

HIGH ROOT CONCENTRATION AND UNEVEN ECTOMYCORRHIZAL DIVERSITY NEAR *SARCODES* *SANGUINEA* (ERICACEAE): A CHEATER THAT STIMULATES ITS VICTIMS?¹

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Sarcodes sanguinea is a nonphotosynthetic mycoheterotrophic plant that obtains all of its fixed carbon from neighboring trees through a shared ectomycorrhizal fungus. We studied the spatial structuring of this tripartite symbiosis in a forest where *Sarcodes* is abundant, and its only fungal and photosynthetic plant associates are *Rhizopogon ellенаe* and *Abies magnifica*, respectively. We found disproportionately high concentrations of *Abies* roots adjacent to *Sarcodes* roots compared to the surrounding soil. *Rhizopogon ellенаe* colonizes the vast majority of those *Abies* roots (86–98%), and its abundance tends to decrease with increasing distance from *Sarcodes* plants. At 500 cm from *Sarcodes* plants we did not detect *R. ellенаe*, and the ectomycorrhizal community instead was dominated by members of the Russulaceae and Thelephoraceae, which are commonly dominant in other California pinaceous forests. The highly clumped distribution of *Abies*–*R. ellенаe* ectomycorrhizas indicates that *Sarcodes* plants either establish within pre-existing clumps, or they stimulate clump formation. Several lines of evidence favor the latter interpretation, suggesting an unexpected mutualistic aspect to the symbiosis. However, the mechanism involved remains unknown.

Key words: *Abies magnifica*; community structure; mutualism; mycoheterotrophy; parasitism; *Rhizopogon ellенаe*; tripartite symbiosis.

The snow plant, *Sarcodes sanguinea* Torrey (Ericaceae), is the largest (up to several kilograms fresh mass) member of the nonphotosynthetic subfamily Monotropoideae. The genus *Sarcodes* is monotypic and restricted to the mountains of the Sierra San Pedro Mártir of Baja California, the southern ranges and Sierra Nevada of California, and the southern ranges of Oregon (Wallace, 1975). Like all other monotropes, the snow plant is a mycoheterotroph: a nonphotosynthetic plant that obtains its fixed carbon from fungi. This is a successful lifestyle as evidenced by over 400 known species in 87 genera, notably in the families Ericaceae and Orchidaceae (Leake, 1994). Many mycoheterotrophs examined to date are linked to surrounding trees via a shared ectomycorrhizal (ECM) fungus (Furman and Trappe, 1971; Vreeland, Kleiner, and Vreeland, 1981; Cullings, Szaro, and Bruns, 1996; Taylor and Bruns, 1997). This interaction is called “epiparasitism” because the mycoheterotroph indirectly parasitizes the trees (Björkman, 1960), thus cheating the ECM mutualism. Furthermore, some ECM photosynthetic angiosperms are now known to engage in facultative epiparasitism. Simard et al. (1997a, b) demonstrated that in nature carbon can be derived from the better competitor tree species by a poorer one if both are colonized by common ECM fungi, thereby providing a novel mechanism

for overcoming competitive exclusion. It appears that by permanently reversing carbon flow in their favor, nonphotosynthetic epiparasites have evolved to one extreme along a continuum of plant strategies for carbon acquisition.

Extreme host specialization appears to be a general pattern among nonphotosynthetic epiparasites; this contrasts with photosynthetic plants, which typically form mycorrhizas with phylogenetically diverse fungi. Recent studies have shown that some epiparasitic orchids (Taylor and Bruns, 1997) and monotropes (Cullings, Szaro, and Bruns, 1996; C. K. Lefevre, and R. Molina, personal communication, Oregon State University) specialize on highly restricted sets of closely related ECM fungal hosts. In fact, the only exception to this pattern of specialization was the snow plant, which appeared to be a generalist (Cullings, Szaro, and Bruns, 1996). However, we have recently determined that the snow plant is specialized over a large area of the Sierra Nevada of California on the ECM fungus *Rhizopogon ellенаe* A. H. Smith (Bidartondo, Kretzer, and Bruns, 1998), a member of the suilloid lineage of the Boletales (Bruns et al., 1998). Over its entire range, the snow plant may actually form a “geographic mosaic of specialization” (Thompson, 1994).

