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In vitro germination of nonphotosynthetic, myco-heterotrophic plants stimulated by fungi isolated from the adult plants

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SUMMARY

Sarcodes sanguinea and *Pterospora andromedea* (Ericaceae, Monotropoideae) are nonphotosynthetic myco-heterotrophic plants. Recent studies have shown that the roots of the adult plants are always associated with closely related but exclusive sets of *Rhizopogon* species (Basidiomycota, Boletales) from section Amylopogon. We have isolated *Rhizopogon* species that were associated with the adult plants and used them to germinate seeds under gnotobiotic conditions. All *Rhizopogon* species isolated from either plant species were capable of stimulating seed germination in both *Sarcodes* and *Pterospora*. Under the primary conditions used, germination varied from 9 to 73% in the case of *Sarcodes* and 0 to 13% in that of *Pterospora*. The single *Rhizopogon* strain that failed to elicit germination in *Pterospora* under these conditions did stimulate germination under slightly different conditions. By contrast, seeds failed to germinate on all media which lacked these *Rhizopogon* species, or in the presence of six other genera of basidiomycetes. Seed germination could be stimulated either through cellophane or at the edge of fungal colonies without direct fungus–seed contact. These results suggest that a diffusible or volatile compound that is unique to *Rhizopogon* stimulates germination of these plant seeds. Seed lots of *Sarcodes* from two successive years had similar germination levels. *Sarcodes* seeds that had overwintered under natural conditions were also stimulated to germinate. These results demonstrate the potential for long-term dormancy. We suggest that a combination of dormancy and the use of specific germination cues might increase the opportunities of these plants for recruitment. In addition, the specific germination response explains at least a part of the specialized associations observed in the adult plants. Nevertheless, the seeds respond to a slightly broader range of *Rhizopogon* species than has been observed to be associated with the adult plants; thus other factors must also be involved with specificity under natural conditions.

Key words: myco-heterotrophy, ectomycorrhizae, epiparasite, symbiosis, specificity.

INTRODUCTION

Sarcodes sanguinea and *Pterospora andromedea* are the only members of two closely related monotypic genera within the Monotropoideae (Ericaceae) (Wallace, 1975; Cullings & Bruns, 1992). Like other members of the subfamily they lack chlorophyll and are assumed to derive all their fixed C from surrounding plants through a shared ectomycorrhizal fungus. Björkman (1960) demonstrated that C could flow from trees to *Monotropa hypopithys* through a shared fungus. He referred to such plants as epiparasites because he viewed them as indirectly parasitic on the surrounding trees. Leake (1994) preferred the broader term, myco-heterotroph, because this does not specify the nature of the interaction beyond its dependence on fungi.

Adult flowering plants of both species are highly specific in their mycorrhizal associations. *Pterospora andromedea* is reported to associate with species in the *Rhizopogon subcaerulescens* group throughout its western North American range (Cullings *et al.*, 1996), and *Sarcodes sanguinea* is restricted to the *R. ellenae* species complex, at least within the part of its geographic range from which our material is derived (Kretzer *et al.*, 2000). It has been proposed that such a high level of fungal specificity is unlikely because it

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would make establishment of new plants difficult (Harley & Smith, 1983). However, all the members of the Monotropoideae that have been studied appear to exhibit high specificity at least within broad geographic regions (Cullings *et al.*, 1996; Kretzer *et al.*, 2000). A similar pattern is found among genera of nonphotosynthetic orchids that associate with ectomycorrhizal fungi; this suggests that specificity is an integral part of many myco-heterotrophic interactions (Taylor & Bruns, 1997, 1999; McKendrick *et al*,. 2000).

How specificity of adult plants for a particular fungus is related to seed germination and seedling establishment remains unknown. Francke reported germination and initial development of *M. hypopithys* seeds under axenic conditions by using an unidentified fungus isolated from the mature plants (Francke, 1934). Recent field work (S. L. McKendrick, J. R. Leake and D. J. Read, unpublished) has shown that seeds of *Monotropa hypopithys* germinate and develop more readily in areas where mature plants occur, and when germination occurred, the fungus found on mature plants was often also associated with the seeds. These results suggest that monotrope seeds might germinate in response to the appropriate fungus. The primary goal of this study is to test this hypothesis; *Sarcodes* and *Pterospora* were chosen because their associated fungi are known and grow readily in culture.

