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Nitrogen availability alters macrofungal basidiomycete community structure in optimally fertilized loblolly pine forests

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Summary

• We investigated the effect of an optimal nutrition strategy designed to maximize loblolly pine (*Pinus taeda*) growth on the rank abundance structure and diversity of associated basidiomycete communities.

• We conducted both small- and large-scale below-ground surveys 10 years after the initiation of optimal nutrition, and used TRFLP of selectively PCR-amplified nrDNA ITS to determine the distribution and abundance of macrofungal basidiomycete species in *c*. 200 soil samples collected from optimally fertilized and unfertilized treatments at the SETRES loblolly pine experimental site, North Carolina, USA.

• Our results indicated an increased relative abundance of *Tylopilus* and *Thelephora* spp. on optimally fertilized stands. Our results also suggested improved mycelial growth of several species, possibly caused by increased connectivity in the forest floor as a result of increased plant growth.

• In addition, our results suggest a trend towards reduced basidiomycete diversity, and that large-scale application of optimal nutrition may need to be sensitive to increased nitrate availability.

Key words: Basidiomycete, loblolly pine (*Pinus taeda*), TRFLP, optimal nutrition, diversity, succession, *Thelephora terrestris*, *Tylopilus*.

Introduction

Loblolly pine (*Pinus taeda*) is the leading commercial timber species in the USA, with over 13 million hectares in production in 15 south-eastern states (Schultz, 1997). Loblolly pine is an early successional species with a high rate of growth and consequently, although tolerant of low-nutrient conditions, the species is highly responsive to improved nutrient availability (Wells & Jorgensen, 1975). Commercially, single time-point nitrogen and phosphorus fertilization is a well established practice for boosting the productivity of short-rotation (< 30-year) loblolly monocultures (Schultz, 1997). Loblolly pine grows least well on droughty, nutrient-deficient, sandy soils, but recent research has shown that a more intensive fertilization strategy, optimum nutrition, can approximately double total biomass production on such soils (Albaugh *et al.*, 1998). Optimal nutrition involves adding N and other limiting nutrients annually at a rate determined through foliar analysis to be optimal for immediate plant growth needs (Albaugh *et al.*, 1998; Bergh *et al.*, 1999). As sandy soils represent approx. 7–10% of the area available for loblolly pine production (Schultz, 1997), optimum nutrition may become strategically important in order to meet global demand for loblolly-derived forest products (Albaugh *et al.*, 1998).

The impact of an optimal nutrition strategy on other components of the loblolly pine soil ecosystem is largely

unknown. Of particular interest, loblolly pine forms ectomycorrhizae with multiple species of basidiomycete and some ascomycete fungi (Menge & Grand, 1977). Ectomycorrhizal communities are typically species-rich, even in small areas of monoculture forest (Bruns, 1995), and often show a successional pattern of shifting species dominance over time that has been linked to variation in life-history strategies (Dighton & Mason, 1985; Abuzinadah & Read, 1986; Last *et al.*, 1987). Individual ectomycorrhizal species differ in their foraging strategies and microhabitat preferences, as well as in their preferred sources of N (Littke *et al.*, 1984; Keller, 1996; Baar *et al.*, 1997; Goodman & Trofymow, 1998; Agerer, 2001). It has been suggested that a decline in mineral N availability and an increase in the importance of the forest floor as a source of organic N affects the relative competitive strength of ectomycorrhizal species, and that this drives succession (Abuzinadah & Read, 1986; Bruns, 1995; Visser, 1995). For example, an increasing number of late-successional ectomycorrhizal species, such as *Piloderma* spp., *Tylospora* spp. and *Russula* spp., are known to have the potential to express peroxidase and/or polyphenol oxidase enzymes and hence may be actively involved in litter decomposition (Chambers *et al.*, 1999; Agerer *et al.*, 2000; Chen *et al.*, 2001). Generally, short-term increases in the availability of mineral N can reduce mycelial growth during the period of enrichment, and may lead to a change in the dominance structure of the community as differential sensitivity alters the outcome of competitive interactions between ectomycorrhizal species (Wallander & Nylund, 1992; Ek, 1997; Kårén & Nylund, 1997; Peter *et al.*, 2001a). Sustained increases in mineral N availability, as provided by an enriched level of atmospheric N deposition, are associated with declines in ectomycorrhizal community richness, possibly caused by the competitive exclusion of late-successional species (Lilleskov *et al.*, 2002a, 2002b). Therefore from the perspective of the soil ecosystem, optimal nutrition is a form of chronic N deposition (Fransson *et al.*, 2000; Peter *et al.*, 2001a) and could potentially alter the structure and possibly retard the development of ectomycorrhizal communities (Fransson *et al.*, 2000). The long-term functional consequences of such changes in ectomycorrhizal community structure are unknown (Leake, 2001); however, a longer period of dominance by early successional species that may be less adapted for saprotrophic activity has the potential to alter nutrient-cycling pathways within the loblolly ecosystem (Carreiro *et al.*, 2000).

