

Seasonal dynamics of ectomycorrhizal fungus assemblages on oak seedlings in the southeastern Appalachian Mountains

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Received: 16 March 2007 / Accepted: 15 January 2008
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Abstract The potential for seasonal dynamics in ectomycorrhizal (EM) fungal assemblages has important implications for the ecology of both the host trees and the fungal associates. We compared EM fungus distributions on root systems of out-planted oak seedlings at two sites in mixed southeastern Appalachian Mountain forests at the Coweeta Hydrologic Laboratory in North Carolina, from samples taken in mid-July and early September. Species level EM fungus type specificity, and identification in some cases, was enabled by direct sequencing of the mycobionts from the seedling roots. Seventy-four EM fungal ITS types were documented, most of which occurred only in the midsummer or early-fall samples, respectively. *Cenococcum geophilum* (morphotyped) was ubiquitously present and accounted for the majority of root tips sampled. Abundance and relative frequency of types other than *C. geophilum* were significantly higher in the July samples, while *C. geophilum* was significantly more frequent and abundant in September. Several generalistic dominants were found fairly equally at both sites and on both sample dates. Other taxa with relatively high frequency were recovered from both sites

and tree seedling species, but were reliable indicators occurring primarily in the July sample (e.g., *Laccaria cf. laccata*). Notable shifts in mycobiont dominance were apparent in relation to sample date, including increases in *Cortinarius* spp. richness, decreases in Thelephoraceae richness, and the disappearance of *Amanita* spp. types in the early fall compared to midsummer samples. However, diversity and rarity were high and differences in overall community composition (other than *C. geophilum*) by season were not significant based on multi-response permutation procedures. Although these results based on a single growing season are preliminary, changes in abundance and frequency, detection of significant indicator species, and the apparent systematic affinities of shifting EM types support the potential for seasonal variability in EM associations in this system.

Keywords Ectomycorrhizal community · Fungal diversity · Molecular ITS typing · *Quercus rubra* · *Quercus prinus* · Temporal dynamics

Introduction

Ectomycorrhizal (EM) fungi form important mutualistic associations with a variety of dominant tree species found in temperate forests (Smith and Read 1997). The general role of EM fungi in providing a number of benefits to their plant hosts, including mineral nutrients, water, and protection from pathogens, is well known (Smith and Read 1997). The high diversity of EM fungi has long been recognized based on sporophore collections (e.g., Bills et al. 1986; Nantel and Neumann 1992). Direct characterization of EM fungal species colonizing root tips by multiple recent molecular studies has further contributed to our under-

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standing of the high level of diversity and spatiotemporal variability in EM fungal communities (reviewed in Horton and Bruns 2001; Izzo et al. 2005). Given this high diversity and potential for specialization, a number of factors suggest that EM fungal communities may be variable throughout the growing season in a mature deciduous forest.

Firstly, edaphic conditions including soil temperature, moisture, and nutrient availability are influenced by the depth and decomposition of the leaf litter layer, which varies seasonally in deciduous forests (Facelli and Pickett 1991). Secondly, colonization by EM fungi occurs on fine root tips (Smith and Read 1997), and fine root tip longevity (which can be highly variable) may be as brief as 1 month (Hendrick and Pregitzer 1992; Gill and Jackson 2000; Gaudinski et al. 2001). Changes in gross (unidentified) EM root tip abundance by season were studied by Blasius et al. (1990), who found variation throughout the growing season and that the period of peak EM root tip abundance differed during separate years. In 1985, peak abundance of EM root tips occurred in May and October, with August being the least abundant time period. This cycle was not repeated in 1986, however, when peak abundance of EM root tips occurred in August and November and low abundance occurred in September and October. Furthermore, it was thought that changes in weather patterns influenced the seasonal variation in abundance cycles across years. Intra-annual variability in EM colonization has also been linked to climatic variation by a number of additional studies (Harvey et al. 1978; Vogt et al. 1980; Rastin et al. 1990; Swaty et al. 1998), although again species-level fungal identification was not provided.

A final study which suggested seasonal patterns in assemblages of EM fungi colonizing roots was conducted in *Salix repens* L. stands in dune ecosystems by van der Heijden and Vosatka (1999). Based on observations of morphotype samples from large numbers of root tips at 16 sites, they hypothesized that relative abundances and composition of taxa changed throughout the season and therefore nutrients, water, or other benefits (e.g., protection from pathogens) provided by the different morphotypes to plants would vary as well.

Interannual temporal variability at the species level has been characterized from root tip samples in many recent molecular studies. However, these studies have not addressed the potential for seasonality or intra-annual variability in EM fungal communities because root samples were collected at a single time of year. Dahlberg and Stenlid (1995) and Izzo et al. (2005) both noted this dearth of information on seasonal dynamics of EM fungi, and strongly promoted the need for better understanding of EM temporal dynamics.

Taken together, the temporally variable nature and high diversity of EM fungal assemblages, along with variability

in edaphic conditions, root tip turnover, gross EM root tip abundance, and morphotype dominance, indicate that seasonality may be important in structuring EM assemblages. Hence, EM fungi with differing environmental tolerances may be better adapted to a given seasonal microhabitat. Seasonal specialization of EM fungi resulting in variable root colonization by differing fungal mycobionts across the growing season should be related to intra-annual variability in carbohydrate expenditures by host trees to EM fungi. Furthermore, the temporal availability of mineral nutrients to the host may be affected. Thus, seasonal dynamics of major resource pools could be strongly tied to seasonal dynamics of EM colonization and persistence. Ultimately, seasonal specialization in EM fungal assemblages may translate into ecological flexibility for the phytobiont by association with fungi that track annual cycles in soil nutrient pools (van der Heijden and Vosatka 1999).

In this study, we compared EM fungal assemblages in midsummer and early fall of a single growing season in a mature mixed forest in the southeastern Appalachian Mountains. To characterize the midsummer and early fall EM fungus assemblages, species level EM fungus typing and frequency data were derived from direct amplification and sequencing of the ITS region of the mycobiont from colonized root tips collected from out-planted oak seedlings. We hypothesized that there would be differences in the abundance, diversity, and community structure between midsummer and early fall assemblages of EM fungi on the seedlings. Observation of a strong seasonal trend would indicate that EM fungal communities may have an additional level of environmental specialization that contributes to both their diversity and functional relationships with host trees and resource pools.