The nature of the interactions between the three partners in the monotrope system, namely the autotrophic plant, the mycorrhizal fungus and the heterotrophic plant, remains unknown. At first sight the monotropes appear to be parasitic, as they are dependent on the interaction and seem to lack resources that would be of use to the other two organisms. The specificity of the monotropes for their fungal associates is also consistent with parasitism, because specificity is a hallmark of parasitism (Price, 1980); it is less common, though not absent, in mutualistic systems (Borowicz & Juliano, 1991). Miller & Allen (1992) stated that the monotrope interaction might be mutualistic, but did not elaborate on the basis for their speculation. Two recent findings support this mutualistic hypothesis. Stimulation of the fungus was observed around and within packets of germinating *Monotropa* seeds (S. L. McKendrick, J. R. Leake, D. J. Read, unpublished), whilst Bidartondo *et al*. (2000) found that the abundance of *Rhizopogon ellenae* on *Abies* mycorrhizae was significantly higher in areas immediately adjacent to *Sarcodes* plants. In this case the increased abundance of both *Abies* roots and *Rhizopogon* appeared to result from stimulation by the monotrope.

A prerequisite for the close examination of the physiological ecology of these tripartite systems is the ability to establish and manipulate them under controlled laboratory conditions. This is our long-term goal, and we view the ability to germinate seeds as an obvious first step towards it.

METHODS

Collection and storage of seeds

Seed capsules of *Pterospora andromedea* Nuttall and *Sarcodes sanguinea* Torrey were collected from mature plants in Sierra National Forest near the Dinkey Creek, CA, workstation of the USDA Forest Service (lat 37°03′46″S, long 119°9′20″W, elev. 1735 m). Capsules were air-dried at room temperature for 2–3 wk in paper envelopes. Seeds were extracted by lightly crushing the capsules and sifting though 2-mm soil screens; they were then placed, along with small debris and silica gel packets, in Ziplock plastic bags (S. C. Johnson & Son Inc., Racine, WI, USA) and stored at 5°C until needed.

Pterospora seeds were collected in August 1998 and *Sarcodes* seeds in August 1997 and August 1998. All three of these seed lots were treated and stored as already described. Unless otherwise stated, all experiments involved only the 1998 seed lots. *Pterospora* and *Sarcodes* seeds were also collected in July 1999 from seed capsules that had overwintered. These were used immediately in a single set of experiments.

Surface sterilization of seeds

Prior to sterilization, seeds were sieved repeatedly through soil screens (0.5-mm mesh for *Sarcodes*, and 0.25-mm for *Pterospora*) to remove chaff. *Sarcodes* seeds were then surface-sterilized for 15 min in 30% H_2O_2 to which a drop of Tween 80 was added, and *Pterospora* seeds were surface-sterilized for 20 min in saturated calcium hypochlorite with Tween 80; this solution was made 1 h before treatment. Seeds of both species were vortexed during the treatment time, filtered, washed with sterile distilled water,and plated onto 2% water agar. The plates were observed for 2–3 wk and contaminated seeds were removed. Seeds that appeared to be intact, turgid and sterile were used immediately in various tests.

Media for attempted asymbiotic germination

The basal medium of Murashige and Skoog with sucrose and agar (Sigma M-9274), Oatmeal Agar (Sigma O-3506), and Phytamax orchid with charcoal and Banana powder media (Sigma P-0156) (Sigma, St. Louis, MO, USA) were made from premixed packages following the manufacturer's instructions. These media were selected on the basis of their ability to stimulate germination in some orchid

seeds. Over 200 seeds of both species were plated onto these media and incubated in the dark at 22°C for 4 months.

Isolation, identification and growth of cultures

Cultures of *Rhizopogon* were isolated from the roots of flowering *Sarcodes* and *Pterospora* plants as follows: roots were washed in tap water to remove some of the soil, then small pieces (5 mm) of root that appeared to be turgid and contained intact fungal mantles were surface-sterilized in 30% H_2O_2 for 10 s, rinsed repeatedly in sterile distilled water, and cut into smaller pieces under a dissecting microscope within a laminar flow hood. Pieces of epidermis < 1 mm thick were selected and placed on Modified Hagem's media (M40 in Stevens *et al*., 1981) supplemented with the following antibiotics: 50 mg l^{-1} chloramphenicol, 50 mg l^{-1} streptomycin sulfate, 50 mg l^{-1} ampicillin, and 1 mg l^{-1} benomyl. Chloramphenicol was added prior to autoclaving; all others were either filter-sterilized (streptomycin and ampicillin) or dissolved in chloroform (benomyl) and added with vigorous stirring after the media had cooled to 42°C. Slow-growing candidate cultures were transferred onto new plates lacking antibiotics. Those conforming to the cultural characteristics expected for *Rhizopogon* were kept for further study.