Until recently descriptions of ectomycorrhizal community structure were based on sporocarp inventories, on the assumption that sporocarp distribution and diversity equalled the distribution and diversity of species below-ground (Vogt & Bloomfeld, 1992). Below-ground investigations suggest that this assumption is not always true, and that a large number of species may be active in the ecosystem (i.e. colonizing root tips) but not present in the sporocarp collection (Gardes & Bruns, 1996; Kõljalg *et al.*, 2000; Peter *et al.*, 2001b). Consequently, most recent surveys have sampled host root systems and identified the ectomycorrhizal species from these, using either morphological or molecular-based approaches (Gehring *et al.*, 1998; Jonsson *et al.*, 1999; Grogan *et al.*, 2000; Peter *et al.*, 2001a, 2001b; Lilleskov *et al.*, 2002a). A limitation of sampling root tips is the time required to process the high numbers recovered in a single sample, which limits the number of samples taken and, in turn, increases the variability in estimates of species relative abundance because of the typically patchy distribution of basidiomycete species (Horton & Bruns, 2001; Taylor, 2002).

Terminal restriction fragment length polymorphism (TRFLP) is a polymerase chain reaction (PCR)-based technique designed to allow genetic diversity to be estimated from complex mixtures of PCR products (Avaniss-Aghajani *et al.*, 1996; Clement *et al.*, 1998). It is possible to use TRFLP to examine the distribution of fungal genes (as an indicator of population diversity) within DNA extracted directly from soil: the technique has been used to assess ectomycorrhizal vertical niche differentiation (Dickie *et al.*, 2002), and fungal community response to elevated CO_2 (Klamer *et al.*, 2002). Generally, the ability to distinguish species with TRFLP is improved by reducing the taxonomic breadth targeted during PCR through the use of taxon-specific oligonucleotide primers, and by employing multiple enzyme digests in parallel (Dunbar *et al.*, 2001). In this study we used a combination of primers specific for the basidiomycete nuclear ribosomal internal transcribed spacer region (nrDNA ITS; Gardes & Bruns, 1993) and the terminal restriction fragment (TRF)-matching approach developed by Dickie *et al.* (2002) to survey and compare the basidiomycete communities of 17-year-old optimally fertilized (0.1 t N ha^{-1} $year^{-1}$) and unfertilized loblolly pine monocultures. We hypothesized that increased nutrient availability would create a shift in basidiomycete dominance patterns, and produce basidiomycete communities with a more early successional character. As little information is available regarding the basidiomycete communities common to managed loblolly pine plantations (Menge & Grand, 1977), we used rank–abundance plots and Hurlbert's probability of interspecific encounter (PIE, Hurlbert, 1971) to assess the relative successional development of the ectomycorrhizal communities (Visser, 1995; Kranabetter & Wylie, 1998). In addition, we used canonical correspondence analysis (ter Braak, 1986) to examine the response of the basidiomycete community to the environmental gradients created by nutritional treatments.

