Germination and infectivity of ectomycorrhizal fungal spores in relation to their ecological traits during primary succession

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Summary

• The spores of ectomycorrhizal fungi (EMF) play critical roles in the population and community development of EMF. Here, the germination and infectivity of EMF spores are examined with reference to the ecological traits of the EMF species.

• Spores were collected from 12 EMF species, whose successional patterns have been studied in the volcanic desert on Mount Fuji, Japan. Spore germination experiments were conducted with host plants (Salix reinii), with nonhost plants (Polygonum cuspidatum), and without plants. The mycorrhizal formation ability of spores was also examined in seven EMF using spore inoculation experiments. To determine the effects of the spore preservation period, both experiments were repeated up to 1 yr after spore collection.

• Spore germination was very low in the absence of host plants. In the presence of hosts, even 30 d after spore collection, spore germination was significantly enhanced in all pioneer EMF (c. 20%) but less so in late-stage EMF (< 5%), except in Hebeloma species. Mycorrhizal formation from spores was also greater in pioneer EMF but was significantly reduced by 1 yr of spore preservation.

• High spore germination and infectivity of pioneer EMF should enable these species to colonize disturbed and isolated areas in accordance with their ecological traits.

Key words: ectomycorrhizal fungi (EMF), Hebeloma, Inocybe, Laccaria, primary succession, Russula, Scleroderma, spore germination.


Introduction

The fine roots of major tree species in many forest ecosystems are associated with ectomycorrhizal fungi (EMF) which substantially improve host performance primarily by enhancing nutrient uptake (Smith & Read, 1997). Because it is difficult for host plants to grow and survive in nature without EMF (Nara & Hogetsu, 2004), they must be colonized by EMF through various means, such as external mycelia and spores.

In developed forests, naturally regenerating seedlings are rapidly colonized by EMF, most of which are common to existing mature trees (Jonsson et al., 1999; Matsuda & Hijii, 2004). In a primary successional area, seedlings were readily colonized by EMF in the vicinity of early-established shrubs through sharing of common EMF mycelia, but EMF colonization itself was quite difficult in patches where EMF mycelia were not available (Nara & Hogetsu, 2004). These studies indicate that external mycelia radiating from pre-existing host trees contribute to EMF colonization of seedlings. The colonization of seedlings from existing mycelia has profound effects on ecosystem processes through the enhancement of host establishment and vegetation succession (Nara, 2006a,b).

There is growing evidence that EMF spores are also important for EMF colonization on hosts, especially in disturbed
areas where external mycelia are absent. For example, following a stand-replacing forest fire, *Rhizopogon* EMF species colonized plants via large stores in the soil spore bank and dominated newly germinated seedlings (Horton et al., 1998; Baar et al., 1999). In a coastal sand dune, spores of *Rhizopogon* and *Suillus* were dispersed in deer feces and colonized seedlings isolated from existing trees (Ashkannejhad & Horton, 2006). Such spore-mediated EMF colonization is not restricted to suillloid fungi. Fungal spores are generally small (c. 10 µm in length; Clémençon et al., 2004) and are usually produced in large amounts (e.g., 1 x 10^8–9 spores per sporocarp in *Suillus bovinus*; Dahlberg & Stenlid, 1994), enabling long-distance (e.g., intercontinental; Nagarajan & Singh, 1990) dispersal by wind or animals (Allen, 1991; Lilleskov & Bruns, 2005). Therefore, spores should provide the main source of EMF colonization of remote seedlings for most EMF species.

EMF spores are also responsible for the development and maintenance of EMF populations. Using molecular markers, several studies have demonstrated that EMF populations are composed of many genetically different units of the same species, or genets (Dahlberg & Stenlid, 1994; Kretzer et al., 2004; Lian et al., 2006). To produce genetically different units, spore-mediated reproduction, including sexual recombination, must occur. Thus, the existence of many genets in an EMF population points to the occurrence of spore-dependent reproduction in the field. Furthermore, the dominance of small genets, which indicates a greater importance of spores relative to mycelia in reproduction, has been frequently observed in a wide variety of EMF taxa, including *Amanita*, *Hebeloma*, *Laccaria*, *Lactarius* and *Russula* species (Fiore-Donno & Martin, 2001; Redecket et al., 2001; Bergemann & Miller, 2002; Guidot et al., 2002; Hirose et al., 2004; Wadud, 2007).