Materials and methods

Site description

The sites for this study were located within the Coweeta Hydrologic Laboratory (Coweeta), part of the NSF Long Term Ecological Research Station network. Coweeta (35° 02' 29" N, 83° 27' 16" W) is located in the Blue Ridge Mountain Physiographic Province in the southwestern corner of North Carolina. Climatically classified as marine and humid, Coweeta experiences relatively high precipitation and mild temperatures. Precipitation is distributed equally throughout the growing season, averaging 180 cm annually (Swank and Crossley 1988). Diversity of ectomycorrhizal fungi is quite high in this area (Walker and Miller 2002; Walker et al. 2005).

Ectomycorrhizal communities were sampled at two locations within Coweeta. The low elevation mesic site (LM site), with a northwestern aspect, was located upslope from Ball Creek at an elevation of approximately 720 m above sea level. The drier high elevation site (HD site) was located above Dryman's Fork at approximately 1,170 m above sea level and had a north–northeasterly aspect. Additional information on site characteristics can be found in Walker et al. (2005) and Beier et al. (2005).

Seedling propagation

Seedlings of two oak species, *Quercus rubra* and *Q. prinus* (a red and a white oak, respectively), were germinated from acorns collected at Coweeta. The acorns were surface sterilized in 10% bleach solution for 10 min and then rinsed with tap water for 5 min before sowing in coarse Vermiculite in a greenhouse. *Pinus rigida* Ait. seeds, also collected at Coweeta, were surface sterilized in hydrogen peroxide for 20 min and germinated in sterilized sand. After germination, the seedlings were transplanted to nursery cells with coarse vermiculite. After 2 months of growth, the seedlings were fertilized weekly with quarter strength Hoagland's solution (Hoagland and Arnon 1950). After 4 months of growth in the greenhouse, the seedlings were planted out at the field sites during the last week of June 2000.

Greenhouse EM contaminants, especially members of the Thelephoraceae, are frequently observed when seedlings are grown in greenhouses. However, EM tree species are rarely grown in the greenhouse we utilized. To test for possible contaminants, 20 seedlings of each oak species were destructively sampled and screened for mycorrhizal colonization at the time of planting. The root systems of the screened seedlings were examined under a dissecting microscope and multiple root tip sections were examined at high magnification under a compound microscope. No evidence of mycorrhizal colonization was observed.

At each site (LM and HD) 60 1×2 m plots were randomly located along four transects oriented cross-slope. At the LM site, four seedlings of each species (*Quercus rubra* and *Q. prinus*) were planted evenly spaced within each 1×2 m plot. At the HD site *Q. rubra* and *Pinus rigida* were planted, again with four seedlings per plot. There were too few *P. rigida* seedlings surviving to analyze after the first growing season. One randomly chosen seedling from each species/site set (*Q. rubra* at LM and HD; *Q. prinus* at LM) was harvested from each plot with surviving seedlings in mid-July and again in early September, 2001. Herbivores eliminated all seedlings from some plots. At the time of harvest, each seedling was carefully removed and bagged with the roots and surrounding soil as intact as possible. After transportation to the lab, the seedlings were stored at approximately 5°C until processed.

Mycobiont sampling

Samples were collected in mid-July and again in early September, 2001. Repeated sampling of the plots over additional seasons was not undertaken because examining seasonality was a secondary objective in a multifaceted project exploring the relationship of EM mycobiont assemblages to site characteristics, host species, and evergreen ericoid shrub presence (Walker et al. 2005; Beier et al. 2005). These additional objectives strongly influenced the development of our experimental design.

From the first harvest (July), half of the plots were systematically chosen for mycobiont sampling by using a seedling from every other plot along each transect (30 plots at each site). For the second harvest, all plots with surviving seedlings were sampled (58 plots at each site). The first harvest seedling sample totals were 30 *Quercus rubra* seedlings from the HD site, 30 *Q. rubra* seedlings from the LM site, and 30 *Q. prinus* seedlings from the LM site. From the second harvest seedling sample totals were 58 *Q. rubra* seedlings from the HD site, 58 *Q. rubra* seedlings from the LM site, and 53 *Q. prinus* seedlings from the LM site.

The soil was removed from the root system of the seedlings manually. Each root system was examined under a dissecting microscope and all mycorrhizal root tips (excluding those colonized by *C. geophilum*) were picked free of debris, removed with tweezers, and stored frozen in 100 µl 2× CTAB buffer. Those colonized by *C. geophilum* were excluded from the molecular characterization because they were quantified reasonably accurately by morphology. All seedlings were processed within 2 weeks from the time of harvest. Due to the high diversity of mycobionts, morphotyping of additional samples was not feasible. We purposely oversampled root tips that showed signs of potential mycorrhization but were not well developed to maximize the recovery of EM fungi from the seedlings.

Excluding *Cenococcum geophilum*, there were 284 and 309 root tip samples (593 total) from the first and second harvests, respectively. Multiple (3–4) PCR attempts were made at various template concentrations for all unamplified samples. Of the 593 root tips sampled, 291 were successfully sequenced (49%). The somewhat low proportion successfully sequenced is reflective of the oversampling of poorly colonized root tips as well as standard issues with amplification and multiple templates in uncloned samples. There were 1,618 and 5,912 *C. geophilum* root tips observed from the first and second harvest, respectively.

DNA was extracted from each root tip using CTAB buffer with chloroform/isoamyl alcohol following standard procedures (Hibbett and Vilgalys 1993). After extraction, the nuclear 5.8S rRNA gene and the flanking internal transcribed spacer regions I and II were amplified by PCR

with primers ITS1F and ITS4 (Gardes and Bruns 1993; White et al. 1990). After purification of the PCR products with QIAquick PCR Purification Kits (QIAGEN, Valencia, CA 91355), sequencing reactions were run using the same primers and ABI PRISM® BigDye™ Terminators Cycle Sequencing Kits (Applied Biosystems, Foster City, CA 94404, USA). Sequences were read in either the forward or reverse direction initially. The complimentary reaction was only run for those samples that produced ambiguous reads in the initial direction. Final amplification products were cleaned and sequenced by the Virginia Bioinformatics Institute Core Lab Facility (Virginia Tech, Blacksburg, VA 24061–0477, USA) using an ABI automated sequencer. Sequences were assembled into sequence types that shared 97% or greater similarity, and were manually edited. Unique ITS-types were compared with sporophore voucher sequences by blast searching against GenBank and private sequence databases (Jeri L. Parrent and Rytas Vilgalys, Department of Biology, Duke University) for identification.