Materials and Methods

Study site

The South-east Tree Research and Education Station (SETRES) site in Scotland County, North Carolina, USA (35° N, 79° W) was established in 1985 to study the interaction of nutrient and water availability on the productivity of loblolly pine (*Pinus*

taeda L.) plantations on well drained, sandy soils (Albaugh *et al.*, 1998). The soil is a siliceous thermic Psammentic Hapludult (Wakula series), and the fertilization treatments are ambient soil conditions and 'optimum nutrition' in a 2×2 factorial design with irrigation as the second factor (King *et al.*, 2002). Optimum nutrition was defined as maintaining dormant-season foliar N concentrations at 1.3% dry mass (Adams & Allen, 1985) with fertilizer added as required, typically annually at the beginning of the growth period in April (P. Anderson, personal communication). In addition to N, other nutrients are added as required to maintain nutrient : N ratios of 0.1 : 1 for P; 0.35 : 1 for potassium; 0.12 : 1 for calcium; and 0.06 : 1 for magnesium. The two irrigation treatments at SETRES are natural precipitation and precipitation + irrigation. Annual precipitation in this region averages 1210 mm, but extended summer droughts are common (Murthy *et al.*, 1996) and irrigation is therefore employed throughout the growing season (March to October) with the aim of maintaining soil moisture between field capacity and 40% field capacity (King *et al.*, 2002). Optimal fertilization began at SETRES in 1992, after the establishment of 16 50×50 m treatment plots in the 8-year-old stand. Optimal fertilization has increased loblolly biomass production by 100% relative to unfertilized controls, and supplemental irrigation increased this growth by a further 20–25% (Albaugh *et al.*, 1998). Nitrogen is the nutrient most limiting to loblolly productivity at SETRES, and during 10 years of optimal fertilization nearly 1 metric tonne of urea has been applied per ha at this study site (Albaugh *et al.*, 1998).

Experimental design

To examine the small-scale distribution of basidiomycete species, two randomly positioned linear transects approx. 3.5 m long were marked between adjacent pine stems in each of the control (NP), fertilized (NR), and fertilized-and-irrigated (NR-w) treatments of one 1 ha SETRES block in April 2000. Eight sampling locations, each 40 cm apart and starting from the northernmost of the two trees, were marked along each transect so that 16 samples were taken per treatment plot, 48 overall. At each sampling location the litter layer was cut and removed, and the thickness of the combined litter, fermentation and humus layers (LFH) determined before taking soil cores (8 cm diameter) from the top of the fermentation horizon to a depth of 5 cm below the soil surface. Field-moist soil and organic material was passed through a nest of sieves (4, 2 and 1 mm mesh size) to separate root sections and woody debris, and fine root (< 2 mm) dry mass determined for each core. The < 2 mm soil and organic material from each core was homogenized and subsequently split into two for chemical and microbiological analyses. For chemical analyses soils were air-dried then stored at 4°C until analysis. Microbiological samples were frozen, subsequently freeze-dried, and stored at −20°C.

To examine the large-scale impact of the optimum nutrition treatments on basidiomycete community structure, soil samples were taken from three replicate plots each of NP, NR and NR-w treatments, using a stratified random scheme, in October 2000. Each 900 m^2 measurement plot was subdivided into a regular grid of 5×5 m subplots, and sample taken from a randomly located position within 18 of these. The October survey (main study) therefore collected 54 samples per treatment, 162 overall. Samples were taken and treated as described.

Laboratory analyses

Soil mineral N ($NH_4^+ + NO_3^-$ as NO_2^-) availability in 2 M KCl extracts using a Lachat microbore flow injection analyzer (method 10-107-06–1A, Lachat Instruments, Milwaukee, WI, USA). Exchangeable Ca^{2+} and Mg^{2+} were determined by optical emission spectroscopy of 1 M NH_4OAc extracts (Suarez, 1996). Acidity (pH) was measured in a 1 : 4 soil: 0.01 M $CaCl_2$ suspension (Hendershot *et al.*, 1993). Moisture content was estimated by loss-of-mass on air-drying for 4 days. Total C and total N were determined by combustion at 600°C (LECO TC/TN Analyzer, Leco Corporation, St Joseph, MI, USA).