Although we follow Björkman (1960) in referring to the monotropes as epiparasites, a net cost to either the photosynthetic plant or the fungal associate remains to be shown (Leake, 1994). Epiparasitism is consistent with (a) the heterotrophic habit of the monotropes; (b) the fact that extreme specialization is a common characteristic of parasitic systems (Price, 1980; Thompson, 1994); and (c) evidence for flow of ¹⁴C-labeled glucose from trees to *Monotropa hypopitys* L. (Ericaceae), a close relative of *Sarcodes* (Björkman, 1960). However, Björkman also found that the growth of a fungus isolated from *Monotropa* mycorrhizas was greatly stimulated by an extract of the plant. Miller and Allen (1992) speculate that

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potential ecophysiological benefits for trees of supplying carbon to *Monotropa* may render the association mutualistic. Research on monotrope symbioses has focused on the mycoheterotrophic plant's nutrition (Björkman, 1960), mycorrhizal ultrastructure (Duddridge and Read, 1982; Robertson and Robertson, 1982), germination (Francke, 1934; S. McKendrick, personal communication, University of Sheffield), flowering (D. Luoma, personal communication, Oregon State University), and associated fungi (Cullings, Szaro, and Bruns, 1996). However, little is known about basic ecological traits of monotropes, and this is partly responsible for our difficulties in understanding the nature of their interactions.

In this study, we investigated the ECM community of a red fir, *Abies magnifica* Andr. Murray (Pinaceae), forest where the snow plant flowers abundantly, and we asked what part *R. ellенаe* played in this community. Although one might expect that the specialization of the snow plant on *R. ellенаe* would result in spatial correlation between these two organisms in nature, at least five different ECM root distributions might be expected: (1) *R. ellенаe* could be free of snow plant infection in localized regions of a site where snow plants are present; (2) *R. ellенаe* could be negatively spatially correlated with the snow plant; (3) *R. ellенаe* could be positively spatially correlated with the snow plant; (4) *R. ellенаe* could be uniformly abundant at a site and randomly associated with the snow plant (particularly since *Rhizopogon* species form rhizomorphs well suited for long-distance physiological transport); (5) *R. ellенаe* ECM roots could be low in abundance and randomly distributed. Low abundance was suggested for *Rhizopogon subcaerulescens* A. H. Smith (Boletaceae) ECM roots with respect to rootballs of the monotrope *Pterospora andromedeae* Nutt. (Ericaceae) (Cullings, Szaro, and Bruns, 1996). Furthermore, low abundance is predicted for suilloid fungi, such as *Rhizopogon* Fr. species, which are known to fruit profusely while making comparatively few connections to trees, and thus are hypothesized to have higher carbon sink strengths than other ECM fungi (Danielson, 1984; Natarajan, Mohan, and Ingleby, 1992; Gardes and Bruns, 1996). To test these hypotheses, we examined the distribution of red fir ECM roots within snow plant rootballs and at several distances from snow plant inflorescences.

MATERIALS AND METHODS

Study site—We sampled a location near Shaver Lake at the Sierra National Forest in the southern Sierra Nevada of California (elevation 2530 m, latitude 37°09'06" N, longitude 119°07'50" W). The study site is a 350–400 yr old, upper-montane, monospecific red fir (*Abies magnifica*) forest that was thinned and burned in 1992. The site came to our attention in 1995 when we observed abundant inflorescences of the snow plant (*Sarcodes sanguinea*). The average density of flowering spikes was 0.41 inflorescences/m² (0.12 SE) in August 1995, and 0.24 inflorescences/m² (0.06 SE) in August 1997, within 14 50-m² permanent circular plots used to monitor population dynamics.