Names for ITS-types are derived from the closest matching sporophore voucher sequence. The taxonomic specificity of the name reflects the authors' opinion based on the amount of sequence data available for the group, the apparent heterogeneity of the ITS regions in the group, and the level of match between the sample and voucher sequences. Additional information and references on identifications can be found by referring to the GenBank accession numbers.

Analytical methods

Due to the much higher frequencies and abundances for *C. geophilum*, differences between the mean abundance and relative frequency of EM root tips from the two sample periods were analyzed separately for *C. geophilum* and all other taxa. A full factorial analysis was not possible because *Q. prinus* was not planted at the HD site. Therefore, *t* tests were used to compare summer vs. early fall samples from each tree species at each site (SPSS 13 for Windows). Abundance of *C. geophilum* was analyzed based on observed morphotype abundance. For taxa other than *C. geophilum*, the abundance of root tip samples sequenced from each seedling was used for the analyses. A number of possible factors could be responsible for the absence of successfully sequenced samples on many of the seedlings (e.g., poor growing conditions, loss of root tips during harvest, poor condition of EM root tips, and amplification problems). Therefore, separate analyses of abundance were conducted both with and without seedlings with only *C. geophilum* present. In addition, the mean relative frequencies (proportion of seedlings with taxa other than *C. geophilum* the type was recovered from) for all EM types identified other than *C. geophilum* in the summer vs.

the early fall were arcsine transformed and compared using a *t* test.

Indicator species analysis, multi-response permutation procedures (MRPP), and detrended correspondence analysis (DCA) were conducted using PC-ORD Multivariate Analysis of Ecological Data version 3.0 for windows (McCune and Medfford 1997). Indicator species analysis was performed using the method of Dufrene and Legendre (1997), which is based both on the abundance and frequency of species in a priori groups. The indicator species analysis uses a Monte Carlo technique to test statistical significance based on repeated randomizations (1,000 in this study) of the dataset, such that *P* values represent the probability of a higher maximum indicator value arising randomly. The indicator value (*I*) is a measure of both the relative abundance and reliability of occurrence of the taxon in the group, and ranges from 0–100 (100 representing perfect indication). Indicator values were compared for all types in the midsummer vs. the early fall.

MRPP (a nonparametric test that compares heterogeneity within predefined groups) was calculated using the Sorensen coefficient. The weighting factor applied to the items in each group was $n/\text{sum}(n)$ where *n* is the number of items in the group. Agreement values (*A*) are defined as $1 - (\text{observed } \Delta / \text{expected } \Delta)$. Agreement values approaching 1 indicate groups with very similar samples, and negative *A* values occur when groups are less similar than expected by chance. *P* values represent the chance of a smaller or equal Δ . The MRPP test was used to compare the overall assemblages in the midsummer vs. the early fall.

For the DCA ordination, the site frequency for each ITS type was defined as the number of plots from which the type was isolated from a seedling at a given site regardless of harvest date. Seasonal frequency was recorded as the number of plots from which the type was isolated on that sample date regardless of site. Frequencies for each type-site and type-sample date were entered as a matrix used for DCA with the “downweight rare species” option. Euclidean distance was used for coefficients of determination (R^2).

Results

Potential seasonal shifts in the EM assemblage on oak seedlings in this system are apparent despite the high diversity in relation to sample size (Figs. 1, 2, and 3). A total richness of 74 EM fungus ITS types were sequenced from oak seedling root tips. Thirty-two types were recovered only in mid-July, 25 types were recovered only in early September, and 17 types were recovered from both harvests (Table 1). Of the 31 types recovered from more than one plot, 45% occurred on only one sample date (Fig. 2). Three *Amanita* types were present in the

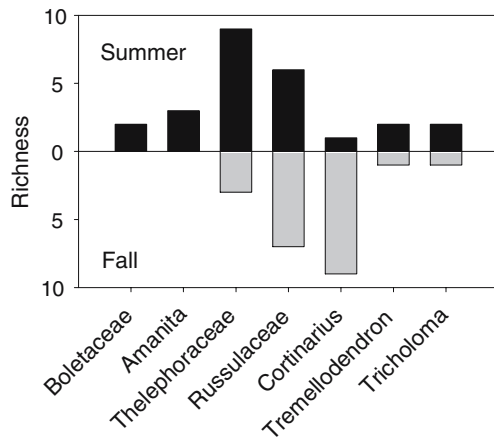


Fig. 1 Richness of ITS types for major ectomycorrhizal groups sequenced from oak seedlings in midsummer and early fall. Summer types are plotted *above the origin*, fall types are plotted *below the origin*. The number of types occurring only in summer or fall samples and in groups with >1 type in either season is shown

midsummer samples from the LM site, while there were none in the late summer harvest (Fig. 1). No bolete types occurred only in the autumn samples (Table 1). Thelephoroid and tomentelloid types (listed as Thelephoraceae if unresolved at the generic level) were three times richer in the midsummer samples, with nine types, than in the late summer (Fig. 2, Table 1). *Cortinariius* types were primarily recovered in the early fall (one type in summer, nine types in fall) (Fig. 2, Table 1). *C. geophilum* was recovered from all seedlings on both sample dates. Previously reported results indicated that there was no difference in EM diversity ($H=2.36$ vs. 2.48) or composition detectable between the two seedling species, and that EM diversity was slightly higher at the LM site compared to the HD site ($H=2.6$ vs. 2.14) (Walker et al. 2005).

Excluding *C. geophilum*, the abundance of EM samples identified per seedling was significantly lower ($P<0.05$) in the early fall for both oak species at the LM site (*Q. rubra*

early fall = 1.8, summer = 2.6; *Q. prinus* early fall = 1.5, summer = 3.4). At the HD site there was also a trend with less abundance in the early fall on the *Q. rubra* seedlings, however, it was not significant (early fall = 2.0, summer = 3.0). These results were congruent in analyses including or excluding seedlings with only *C. geophilum*. In contrast, for *C. geophilum*, significantly more colonized root tips were observed per seedling in the early fall on both oak species at the LM site (*Q. rubra* early fall = 24.9, summer = 16.5; *Q. prinus* early fall = 45.3, summer = 23.4; $P<0.01$) and on *Q. rubra* at the HD site (early fall = 36.6, summer = 13.5; $P<0.01$). The average relative frequency of all identified EM types other than *C. geophilum* (excluding seedlings with only *C. geophilum*) was significantly higher in the summer (3.5%) than in the early fall (2.0%; $P<0.05$). The relative frequency of *C. geophilum* was 100% on both sample dates.