Identities of all *Rhizopogon* cultures used in this paper were confirmed by RFLP of the polymerase chain reaction amplified ITS region following the method of Gardes & Bruns (1996); the ITS RFLPs were compared with those derived from identified sporocarp collections. Ultimately the ITS region from all unique RFLP types was sequenced and compared with a *Rhizopogon* sequence database produced by Grubisha (1998) which has been supplemented by a few additional species by Kretzer *et al*. (2000). In this way all of the *Sarcode*s isolates were confirmed to be within the *R. ellenae* clade. By the same method, the *Pterospora* isolates were found to lie outside the *R. ellenae* group, and were placed in the monophyletic groups 1 and 4 of Kretzer *et al*. (2000) within section *Amylopogon*. Although named sporocarp collections fall within group 1, we refrain from applying any of these names because a preliminary study of holotype collections suggests that there has been much confusion in the application of species names in section *Amylopogon* (M. I. Bidartondo, unpublished). For this reason we refer to these *Rhizopogon* isolates only by genus and group number.

Other fungal cultures were isolated from sporocarps. *Suillus bovinus* (L: Fr.) O. Kuntze, *S. variegatus* (Swartz: Fr.) O. Kuntze, *Lactarius rufus* (Scop.: Fr.) Fr., *Laccaria proxima* (Boud.) Pat., and *Lycoperdon foetidum* Bonorod. were identified and isolated by the first author. *Lactarius* and *Laccaria* isolates were derived from collections made at Pt. Reyes National Seashore, CA, USA, others were derived from material collected in Derbyshire and North Yorkshire, UK. *Tricholoma flavobrunneum* (Fr.) Kummer was collected in Sheffield, UK, and identified, and isolated by Jesus Perez-Moreno. *Suillus* species were chosen because this genus is known to be the sister group to *Rhizopogon* (Kretzer *et al*. 1996, 2000). *Lactarius*, *Laccaria* and *Tricholoma* species were chosen as representatives of unrelated ectomycorrhizal basidiomycetes. *Lycoperdon* was selected as representative of a saprophytic basidiomycete that is common in forest settings.

Fungal cultures, with or without seeds added, were incubated in the dark at 20°C on either 1% malt extract agar (MEA) or 1/10 Modified Melin Norkrans (1/10 MMN): glucose 2.5g, malt extract 10 g, $CaCl_2 \cdot 2(H_2O)$ 0.5 g, NaCl 0.025 g, $MgSO_4 \cdot 7(H_2O)$ 0.015 g, $(NH_4)_2$ HPO_4 0.025 g, KH_2PO_4 0.5 g, $FeCl_2 \cdot 6(H_2O)$ 0.01 g, Thiamin HCL 0.001 g and H_2O to 1 l; the pH was adjusted to 4.7 with 1% HCl before autoclaving. Both media contained 2% agar to promote growth of aerial mycelium.

Symbiotic seed germination experiments

In initial experiments seeds were placed in front of the advancing fungal mycelium; however, in all later experiments they were placed directly onto the fungal culture a few millimeters behind the advancing edge. The second approach had two advantages: all seeds achieved contact with the test fungus at the same time; seeds were not overgrown as completely and so were easier to locate at the end of the experiment. Seeds were incubated for 2 months after contact with the fungus had been made. This period was selected because germination was initially observed in both *Sarcodes* and *Pterospora* after 30 d; this was doubled to ensure that sufficient time was provided for germination to become evident. At the end of the experiments seeds were examined with a dissecting microscope at a magnification of 40×. Fine needles were used to turn seeds over so that all sides could be checked for signs of germination.

Initially, both 1% MEA and 1/10 MMN were tested as candidate media with a subset of *Rhizopogon* isolates, but results using both media were almost identical for all isolates originally tested. For subsequent tests 1/10 MMN was used because many fungi grow faster on it. In the cases of *L. rufus* and *L. foetidum*, neither medium produced significant growth, so full-strength MMN was used.

