

Ectomycorrhizal networks and seedling establishment during early primary succession

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Summary

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- Ectomycorrhizal (ECM) fungal mycelia are the main organs for nutrient uptake in many woody plants, and often connect seedlings to mature trees. While it is known that resources are shared among connected plants via common mycorrhizal networks (CMNs), the net effects of CMNs on seedling performance in the field are almost unknown.
- CMNs of individual ECM fungal species were produced in an early succession volcanic desert by transplanting current-year seedlings of *Salix reinii* with ECM mother trees that had been inoculated with one of 11 dominant ECM fungal species.
- Most seedlings were connected to individual CMNs without being infected by other ECM fungi. Although control seedlings showed poor growth under severe nutrient competition with larger nonmycorrhizal mother trees, nutrient acquisition and growth of seedlings connected to CMNs were improved with most fungal species.
- The positive effects of CMNs on seedling performance were significantly different among ECM fungal species; for example, the maximum difference in seedling nitrogen acquisition was 1 : 5.9. The net effects of individual CMNs in the field and interspecific variation among ECM fungal species are shown.

Key words: ectomycorrhizal fungi, facilitation, field experiment, *Inocybe*, interspecific differences, mycelial connections, nutrient competition, *Russula*.

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Introduction

Ectomycorrhizal (ECM) mycelia radiating from ectomycorrhizae function as primary organs for absorption of nutrients in many host plants (see review by Leake *et al.*, 2004), and many woody plants in forest ecosystems are dependent on ECM fungi for their growth and survival. In addition, ECM mycelia radiating from a plant function as a source of ECM infection for neighbouring host plants, and form common mycorrhizal networks (CMNs) that connect a number of different host plants (Newman, 1988; Read, 1997). Some *in vitro* experiments have shown that carbon and nutrients are shared among connected host plants (Finlay & Read, 1986a, 1986b; Arnebrant *et al.*, 1993; He *et al.*, 2004). Therefore, CMNs may affect the growth of host plants in the field (see review by Simard & Durall, 2004).

There is limited information on the role and effect of CMNs of ECM fungi in the field. In forests, the majority of ECM fungi are shared among different canopy trees (Horton & Bruns, 1998; Cullings *et al.*, 2000); canopy trees and understory plants (Horton *et al.*, 1999; Kennedy *et al.*, 2003); and mature and juvenile plants (Jonsson *et al.*, 1999; Matsuda & Hijii, 2004). CMNs may therefore be widespread. However, sharing of the same ECM fungal species by different hosts does not necessarily indicate a direct connection among the host plants. In most ECM habitats, genetically different units, or genets, of the same ECM fungal species usually exist in close proximity (Redecker *et al.*, 2001). Different genets of the same ECM fungal species may therefore colonize neighbouring hosts. In addition, even if the mycobionts on neighbouring hosts belong to the same genet, the genet may be isolated physiologically by fragmentation (Wu *et al.*, 2005). To date,

the abundance and frequency of CMNs in the field are largely unknown.

It is difficult to examine accurately the effects of CMNs on plant growth in natural ecosystems. Soil trenching can be used to sever host plants from CMNs, and may provide information concerning the effects of CMNs on plant growth (Simard *et al.*, 1997; Booth, 2004). However, the results of soil-trenching experiments are confounded by the reduced underground competition resulting from cutting roots and ECM mycelia of neighbouring plants. This is of considerable significance, because below-ground competition for nutrients is an important determinant of seedling growth in natural settings (Berntson & Wayne, 2000). Soil trenching also alters the movement of soil organisms and the status of pathogenic and saprotrophic fungi (Fisher & Gosz, 1986). Other approaches, such as soil transfer (Perry *et al.*, 1989), or comparison of transplants in different positions (Newbery *et al.*, 2000; Dickie *et al.*, 2002; Nara & Hogetsu, 2004), may reveal possible effects of CMNs, but are also confounded by differences in soil, light and competitive interactions. Moreover, all previous studies using these approaches were affected by the formation of new ectomycorrhizae from spores. Such ECM contamination makes it difficult to evaluate the net effects of CMNs in the field, because the net CMN effects cannot be separated from the effects of ECM contamination. Determination of the net effects of CMNs in the field requires an experiment that reproduces CMNs naturally at sites where ECM infection from spores does not occur. However, such ideal conditions are rarely found; even at the foot of receding glaciers or in severely burned forests, seedlings of ECM host species are quickly colonized by ECM fungi from spores (Horton *et al.*, 1998; Helm *et al.*, 1999). Therefore, no information is currently available for accurate evaluation of the effects of CMNs on plant growth and vegetation development in natural ecosystems.