For microbial analysis, DNA was extracted from two 0.5 g subsamples from each core. Soil was soaked in 1 ml fungal lysis buffer Y (Bio 101, Vista, CA, USA) overnight at 4°C before bead-beating, and then processed with the Bio 101 FastDNA S kit following the manufacturer's instructions, except that a 1 h digest with 20 U proteinase-K (Boehringer Mannheim, Indianapolis, IN, USA) was added between the bead-beating and protein precipitation steps to improve DNA yield. DNA from both subsamples was combined and further purified with Geneclean (Bio 101). All DNA was stored at −20°C until required. TRFLP was performed as described in the following section, with 50–200 ng soil bulk DNA as template for PCR.

Determination of operational TRFLP resolution

Sporocarps were collected at SETRES between August and October 2000, sorted, identified to genus in the field based on morphological characters (Largent & Thiers, 1977; Arora, 1986), and subsequently divided into TRFLP types defined by a unique three character *Hin*fI–*Taq*I–*Hae*III TRF profile. Dried specimens were deposited in the Purdue University Herbaria, West Lafayette (PUL). Each unique TRFLP type was subsequently sequenced, and the sequence alignment used to determine the operational resolution of the TRFLP approach.

Sporocarp DNA was extracted from 10–20 mg dried pileal tissue using the Bio 101 FastDNA kit according to the manufacturer's instructions. DNA extracts were subsequently cleaned with Geneclean (Bio101) and purity checked by UV absorbance at 260/280 nm (Sambrook *et al.*, 1989). A

Geneclean step was found to be necessary for many of the darkly pigmented specimens to ensure efficient recovery of DNA. The basidiomycete nuclear rDNA internal transcribed spacer (ITS) was amplified for TRFLP using the basidiomycete-specific primer pair ITS1F–ITS4B (Gardes & Bruns, 1993) with the ITS1F primer 5′ labeled with the dye 6-FAM. PCR cocktails contained 1–10 ng sporocarp DNA, 1× PCR buffer (Promega, Madison, WI), 200 nM dNTPs, 0.1 mg ml^{-1} BSA, 1.5 mM KCl, 0.5 μmol of each primer and 1 U *Taq* DNA polymerase (Promega) in a 50 μl final volume. The thermal PCR profile consisted of an initial denaturation step of 1 min at 94°C followed by 35 cycles consisting of 35 s denaturation at 94°C, annealing for 55 s at 55°C, and elongation at 72°C. The length of the elongation step was 45 s for cycles 1–12, 2 min for cycles 13–26, and 3 min for cycles 27–35 (Gardes & Bruns, 1993). The elongation step in the final cycle was extended to 13 min. PCR reactions were performed in an Eppendorf Mastercycler gradient thermal cycler (Eppendorf GmbH, Hamburg, Germany), and amplification success was checked by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Before restriction digestion, Wizard PCR Preps (Promega) were used according to the manufacturer's instructions to remove excess primer and reaction components from PCR products. Aliquots (10 μl) of the purified products were digested for 8 h with 5 U *Hin*fI, *Taq*I or *Hae*III in the appropriate buffer (Promega). A 1 μl aliquot of each digest was combined with 1 μl TAMRA 50–500 internal standard (Applied Biosystems, Warrington, UK), 1 μl gel-loading dye and 2.5 μl electrophoresis-grade formamide (FisherBiotech, Fair Lawn, NJ, USA). The mixtures were denatured at 95°C for 3 min, then immediately placed on ice until loading the gel. Electrophoresis was carried out on a 36-lane, 36 cm 8% polyacrylamide gel containing 8.3 M urea and 1× TBE buffer for 3 h in an ABI 377 automated sequencer (Applied Biosystems). Each sample was prepared in triplicate and 2 μl manually loaded into each of three adjacent lanes. Electropherogram traces for the three replicate lanes for each sample were overlaid, and only fragments occurring in all three lanes scored. Fragment sizes were calculated with respect to internal standard fragment migration time using GENESCAN software (Applied Biosystems) and a local Southern algorithm.