Another important aspect of EMF spores in nature involves their role in the development and succession of EMF communities. In early successional stages, EMF communities are simple and composed of a small number of EMF species (Jumpponen et al., 2002; Nara et al., 2003a,b; Cázares et al., 2005). After long successional processes, many new EMF have recruited and accumulated in EMF communities. Developed EMF communities are often species-rich and can include hundreds of EMF species (Tedersoo et al., 2006; Ishida et al., 2007). During these successional and community development processes, new EMF species are likely to be introduced from outside the system in the form of spores.

Despite the potential importance of EMF spores in many ecological processes, the available information regarding spore germination and infectivity is quite limited. Although host roots and root exudates improve EMF spore germination (Bowen, 1994, and references therein), the extent of the host effect on spore germination varies considerably (Fries, 1987). For example, pine (*Pinus radiata*) seedlings enhanced spore germination by up to 34 and 69% in species of *Suillus* and *Rhizopogon*, respectively (Theodorou & Bowen, 1987), whereas germination was as low as 1% in *Hebeloma* species (Fries & Biraux, 1980). To date, the causes of such variation in EMF spore germination are poorly understood.

An additional area requiring further research is the length of time for which EMF spores maintain their activity. The length of the active period would substantially affect the ecology of EMF and their hosts. A few studies have examined the length of the active period using a small number of EMF species with unique ecological strategies, demonstrating that EMF spores can maintain activity for long periods of time and can develop spore banks in the soil (Bulmer & Beneke, 1964; Theodorou & Bowen, 1987; Ashkannejhad & Horton, 2006). However, we cannot apply such specific results to all EMF species. To better understand the ecological functions of EMF spores and their interspecific variation, spore germination and infectivity should be examined over time in many EMF species that have different ecological strategies.

In the volcanic desert ecosystem on Mount Fuji, Japan, > 10 000 EMF sporocarps associated with dwarf shrubs (comprising only 1% cover) were recorded in an area of 5.5 ha during a 2-yr survey (Nara et al., 2003a). Intensive studies of EMF communities in this desert have revealed several distinct ecological strategies among EMF species (Nara et al., 2003a,b). Three pioneer EMF species (*Inocybe lacera*, *Laccaria amethystina* and *Laccaria lacata*) can effectively colonize non-mycorrhizal habitats. Other EMF species are sequentially recruited with host developmental stages. For example, *Laccaria marina* and *Scleroderma bovista* are secondary colonizers (following the three pioneer EMF), whereas species of *Russula* and *Cortinarius* are observed during late stages in the sere. During this successional or community development process, new species should arrive in the form of spores, because individual host islands are separated by large, bare areas of scoria, which prevent EMF mycelia from spreading between host islands. Therefore, the characteristics of these fungal spores may correspond to the observed successional pattern. The unique conditions of this desert site (i.e., abundant sporocarp production and considerable background information on EMF ecology) provide a perfect opportunity to address the relationship between spore characteristics and ecological traits of EMF.

We examined spore germination and infectivity of all dominant EMF (12 species) in the volcanic desert on Mount Fuji. We focused on the effects of plant (host and nonhost species) roots on spore germination and how these effects varied among the 12 EMF species. The infectivity of spores on host seedlings (and EMF interspecific variation therein) was also investigated in spore inoculation experiments using seven EMF species. Furthermore, to determine the length of EMF spore activity periods, both germination and inoculation experiments were repeated up to c. 1 yr after spore deposition. We also discuss spore-related characteristics and their crucial role in EMF community development and succession.
Materials and Methods

Study site and background information on dominant EMF

The study site was located in the volcanic desert ecosystem on Mount Fuji, Japan, as described in detail by Nara et al. (2003a,b) and Nara (2006b). In short, the research site (35°20′N, 138°48′E; 1500–1600 m above sea level) is located on the southeast slope of the mountain. The current vegetation is patchily distributed, forming isolated vegetation islands in a sea of volcanic desert. *Salix reinii* Franch. & Savat. (hereafter *Salix*), an alpine dwarf willow species, is the pioneer ectomycorrhizal host species and exclusively dominates this desert habitat (> 99% of the total EMF host coverage area).