The south-east slope of Mount Fuji, Japan, resembles a sea of volcanic desert with islands of vegetation. The total vegetation cover is only 5–6% at elevations of 1500–1600 m, suggesting that this area is at an early stage of primary succession. At this site, *Salix reinii* (an alpine dwarf willow) is found in some vegetation patches and is the dominant ECM host, contributing over 99% of the total ECM host cover (Nara *et al.*, 2003a). Seedlings of *S. reinii* and two timber tree species cannot form any ectomycorrhizae unless they are located near established *S. reinii* shrubs (Nara & Hogetsu, 2004). Furthermore, ECM fungal colonization improves the growth of *S. reinii* seedlings near the established shrubs (Nara & Hogetsu, 2004). This facilitative effect of mature *S. reinii* on seedling establishment results in the formation of *S. reinii* patches (Lian *et al.*, 2003), and drives vegetation succession in this volcanic desert (Nara & Hogetsu, 2004). This suggests that CMNs of established shrubs may play an important role in seedling recruitment, although the effects of CMNs have not been distinguished from the effects of ECM colonization from spores.

Moreover, the effect of each fungal species remains unknown because several fungal species are often found on a seedling near naturally established shrubs (Nara & Hogetsu, 2004).

A field experiment was conducted using CMNs of 11 different ECM fungal species isolated from the study site to determine the net effects of mycelial networks of individual ECM fungal species on *S. reinii* seedling establishment in the volcanic desert of Mount Fuji, Japan. CMNs between current-year seedlings and large juveniles of *S. reinii* were reproduced successfully in all 11 fungal treatments without being infected by other ECM fungi. The net effects of CMNs on seedling establishment in the field were determined and compared among the 11 ECM fungal species.

Materials and Methods

Research site

The field experiment was conducted in a 100 × 550-m quadrat described previously by Nara *et al.* (2003a, 2003b); Nara & Hogetsu (2004). The quadrat was located 1500–1600 m above sea level on the south-east slope of volcanic Mount Fuji. The last eruption, which occurred in 1707, was intense and completely destroyed the vegetation on the south-east slope of the volcano. Although the annual precipitation in this area is nearly 500 cm, vegetation recovery is very slow because of the unstable and nitrogen-poor scoria substrate. Consequently, the tree line is located at *c.* 1300 m on the south-east slope, whereas it is located at *c.* 2500 m on other aspects of the mountain. Vegetation in the quadrat was patchily distributed and formed islands of vegetation in the volcanic desert. Total vegetation cover in the quadrat was *c.* 5%. Although N-fixing plants sometimes play an important role at early primary succession sites in glacial areas (Chapin *et al.*, 1994; Hobbie *et al.*, 2000) and volcanic deserts (Walker & del Moral, 2003), this did not occur on Mount Fuji. There was no N-fixing alder in the study quadrat. Only a relatively late-colonizing leguminous plant species, *Hedysarum vicioides*, was present in 22 of 159 vegetation patches (Nara *et al.*, 2003a); however, the cover contributed by this plant was less than 1% of each vegetation patch. A more detailed description of the research site is given by Nara *et al.* (2003a, 2003b); Nara & Hogetsu (2004).

The alpine dwarf willow *S. reinii* is the pioneer ECM host species at this site, and comprises *c.* 20% of the total vegetation cover. *Salix reinii* of a range of sizes inhabited 37 of the 159 vegetation patches in the quadrat. All established *S. reinii* individuals were intensively colonized with ECM fungi, but only rarely with arbuscular mycorrhizal fungi. Seedlings transplanted close to established *S. reinii* shrubs readily formed ectomycorrhizae, while most seedlings transplanted into bare ground or vegetation patches containing no *S. reinii* lacked ectomycorrhizae after an entire growing season (Nara & Hogetsu, 2004). The natural establishment of *S. reinii* always occurs at the periphery of vegetation patches; these events are

Table 1 Fungal strains of ectomycorrhizal fungi used in a field experiment in an early successional volcanic desert on Mount Fuji, Japan

Strain	Species	Accession no.*
CeG02	<i>Cenococcum geophilum</i>	AB211277
HeL01	<i>Hebeloma leucosarx</i> Orton	AB211268
HeM01	<i>Hebeloma mesophaeum</i> (Pers.) Quél.	AB211272
HeP131	<i>Hebeloma pusillum</i> Lange	AB211274
InL83	<i>Inocybe lacera</i> (Fr.) Kumm.	AB211269
LaA01	<i>Laccaria amethystina</i> Cooke	AB211270
LaL01	<i>Laccaria laccata</i> (Scop. Fr.) Berk. & Br.	AB211273
LaM90	<i>Laccaria murina</i> Imai	AB211271
RuP01	<i>Russula pectinatoides</i> Peck	AB211276
RuS01	<i>Russula sororia</i> (Fr.) Romell	AB211275
ScB84e	<i>Scleroderma bovista</i> Fr.	AB211267

*Sequence data of nuclear ribosomal DNA, including complete regions of internal transcribed spacer (ITS)1, 5.8S ribosomal RNA and ITS2, were submitted to the DNA Data Bank of Japan (DDBJ).

rare, probably because of low natural infection rates by ECM fungi. However, this observation indicates that invading seedlings can survive in this position once ECM colonization occurs. The peripheries of vegetation patches lacking *S. reinii* are therefore ideal for testing the effects of target ECM fungi in the field without unwanted ECM contamination. The transplant experiment was conducted on the periphery of three vegetation patches that lacked *S. reinii*.