Design—We sampled a total of five snow plants on either 16 July or 20 August 1997 from outside of our circular plots. We selected inflorescences for sampling based on the criterion that they had to be separated by at least 5 m from any other flowering snow plant. Each selected inflorescence became the center for a linear transect. We removed two soil cores at each of four distances (10, 50, 100, and 500 cm) away from each selected inflorescence. Cores removed at 5 m were also at least 5 m away from neighboring snow plant inflorescences. The soil cores were 4.6 cm in diameter and they were as deep as the rocky ground allowed, but at least 20 cm and at most 40 cm. Then we excavated the rootball of each snow plant. The rootball is a con-

gested and dense mass of brittle, succulent, highly branched monotropoid mycorrhizal roots from which adventitious inflorescence axes emerge (Wallace, 1975). In one case, we were unable to find the rootball in the soil after the inflorescence axis accidentally broke off. All soil cores and rootballs were kept at 4°C and processed within 3 wk of field sampling.

We sprayed each soil core vigorously with tap water over 2 mm and 500- μ m mesh stacked sieves to separate coarse and fine soil fractions. The *Sarcodes* rootballs were manually broken into small pieces and otherwise treated in the same manner. All the washed soil and roots collected in both sieves were spread thinly in petri dishes and examined using stereo microscopes. All *Abies* roots were collected from each individual core or rootball and sorted into morphotypes according to gross mantle characters (color, color changes, branching pattern, presence of rhizomorphs, mantle surface, thickness). We discarded degraded roots and placed recognizable ECM roots that were partially degraded and/or not turgid in separate morphotypes. We did not attempt to identify identical morphotypes among different cores based on morphological characters alone, but instead relied on molecular analysis to determine identity among samples from different cores. We did not collect the monotropoid mycorrhizas of the snow plant itself for this study. All ECM roots were then lyophilized, and the dry mass of each morphotype was determined. We calculated approximate soil volumes sampled from the core diameter and the soil depth reached. For the rootballs, we determined the volume displaced in water by the snow plant roots after these had been broken up and examined.

Ecotomycorrhizal identification—We identified ECM fungi using methods described by Gardes and Bruns (1996). Briefly, we extracted DNA from individual ECM roots of each morphotype and we amplified the internal transcribed spacer (ITS) of the nuclear ribosomal repeat by the polymerase chain reaction (PCR) with the fungal-specific primers ITS1F and ITS4B, or ITS1F and ITS4 (White et al., 1990; Gardes and Bruns, 1993). PCR products were then screened by restriction fragment length polymorphisms (RFLP) using first the restriction endonuclease (RE) *Alu-I* (New England Biolabs Inc., Beverly, Massachusetts, USA). When types were redundant within a core by *Alu-I* RFLP only one of those types was analyzed further. We then screened with the RE *Hinf-I*. On average, we extracted and amplified each morphotype 2.6 times, with a bias for more abundant types. We estimated the molecular size of the restriction fragments obtained with *Alu-I* and *Hinf-I* using the program GelReader v.2.0.5 (National Center for Supercomputing Applications, Champaign, Illinois) and sorted the morphotype fragment sizes in various combinations in Excel 4.0 spreadsheets (Microsoft Co., Redmond, Washington, USA) to identify groups of morphotypes that matched for both restriction endonucleases. We checked that matching morphotypes were compatible according to our descriptions of their gross morphology. From previous studies we knew that ECM roots of closely related *Rhizopogon* section *Amylopogon* species are virtually indistinguishable by morphology alone. They are also difficult to differentiate by ITS RFLP with the two restriction enzymes mentioned above. Thus, we digested the ITS PCR products of all *Rhizopogon*-like types with a third RE, *Cfo-I*, which differentiates *R. ellенаe*.

Lastly, we examined the phylogenetic distribution of the ITS RFLP groups obtained. Because fungal fruiting at our site is rare and sporadic, direct RFLP matching to fungal sporocarps was not an option. Instead, we ranked ITS RFLP types according to their dry biomass pooled over all samples, and we selected those types with highest biomass for PCR amplification and sequencing of a fragment of the fungal mitochondrial large subunit (mtLSU) rDNA (Bruns et al., 1998). In most cases, the primer combination ML5/ML6 was used; in cases where PCR amplification was weak, or if sequencing proved difficult possibly due to the presence of introns, we attempted amplification with the primer combinations MLIN5R/ML5, CML5.5/ML6, or MLIN3/ML5.5 (Bruns et al., 1998). Sequencing of both strands was performed with an ABI model 377 Sequencer (Applied Biosystems Co., Foster City, California, USA) using an ABI PRISM™ Dye Terminator Cycle Sequencing Core Kit (Perkin Elmer Co., Foster City, California, USA) or a Thermo Sequenase™ Dye Terminator Cycle Sequencing Pre-Mix Kit (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). We used DNA Sequencing Analysis v.2.1.2 and Sequence Navigator v.1.0.1 (Applied Biosystems Co., Foster City, California, USA) for processing raw data. The nearest rel-