Isolation of seeds from fungi via cellophane

Cellophane film (type 350 P 00, UCB Films, Somerset, UK) was cut into circles the size of Petri dishes, boiled three times in distilled water, and

autoclaved before use. Approximately 20 seeds were placed near the centre of each plate, the agar and seeds were covered with sterilized cellophane, and *R. ellenae* (SNF-371) and *R.* sp. group 1 (SNF-379) were inoculated on the *Sarcodes* and *Pterospora* plates, respectively. Initially, eight plates of each plant species were started; final plate counts differed because those that became contaminated with other fungi were discarded. An equivalent number of control plates without fungal inoculum were also set up. The plates were incubated for 2 months after the fungi had grown over the cellophane above the seeds. The cellophane was then removed, the agar was checked to ensure no fungal penetration had occurred, and the seeds were examined for germination. In the second set of tests, *R. ellenae* (SNF-371) was inoculated onto cellophane without *Sarcodes* seeds, and grown for 2 months. The cellophane and fungus were removed, then *Sarcodes* seeds were placed on the agar media, incubated for 2 months, and examined to determine whether residual fungal compounds had stimulated germination.

Effects of seed age on germination of Sarcodes *and* Pterospora

Ten 1/10 MMN plates were inoculated with *R. ellenae* (SNF-371), and 11–18 *Sarcodes* seeds were placed just behind the actively growing margin. Seeds from the 1997 and 1998 seed lots were placed on different halves of each plate. Germination was assessed every 2–4 d through the unopened lids of the Petri dishes with the aid of a dissecting microscope. Seeds that were observed to germinate were recorded and marked on the lid of the dishes to avoid recounting. After 40 d the Petri dishes were opened and all seeds were examined for signs of germination. A similar test was not attempted with *Pterospora* because the small size of seeds made it too difficult to score germination without disturbance.

Seeds of both species were also collected in July 1999 from capsules that had overwintered in the field. A total of 64 and 83 seeds of *Sarcodes* and *Pterospora*, respectively, were surface-sterilized as already described and placed onto growing cultures of *R. ellenae* SNF-371 and *R.* sp. group 1 SNF-379, respectively. After 2 months the seeds were examined for germination.

Statistics

The proportion of seeds germinated on each Petri dish was treated as a replicate for a given experiment. Means and standard deviation were calculated for these proportions by standard methods using Excel 98 (Microsoft Corp., Redmond, WA, USA). For multiple comparisons between means, proportions were transformed by the arcsine square root method of Freedman and Tukey (1950) as suggested by Zar (1999); a one-way ANOVA and a Tukey test were then used to compare the transformed means.

RESULTS

All cultures of *Rhizopogon* stimulated germination of both *Sarcodes* and *Pterospora*, whilst none of the other fungi used stimulated germination of either plant (Table 1). Similarly, no germination occurred on any synthetic media that lacked a *Rhizopogon* culture. Approximately 600 seeds of each species were assayed on media used to germinate orchid seeds (oatmeal agar, Phytamax orchid media and Murashige and Skoog basal medium) but none of these seeds germinated in the absence of a *Rhizopogon* associate. Similarly, none of the seeds of either species germinated on either 1% MA or 1/10 MMN, the two media used for all of the *Rhizopogon*-induced germination trials.

Stages and timing of germination

We recognized three stages of germination in *Sarcodes* and *Pterospora*. Stage 1 was characterized by a crack in the seed coat, generally a single longitudinal slit, but sometimes more randomly oriented or more complex. In *Sarcodes* this stage was easy to observe because of the contrast between the thick reddish-brown reticulate seed coat and the smooth white endosperm. Furthermore, *Sarcodes* seeds were seldom broken during surface sterilization, and if so, not by a longitudinal crack. In *Pterospora*, stage 1 was often more difficult to discern, because the seed coat is thinner, paler, and partially translucent, with faint longitudinal ribs. Furthermore it was often longitudinally cracked during sterilization. For these reasons we did not score stage 1 in *Pterospora* as being germinated in Table 1. Therefore, we present a conservative estimate of the true germination rate for *Pterospora*.