Isolation and maintenance of ECM fungal strains

Modified Melin–Norkrans (MMN) agar plates with chlorotetracycline (30 mg l⁻¹) were used for the isolation of 11 species of ECM fungi (Table 1). All strains except *Cenococcum geophilum* were isolated from sporocarps collected at the research site in 1999 and 2000. *Cenococcum geophilum* was isolated from a sclerotium collected at the research site. The identities of isolated strains were confirmed by comparing DNA sequences of internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (rDNA), including the complete region of ITS1 and ITS2, with those of the original sporocarps and sclerotium. The details of this molecular analysis are described below. Sequence data were submitted to the DNA Data Bank of Japan (DDBJ), and accession numbers are listed in Table 1. All isolated strains of the 11 ECM fungal species were maintained on MMN agar plates at 20°C and subcultured every 3 months until use. These fungal strains are available from the author on request.

Production of ECM mother trees

Salix reinii seeds were collected near the research site from late June to early July 2001, and stored at 4°C until use. Seeds were placed on an autoclaved (121°C, 180 min) mixture of Shibano soil and Tanashi nursery soil (1 : 1 vol) after surface sterilization in 1.0% NaClO solution (Wu *et al.*, 1999).

Seedlings were maintained in a growth chamber for 1 month (25°C, 300 µmol photon m⁻² s⁻¹, 15 h light/9 h dark).

Each fungal strain was cultured for 2 months at 25°C in rectangular polystyrene plates (140 × 100 × 15.5 mm) containing 25 ml MMN agar medium. MMN agar plates containing no fungal mycelia were prepared as a control treatment. After the culture period, one short side of each plate was removed to form an open-topped container for plants.

The root systems of five 1-month-old seedlings were placed on the fungal mycelium cultured on MMN agar in each container. The container was then filled with the autoclaved soil mixture and maintained in a growth chamber for 10 months. Above-ground parts of the seedlings remained outside the containers. Two replicate containers were prepared for each fungal species. Although all seedlings formed extensive ECM tips, seedling size varied among individuals and fungal species. Three similarly sized seedlings were used as ECM mother trees for each fungal species, or as nonmycorrhizal mother trees for the control treatment.

Co-transplanting ECM mother trees and current-year seedlings of *S. reinii*

Empty containers were established from new rectangular plates as described above. In early July 2002, an ECM mother tree or a nonmycorrhizal mother tree was planted in the centre of each container, and containers were filled with autoclaved scoria substrate collected from the research site. At the same time, 20 surface-sterilized seeds of *S. reinii* were sown around the mother tree in each container. Three replicate containers were prepared for each fungal species, and were maintained in a temperature-regulated glasshouse for 4 wk until the germinants were of sufficient size to tolerate transplantation to the field (Fig. 1).

At 3 wk after sowing, the number of germinants in each container was reduced to five. In early August 2002, each container that had five germinants and a mother tree was buried in the scoria ground as a transplanting unit. One large side of each container was removed to allow roots and mycelia to grow into the field scoria. Great care was taken not to disturb the tiny root systems of the germinants and the developing ECM mycelia. Containers were placed at 20-cm intervals along the periphery of vegetation patches lacking established willow shrubs. Three replicate units for each fungal species or control were transplanted to three different vegetation patches. In total, 36 mother trees and 180 current-year seedlings were transplanted into the volcanic desert.

Analysis of transplanted seedlings

In late October 2002, the numbers of surviving current-year seedlings were counted. In early November 2002, all transplanted current-year seedlings, including the entire root system, were removed. The transplants were washed carefully with tap water, and scoria particles and debris were removed from the root systems under a dissecting microscope. The numbers of root

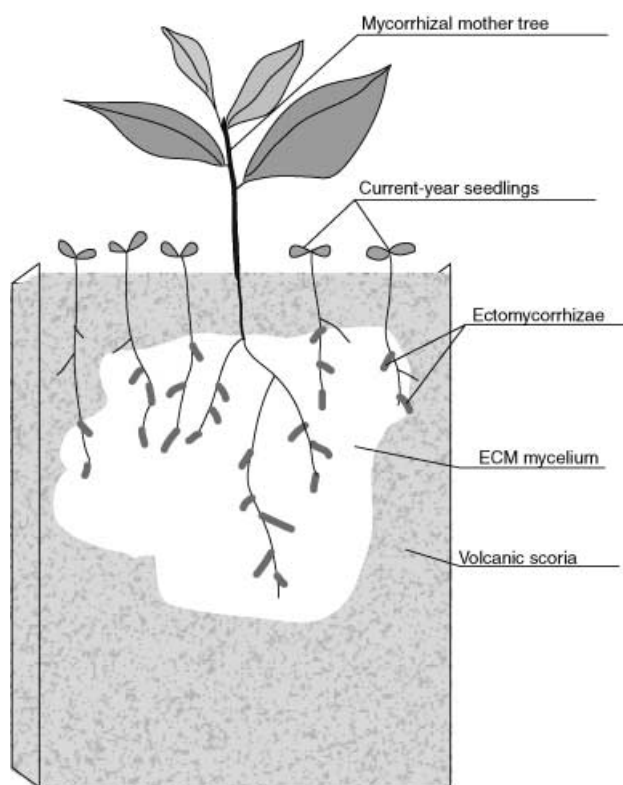


Fig. 1 Example of a common mycorrhizal network of an ectomycorrhizal fungus reproduced in an early successional volcanic desert.

