uniflora* plants mycorrhizal with *R. decolorans* perform best when they become mycorrhizal with *R. decolorans*. Similarly, seeds obtained from plants mycorrhizal with *R. nitida* perform best when they become mycorrhizal with *R. nitida* (Fig. 4). Thus, secondary constraints during seedling development can maintain, and perhaps contribute to generate, sympatric intraspecific mycorrhizal differentiation following host shifts to related fungi.

Ideally, we would like to use a full-factorial experimental design with all seed sources and all monotrope mycorrhizal fungi to systematically examine local mycorrhizal adaptation, but this was unfeasible because of logistical reasons. Nevertheless, in those cases where germination was obtained from seeds buried outside their location of origin, our results suggest that local adaptation can take place. Seedlings of *M. uniflora* plants mycorrhizal with *Russula brevipes* performed best when they were colonized by *R. brevipes* at a site in California that is outside the geographical range of the plant. However, seeds from that same source or from *M. uniflora* from Maryland were also able to develop when colonized by other local *Russula* not known to form mycorrhizas with mature *M. uniflora* anywhere we have sampled (Fig. 3). In addition, seedlings from *M. uniflora* plants associated with *R. brevipes* in California performed best when colonized by *Russula vesca* at one of the southernmost California populations of *M. uniflora* where

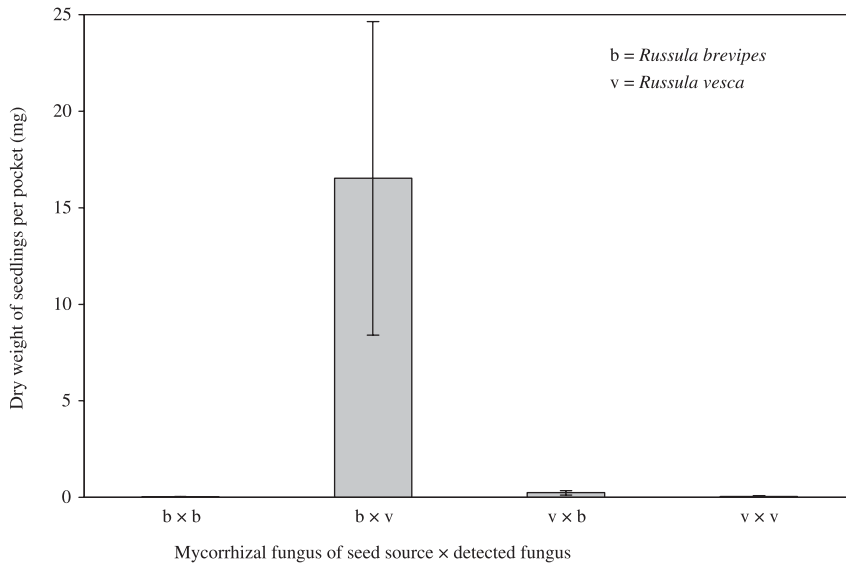


Fig. 5 *Monotropa uniflora* seed germination at Del Norte County, California.

plants are mycorrhizal with *R. vesca* (Fig. 5). *M. uniflora* merits further investigation on this allopatric geographical scale and it is noteworthy that, at least in this study, it was a much more rewarding investment than any of the *Tricholoma*-associated Monotropoideae.

A caveat to our model is that switching mycorrhizal fungi during development may occur in some cases, but we did not find evidence for this phenomenon in the form of seedlings simultaneously colonized by more than one fungus. We have not found this situation either in several hundred mature plants examined (with the exception of one stunted *Cenococcum*-colonized root tip in an otherwise entirely *Tricholoma*-colonized *Monotropa hypopithys* root system from Michigan).

Two factors should conspire against, but clearly do not preclude, the evolution of monotrope specificity to fungi: cheaters are expected to have minimal fitness costs (relative to pathogens) and the mycorrhizal symbiosis does not directly mediate reproduction (as pollination does). Although germination appears to be a positive interaction in which seeds respond to a specific fungal cue, it is unclear whether development is dominated by positive interactions between matching plant and fungal genotypes leading to a stable symbiosis, or negative interactions leading to recognition and rejection of the plant by the fungus in the form of a co-evolutionary arms race. However, the arms race model could account for the evolution of specialized host races, for instance within *P. andromeda* at Dinkey Creek and perhaps *M. uniflora* in Maryland. It would also predict that host shifts are more likely when seeds encounter naïve fungal populations, e.g. after rare dispersal events, as suggested by the results for *M. uniflora* at Mount Tamalpais (Fig. 3) and Del Norte County (Fig. 5).

In addition, although among eukaryotes most hosts and parasites reproduce sexually and this may be needed to

stay ahead on an arms race (Kurtz 2003), there is evidence for autogamy in the Monotropoideae such as high seed set in every fruit even when pollinators are excluded (Wallace 1975). In fact, autogamy is a characteristic trait of myco-heterotrophic plants (Dressler 1981; Benzing & Atwood 1984; Leake 1994). Some Monotropoideae (notably *Pterospora*) have morphological features to facilitate wind dispersal, but the forest floor habitat of these plants should provide few opportunities for it. Thus, the seed rain shadow of individual plants must be strongly leptokurtic. Because myco-heterotrophic plants do not compete for light but instead for spatially discrete fungal and root resources, establishment can be more spatially clumped than for photosynthetic plants. In fact, within seed packets we often observed clusters of seedlings at different stages of development bound by a common mycorrhizal fungal mantle (Fig. 1). A mature root mass may have tens to hundreds of inflorescences at different stages of development and the root mass may reach from centimetres up to a metre in diameter. We hypothesize that these densely congested clusters of fine roots may often represent several cohorts of very closely related plants, perhaps fused into a physiologically continuous genetic mosaic, colonized by a single clonally spreading fungal individual.

Symbiotic germination generates interest because it places strong constraints upon plant distributions and efforts aimed at plant conservation (McKendrick *et al.* 2000b; Batty *et al.* 2001; Bowles *et al.* 2002; Stewart & Zettler 2002; Sharma *et al.* 2003; Leake *et al.* 2004; McCormick *et al.* 2004; Otero *et al.* 2004). The monotropoid mycorrhizal symbiosis is the focus of some conservation efforts; in the United States, *Allotropa* is a 'sensitive' species, *Pityopus* and *Monotropopsis* are 'critically imperiled', and in parts of Europe *Monotropa hypopithys* is 'declining' or 'threatened'. Some monotrope species are old-growth-forest associates that disappear following

forest management practices (Lichthardt & Mancuso 1991; Wogen & Lippert 1998; Moola & Vasseur 2004). Thus, understanding the life cycles of myco-heterotrophic plants and the major constraints to their establishment is critical. A handful of recent *in vitro* studies suggest that plants that rely upon symbiotic germination may prefer some fungi over others (Zettler & Hofer 1998; McCormick *et al.* 2004; Otero *et al.* 2004). In this study, we have tested this hypothesis *in situ*. We have also shown that there is realistic potential for reintroduction to native forest settings of myco-heterotrophic plants, the largest group of plants that has so far not been successfully brought into cultivation.

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