Stage 2 was characterized by swelling and emergence of the seedling from the apical end of the seed, and in *Pterospora* the seed coat was often partially or completely shed (Fig. 1). At stage 3 the width of the emergent seedling was approximately equal to that of the ungerminated seed (Fig. 1). About half the seeds at this stage were partially or completely obscured by fungal hyphae. Fungal rhizomorphs were often associated with such seeds, but, as discussed later in this section, hyphal contact was not necessary for development to this stage. In *Sarcodes*, development did not progress beyond stage 3. If seeds were left longer than the normal 2-month observation period the white emergent part of the seedling often became brown and hardened. In *Pterospora*, the volume of eight of 99 germinated seedlings was *c.* 10-fold their ungerminated size

Table 1. *Seed germination percentages for* Sarcodes *and* Pterospora *with selected fungi*

Fungus	*Sarcodes**	*Pterospora*
Rhizopogon ellenae SNF-356	73 ± 22 (7, 56)a	5.4 ± 9.0 (9, 73)
R. ellenae SNF-371	66 ± 14 (7, 104)a,b	1.8 ± 4.7 (7, 59)
R. sp. group 1 SNF-379	46 ± 22 (9, 72)b	0.0 (8, 58)†
R. sp. group 4 SNF-378	14 ± 8.7 (6, 52)c	13 ± 22 (3, 26)
R. sp. group 4 SNF-387	9.0 ± 9.7 (101, 7)c,d	9.4 ± 12 (4, 32)
Suillus variegatus TDB-UK12	0.0 (46, 5)d	0.0 (5, 54)
S. bovinus TDB-UK7	0.0 (10, 106)d	0.0 (5, 64)
Lactarius rufus TDB-2181	0.0 (5, 45)d	0.0 (5, 49)
Laccaria proxima TDB-2332	0.0 (5, 44)d	0.0 (6, 71)
Tricholoma flavobrunneum USTF97	0.0 (4, 37)d	0.0 (7, 86)
Lycoperdon foetidum TDB-UK1	0.0 (5, 46)d	0.0 (5, 52)
1/10 MMN controls	0.0 (4, 41)d	0.0 (6, 57)
1/10 MMN controls (under cellophane)	0.0 (4, 42)d	0.0 (5, 53)

*Mean % seed germination ± SD, number of Petri-dish trials and total seed numbers are given in parentheses. Means that are not significantly different ($P < 0.05$, Tukey test following Freeman & Tukey, 1950, Arcsine square root transformation) among the *Sarcodes* trials share a common lowercase letter. None of the *Pterospora* means is significantly different. All trials were conducted on 1/10 MMN except for *Lactarius rufus* and *Lycoperdon foetidum* for which full-strength MMN was used. †*Pterospora* germination of 26 ± 21% (8, 65) was observed with SNF-379 on 1% malt agar.

Fig. 1. *Sarcodes* and *Pterospora* at various stages of seed germination and seedling development. (a) Germination of *Sarcodes* seeds on a Petri dish with *R. ellenae* SNF-371. Arrow, one of the many seeds that are enveloped by mycelium and associated with rhizomorphs. (b) Close-up of one seed from plate. (c) Germination of *Sarcodes* to stage 3 without obvious mycelial envelopment or rhizomorph development. (d) Germinating *Pterospora* seed at stage 2. (e) Germinating *Pterospora* seed at stage 3. (f) *Pterospora* seedling at stage 4 (top arrow) with ungerminated *Pterospora* seed for comparison of size (bottom arrow). Ungerminated *Sarcodes* and *Pterospora* seeds are approximately 0.8–1.3 mm and 0.2–0.5 long, respectively.

(Fig. 1). Development to this extent was never observed in *Sarcodes*, and in *Pterospora* it was only achieved in association with *R.* sp. SNF-379, and *R.* sp. SNF-387, both of which were isolated from *Pterospora* roots.