tips and ECM tips were counted under a dissecting microscope. After all ECM tips had been morphotyped (Nara *et al.*, 2003b), two ECM root tips per morphotype for each seedling were placed in two different 2.0-ml tubes and dried in a vacuum (FDU-540, EYELA, Tokyo, Japan) for molecular identification, as described below. The number of winter buds was also recorded. Seedling shoot and root dry weights were measured using an ultra-microbalance (UMT2, Mettler Toledo, Greifensee, Switzerland) after drying in a vacuum. Individual shoot and root N and phosphorus contents were measured colorimetrically after digestion with sulfuric acid and hydrogen peroxide, using the indophenol blue method and the ascorbic acid deoxidizing molybdenum blue method, respectively. Nitrogen and P contents of five *S. reinii* seeds were also measured in four replicate sets.

ITS terminal RFLP analyses

The methods used in DNA extraction, PCR amplification, and terminal restriction fragment length polymorphism (T-RFLP) analyses for ECM tips and isolated ECM fungal strains were as described by Nara *et al.* (2003b). Briefly, DNA was extracted from individual ECM root tips using the cetyl trimethyl ammonium bromide (CTAB) method. DNA was also extracted from a mycelial disk from each ECM isolate that had been cultured on MMN agar plates for 1 month, and

from a small piece (*c.* 1 mm³) of each sporocarp used for the isolation. Extracted DNA was dissolved in 200 µl TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and stored at -30°C until use. PCR amplification was conducted using AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) with an annealing temperature of 51°C, and following the manufacturer's instructions for all other PCR conditions. PCR products (ITS₃₋₄) from primers ITS3 and ITS4 (White *et al.*, 1990; Gardes & Bruns, 1993), where the primer ITS4 was labelled with Texas Red, were diluted 15 times with TE buffer and used for the first T-RFLP analysis. For a second T-RFLP analysis, 2 µl of each PCR product amplified using ITS1F and ITS4, where the primer ITS1F was labelled with Texas Red, were digested with 8 µl *Hinf*I solution (1061A, Takara Shuzo Co., Shiga, Japan) at 37°C for 8 h, following the manufacturer's instructions. The digested solution containing the fragment from the ITS1F primer site to the *Hinf*I digestion site (ITS_{1F-Hinf}I) was diluted twice with TE buffer. Each fragment (ITS₃₋₄ or ITS_{1F-Hinf}I) was electrophoresed in a DNA sequencer (SQ-5500E, Hitachi Electronics Engineering Co., Tokyo, Japan), and the length of each fragment was estimated from size standards using FRAGLYS 3.0 software (Hitachi). Fragment sizes from ECM tips were compared with those of isolated fungal strains. In total, 312 root tips were included in this molecular analysis, and 245 provided results in both T-RFLP analyses.

Sequence analysis

ITS regions of DNA templates from the isolated strains and sporocarps, described above, were amplified using nonlabelled ITS1F and ITS4 primers. PCR products were purified using a PCR Product Pre-Sequencing Kit (PN:70995, USB Co., Cleveland, OH, USA) in accordance with the manufacturer's instructions. Then 3.5 µl of each purified product was mixed with 4 µl DTCS Quick Start Master Mix (PN:608120, Beckman Coulter Inc., Fullerton, CA, USA), 1 µl of a sequencing primer solution (1.6 µM), and 1.5 µl pure water in a 0.2-ml PCR tube. ITS1F and ITS3 sequencing primers were used initially, and followed by ITS2 and ITS4 primers, to obtain the complete sequence of the internal transcribed spacer 1 and 2 regions and the 5.8 S ribosomal RNA region. Conditions for sequencing reactions in a thermal cycler, sample plate preparation, and conditions for electrophoresis in a capillary sequencer (CEQ8800, Beckman Coulter) followed standard methods described in the Beckman User's Manual.

Statistics

Values are presented as mean ± SE unless otherwise specified. All statistical analyses were conducted using SPSS ver. 11.5 with Exact Test option (SPSS Inc., Chicago, IL, USA). Differences among the 11 fungal treatments were examined using Kruskal-Wallis tests for the number of winter buds, number of ECM tips, total number of root tips, shoot and root dry weights,

shoot and root N contents, and shoot and root P contents. Control and individual fungal species were compared using Mann–Whitney tests. Shoot and root dry weights of mother trees, including nonmycorrhizal mother trees, were compared among treatments using Kruskal–Wallis tests.

Results

At the end of the growing season, current-year seedlings accompanying nonmycorrhizal mother trees (control treatment) had not formed any ECM root tips (Table 2). In contrast, all surviving current-year seedlings accompanying mycorrhizal mother trees formed ectomycorrhizae, although the number of ECM root tips per seedling varied among fungal species ($P < 0.001$, Table 2). The mycobiont on each seedling was confirmed to be the same as that on the mycorrhizal mother trees using T-RFLP analyses. Mycobiont identities were also confirmed to be identical to those of the fungal inoculation isolates in all treatments using T-RFLP analyses. Furthermore, colonization of other ECM fungi was not detected on any of the seedlings. These results indicate that all fungal species formed CMNs between mycorrhizal mother trees and current-year seedlings without contamination by other ECM fungi.