Because of the reduced abundance, we were able to sample more plots in the early fall within the time constraint for DNA preservation. This spatial bias dramatically increased the richness of EM fungi recovered in the early fall. Aside from richness, however, there was no evidence of spatial bias in our estimation of seasonal dynamics based on all samples. Ninety percent of the *Cortinariius* types recovered only in the early fall were sampled from plots that were also sampled in the summer (Fig. 2). Of the types recovered in both seasons, half were from plots in the early fall that were not sampled in the summer (Fig. 2). Recovery of groups notably absent in the early fall would have been more likely due to the increased area sampled in the early fall, whereas the higher abundance and frequency in the summer would have enhanced the likelihood of finding early fall groups in the summer.

Significant indicator species for summer included Corticiaceae #01 (I for summer = 10, early fall = 0; $P=$

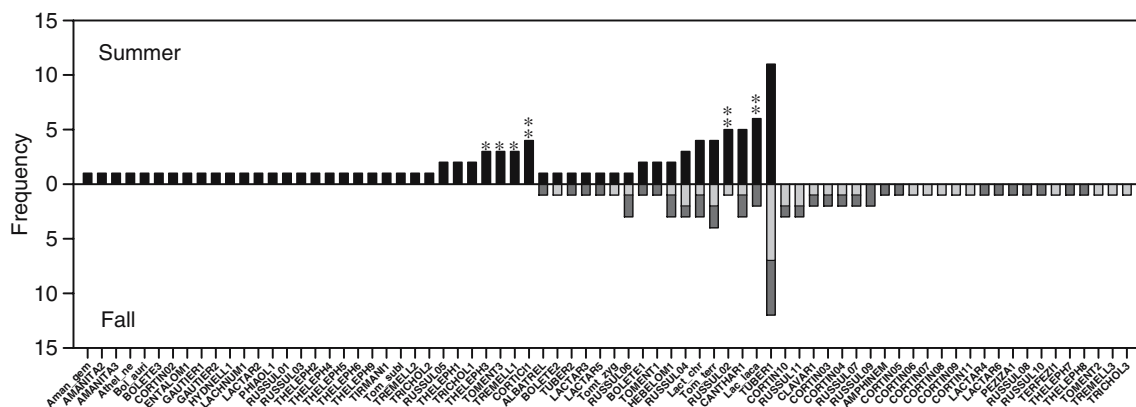
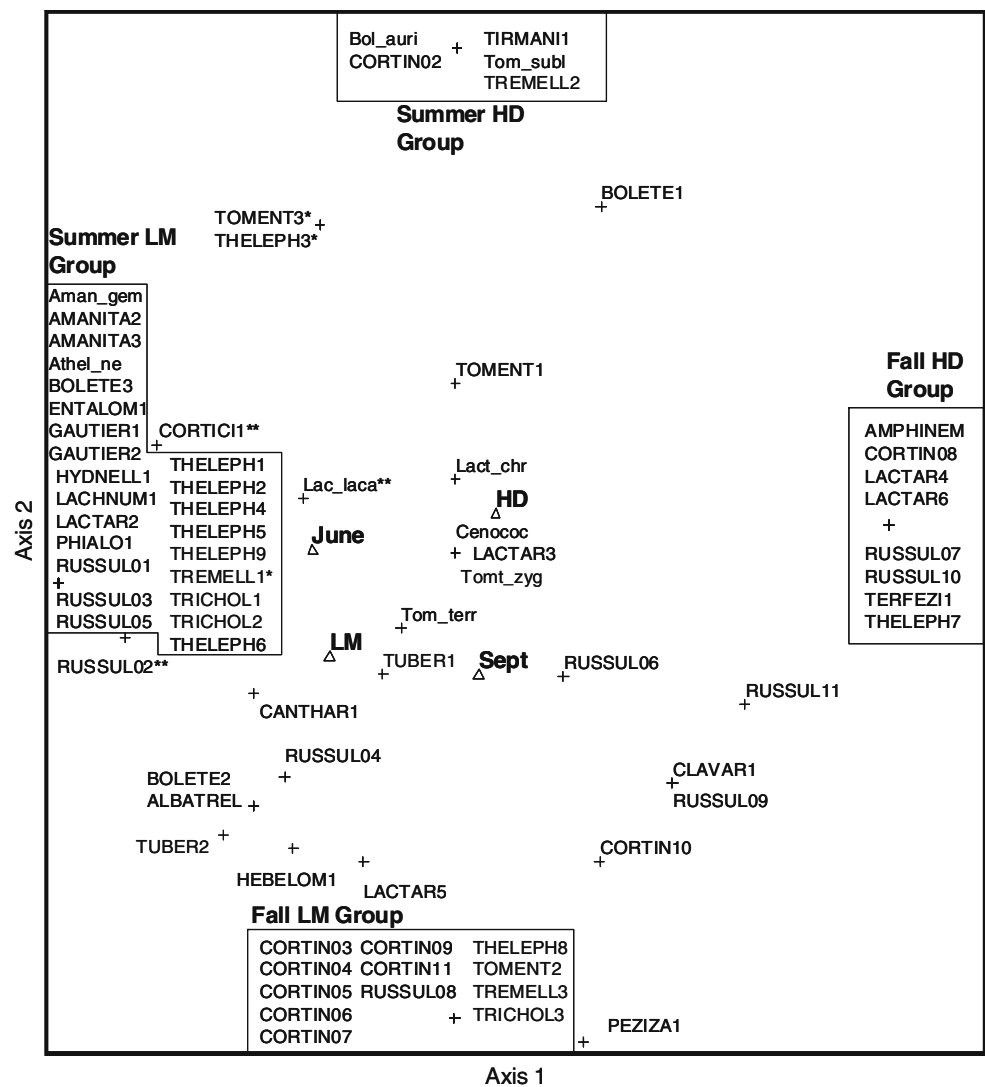