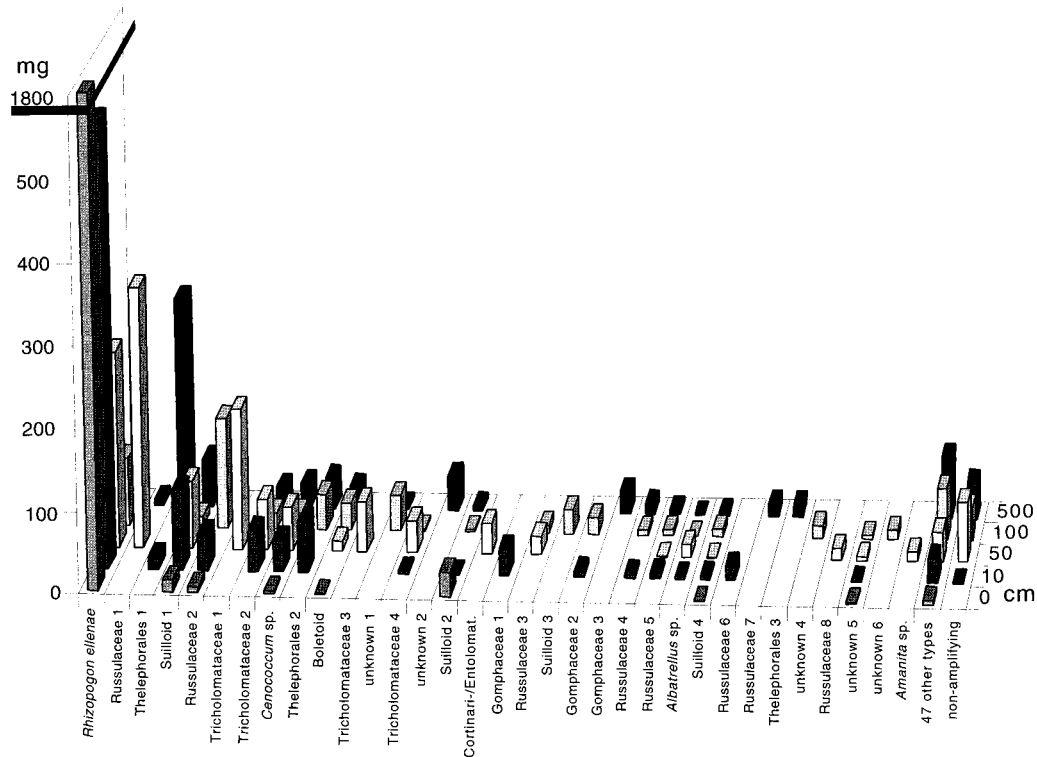


Fig. 1. Total dry mass (mg) of red fir (*Abies magnifica*) ectomycorrhizas (y axis) in soil cores removed at four distances (10, 50, 100, and 500 cm) from five inflorescences of the snow plant, *Sarcodes sanguinea*, and within four snow plant rootballs (z axis). Each ITS type (designated by a unique letter and number combination) is ranked according to its total dry mass over all distances (x axis). Inferred mtLSU phylogenetic affinity is provided as a basidiomycete family name whenever available. Note that the dry mass axis is truncated.

atives of each mycorrhizal type were inferred with the neighbor-joining algorithm implemented in the program PAUP*d64 (Swofford, 1993) using a database by Bruns et al. (1998)

RESULTS

Among the four *Sarcodes* rootballs and 36 soil cores removed across an area of ~1800 m², we found a total of 80 different ectomycorrhizal types defined by unique combinations of characters (gross morphology and ITS RFLP). We found 47 ECM types that occurred in single soil cores (59% of all ECM types, 7% of all ECM biomass sampled). For ease of presentation, we pooled the data for soil cores taken at the same distance from each of the five snow plants sampled. Analysis considering each snow plant and its surrounding soil cores as independent units leads to essentially the same conclusions.