Unlabelled ITS1F–ITS4B PCR products for subsamples of each TRFLP type were reamplified with ITS1F–ITS4 (2 μl PCR product in a 20 μl PCR reaction, PCR mixture and thermocycle parameters as above), cleaned as above, and sent to the Purdue Agricultural Genomics Core Laboratory for sequencing. Sequences were aligned and percentage sequence similarity determined using DNAMAN ver. 4.13 (Lynnon Biosoft, Vaudreuil, Quebec). TRFLP type sequences were compared with GenBank and EMBL nucleotide databases using the BLAST tool (NCBI) to determine the species or species group of closest identity. The nucleotide sequences of each SETRES TRFLP type have been submitted to GenBank (Accession numbers AY456332 to AY456377).

Terminal restriction fragment-matching procedure

Sporocarp TRFLP types were used to create a SETRES-specific version of the T-RFLP ANALYSIS MATCHING PROGRAM (TRAMP) (Dickie *et al.*, 2002) to aid the interpretation of soil TRFLP profiles. For each soil sample separately, all scored *Hin*fI and *Taq*I TRF fragments > 2% mean relative signal intensity were used as TRAMP input, with measurement precision set at ±2 bp precision (Dickie *et al.*, 2002). TRAMP uses an array formula to compare input TRF against the reference collection, and we considered the TRAMP results as putative matches for basidiomycete species known to be present at SETRES. For each putative match the mean relative intensities of the appropriate *Hin*fI and *Taq*I TRF were compared, and when these did not differ significantly (*t*-test, alpha level 0.05), the TRAMP match was accepted. When *Hin*fI and *Taq*I TRF alone could not distinguish unambiguously between putative matches, a further *Hae*III digest was performed on the soil PCR product, and the TRFLP output inspected for *Hae*III TRF of the correct size. *Hin*fI and *Taq*I TRF observed in soil samples but not represented by species in the TRAMP database were grouped together based on their mean relative signal intensity, and assigned numbers (e.g. TRFLP type 1). All TRFLP types were treated as species for analysis.

Statistical analysis

A repeated-measures analysis was used to examine the small-scale spatial influence of trees on the environmental parameters litter depth and root mass (SAS ver. 8.2, SAS Institute, Cary, NC, USA). Four distance classes were defined (40, 80, 120 and 160 cm) corresponding to the distance to the nearest tree of the samples taken along the small transects. The influence of treatment on the species similarity of adjacent cores along transects was assessed with one-way ANOVA of Dice similarity index scores (SAS ver. 8.2).

For the main study, ANOVA was used to determine the effect of optimal fertilization treatments on the soil variables and TRFLP type richness using a randomized complete block design with three treatments (unfertilized, NP; optimally fertilized, NR; optimally fertilized and irrigated, NR-w) and three replicates of each treatment. Normality and equivalence of variance assumptions were checked graphically, and variables transformed (typically $\log_{10}$) when necessary for analysis. The significance of changes in the relative abundance of individual TRFLP types was also assessed with ANOVA. However, many TRFLP types were absent from over half the replicate plots, and hence exhibited highly skewed distributions that could not be improved through transformation. For these TRFLP types we did not perform ANOVA. Significant treatment differences were assessed with Tukey's honestly significant difference (HSD) at an alpha level of 0.05.

Canonical correspondence analysis (CCA, ter Braak, 1986) was used to determine the relationship between the relative

abundance of each TRFLP type and the underlying environmental gradients created by the fertilization treatments. The statistical model underlying CCA is that species' abundance exhibits a unimodal response to linear combinations of environmental values. CCA creates an ordination diagram that simultaneously displays the main patterns of community variations and the main pattern in the weighted averages of species relative abundances with respect to the environmental parameters (ter Braak & Prentice, 1988). The abundance of TRFLP types was estimated as their spatial frequency, and the relative abundance of species by:

$$\text{relative abundance of species}_i = \{[(\text{number of observations}_i)/\text{total number of observations}] \times 100\}$$