In total, 31 EMF species have been identified on *Salix* at this site, and these species comprise a clear successional sere (Nara et al., 2003a,b). Newly established *Salix* in nonmycorrhizal islands are associated with one of three first-stage fungi: *Laccaria amethystina* Cooke, *Laccaria laccata* (Scop.: Fr.) Berk. & Br., and *Inocybe lacera* (Fr.) Kumm. With growth of *Salix*, other first-stage fungi as well as two second-stage fungi, *Laccaria murina* Imai and *Sclerodermum bovista* Fr., colonize the host. With subsequent host growth, EMF communities become more diverse, with many third-stage fungi, such as species of *Cortinarius*, *Hebeloma*, *Russula* and *Tomentella*.

The fruiting seasons differ among the EMF species in this ecosystem (Nara et al., 2003a). All species of *Laccaria* and *Russula* exhibit peak sporocarp production in the summer, and *I. lacera* and *S. bovista* continuously produce sporocarps during summer and autumn. Fruit production in *Hebeloma* and *Cortinarius* species peaks in late autumn, near the end of the plant growing season.

Some EMF species prefer specific habitats within the study site (Nara et al., 2003a,b). For example, sporocarps and ectomycorrhizas of all species of *Cortinarius*, *Hebeloma* and *Russula* are usually found inside large vegetation islands, indicating preferences for relatively developed soil that contains more organic substrates and higher nutrient concentrations. The opposite trend is exhibited by *I. lacera*, *L. murina* and *S. bovista*, which usually appear outside of the vegetation islands (Nara et al., 2003a,b).

Spore germination experiments

Spore preparation Of the 23 EMF species that produce sporocarps in this desert (Nara et al., 2003a), 12 dominant species were used for spore germination experiments (Table 1). From late August to early October 2006, we collected c. 20 fully matured sporocarps of each species, although the number of sporocarps collected for rare EMF species was much smaller (Table 1). To obtain genetically different sporocarps of the

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Sporocarps were collected from as many different vegetation islands as possible to secure the maximum genetic diversity in the volcanic desert on Mount Fuji.

*See Nara et al. (2003a,b) for details.

†Spores of each sporocarp were used separately in the experiment.

‡Spores were mixed within species before the experiment.
same species, replicate sporocarps were collected from as many different vegetation islands as possible. To obtain spore prints, pilei of individual sporocarps were placed on 3% agar film (c. 2 mm thick; without antibiotics) overnight at room temperature. Spores of *S. bovista*, a gasteromycetous fungus, were collected directly from the gleba using forceps and were spread on the agar film. Spore prints were overlaid by another 3% agar film to prevent desiccation and were kept in sealed boxes at 4°C until use. In total, we used separate spore prints of 246 sporocarps from 12 EMF species.

**Plant materials** We prepared nonmycorrhizal seedlings of *Salix* for the ‘host treatment,’ in which EMF spores were co-cultured with host roots. In late June 2006 and early July 2007, *Salix* seeds were collected from several trees, mixed, surface-sterilized with a 1% sodium chloride solution for 30 s, rinsed four times with tap water, and stored at 4°C for 1–2 months before the seedlings were used. *Salix* seeds were sown in an autoclaved (121°C for 180 min) mixture of sieve-selected loam (Shibanome soil; Setogahara Co., Gunma, Japan) and nursery soil from the Tokyo University Forest at Tanashi (1 : 1 by volume). Seedlings were fertilized with 1/500 strength Hyponex 6-10-5 (HYPONeX Japan Co., Osaka, Japan) at weekly intervals and were maintained in a growth chamber (24°C, light:dark 16 : 8 h; 150 µmol m−2 s−1 photosynthetic photon flux rate (PPFR); MLR-350; Sanyo, Osaka, Japan).

*Polygonum cuspidatum* Sieb. & Zucc. seedlings were collected at the study site from late August to mid-September 2006 for the ‘nonhost treatment,’ in which EMF spores were co-cultured with roots of small seedlings (<15 cm in height). This perennial herbaceous species is nonmycorrhizal and plays an important role in initiating vegetation islands. Collected seedlings were transplanted into plastic containers containing scoria soil and were maintained in a growth chamber until use.