We identified 28 of the 80 ITS RFLP types to family, genus, or species level, corresponding to 36% of all ECM types and 89% of all ECM biomass sampled. The rest of ECM types are labeled “unknowns.” Types labeled “nonamplifying” (2.9% of all ECM biomass sampled) were partially degraded and/or nonturgid types that failed to PCR-amplify after at least three independent DNA extractions and several attempts at PCR of both the ITS and mtLSU regions. The cumulative dry mass of each ITS RFLP type at each distance is shown in Fig. 1. *Rhizopogon ellenaee* is the predominant type in the *Sarcodes* rootballs and 10 cm away from the inflorescences.

The average concentration of red fir ECM roots within the snow plant rootballs is significantly greater than at any distance sampled away from the snow plant inflorescences (*P* =

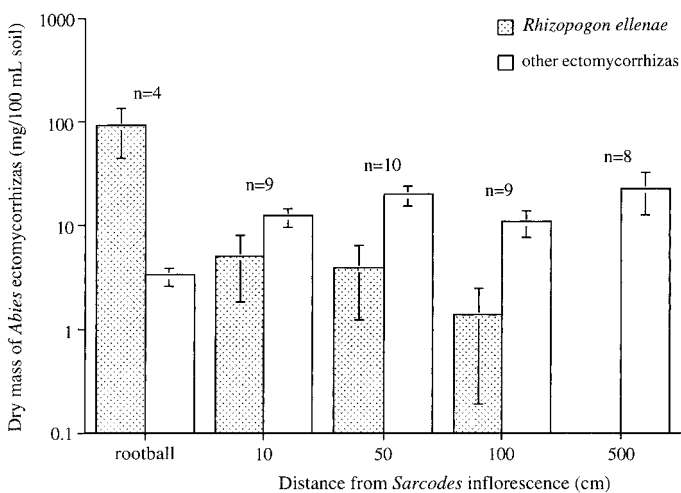


Fig. 2. Estimated concentration (mg/100 mL soil) of red fir (*Abies magnifica*) ectomycorrhizas in soil cores removed at four distances (10, 50, 100, and 500 cm) from five inflorescences of the snow plant, *Sarcodes sanguinea*, and within four snow plant rootballs. All non-*Rhizopogon ellenaee* ectomycorrhizas are displayed as a single bar. *Rhizopogon ellenaee* ectomycorrhizas were not detected at 500 cm. Error bars correspond to SE. Note that the concentration axis (y axis) scale is logarithmic.

TABLE 1. GeneBank accession numbers for fungal mtLSU sequences obtained from 26 ITS restriction length polymorphism (RFLP) types of *Abies magnifica* ectomycorrhizae. Inferred phylogenetic group names follow Bruns et al. (1998). Order corresponds to decreasing overall biomass rank (top: highest biomass) with *Rhizopogon ellенаe*, *Cenococcum* sp., and unidentified types removed.

ITS RFLP type	GeneBank accession ^a
Russulaceae 1	GBAN-AF177709
Thelephorales 1	GBAN-AF177725
Suilloid 1	GBAN-AF177721
Russulaceae 2	GBAN-AF177710
Tricholomataceae 1	GBAN-AF177717
Tricholomataceae 2	GBAN-AF177718
Thelephorales 2	GBAN-AF177726
Boletoid	GBAN-AF177702
Tricholomataceae 3	GBAN-AF177719
Tricholomataceae 4	GBAN-AF177720
Suilloid 2	GBAN-AF177722
Cortinari-/Entolomataceae	GBAN-AF177705
Gomphaceae 1	GBAN-AF177706
Russulaceae 3	GBAN-AF177711
Suilloid 3	GBAN-AF177723
Gomphaceae 2	GBAN-AF177707
Gomphaceae 3	GBAN-AF177708
Russulaceae 4	GBAN-AF177712
Russulaceae 5	GBAN-AF177713
Albatrellus sp.	GBAN-AF177704
Suilloid 4	GBAN-AF177724
Russulaceae 6	GBAN-AF177714
Russulaceae 7	GBAN-AF177715
Thelephorales 3	GBAN-AF177727
Russulaceae 8	GBAN-AF177716
Amanita sp.	GBAN-AF177703

^a The prefix GBAN- has been added to link the online version of *American Journal of Botany* to GeneBank but is not part of the actual accession number.