Germination rate, viability and germination after overwintering in Sarcodes

The first *Sarcodes* seeds were observed to germinate 13 d after placement on *R. ellenae* mycelium;

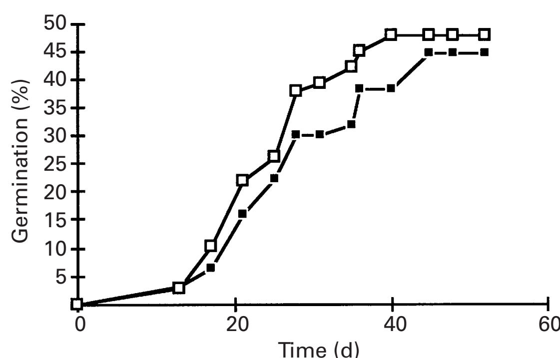

Fig. 2. Germination time course for *Sarcodes* seeds. Cumulative germination is shown for two different *Sarcodes* seed lots (1998, open squares; 1997, closed squares). Seeds were placed on the growing edge of *R. ellenae* SNF-371 on 1/10 MMN plates on day 0.

germination continued throughout the early period of observation and reached a plateau after about 43 d (Fig. 2); this shows that the 2-month period used in other parts of the study was sufficient for estimating percentage germination. After 52 d the Petri dishes were opened and a final intrusive examination of the seeds was made. The germination percentages were 58 and 55 for 1997 and 1998, respectively, about 15% more than the non-intrusively observed percentages of 44 and 49 (Fig. 2). The latter were underestimated because fungal mycelium obscured some seeds and the orientation of others made it difficult to observe early germination.

Sarcodes seeds collected from overwintered capsules also germinated, but at a significantly lower rate (21%) than the 1997 (58%) and 1998 (55%) seed lots, which were stored under laboratory conditions ($P < 0.05$, one-way ANOVA with Tukey test). Germination differences between the 1997 and 1998 seed lots were not significant. No germination (i.e. stage 2 or later) was observed in the overwintered *Pterospora* seeds ($n = 84$).

Germination in the vicinity of Rhizopogon *but without direct contact*

Development up to stage 3 could occur in both plant species without direct fungal contact. We first observed this in a set of tests in which seeds were placed on malt-extract agar plates in front of advancing fungal colonies. Two of the 179 *Pterospora* seeds that were never reached by the fungi germinated and reached stage 2 (i.e. swelling and partial emergence). These two seeds were within 1.5 mm of the edge of the fungal colony; most seeds that did not germinate were further away. This result suggests that a diffusible or volatile substance might be involved. To test this we placed seeds of *Sarcodes* and *Pterospora* on agar media, overlaid them with sterile cellophane, and inoculated *Rhizopogon* isolates on top of the cellophane. *Sarcodes* seeds exhibited a mean germination rate of 38% under these conditions (SD = 24, 4 plates, 83 seeds in total) and 10 of the 31 germinated seeds reached stage 2 or 3. Only one of 169 *Pterospora* seeds germinated under cellophane, and it reached stage 3. Four additional seeds exhibited cracks, but were not scored as germinated. No seeds on the uninoculated cellophane control plates germinated.

To test whether some stable germination-inducing compound was exuded into the agar media, *R. ellenae* cultures were grown for 2 months on cellophane above the agar media, but were then removed along with the cellophane. *Sarcodes* seeds were placed on these plates and incubated for an additional 2 months, but none of the 117 seeds germinated.

DISCUSSION

To our knowledge this is the first report of germination in either *Sarcodes* or *Pterospora*. Bakshi (1959) failed to germinate *Pterospora* seeds on various agar media in the absence of fungal associates, a negative result that we have reproduced here. Bakshi also used tetrazolium tests to assay the viability of *Pterospora* seeds, and from the results he claimed that the seeds were initially highly viable, but that after 3–9 wk their viability dropped to zero, and suggested that seeds in nature are short-lived. Most of our experiments were conducted with seeds that had been stored for 6–8 months, yet we observed germination rates as high as 13% (Table 1). However, we were not able to induce seeds which had overwintered in the field to germinate, which might indicate that Bakshi was at least partly correct. Bakshi also noted that the small seeds of *Pterospora* are easily killed by surface sterilization. We observed that the sterilization treatment often resulted in visible damage to the seed coat, and that the frequency of such damage appeared to be much higher in the older, overwintered seed lot. This side effect of the sterilization process might well have resulted in much lower germination rates than could be achieved under natural conditions.

Sarcodes seeds retained high viability for at least 2 yr under laboratory conditions (Fig. 2), and remain viable after overwintering in the field. Similarly, it has been shown (S. L. McKendrick, J. R. Leake, D. J. Read, unpublished) that *M. hypopithys* seeds can germinate in nature at least 2 yr after planting. These results, which are based on real germination rather than biochemical tests of viability, show that seeds of these monotropes are viable for much longer than previously supposed. This is likely to be functionally significant to these plants, because it enables them to lie dormant until they encounter the correct fungus.