Roots of host and nonhost plants were carefully washed in tap water before co-culture with EMF spores. Before experiments, the absence of EMF colonization in seedlings was verified under a stereomicroscope.

**Germination treatments** Just before germination experiments, a small piece (c. 25 mm²) of double-layered agar film on which spores had been deposited was cut from each spore print (spore film). The spore films were used without adjustments to spore density. In germination experiments, we initially conducted three (host, nonhost and control) treatments (Table 1). In the host treatment, *Salix* roots were sandwiched between the layers of spore film. The entire root system and attached spore film were further sandwiched between two sheets (80 × 100 mm) of filter paper (26-WA; Advantec MFS, Inc., Dublin, CA, USA; Fig. 1). To prevent seedlings from drying, filter paper was further overlaid with a wet paper towel (PN 61000; Nippon Paper Crecia Co., Tokyo, Japan). The assemblage was then placed in a plastic bag (90 × 120 mm). Each seedling was co-cultured with one to four spore films derived from different sporocarps of the same species. In the nonhost treatment, *P. cuspidatum* seedlings were used in place of *Salix* seedlings. In the control treatment, no plant was used, and one to four spore films were cultured between the filter paper using the procedure described above. All assemblages were incubated in a growth chamber (24°C; light:dark 16 : 8 h; 150 µmol m−2 s−1 PPFR) for 14 d.

After incubations, each spore film was transferred onto a glass slide, covered with a coverslip, and lightly squeezed. The numbers of germinated and ungerminated spores were counted for a total of 50–155 spores per spore film under a differential interference contrast microscope (BX60F; Olympus Co., Tokyo, Japan). Spores were considered as germinated when the germ tube emerged from the spores, irrespective of the length of the germ tubes. As the experiment was conducted under nonaseptic conditions, bacterial contamination was frequent, while other microbial contamination was rare. In any case, the microbial contamination did not affect the observation of spores (Fig. 2).

To determine the effect of the spore preservation period on germination, germination experiments were conducted after
maintaining spores for 30 d or c. 1 yr (350–390 d) in a refrigerator (4°C). As a consequence of various unpredicted problems (drying, contamination, and mixing with dew drops) during the spore preservation period, the number of replicates was reduced in the 1-yr treatment (Table 1).

Statistical analyses for spore germination experiments
Arcsine square root-transformed germination rates were used in all statistical analyses. Tukey’s Honestly Significant Difference (HSD) tests conducted in jmp 6.0 (SAS Institute, Cary, NC, USA) were used to test for interspecific differences in each treatment and to determine the significance of treatment effects.

To identify important factors and their contribution to variance in spore germination in host-effect treatments, we applied general linear models (GLM; using jmp 6.0) to data from control and host treatments. Because the effect of nonhost presence was not the focus of this analysis, data from the non-host treatment were excluded. We designed models including EMF species, treatments, and their interaction as fixed factors. To examine the factors affecting spore germination in preservation treatments (cultured with host after 0 d, 30 d, and 1 yr of preservation), we also used similar GLM analyses as described above, with preservation period included as a categorical variable. In both GLM analyses, Hebeloma pusillum Lange and Inocybe dulcamara (Pers.) Kumm. were excluded because of lack of data or insufficient replicates in some treatments.

We also analyzed the effect of host individuals (a nested, random factor) on spore germination using GLMs and data from the host treatment (0 d). Because intraspecific variation was the focus of this analysis, we separately applied the analysis to data from six fungal species (I. lacera, L. amethystina, L. laccata, Hebeloma mesophaeum (Pers.) Quel., Hebeloma leucosarx Orton and L. murina) that exhibited large intraspecific variation (range of > 15% between highest and lowest values).

Spore inoculation experiment
Preparation of spore inoculation tubes
To determine the infectivity of EMF spores and interspecific variation thereof, seven EMF species were tested in inoculation experiments. Fully matured sporocarps were collected from many vegetation islands (as in experiment 1) in late August or early October 2005 (Table 1). To obtain spore prints, pilei of all sporocarps (with the exception of S. bovista) were placed on 1% agar plates for 1–4 d. For each species, spore suspensions were obtained by washing the spore prints with distilled water containing a trace of Tween 40. Spores of S. bovista were collected from gleba using forceps and suspended in distilled water containing Tween 40. The spore density in each suspension was measured under a microscope and adjusted to $2.0 \times 10^4$ spores ml$^{-1}$ using distilled water.