Fig. 2 Frequency of ITS types for ectomycorrhizal fungi sequenced from oak seedlings in midsummer and early fall. Summer types are plotted *above the origin*, early fall types are plotted *below the origin*. For early fall samples, the shading indicates spatial overlap with

summer plots—frequency of early fall types recovered from plots that were also sampled in the summer are *lighter gray*; frequency from plots only sampled in the early fall are *darker gray*. Asterisks represent significant indicator species; * $P<0.10$; ** $P<0.05$

Fig. 3 Detrended correspondence analysis (DCA) ordination of EM fungus ITS types from oak seedlings out-planted in two mature mixed forest sites in the southern Appalachian Mountains. Grouping is delineated (boxes) within sites (low mesic, *LM*; high drier, *HD*) by season (July, September). Types (crosses) are named by code (see Table 1) and separated by site (triangles, LM and HD) and by sample date (triangles, July and Sept). Axis 1 $R^2=0.535$, axis 2 $R^2=0.329$. Asterisks represent statistically significant indicator species; * $P<0.10$; ** $P<0.05$



0.0250), *Laccaria cf laccata* (I for summer = 12, early fall = 1; $P=0.044$), and *Russula* #02 (I for summer = 11, early fall = 0; $P=0.0260$). Two of the significant summer indicators showed no site affinity (Corticaceae #01 and *Laccaria cf laccata*). These taxa were recovered from both sites and both seedling species, yet were primarily recovered only in the summer. *Russula* #02, also a significant summer indicator, was recovered primarily from the LM site, but also occurred on both oak species. Note that *Russula* #02 had a frequency of one in the early fall (Fig. 2) even though the I value based on the relative abundance and relative frequency rounds to zero. Marginally significant indicator species included Thelephoraceae #03 (I for summer = 8, early fall = 0; $P=0.0610$), *Tomentella* #03 (I for summer = 8, early fall = 0; $P=0.0690$), and *Tremellodendron* #01 (I for summer = 8, early fall = 0; $P=0.0620$). There were no significant indicator species for early fall. Potential early fall indicators with distributions similar to the significant summer indicators

were not statistically supported because more seedlings were sampled in the early fall. At the overall group level (based on all taxa with no taxonomic constraints), MRPP showed no difference in assemblages by season ($A=-0.005$, $P=0.86$).

Grouping of EM types is apparent at the site level and within sites at the sample date level in the ordination; however, these groups are primarily composed of very infrequent taxa (Fig. 3). Generalistic fungi lacking site affinity and seasonality are located in the center of the ordination (Fig. 3). Several taxa were recovered primarily in the summer (Corticaceae #01 and *Laccaria cf laccata*) or early fall (e.g., Clavariaceae #01 and *Russula* #09) but showed little site affinity. The DCA ordination coefficients of determination for the correlations between ordination distances and distances in the original n -dimensional space (an index of the percent of variation in the distance matrix explained by the axis) were axis 1 $R^2=0.535$, axis 2 $R^2=0.329$, and axis 3 $R^2=-0.015$. The cumulative R^2 for the

Table 1 ITS types for ectomycorrhizal fungi sequenced from oak seedlings in midsummer and early fall

Summer Only	Fall Only	Summer and fall
<i>Amanita</i> #02 (AY656923; AMANITA2)	<i>Amphinema</i> #01 (AY656917; AMPHINEM)	<i>Albatrellus</i> #01 (AY656960; ALBATREL)
<i>Amanita</i> cf <i>gemmata</i> (AY656924; Aman_gem)	Clavariaceae #01 (AY730686; CLAVAR1)	Bolete #01 (AY656925; BOLETE1)
<i>Amanita</i> DFMO1078 ^a (AY656916; AMANITA3)	<i>Cortinarius</i> #03 (AY656929; CORTIN03)	Bolete #02 (AY656926; BOLETE2)
<i>Athelia</i> cf <i>neuhoffii</i> (AY656918; Athel_ne)	<i>Cortinarius</i> #04 (AY656930; CORTIN04)	Cantharellaceae #01 (AY656927; CANTHAR1)
Bolete #03 (AY656922; BOLETE3)	<i>Cortinarius</i> #05 (AY656965; CORTIN05)	<i>Hebeloma</i> #01 (AY730685; HEBLOM1)
<i>Boletus auriporus</i> (AY656919; Bol_auri)	<i>Cortinarius</i> #06 (AY656966; CORTIN06)	<i>Laccaria</i> cf <i>laccata</i> (AY656938; Lac_laca)
cf <i>Tirmania</i> #01 (AY656920; TIRMANI1)	<i>Cortinarius</i> #07 (AY656967; CORTIN07)	<i>Lactarius</i> #03 (AY656971; LACTAR3)
Corticiaceae #01 (AY656928; CORTICI1)	<i>Cortinarius</i> #08 (AY656968; CORTIN08)	<i>Lactarius</i> #05 (AY656973; LACTAR5)
<i>Cortinarius</i> #02 (AY656964; CORTIN02)	<i>Cortinarius</i> #09 (AY656969; CORTIN09)	<i>Lactarius</i> <i>chrysotheus</i> (AY656937; Lact_chr)
Entolomataceae #01 (AY656932; ENTALOM1)	<i>Cortinarius</i> #10 (AY656961; CORTIN10)	<i>Russula</i> #02 (AY656942; RUSSUL02)
<i>Gautieria</i> #01 (AY656933; GAUTIER1)	<i>Cortinarius</i> #11 (AY656931; CORTIN11)	<i>Russula</i> #04 (AY656944; RUSSUL04)
<i>Gautieria</i> #02 (AY656970; GAUTIER2)	<i>Lactarius</i> #04 (AY656972; LACTAR4)	<i>Russula</i> #06 (AY656962; RUSSUL06)
<i>Hydnellum</i> #01 (AY656934; HYDNELL1)	<i>Lactarius</i> #06 (AY656974; LACTAR6)	<i>Tomentella</i> #01 (AY656951; TOMENT1)
<i>Lachnum</i> #1 (AY656935; LACHNUM1)	<i>Peziza</i> #01 (AY656939; PEZIZA1)	<i>Tomentella</i> cf <i>terrestris</i> (AY656954; Tom_terr)
<i>Lactarius</i> #02 (AY656936; LACTAR2)	<i>Russula</i> #07 (AY656975; RUSSUL07)	<i>Tomentellopsis</i> <i>zygodesmoides</i> (AY656952; Tomt_zyg)
<i>Phialophora</i> #1 (AY656940; PHIALO1)	<i>Russula</i> #08 (AY656946; RUSSUL08)	cf <i>Tuber</i> #01 (AY656958; TUBER1)