0.01, Student's *t*). The vast majority of these roots was colonized by *R. ellенаe* (86–98%) (Fig. 2). In fact, the concentration of *R. ellенаe* ectomycorrhizas in the rootballs is between three and ten times greater than at any distance away from them. There is a trend for decreasing concentration of *R. ellенаe* ectomycorrhizas from 10 to 100 cm. At 500 cm, we did not find any *R. ellенаe*. In contrast, the concentration of all other ectomycorrhizas combined is lowest in the rootballs compared to any distance ($P = 0.01$, Student's *t*) and shows a trend for increase from 0 to 50 cm.

We must note that expressing our data as concentration values (Fig. 2) does not imply that ECM roots are homogeneously distributed either in the soil or within the rootball. In fact, clumping is common and root density often varies with soil depth as well as in the rootball. In addition, since soil and rootball volumes were determined by different methods, rootball volume (90–700 cm³) was slightly underestimated because some of the soil was washed away prior to measurement. Nevertheless, this source of error is minimal since soil is a minor component of the densely coralloid snow plant rootballs.

DISCUSSION

This is the first study to report on the structure of an ECM community associated with red fir (*Abies magnifica*). Few ECM fungi associated with the genus *Abies* have been previously described (Comandini, Pacioni, and Rinaldi, 1998). Despite our *Sarcodes sanguinea*-biased sampling, we can infer

that members of the Russulaceae and Thelephoraceae dominate at distances farthest away from *Sarcodes* at our study site. These fungal families are also dominant in other pinaceous Californian forests (Gardes and Bruns, 1996; Horton and Bruns, 1998; Stendell, Horton, and Bruns, 1999). The high diversity and patchiness of the ECM community at our site (Fig. 1) resemble that found in a *Pinus ponderosa* Laws. (Pinaceae) forest located also in the Sierra National Forest (Stendell, Horton, and Bruns, 1999) as well as in old-growth forests (Dahlberg, Jonsson, and Nylund, 1997; Jonsson et al., 1999). In contrast, the ECM community of *Pinus muricata* (Pinaceae) forests in coastal California appears generally less diverse (Gardes and Bruns, 1996).

The distribution of red fir root tips colonized by *R. ellенаe* at our site was greatest directly on the snow plant rootballs and decreased sharply away from them. This finding suggests that most physiological transfer between snow plant roots and *R. ellенаe*-*Abies* ECM occurs over short distances (i.e., <10 cm) despite the presence of *R. ellенаe* rhizomorphs, which can exceed that length. It is interesting that another suilloid fungus (Suilloid 1; Fig. 1), which appears more uniformly distributed and ranks fourth overall, does not associate with the snow plant. *Rhizopogon ellенаe* was not detected in any of the nine soil cores we removed 500 cm away from snow plants. Thus, the snow plants sampled appear to occupy dense islands of *R. ellенаe* ECM roots. Despite the unevenness observed, ECM type richness within the snow plant rootballs was not significantly different than that in the surrounding soil when we take into account differences in sampled volume between rootballs and soil cores.

If a fungus is necessary for seed germination (Leake, 1994), our results are consistent with data indicating that most seeds of the obligate mycoheterotrophs *Monotropa hypopithys* L. (Ericaceae) and *Neottia nidus-avis* L. (Rich) (Orchidaceae) germinate close to prerecorded positions of adult plants (S. McKendrick, personal communication, University of Sheffield). Snow plant seeds germinate in the laboratory when grown axenically within *R. ellенаe* cultures, and field germination trials have recently been successful (T. D. Bruns, unpublished data).