To prepare spore inocula, 5 ml of a spore suspension (equivalent to $1.0 \times 10^5$ spores) was mixed with 40 ml of autoclaved (121°C for 180 min) scoria in a 50-ml plastic tube. A drain hole was bored 2 cm above the bottom of the tube, which was then wrapped in aluminum foil for shading. All replicate inoculation tubes were watered once a week and kept in a glasshouse (25 : 23°C day:night) with sunshade until use.

Seedling preparation and spore inoculation
Salix seeds were collected in early July 2005, and nonmycorrhizal seedlings were raised using the same method as in the germination experiments. After 0, 10 and 90 d, and c. 1 yr (292–352 d)
from the time of inoculum preparation, a 1–2-month-old nonmycorrhizal Salix seedling was transplanted into each inoculation tube. Transplanted seedlings were watered once a week and maintained in the glasshouse for 90 d. Seven to ten replicates were prepared for each EMF species for each experimental time period.

**Ectomycorrhizal assessment** After the 90-d inoculation period, seedlings were removed from the inoculation tubes. Root tips of the seedlings were observed under a stereomicroscope after careful washing with tap water. To confirm the identity of the EMF species on ectomycorrhizal root tips, all ectomycorrhizal root tips were morphotyped, and a representative root tip from each morphotype in each seedling was subjected to molecular identification. The molecular identification methods were described by Ishida *et al.* (2007). In brief, terminal restriction fragment length polymorphism (T-RFLP) analysis of the internal transcribed spacer (ITS) region in rDNA was conducted using a capillary sequencer (CEQ8800; Beckman Coulter, Fullerton, CA, USA) after digesting fluorescent-dye-labeled PCR products (ITS1F–ITS4) with *HinfI* (Takara Shuzo, Shiga, Japan, or Toyobo, Osaka, Japan). T-RFLP patterns and morphological characteristics were compared with those of species listed by Nara *et al.* (2003b) and Nara (2006b). When DNA fragments could not be assigned to any species on the list, the PCR product was purified using a PCR product pre-sequencing kit (USB Co., Cleveland, OH, USA), sequenced, and compared with the sequences of known species in GenBank using a BLAST search.

**Statistical analyses** In all analyses, the percentage of infected seedlings was arcsine square root-transformed. To examine the effects of EMF species and preservation period on EMF infectivity, we used GLM analysis in *JMP* 6.0. We initially constructed the model including species, preservation period (a continuous variable), and their interaction as fixed factors. To test pairwise differences between species, we applied Tukey’s HSD tests to pooled data for the same experiment.

**Results**

**Spore germination**

Germinated spores were easily distinguished from ungerminated spores by the presence of germ tubes (Fig. 2). Some germ tubes branched immediately after germination (e.g. Fig. 2a), while others elongated straight without branching (e.g. Fig. 2d).

In the absence of plant roots (control treatment), germination of EMF spores was very low, and mean germination rates were less than 1% in 11 of 12 species (Fig. 3a). Although spore germination slightly increased in most EMF species when co-cultured with the nonhost plant, *Polygonum cuspidatum* (Fig. 3b), the change was not significant for any of the EMF species (Tukey’s HSD tests, *P* > 0.05), except *L. lacera*.