Table 1 (continued)

Summer Only	Fall Only	Summer and fall
<i>Russula</i> #01 (AY656941; RUSSUL01)	<i>Russula</i> #09 (AY656978; RUSSUL09)	cf <i>Tuber</i> #02 (AY656959; TUBER2)
<i>Russula</i> #03 (AY656943; RUSSUL03)	<i>Russula</i> #10 (AY656976; RUSSUL10)	
<i>Russula</i> #05 (AY656945; RUSSUL05)	<i>Russula</i> #11 (AY656977; RUSSUL11)	
Thelephoraceae #01 (AY656947; THELEPH1)	cf <i>Terfezia</i> #01 (AY656921; TERFEZI1)	
Thelephoraceae #02 (AY656979; THELEPH2)	Thelephoraceae #07 (AY656981; THELEPH7)	
Thelephoraceae #03 (AY656948; THELEPH3)	Thelephoraceae #08 (AY656982; THELEPH8)	
Thelephoraceae #04 (AY656980; THELEPH4)	<i>Tomentella</i> #02 (AY656984; TOMENT2)	
Thelephoraceae #05 (AY656949; THELEPH5)	<i>Tremellodendron</i> #03 (AY656985; TREMELL3)	
Thelephoraceae #06 (AY656950; THELEPH6)	<i>Tricholoma</i> #03 (AY656988; TRICHOL3)	
Thelephoraceae #09 (AY656983; THELEPH9)		
<i>Tomentella</i> #03 (AY656963; TOMENT3)		
<i>Tomentella</i> cf <i>sublilacina</i> (AY656953; Tom_subl)		
<i>Tremellodendron</i> #01 (AY656955; TREMELL1)		
<i>Tremellodendron</i> #02 (AY277943; TREMELL2)		
<i>Tricholoma</i> #01 (AY656986; TRICHOL1)		
<i>Tricholoma</i> #02 (AY656987; TRICHOL2)		

Genbank accession number and type code used for figures are listed in parentheses below each type.

^a Duke Forest Mycological Observatory sporophore voucher number

first two axes shown (Fig. 3) equaled 0.848. Neither axis was directly aligned with the variability across seasons or sites.

Discussion

These data are all congruous with the idea of alternate EM fungus assemblages for the early and late portions of the growing season. However, the results must be considered preliminary due to the lack of replication over multiple years and the lack of statistical power due to the high diversity and rarity of EM types recovered. Even if the results of our study represent systematically structured intra-annual variation that is not directly related to factors that vary consistently across the growing season, they suggest that EM fungus associations are even more dynamic than typically considered. More importantly, these results indicate that EM communities in this system cannot be adequately characterized based on samples taken at a single time in the growing season.

Community composition

Cenococcum geophilum, known to be a generalistic mycobiont, was present on all the seedlings in both midsummer and fall but was most abundant in the early fall. Dominance by *C. geophilum* has commonly been found in variety of forest ecosystems (Bird and McCleneghan 2005; Izzo et al. 2005; Peter et al. 2001; Jonsson et al. 1999). However, it is unclear what effect *C. geophilum* may have had on the diversity or abundance of other EM types. In addition, previous work in this system and others has indicated that *C. geophilum* colonization levels in some cases are dependant on non-seasonal factors such as the presence of dense ericoid shrub thickets (Walker et al. 1999), host taxa (Walker et al. 1999; Malloch and Malloch 1981), and disturbance Antibus (1980) or drought (Pigott 1982; Worley and HacsKaylo 1959).

In contrast, types other than *C. geophilum* were more abundant and frequent in the midsummer EM samples. Higher abundance and relative frequency of taxa other than *C. geophilum* in the midsummer vs. the early fall may have been driven by weather patterns or other environmental factors that vary non-cyclically among seasons, as was found for example by Blasius et al. (1990). Given that EM root tip turnover would likely be necessary for seasonal patterns in mycobiont community composition to develop, long-term studies of EM root tip dynamics are needed to separate effects of seasonally-dependant from seasonally-independent factors.

In these preliminary results, the major groups of EM fungi seemed to change between summer and early fall samples.

Although these trends clearly cannot be extrapolated to higher taxonomic groups because of the relatively small number of taxa involved, it appeared that in some cases, different groups of closely related species were present in the midsummer vs. the early fall. The dominant group appeared to shift from members of the Thelephoraceae in the summer to *Cortinarius* spp. in the early fall (Figs. 1 and 2). In the genus *Cortinarius*, of seven types that occurred only once, six occurred only in the early fall. In addition, no *Cortinarius* type occurred more than once in the summer, whereas three did in the early fall. Members of the Russulaceae were distributed equitably among seasons, however, *Amanita* spp. and boletes were found predominantly in the summer (Figs. 1 and 2).

Hence, the apparent changes in composition of the EM assemblages between sample dates seem to follow the systematic affinities of the shifting types, which is noteworthy because closely related taxa are likely to have similar environmental tolerances and growth strategies. In previously reported results, no systematic patterns were apparent when comparing the structure of EM assemblages at the two sites and on the two oak species (Walker et al. 2005). We are not aware of any previous reports based on species level identification of EM types where high temporal variability appears to be systematically structured. For example, Stendell et al. (1999) sampled EM fungus types (by soil cores) in May in consecutive years and found apparent changes in the assemblage, even though samples from consecutive years were frequently only 25 cm apart. However, the changes in species composition (Stendell et al. 1999) appeared random and did not reflect an apparent systematic shift in dominant groups such as we report.