Unexpectedly, the snow plant rootballs sampled were sites of disproportionately high concentrations of red fir roots compared to any soil core, and *R. ellенаe* colonized the vast majority of those roots (86–98%; Fig. 2). For the related monotrope, *Pterospora andromedea*, Cullings, Szaro, and Bruns (1996) documented the scarcity of ECM roots of its symbiont, *Rhizopogon subcaerulescens*, in one rootball and in soil cores removed at 0.5 m from three inflorescences. Because the *P. andromedea* rootball data disagree with our results for *Sarcodes* rootballs, we examined two additional *P. andromedea* rootballs. These were densely covered with ECM roots with a morphology consistent with *Rhizopogon*-*Pinus* mycorrhizas, similar to what we have observed for *Sarcodes*.

Two possible explanations exist for the association of *Sarcodes* with dense clumps of *R. ellенаe*. Either *Sarcodes* plants preferentially establish in pre-existing clumps, or they create them. We favor the second explanation for two reasons. First, the *Sarcodes* rootball itself has relatively few ECM roots in it; instead most roots are found on the outside surface. This distribution suggests that *Abies*-*R. ellенаe* ECM roots form as the *Sarcodes* rootball develops. If instead *Sarcodes* roots were finding such clumps and establishing around them, one would expect the *Abies*-*R. ellенаe* ECM clumps to be predominantly

internal to the rootballs. Second, these clumps are much larger than any we have observed for related *Rhizopogon* species in a variety of pinaceous forests. Most, perhaps all, *Rhizopogon* species have the ability to form coralloid ectomycorrhizae, which are essentially small compact areas of dense root proliferation that are colonized by the fungus (Molina and Trappe, 1994). But the scale of the *Abies*–*R. ellенаe* clumps associated with *Sarcodes* is far beyond any aggregations that we have observed. In fact, the *Sarcodes* rootball itself could be viewed as a giant mycorrhiza, one that can be 1000 cm³ or more rather than the typical size of <1 cm³.

If, for the moment, we assume that *Abies*–*R. ellенаe* clumps develop in response to *Sarcodes* plants, this creates an apparent mechanistic conundrum. How can a plant that lacks its own carbon source stimulate both its mycorrhizal associate and the roots of the photosynthetic plant to which the fungus is attached? This problem is not as great as it may seem. Growth stimulation of host tissues is a common pattern in parasitic interactions. Generally, abnormal cell enlargement and/or division are mediated by hormonal imbalance associated with infection (Agrios, 1997). Examples include branch swelling caused by mistletoes, tumors by *Agrobacterium*, cankers by some rust fungi, and a variety of galls by flies, aphids, and wasps.

Yet these examples differ in at least two ways from the *Sarcodes* system. First, both *R. ellенаe*, which is directly connected to *Sarcodes*, and *Abies* roots, which are not directly connected to *Sarcodes*, have proliferated. If *Sarcodes* seeds germinate near pre-existing clumps of *R. ellенаe* ECM, the snow plant could subsequently alter: (a) a *R. ellенаe* mycorrhization pathway that stimulates *Abies* roots indirectly or (b) an *Abies* root proliferation pathway that stimulates *R. ellенаe* indirectly. A mechanism for the former process is suggested by indole-3-acetic acid (IAA)-overproducing mutant strains of the ECM fungus *Hebeloma cylindrosporum* Romagnesi, which can form three to six times more ECM roots with pine hosts than wild-type strains (Durand et al., 1992; Gay et al., 1994). A second, and more important difference of the *Sarcodes* system, is that growth stimulation is likely to benefit *R. ellенаe*. Instead, parasite-induced growth reduces host fitness (e.g., “parasitic castration” of mollusks by trematodes; Sorensen and Minchella, 1998). In this study, it seems that *R. ellенаe* benefits; it colonizes a vastly larger proportion of *Abies* roots relative to its competitors in a diverse ECM community. This must in turn benefit the specialized *Sarcodes*. In this aspect the relationship between *Sarcodes* and *R. ellенаe* appears mutualistic rather than parasitic.

In summary, we found both fungal and photosynthetic hosts in disproportionate concentrations in rootballs of *Sarcodes* compared to the adjacent soil. Thus, the ectomycorrhizal community differs significantly where *Sarcodes* roots are present in a manner that appears beneficial, at least over the short term, to the fungal symbiont. However, we do not know whether there is any trade-off incurred by *R. ellенаe* as a result of its association with *Sarcodes*. This will be a critical piece of information to acquire if we are to understand the nature of this tripartite symbiosis.

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