By contrast, spore germination was drastically enhanced in the presence of host roots. Germination rates of nine EMF species, belonging to five different genera, were significantly higher than in control and/or nonhost treatments (Fig. 3c; Tukey’s HSD tests, *P* < 0.05). The degree of increase exhibited significant interspecific variation and was generally high in first-stage fungi: *L. lacera* (21.4 ± 3.9%; mean ± 1 SE), *L. amethystina* (22.3 ± 3.1%), and *L. lacata* (16.0 ± 1.9%). Spore germination of second-stage fungi, *L. murina* (6.3 ± 0.8%) and *S. bovista* (2.4 ± 0.5%), also significantly increased in the presence of host-plant roots, but the germination rates of these species were lower than those of first-stage fungi (Fig. 3c). Species of third-stage fungi exhibited two general responses. In *Hebeloma* species, germination significantly increased compared with control and/or nonhost treatments (Tukey’s HSD tests, *P* < 0.05), and germination rates of *H. mesophaeum, H. leucosarx* and *H. pusillum* were 31.2 ± 4.2, 19.0 ± 5.1 and 34.7 ± 8.8%, respectively. These values were higher than those of second-stage fungi and similar to those of first-stage fungi (Fig. 3c). The remaining species of third-stage fungi exhibited weak responses to host roots, and the germination rates of these EMF were low. Although the germination rate in *Russula pectinatoides* Peck (3.7 ± 1.1%) was significantly higher than in controls, no spore germination occurred in *Cortinarius decipiens* (Pers.: Fr.) Fr. or *I. dulcamara*.

GLM analysis for host-effect treatments (control and host (0-d) treatments) indicated that EMF species (df = 9, *F* = 28.57, *P* < 0.0001), host-plant treatment (df = 1, *F* = 341.14, *P* < 0.0001), and their interaction (df = 9, *F* = 24.95, *P* < 0.0001) had significant effects on spore germination. This model (two main factors and their interaction) explained 72% of the variability in spore germination data.

In the preservation experiments, all first-stage fungi maintained high activity after 30 d (Fig. 3d). The germination rates of these species declined after 1 yr, but germination was still detected in all first-stage fungi (Fig. 3e), namely *I. lacera* (4.4 ± 1.6%), *L. amethystina* (0.8 ± 0.3%) and *L. lacata* (1.1 ± 0.8%). Germination of second-stage fungi significantly decreased after 30 d (Fig. 3d), and no germination was observed after 1 yr (Fig. 3e). In third-stage fungi, spore germination of *H. mesophaeum* significantly declined after 30 d (Fig. 3d), although its germination rate after 1 yr (11.0 ± 3.5%) remained high, as did that of *H. leucosarx* (14.1 ± 4.0%).

GLM analysis of the preservation period experiments (treatments in which spores were cultured with the host after 0 d, 30 d, and 1 yr of preservation) indicated that EMF species (df = 9, *F* = 40.14, *P* < 0.0001), preservation period (df = 2, *F* = 35.58, *P* < 0.0001), and their interaction (df = 18, *F* = 3.90, *P* < 0.0001) significantly affected spore germination. The full model (two main factors and their interaction) explained 61% of the variability in spore germination data.

Surprisingly, we observed a large amount of intraspecific variation in the host treatment. For example, the ranges of germination rates in *I. lacera, L. amethystina, L. lacata* and
H. mesophaeum were 0–56.7, 4.6–67.9, 3.9–34.5 and 0–60.0%, respectively (Fig. 3c). GLM analysis for each of the six EMF species that exhibited wide ranges of germination rates revealed that host individuals significantly \( (P < 0.005) \) affected spore germination in L. amethystina, L. murina and H. mesophaeum, whereas the effect was marginal in I. lacera \( (P = 0.056) \) and H. leucosarx \( (P = 0.061) \). On average, 48% of the intraspecific variability in spore germination of these six EMF was explained by the effect of host individuals.

**Spore infectivity**

The spore inoculation experiment was conducted using 326 Salix seedlings. Unfortunately, 107 seedlings died during the experiment and were removed from the analysis. Most seedlings were colonized by at least one EMF species used in inoculations; however, 10 seedlings were colonized by EMF that were not used as infective agents. Molecular identification revealed that these seedlings were colonized by either *Tomentella* sp. or unidentified ascomycetes. These 10 seedlings were included in the analysis after excluding the contaminated root tips.