Although mycorrhizal and nonmycorrhizal mother trees were 16 months old at the end of the transplant experiment, their size was far larger than naturally established seedlings of the same age. This was because of the nutrient-rich nursery soil and additional nutrients from the MMN used in mother tree preparation. Shoot dry weight of individual mother trees ranged from 20 to 96 mg. The shoot dry weight was not significantly different among treatments, including the control treatment ($P = 0.141$), and the mean shoot dry weight of all mother trees was 48 ± 3 mg. Root dry weight of mother trees was also not significantly different among treatments ($P = 0.133$). The mean root dry weight of all mother trees was 224 ± 16 mg, 70 times greater than the mean root dry weight of all current-year seedlings.

The dry weight of roots was always greater than that of shoots in all current-year seedlings. Moreover, root dry weight was two or more times greater than shoot dry weight in most treatments, including the control (Table 2). Root-to-shoot ratios of current-year seedlings did not differ among treatments ($P = 0.120$).

Current-year seedlings in the control treatment grew poorly and had the smallest number of winter buds and the lowest shoot dry weight (Table 2). In contrast, the growth of seedlings connected to mycelial networks was greater than that of controls for most fungal species treatments, as indicated by larger numbers of winter buds and greater shoot dry weights (Table 2). For example, mean dry weights of seedlings connected to CMNs of *Hebeloma leucosarx*, *C. geophilum* and *Russula sororia* were 7.5 ± 0.7 , 6.9 ± 0.5 and 6.6 ± 1.0 mg, respectively. These dry weights were 4.1, 3.8 and 3.6 times that of control seedlings (1.8 ± 0.2 mg), respectively. Seed-

lings connected to *Laccaria amethystina* were the exception to this improvement in growth; all growth parameters were not significantly different from those of control seedlings for this ECM fungal species ($P > 0.05$, Table 2). Therefore, the degree of improvement in seedling growth varied among ECM fungal species in numbers of winter buds ($P < 0.001$), shoot dry weights ($P < 0.001$) and root dry weights ($P < 0.001$).

The range of dry weights was wider in seedlings associated with ECM fungi than in control seedlings. In particular, *R. sororia*, *H. leucosarx* and *Inocybe lacera* treatments produced the largest seedlings, at 7.5, 7.4 and 6.4 times, respectively, the mean size of control seedlings (Fig. 2a).

Nitrogen and P contents of *S. reinii* seeds were 6.2 ± 0.7 and 1.2 ± 0.1 μg per seed, respectively. Thus, all seedlings, including those in the control treatment, absorbed both nutrients during growth (Fig. 2b,c). Seedling N and P contents differed significantly among the 11 ECM fungi ($P < 0.001$). Most ECM fungal species significantly improved the absorption of both nutrients (Table 2). For example, the amount of N in each seedling was 103 ± 13 and 96 ± 14 μg in *H. leucosarx* and *R. sororia* treatments, respectively, while that in control seedlings was 19 ± 4 μg . Similarly, the amount of P in each seedling was 15.8 ± 2.1 and 12.8 ± 2.9 μg in *H. leucosarx* and *R. sororia* treatments, respectively, while that in control seedlings was 2.9 ± 0.3 μg . In contrast, *L. amethystina* treatment did not improve the P and N status of seedlings over that of control seedlings (Table 2).

Discussion

Fungal species used in the field experiment

The relative abundance of the 11 ECM fungal species examined amounted to 93% of all ECM sporocarps (Nara *et al.*, 2003a); 84% of the underground ECM root tips in naturally established shrubs (Nara *et al.*, 2003b); and 99% of the ECM root tips on transplanted seedlings (Nara & Hogetsu, 2004) in this early successional volcanic desert. These 11 ECM fungal species are therefore appropriate representatives of the ECM fungi present at this site. This exhaustive approach, rather than the use of a small number of easily cultured ECM fungi, was necessary to evaluate accurately the effects of ECM fungal species in the field.

ECM fungi in the genus *Russula* are thought to grow poorly or not at all in major types of culture media, and have rarely been used in inoculation experiments (Taylor & Alexander, 1989; Dunabeitia *et al.*, 1996). The genus *Inocybe* has also rarely been studied, having been isolated, cultured and used in inoculation in only one experiment (Cripps & Miller, 1995). In the present study, two *Russula* species and *I. lacera* were successfully isolated from sporocarps. Furthermore, ectomycorrhizae of these fungal strains were formed with *S. reinii* during mother tree preparation. The identity of these strains was confirmed by comparison of the rDNA sequence in the ITS

Table 2 Performance of transplanted current-year *Salix reinii* seedlings in connection with common mycorrhizal networks of individual ectomycorrhizal fungal species in an early successional volcanic desert on Mount Fuji, Japan