There is a single previous report of fungal stimulation of germination in monotropes. Francke (1934) germinated seeds of *M. hypopithys* under gnotobiotic conditions by using an unidentified fungus which he had isolated from the roots of mature *Monotropa*. Development of the *Monotropa* seedlings appears to have reached a stage similar to that which we observed in *Pterospora* and *Sarcodes*. Our results differ from Francke's in two ways: first, we show that fungus does not need to be in direct contact with the seed in order to stimulate germination; second, we show that the stimulation is fairly specific. Only fungi isolated from the roots of these plants stimulated germination.

The fact that fungal contact is not necessary for seed germination suggests that some type of diffusible or volatile compound is involved, and that this compound is apparently either not stable or not available on plates on which the fungus has grown. In addition, the fact that seedlings could reach development stage 3 without contact suggests that seed reserves alone might be adequate to sustain growth to this stage. However, we cannot eliminate the possibility that seedlings obtain some sugars from the media.

As we hypothesized, the germination reaction was fairly specific; most fungi that are not associated with the adult plants did not stimulate seed germination (Table 1). The only exception that we found concerns the ability of *Rhizopogon* species isolated from each plant species to stimulate seed germination of both plants. In the case of *Sarcodes*, 64 adult plants have been sampled in two regions of the Sierra Nevada mountains, including the site from which the seeds and cultures were derived, and only *R. ellenae* has been found associated with them (Kretzer *et al.*, 2000). Thus, our finding that *Rhizopogon* species from groups 1 and 4 stimulate seed germination in *Sarcodes* means that requirements for initial seed germination are less specific than those enabling associations between the fungus and adult plants. In *Pterospora* this same pattern appears to occur, but it is not as clear, because it is less certain which *Rhizopogon* species are associated with *Pterospora* adults. Although Cullings *et al.* (1996) reported that all 31 *Pterospora* individuals sampled from across its western North American range were associated with the *R. subcaerulescens* species group, their concept of this group included *R. ellenae* and groups 1 and 4. The sequence data available at that time were insufficient to allow separation of the various lineages now apparent. More recently the fungal associates of 26 adult *Pterospora* plants were examined in the Sierra National Forest region where *Sarcodes* had been intensively sampled, and all *Pterospora* plants were found to be associated with either group 1 or 4 species, but none was associated with the *R. ellenae* group (M. I. Bidartondo, T. D. Bruns, unpublished), in spite of the fact that *R. ellenae* stimulates germination of *Pterospora* seeds (Table 1). These results are especially interesting because *Pterospora* and *Sarcodes* frequently grow in close proximity throughout the region studied, and some of the cultures that we used were derived from sites where *Pterospora* and *Sarcodes* are sympatric; thus the seeds of each are likely to encounter the fungi of the others.

How then does this observed laboratory behaviour of the seeds relate to recruitment of the plants in nature? We know from many studies that the below-ground ectomycorrhizal community is rich in species and that the distribution of individual species is often patchy (Gardes & Bruns, 1996; Dahlberg *et al.*, 1997; Gehring *et al.*, 1998; Jonsson *et al.*, 1999). We also know that in Californian pinaceous communities, including those from the study area, the most abundant species are members of the Russulaceae and Thelephoraceae; *Rhizopogon* species, though common, are seldom abundant, except in post-fire settings (Gardes & Bruns, 1996; Horton & Bruns, 1998; Horton *et al.*, 1998; Baar *et al.*, 1999; Horton *et al.*, 1999; Stendell *et al.*, 1999; Bidartondo *et al.*, 2000). Given this spatial patterning, the ability to lie dormant until the correct fungus approaches the seed would substantially increase the odds of new recruitment, particularly if the nonhost fungi are more common than the host fungi. Thus, specific germination cues coupled with dormancy might make specificity of the adult plants a feasible strategy, because little cost other than delay would result from the inability to associate with other potential hosts.

Occasional mistakes, such as *Sarcodes* germination in response to *Rhizopogon* species in groups 1 and 4, or *Pterospora* germination in response to *R. ellenae*, could be caused by similarity of the chemical signals in these closely related fungi. The fate of these encounters in nature is unknown; they could either be aborted, at a cost to the plant, or transient, with an eventual switch to the correct fungus before plant maturation.

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