EMF infection was observed in the majority of seedlings in all first-stage fungi, even after keeping spores in tubes for 90 d before inoculation (Fig. 4). Although the frequency of ectomycorrhizal seedlings declined after 1 yr, ectomycorrhizas were confirmed in one of 10, four of nine, and four of 10 seedlings in the *I. lacera*, *L. amethystina* and *L. laccata* treatments, respectively. Infectivity of *S. bovista* spores was also generally high, with spores retaining some infectivity after 1 yr. Among third-stage fungi, infectivity of *Russula sororia* \((\text{Fr.})\) Romell was never confirmed. The infectivity of spores of *C. decipiens* was also low, and ectomycorrhizal formation was observed on only one seedling at the first inoculation. The infectivity of *H. mesophaeum* spores was highest among the third-stage fungi, and this species maintained high infectivity up to 30 d after spore preparation. However, no EMF colonization was observed after 1 yr. Because the interaction of species and germination attributable to nonhost (b) and host (c) roots are shown in the shaded boxes at the bottoms of (b) and (c), respectively; indicated by an upward arrow \( (b \rightarrow a) \), an upward single arrowhead \( (c \rightarrow a) \), an upward double arrowhead \( (c \rightarrow a, \text{and } c \rightarrow b) \), and ‘ns’ not significant by Tukey’s HSD test \( (P > 0.05) \). ‘na’ indicates that Tukey’s HSD test was not applied because of data deficiency. Similarly, in comparisons of data for the same species among the three host treatments (c, d and e) by Tukey’s HSD test, significant decreases \( (P < 0.05) \) in spore germination with preservation period are indicated by a downward arrow \( (d \rightarrow c) \), a downward single arrowhead \( (e \rightarrow c) \), and a downward double arrowhead \( (e \rightarrow c, \text{and } e \rightarrow d) \) in the dark boxes at the bottoms of (d) and (e). Il, *Inocybe lacera*; La, *Laccaria amethystina*; Li, *Laccaria laccata*; Lm, *Laccaria murina*; Sb, *Scleroderma bovista*; Rp, *Russula pectinatoides*; Cd, *Cortinarius decipiens*; I2, *Inocybe sp. 2*; Id, *Inocybe dulcamara*; Hm, *Hebeloma mesophaeum*; Hl, *Hebeloma leucosarx*; Hp, *Hebeloma pusillum*; ND, no data.
and significantly greater than those of third-stage fungi (among species. The infectivities of first-stage fungi were significantly effects on EMF infection and together explained 76% of the variability in the infectivity data. Pairwise comparisons also revealed clear differences in spore infectivity among species. The infectivities of first-stage fungi were significantly greater than those of third-stage fungi (C. decipiens and R. sororia; Fig. 4).

Discussion

Spore germination of EMF species is often enhanced by host roots (Bowen, 1994, and references therein), particularly at short distances (<1 mm) from roots (Ali & Jackson, 1988). Several chemicals released by plant roots have been identified as triggers of EMF spore germination (Fries et al., 1987; Kikuchi et al., 2007). In this study, spore germination significantly increased in nine of 12 EMF species in the presence of host roots, but similar stimulatory effects were not observed in the presence of nonhost roots. Thus, specific chemicals released from host plants probably enhance spore germination in a wide range of, but not all, EMF species. Several chemicals released by plant roots have been identified as triggers of EMF spore germination (Fries et al., 1987; Kikuchi et al., 2007). In this study, spore germination significantly increased in nine of 12 EMF species in the presence of host roots, but similar stimulatory effects were not observed in the presence of nonhost roots. Thus, specific chemicals released from host plants probably enhance spore germination in a wide range of, but not all, EMF species. In fact, no spore germination was observed in any of the replicates of C. decipiens and I. dulcamara, even in the presence of host roots.

Spore germination in the presence of host roots significantly differed among EMF species, and the observed patterns corresponded to the EMF successional sere in this desert ecosystem. The germination rates of all first-stage fungi in the primary successional sere were among the highest of all EMF species examined (Fig. 3c). The first-stage fungi also exhibited high infectivities in the spore inoculation experiments. Our results strongly support the hypothesis that high germination rates and high infectivities of spores are important prerequisites for initial colonization of nonmycorrhizal habitats. Similarly, Fox (1986) and Bowen (1994) suggested that spores of ‘early-stage fungi’ respond well to hosts. This general idea has rarely been rigorously tested; thus, our study, which used many replicates from a wide range of EMF species, provides the first convincing and direct evidence in support of this hypothesis.