Fungal species	Survival† (of 15)	Winter buds (per seedling)	ECM tips (per seedling)	Total tips (per seedling)	Shoot DW (mg per seedling)	Root DW (mg per seedling)	Shoot N (µg per seedling)	Root N (µg per seedling)	Shoot P (µg per seedling)	Root P (µg per seedling)
Control	8 (6)	2.2 ± 0.5	0 ± 0	37 ± 5	0.56 ± 0.09	1.27 ± 0.16	6.3 ± 1.4	12.9 ± 2.5	1.2 ± 0.1	1.7 ± 0.3
<i>Cenococcum geophilum</i>	15 (8)	4.4 ± 0.3**	30 ± 10***	122 ± 14***	1.99 ± 0.17**	4.90 ± 0.36***	30.2 ± 2.4***	52.7 ± 4.4***	3.4 ± 0.4***	8.0 ± 0.6***
<i>Hebeloma leucosarx</i>	13 (14)	4.5 ± 0.3**	71 ± 10***	96 ± 8***	2.49 ± 0.21***	5.02 ± 0.57***	38.4 ± 5.3***	65.0 ± 9.2***	5.5 ± 0.8***	10.3 ± 1.4***
<i>Hebeloma mesophaeum</i>	15 (8)	3.8 ± 0.4*	40 ± 13***	86 ± 15**	1.30 ± 0.19**	2.68 ± 0.54**	14.4 ± 2.1*	22.1 ± 4.6*	1.9 ± 0.2*	3.5 ± 0.7*
<i>Hebeloma pusillum</i>	14 (13)	3.8 ± 0.3*	57 ± 8***	85 ± 8***	1.63 ± 0.09***	3.25 ± 0.32***	23.1 ± 1.6***	40.8 ± 3.5***	3.1 ± 0.2***	6.1 ± 0.5***
<i>Inocybe lacera</i>	14 (10)	4.0 ± 0.4*	72 ± 13***	123 ± 15***	1.88 ± 0.26***	4.16 ± 0.76**	26.3 ± 6.0***	41.4 ± 9.4**	4.3 ± 1.0***	8.4 ± 2.0**
<i>Laccaria amethystina</i>	14 (13)	2.4 ± 0.2	32 ± 3***	41 ± 3	0.66 ± 0.05	1.25 ± 0.13	8.8 ± 1.3	9.3 ± 1.3	1.5 ± 0.2	2.7 ± 0.7
<i>Laccaria laccata</i>	13 (7)	3.4 ± 0.6	66 ± 12***	90 ± 17**	1.47 ± 0.19**	2.89 ± 0.67*	19.0 ± 4.3**	21.6 ± 4.9	2.8 ± 0.8	5.0 ± 2.3
<i>Laccaria murina</i>	11 (10)	3.6 ± 0.4*	60 ± 6***	85 ± 11**	1.39 ± 0.16**	2.61 ± 0.34*	19.3 ± 3.2**	26.6 ± 5.3	2.8 ± 0.3**	4.6 ± 0.6**
<i>Russula pectinatoides</i>	14 (10)	3.1 ± 0.3	22 ± 5***	54 ± 7	1.10 ± 0.19*	2.38 ± 0.36*	14.8 ± 3.9	28.6 ± 7.1*	1.9 ± 0.3	4.4 ± 0.8*
<i>Russula sororia</i>	13 (12)	4.2 ± 0.3**	4 ± 2*	78 ± 11**	2.42 ± 0.30***	4.15 ± 0.70***	33.7 ± 4.4***	62.0 ± 10.1***	3.9 ± 0.6***	8.0 ± 1.4***
<i>Scleroderma bovista</i>	13 (13)	3.7 ± 0.3*	9 ± 3**	83 ± 9***	1.80 ± 0.15***	3.21 ± 0.25***	27.3 ± 3.0***	41.2 ± 4.7***	3.5 ± 0.6***	7.3 ± 1.1***
Kruskal–Wallis <i>P</i> value‡	–	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Values are mean ± SE; *, **, *** indicate statistically significant differences compared with the control treatment (Mann–Whitney tests) with $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

†Number of seedlings surviving out of 15 transplanted seedlings followed by number of undamaged seedlings in parentheses. Only undamaged seedlings were used in this study.

‡Differences among the 11 ECM fungal treatments were tested using Kruskal–Wallis tests. *P* values shown are from tests excluding the control treatment; similar results were obtained when the control treatment was included.