However, because of the high level of spatiotemporal variation and the large number of rare species, this apparent systematic trend must be interpreted cautiously. Ultimately, much larger sample sizes will be needed to properly assess the importance of these trends based on groups of infrequent but closely related taxa. There was no statistical support for differences at the overall assemblage level by season (MRPP), and the high diversity and low frequency of taxa other than *C. geophilum* severely limited the power of the analysis. Therefore, it remains somewhat unclear whether the apparently obvious systematic trend is an artifact that arose randomly or whether the lack of significance is merely due to low statistical power. Spatiotemporal variation was exceptionally high in this system (Walker et al. 2005) and this type of difficulty in interpretation due to high levels of spatiotemporal variation is often encountered in EM community studies (Horton and Bruns 2001).

Although much additional work is needed to adequately characterize the reliability of systematic seasonal patterns in this system, the appearance of different clusters of closely

related infrequent taxa on the two sample dates implies potential partitioning of the EM community associated with the seedlings within the growing season. Additional research testing whether this pattern can be observed in repeated samples over multiple seasons would be a valuable addition to our understanding of potential seasonal dynamics. For this system, by targeting the groups and taxa indicated and sequenced by this study (e.g., the thelphoid and cortinarioid groups) with molecular probes or microarrays, it may be possible to generate substantially larger sample sizes and a more intensive sampling scheme throughout the season.

The morphotyping work by van der Heijden and Vosatka (1999) also indicated seasonal shifts in generic types; however, they did not emphasize these results because of the potential for spatial variation within field sites and unknown composition of the generic morphotypes. Data were only presented for a handful of dominant types, whereas 15 types were documented and 78 sporophore species were present. In a separate publication, van der Heijden et al. (1999) posit that their *Cortinarius* type might comprise up to 30 species. Of particular interest, however, is their observation that the *Cortinarius* spp. group only formed abundant mycorrhizae in the autumn. This corroboration between the fine scale results in our study and broader ranging coarser scale results from *S. repens* stands suggests that seasonal dynamics in EM communities may occur in at least two disparate ecosystems.

Indicator species

There is a high proportion of rare species in our data, and especially rare types should not be considered to have high seasonal fidelity based on their placement in the ordination (Fig. 3). Much larger sample sizes will be required to further develop our understanding of seasonal dynamics of these rare types. For more common types, several taxa were statistically supported as indicators of the midsummer sampling. Of these, *Laccaria cf. lacatta* and Corticiaceae #1 (cf. *Piloderma bicolor*) are typically considered as generalists, and these taxa occurred on both seedling species and at both sites. However, based on these distribution patterns and the significance of the indicator values, these taxa seemed to have formed EM associations primarily in the midsummer. Although these results are congruent with seasonally partitioned variation, whether this pattern is repeatable over multiple seasons and at larger geographic scales needs to be assessed.

Several other individual dominant types did not follow a seasonal pattern (e.g., *Tuber* #01). Types that were recovered in both the midsummer and early fall harvests were also very frequently recovered at both sites (Figs. 2 and 3). A comparison of the environmental tolerances of

these ubiquitous types to those of the significant indicators would add greatly to our understanding of potential seasonal dynamics in this system. In addition, rare types may likely be more specialized and therefore may have the greatest potential as seasonal indicators but will require much larger sample sizes to document.

Ecological considerations

The implication of seasonal dynamics in EM fungus communities impacts how we think of nutrient flux between plants and fungi involved in EM associations. Based on the number of EM types per seedling in our study, seasonal carbohydrate drain by EM fungi may peak in the in midsummer or earlier, but could be extended throughout the season by a progression of fungi. Shifting EM fungi throughout the growing season could provide the phytobiont with an adaptable assemblage with greater plasticity with regard to associated changes in environmental conditions over the course of the growing period, potentially enhancing nutrient and water uptake for the plant. van der Heijden and Vosatka (1999) also hypothesized that seasonal shifts among EM types (and among AM and EM fungus types) by *Salix repens* contributes to the ecological plasticity of the phytobiont. Thus, it appears likely that the adaptability of the root-soil interface, which is mediated by EM fungi in this system, would be enhanced by seasonal dynamics of the fungi.

A possible mechanism for temporal shifts in EM assemblages has previously been discussed by Bruns (1995) and Izzo et al. (2005). They propose that root turnover, stochasticity and fine scale disturbance associated with root tip emergence, and mycelial dieback could be factors that lead to temporal transitions. In association with these factors, intra-annual variability in EM assemblages (whether seasonal or non-cyclical) could also be driven by differences in environmental tolerances of EM types or differences in foraging strategies (Agerer 2001). Changes in the relative size or activity level of the EM thalli could result in variable colonization levels even if the thalli do not completely die off over the season.

Seasonal patterns of sporophore production are well known for many EM fungus species. Virtually nothing is known, however, regarding the relationship between timing of sporophore production and root colonization and turnover events. The increase in *Cortinarius* types in the early fall (Table 1) we report is remarkably consistent with generally known seasonal sporophore production patterns for the region. Species-specific identifications of our ITS types as additional sporophore voucher sequences become available would help clarify these patterns.

Acknowledgements The authors would like to thank the Coweeta Hydrological Lab for use of facilities and site locations and the

Department of Biology at Virginia Tech. Preston Galusky, Dr. Erik Nilsen, Colin Beier, and Barry Clinton are thanked and acknowledged for assistance with field and laboratory work. This work was supported by USDA-NRI grant renewal #9502486 and an SPIRES grant from Virginia Tech.