Fox (1986) and Bowen (1994) also suggested that ‘late-stage fungi’ respond weakly to hosts. As predicted, all third-stage fungi (except Hebeloma species) in the primary successional sere in this desert ecosystem exhibited weak responses to host roots. However, germination in Hebeloma species exhibited high germination rates and high infectivities comparable to those of first-stage fungi. This apparent contradiction indicates that other factors appear to constrain the colonization of Hebeloma species. In the desert habitat on Mt Fuji, all Hebeloma species prefer relatively developed soils (rich, organic conditions) to poorly developed pioneer habitats. In addition, Hebeloma sporocarps are usually produced near the end of the plant growing period (Nara et al., 2003a,b). This trend makes it impossible for current-year Salix seedlings to use current-year Hebeloma spores, because Salix seeds are dispersed and germinate in the spring. Therefore, the soil preferences and fruiting season of Hebeloma species may prevent them from being first-colonizers at this study site. Interestingly, some Hebeloma species could be ‘early-stage fungi’ in certain secondary successional settings, in which the soil has already developed and host trees have already been transplanted (Deacon et al., 1983; Fleming, 1983; Last et al., 1983).

The germination rates and infectivities of second- and third-stage fungi, excluding Hebeloma spp., were lower than those of first-stage fungi. These results may explain why these are not pioneer EMF. However, the weak responses of these late colonizers to host roots do not explain why they colonize during later stages. In the present study, the effect of host roots on spore germination was studied by using young seedlings,
not mature trees. Thus, it may be possible that stimulants released only from mature trees improve spore germination. In addition to the effects of hosts, it is also known that spore germination of some EMF species increases with the presence of bacteria and fungi isolated from sporocarps (Ali & Jackson, 1989), conspecific fungal mycelia (Iwase, 1992), and mineral nutrients (Theodorou, 1993). Therefore, together with the possible stimulation by older host trees, factors other than host-derived stimulants are probably involved in the colonization processes of late colonizers. It might be interesting to study the effects of pre-existing mycelia and mycorrhizas of early stage EMF on the spore germination of late stage EMF, particularly in the context of the ecological strategies and successional positions of these species.

Unexpectedly, we observed a considerable intraspecific variation in spore germination in the presence of host roots (especially in the 0-d and 30-d treatments). GLM analyses indicated that this variability was attributable in part to variance among host individuals (which explained an average of 48% of intraspecific variability in spore germination), whereas the remaining variability included variance attributable to sporocarp individuals. These results indicate that genetic differences and the physiological condition of hosts as well as differences in sporocarp condition (e.g. dryness) and sporocarp genotypes probably affect EMF spore germination.

We also found that the majority of spores decreased in activity level (both germination and infectivity) with time, especially after 1 yr, in all EMF species examined. Because the environmental conditions in the field are more variable than the experimental conditions in this study, spore activity should decline even more quickly in nature. Thus, the establishment of a spore bank may be difficult for most EMF species in this desert environment. In fact, a spore bank was not available for most seedlings transplanted at this site (Nara & Hogetsu, 2004). Miller et al. (1994) also found that most EMF spores observed in the fall disappear until the following spring. Although the existence of a spore bank has been confirmed in some EMF taxa, especially suillusoid species (Horton et al., 1998; Ashkannejhad & Horton, 2006), most EMF taxa may rely more on instantaneous colonization after spore dispersal than on sporocarps. This particular strategy of EMF spores is disadvantageous for colonizing nonmycorrhizal habitats in this desert (see Nara & Hogetsu, 2004; Nara, 2006b), although spores are effective EMF sources for hosts that have already established.

In conclusion, our study demonstrated that spore characteristics (germination and infectivity) of EMF species (especially first-stage fungi) corresponded well with their ecological traits in the primary successional sere in the desert ecosystem on Mount Fuji. Although there is growing indirect evidence that EMF spores are important to the development and maintenance of EMF populations and communities, unequivocal direct evidence is scarce. By analyzing spores of all dominant EMF species in a primary successional desert, we revealed that spore germination and infectivity were significantly affected by the EMF species, host roots, preservation period, and the interactions of these factors. We also quantified the extent to which these factors explained variation in spore germination and infectivity data, thus providing initial data for subsequent EMF successional model development. Our results clearly indicated that EMF spores are key to understanding the ecology of EMF fungi and warrant further investigation.

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