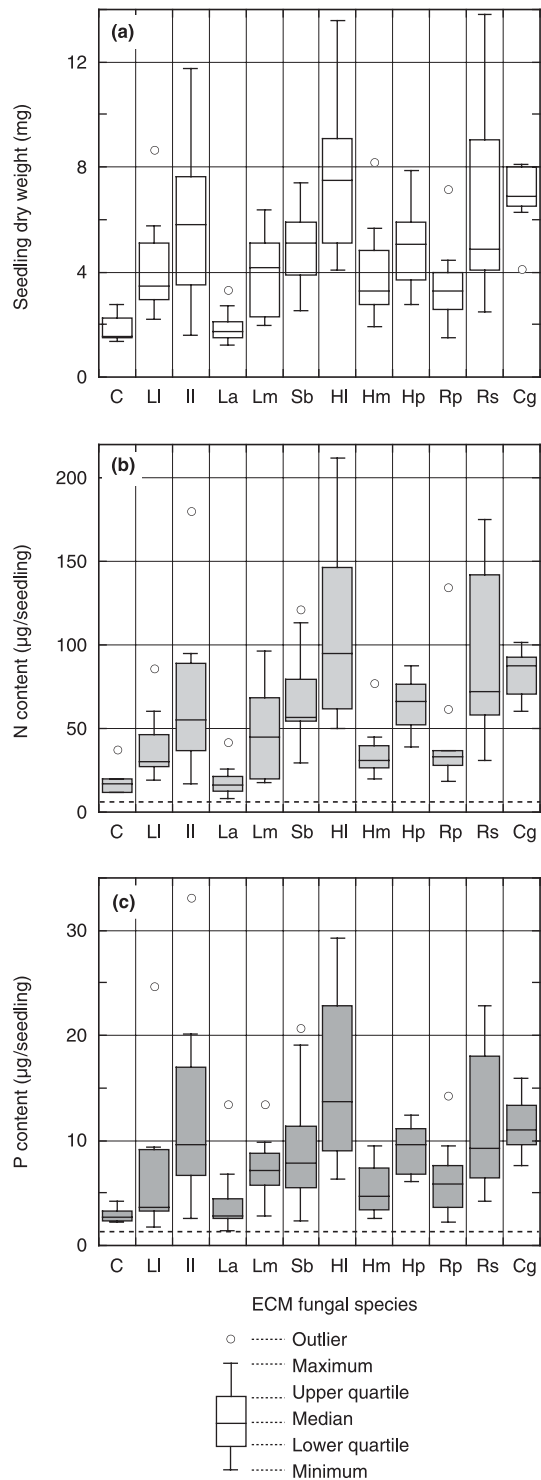


Fig. 2 Effects of common mycorrhizal networks of individual ectomycorrhizal fungal species on (a) dry weight; (b) nitrogen content; (c) phosphorus content of current-year *Salix reinii* seedlings in an early successional volcanic desert on Mount Fuji, Japan. C, Control; LI, *Laccaria laccata*; II, *Inocybe lacera*; La, *Laccaria amethystina*; Lm, *Laccaria murina*; Sb, *Scleroderma bovista*; HI, *Hebeloma leucosarx*; Hm, *Hebeloma mesophaeum*; Hp, *Hebeloma pusillum*; Rp, *Russula pectinatoides*; Rs, *Russula sororia*; Cg, *Cenococcum geophilum*. Broken lines indicate the mean for *S. reinii* seeds.

regions of the cultured mycelia and sporocarps. Moreover, the ITS sequences of these fungal cultures had the highest BLAST values compared with other ITS sequences of the same species or closely related species in DDBJ/EMBL/GenBank, indicating that these strains were correctly identified (Table 1; BLAST results not shown). *Russula* is one of the dominant ECM fungi in many forest ecosystems (Horton & Bruns, 2001; Peter *et al.*, 2001; Matsuda & Hijii, 2004); *Inocybe* is widely distributed from the Arctic to the tropics, and is sometimes dominant in harsh environments and nurseries (Cullings & Makhija, 2001). Although the relative abundance of ectomycorrhizae may not always indicate the functional significance of ECM fungi, more attention should be paid to the functional aspects of *Russula* and *Inocybe*. This study is an important step in this direction.

Ecological significance of CMNs

In previous field studies of the ecological function of CMNs, there was no evidence that plants were directly connected with mycelia belonging to the same genet of ECM fungus. However, direct connection to CMNs is apparent in some *in vitro* microcosm experiments in which mycelia radiating from an ECM plant were used as inocula for surrounding seedlings (Finlay & Read, 1986a, 1986b; Wu *et al.*, 1999). I applied this *in vitro* approach to the field experiments in an attempt to evaluate the ecological functions of CMNs. To evaluate accurately the effects of individual CMNs, it is important that they be kept free from natural colonization by other ECM fungal species. This is impossible in many field sites. However, in the volcanic desert on Mount Fuji, transplanted *S. reinii* seedlings do not form ectomycorrhizae in the absence of established willow shrubs (Nara & Hogetsu, 2004). By applying this new approach at this field site, CMNs were established in all ECM fungal treatments without other ECM contamination. Therefore, the effects of individual CMNs on seedling establishment could be evaluated accurately.

Although seedling dry weight correlated significantly with P content ($r = 0.889$, $P < 0.001$), a stronger correlation was observed between seedling dry weight and N content ($r = 0.935$, $P < 0.001$). Photosynthetic activity of naturally established *S. reinii* is closely correlated with leaf N concentration in this volcanic desert (Nara *et al.*, 2003a). Nitrogen availability appears to be the most important growth-limiting factor at this site, an observation that has been reported from many other primary succession sites (Walker & del Moral, 2003).

In the volcanic desert on Mount Fuji, *S. reinii* seedlings are always found close to established plants, especially established *S. reinii* shrubs (Lian *et al.*, 2003; Nara & Hogetsu, 2004). Thus, newly recruited *S. reinii* seedlings must undergo N competition with larger established plants (Berntson & Wayne, 2000). In our study, control seedlings in competition with nonmycorrhizal mother trees showed poor growth and low N

content. In contrast, 101 of 105 seedlings in CMNs of ECM fungi (other than *L. amethystina*) contained higher levels of N than did control seedlings. This suggests that seedlings lacking the support of ECM fungi could not acquire sufficient N when in competition with larger trees. Therefore, it appears that CMNs generally alleviate competition with larger trees for N.