References

- Agerer R (2001) Exploration types of ectomycorrhizae: a proposal to classify ectomycorrhizal systems according to their patterns of differentiation and putative ecological importance. *Mycorrhiza* 11:107–114
- Antibus RK (1980) Mechanisms of acclimation to lowered growth temperatures in isolates of arctic and temperate ectomycorrhizal fungi. Ph.D. Thesis, Virginia Polytechnic Institute and State Univ., Blacksburg, Virginia
- Beier CM, Horton JL, Walker JF, Clinton BD, Nilsen ET (2005) Carbon limitation leads to suppression of first year oak seedlings beneath evergreen understory shrubs in Southern Appalachian hardwood forests. *Plant Ecol* 176:131–142
- Bills GF, Holtzman GI, Miller OK Jr (1986) Comparison of ectomycorrhizal-basidiomycete communities in red spruce versus northern hardwood forests of West Virginia. *Can J Bot* 64:760–768
- Bird C, McCleneghan C (2005) Morphological and functional diversity of ectomycorrhizal fungi on Roan Mountain (NC/TN). *Southeast Nat* 4:121–132
- Blasius D, Kottke I, Oberwinkler F (1990) Spatial and seasonal dynamics of ectomycorrhizae of *Picea abies* (L.) Karst. in the Black Forest. *Agric Ecosyst Environ*, Special Issue Part A 28:27–30
- Bruns TD (1995) Thoughts on the processes that maintain local species diversity of ectomycorrhizal fungi. *Plant Soil* 170:63–73
- Dahlberg A, Stenlid J (1995) Spatiotemporal patterns in ectomycorrhizal populations. *Can J Bot* 73:S1222–S1230
- Dufrene M, Legendre P (1997) Species assemblages and indicator species: the need for a flexible asymmetrical approach. *Ecol Monogr* 67:345–366
- Facelli JM, Pickett STA (1991) Plant litter—its dynamics and effects on plant community structure. *Bot Rev* 57:1–32
- Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for Basidiomycetes—application to the identification of mycorrhizae and rusts. *Mol Ecol* 2:113–118
- Gaudinski JB, Trumbore SE, Davidson EA, Cook AC, Markewitz D, Richter DD (2001) The age of fine-root carbon in three forests of the eastern United States measured by radiocarbon. *Oecologia* 129:420–429
- Gill RA, Jackson RB (2000) Global patterns of root turnover for terrestrial ecosystems. *New Phytol* 147:13–31
- Harvey AE, Jurgensen MF, Larsen MJ (1978) Seasonal distribution of ectomycorrhizae in mature Douglas-fir/Larch forest soil in Western Montana. *For Sci* 24:203–208
- Hendrick R, Pregitzer K (1992) The demography of fine roots in a northern hardwood forest. *Ecology* 73:1094–1104
- Hibbett DS, Vilgalys R (1993) Phylogenetic relationships of *Lentinus* (Basidiomycotina) inferred from molecular and morphological characters. *Syst Bot* 18:407–433
- Hoagland DR, Arnon DI (1950) The water culture method for growing plants without soil. *California Agricultural Experiment Station Circular* 347
- Horton TR, Bruns TD (2001) The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. *Mol Ecol* 10:1855–1871
- Izzo A, Agbowo J, Bruns TD (2005) Detection of plot-level changes in ectomycorrhizal communities across years in an old-growth mixed-conifer forest. *Can J Bot* 166:619–630
- Jonsson L, Dahlberg A, Nilsson MC, Kären O, Zackrisson O (1999) Continuity of ectomycorrhizal fungi in self-regenerated boreal *Pinus sylvestris* forests studied by comparing mycobiont diversity on seedlings and mature trees. *New Phytol* 142:151–162
- Malloch D, Malloch B (1981) The mycorrhizal status of boreal plants: species from northeastern Ontario. *Can J Bot* 59:2167–2172
- McCune B, Medford MJ (1997) *Multivariate Analysis of Ecological Data*. Version 3. MJM Software
- Nantel P, Neumann P (1992) Ecology of ectomycorrhizal-basidiomycete communities on a local vegetation gradient. *Ecology* 73:99–117
- Peter M, Ayer F, Egli S, Honegger R (2001) Above- and below-ground community structure of ectomycorrhizal fungi in three Norway spruce (*Picea abies*) stands in Switzerland. *Can J Bot* 79:1134–1151
- Pigott CD (1982) Survival of mycorrhiza formed by *Cenococcum geophilum* Fr. in dry soils. *New Phytol* 92:513–517
- Rastin N, Schlechte G, Hüttermann A, Rosenplänter K (1990) Seasonal fluctuation of some biological and biochemical soil factors and their dependence on certain soil factors on the upper and lower slope of a spruce forest. *Soil Biol Biochem* 22:1049–1061
- Smith SE, Read DJ (1997) *Mycorrhizal Symbioses*, 2nd edn. Academic, London
- Stendell ER, Horton TR, Bruns TD (1999) Early effects of prescribed fire on the structure of the ectomycorrhizal fungus community in a Sierra Nevada ponderosa pine forest. *Mycol Res* 103:1353–1359
- Swank WT, Crossley DA (1988) Introduction and site description. In: Crossley DA (ed) *Forest hydrology and ecology at Coweeta*. Springer, New York, pp 3–16
- Swaty RL, Gehring CA, Van Ert M, Theimer TC, Keim P, Whitham TG (1998) Temporal variation in temperature and rainfall differentials affects ectomycorrhizal colonization at two contrasting sites. *New Phytol* 139:733–739
- van der Heijden EW, Vosatka M (1999) Mycorrhizal associations of *Salix repens* L. communities in succession of dune ecosystems. II. Mycorrhizal dynamics and interactions of ectomycorrhizal and arbuscular mycorrhizal fungi. *Can J Bot* 77:1833–1841
- van der Heijden EW, Vries FW, Kuyper TW (1999) Mycorrhizal associations of *Salix repens* L. communities in succession of dune ecosystems. I. Above-ground and below-ground views of ectomycorrhizal fungi in relation to soil chemistry. *Can J Bot* 77:1821–1832
- Vogt KA, Edmonds RL, Grier CC, Piper SR (1980) Seasonal changes in mycorrhizal and fibrous-textured root biomass in 23- and 180-year-old pacific silver fir stands in western Washington. *Can J For Res* 10:523–529
- Walker JF, Miller OK Jr (2002) Ectomycorrhizal sporophore distributions in a southeastern Appalachian mixed hardwood/conifer forest with thickets of *Rhododendron maximum*. *Mycologia* 94:221–229
- Walker JF, Miller OK Jr, Horton JL (2005) Hyper-diversity of ectomycorrhizal fungus assemblages on oak seedlings in mixed forests in the southern Appalachian Mountains. *Mol Ecol* 14:829–838
- Walker JF, Miller OK Jr, Semones S, Lei T, Nilsen E, Clinton BD (1999) Suppression of ectomycorrhizae on canopy tree seedlings in *Rhododendron maximum* L. (Ericaceae) thickets in the southern Appalachians. *Mycorrhiza* 9:49–56
- White TJ, Bruns TD, Lee SB, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: White TJ (ed) *PCR protocols: a guide to methods and applications*. Academic, London, pp 315–322
- Worley JF, Hacsckaylo E (1959) The effect of available soil moisture on the mycorrhizal association of Virginia pine. *For Sci* 5:267–268