The number of ECM root tips on a seedling was significantly different among the 11 ECM fungal treatments. This may be caused partly by mycorrhizal morphology. ECM fungi that produce extensive extramatrical mycelia, such as *Hebeloma* spp., formed many ECM tips on neighbouring seedlings in comparison with *Russula* spp., which produce fewer extramatrical mycelia, and *Scleroderma bovista*, which produce developed rhizomorphs but fewer infective mycelia.

The number of ECM root tips did not appear to be an important factor in the growth of seedlings. In the case of the *R. sororia* treatment, only 4 ± 2 ECM root tips significantly improved nutrient acquisition and seedling growth. Radial thickening of root tips and a fully developed mantle do not always accompany the initial stage of ECM colonization. It is also likely that ECM fungi may colonize the nonsuberized region of long roots, as well as the root tips (Jones *et al.*, 1990; Püttsepp *et al.*, 2004). Therefore, ECM infection may occur at locations, other than the root tips, that were not examined under a dissecting microscope. However, results from the present study indicate that only a small number of ECM connections to CMNs are enough to improve the growth of *S. reinii* seedlings if the carbon demand of extensive CMNs is supplied by larger established trees.

Interspecific differences in CMN-mediated facilitation among ECM fungi

The quantity of N in *S. reinii* seedlings differed significantly among the 11 ECM fungal treatments ($P < 0.001$). This is partly the result of variation in preferred N forms and ability to use N among ECM fungal species (Abuzinadah & Read, 1986, 1988). In addition, N transfer from fungi to hosts may differ in magnitude and quality among fungal species (Hobbie *et al.*, 2000).

The ECM fungus *L. amethystina* was not effective in improving seedling N acquisition through shared CMNs with ECM mother trees. However, this same strain improves seedling growth when inoculated as cultured mycelia (data not shown). Thus, N absorbed by *L. amethystina* CMNs may be transferred preferentially to the larger mother trees that supply most of the carbon. In a glasshouse experiment, Kytöviita *et al.* (2003) also demonstrated that the growth-promoting effects of some arbuscular mycorrhizal fungi were invalidated by competition with larger adult plants within a CMN. Therefore, the pattern of N allocation within an individual mycelial network may be affected by the competitive interac-

tions between plants, in addition to significant interspecific variation among ECM fungi.

Another major difference among the ECM fungi is shown in the distribution of data in Fig. 2. For example, the quantity of N in seedlings with *L. amethystina*, *Hebeloma mesophaeum* and *Russula pectinatoides* varied slightly among individuals. In contrast, a few exceptionally large seedlings contained large quantities of N with *H. leucosarx* and *R. sororia*. Although it is unclear how such exceptionally large seedlings were produced, an earlier connection to CMNs or preferential movement of nutrients to a specific seedling in each CMN may have occurred. Current-year seedling size is an important factor predicting survival during the severe winters at this site, as demonstrated in other plant species on Mount Fuji (Maruta, 1983). While most current-year seedlings of *S. reinii* do not survive the first winter in this volcanic desert, some larger seedlings can occasionally survive the winter. Therefore, exceptionally large seedlings associated with some fungal species may be ecologically important.

In the present study, a CMN was examined for each fungal species. A previous study, in which nonmycorrhizal *S. reinii* seedlings were transplanted beside naturally established willow shrubs, showed a significant correlation between seedling growth and the number of colonized ECM fungal species (Nara & Hogetsu, 2004). Dickie *et al.* (2002) also found that increased diversity of ECM infection may contribute to increased N acquisition in *Quercus* seedlings. Although the CMN contribution to ECM infection in these studies was not separated, the CMN of an ECM fungus may be even more effective in combination with other ECM fungi. Allen *et al.* (2003) proposed a matrix model that integrates many different functional parameters of individual mycorrhizal fungi to predict ecosystem functioning; however, a number of functional parameters are not currently available. The present study contributes some reliable functional parameters to the model, particularly regarding the CMN functions of individual ECM fungi.

In conclusion, the CMN functions of most of the dominant ECM fungal species present in a natural ecosystem were examined in a field experiment. Each treatment was free from natural contamination by other ECM fungal species in the field throughout the growth period, allowing for accurate determination of the effects of each ECM fungal species on plants in their natural environment. Thus, the ecological significance of the ECM mycelial network of each fungal species was examined individually. The results generated by this unique experimental design made some important and original contributions to our knowledge in this area. Mycelia spreading from ECM mother trees infected neighbouring seedlings by forming CMNs in all the fungal species examined. Thus, CMNs are not restricted to specific groups of fungal taxa, and may occur in all ECM fungal taxa. Although the CMNs of most ECM fungi alleviated nutrient starvation of seedlings in competition with larger trees, their effects on

seedling growth differed significantly among individual ECM fungal species. These results suggest that CMNs, and their interspecific differences in facilitative effects, are important determinants of seedling establishment and plant community structure during early primary succession.

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