CCA with the model optimal fertilization + irrigation was performed in CANOCO ver. 4.5 (ter Braak & Smilauer, 2002). Soil variables characterizing the forest floor environments were added as supplementary 'passive' variables to allow an alternative interpretation of the main treatment effects (Baar & ter Braak, 1996). To limit the influence of rare species, the CCA species matrix was limited to those species that were observed at least twice in the main survey. The statistical significance of the first canonical axis, and of both constrained axes together, was assessed through Monte Carlo simulations with 499 permutations of the residuals. Monte Carlo tests do not require normality of the errors (Manly, 1990). Generalized linear models (GLM) were used to assess the significance of apparent changes in individual TRFLP type relative abundance along the primary CCA-defined constrained axis.

Basidiomycete community rank–abundance distributions were determined from pooled replicate treatment data. Values of Hurlbert's PIE (Hurlbert, 1971) were calculated for each plot, and the significance of difference between treatments determined with one-way ANOVA. PIE was calculated using the species-richness module of ECOSIM (Gotelli & Entsminger, 2001) as $\text{PIE} = (N/N-1)(1-\Sigma\, p_i^2)$, where N equals the total number of species in the sample, and p_i is the proportion of the sample represented by species i. Rarefaction was used to balance the number of observations in each sample before calculating PIE.

Results

Environmental variation between fertilization treatments

ANOVA revealed that the environmental variables could be divided into three groups in terms of their response to the optimal fertilization programs: those that showed a significant response to fertilization; those that showed a significant response to fertilization and a differential response between irrigated and nonirrigated treatments; and those that showed no significant response. Total depth of forest floor (litter layer + combined fermentation and humus layers), total N, and fine root mass all showed a general response to fertilization, being typically greater on fertilized plots (Table 1). The concentrations of potentially available NH_4^+, NO_3^-, Ca^{2+} and Mg^{2+} also increased on fertilized plots (Table 1), although concentrations of calcium and magnesium were significantly higher on NR than NR-w plots ($P < 0.005$), and nitrate concentrations tended to be higher on NR than NR-w. By contrast, ammonium concentrations tended to be higher in NR-w plots than in NR, and soil moisture content was also highest in these plots (Table 1). Neither mineral soil pH nor total organic carbon showed a significant response to fertilization, although both tended to increase (Table 1).

Table 1 Mean values and coefficient of variation (%) for environmental variables on control, fertilized and fertilized + irrigated treatment plots at the SETRES loblolly pine (*Pinus taeda*) plantation in North Carolina, USA in October 2000

Parameter	Control (NP)	Fertilized (NR)	Fertilized and irrigated (NR-w)
Parameters showing a general response to fertilization			
Litter depth (cm)	0.91 b† (78)	2.34 a (38)	2.22 a (42)
Total N (%)	0.062 b (16)	0.083 a (7.2)	0.083 a (9.6)
Fine roots (g per core)‡	1.26 ab (69)	2.01 a (32)	2.26 a (17)
Parameters showing a differential response to specific fertilization treatments			
Soil moisture (%)	2.46 b (16)	3.00 b (31)	7.00 a (42)
FH layer (cm)	0.21 b (81)	0.56 ab(71)	0.83 a (72)
NH_4^+ (mg kg^{-1})	3.46 b (76)	4.97 ab (55)	6.79 a (79)
NO_3^- (mg kg^{-1})	0.28 b (135)	0.58 a (90)	0.47 ab (87)
Ca^{2+} (mg kg^{-1})	17.4 c (70)	48.5 a (77)	30.1 b (99)
Mg^{2+} (mg kg^{-1})	2.27 c (70)	8.10 a (82)	4.33 b (59)
Parameters showing no significant response to fertilization			
pH	3.39 a (7.6)	3.51 a (6.3)	3.41 a (6.7)
Total organic C (%)	2.92 a (14)	3.26 a (8.6)	3.18 a (8.5)

†Values followed by different letters in the same row are significantly different at $\alpha = 0.05$ as determined by Tukey's HSD. ‡Core area 50 cm^2, volume variable because of inclusion of forest floor material.

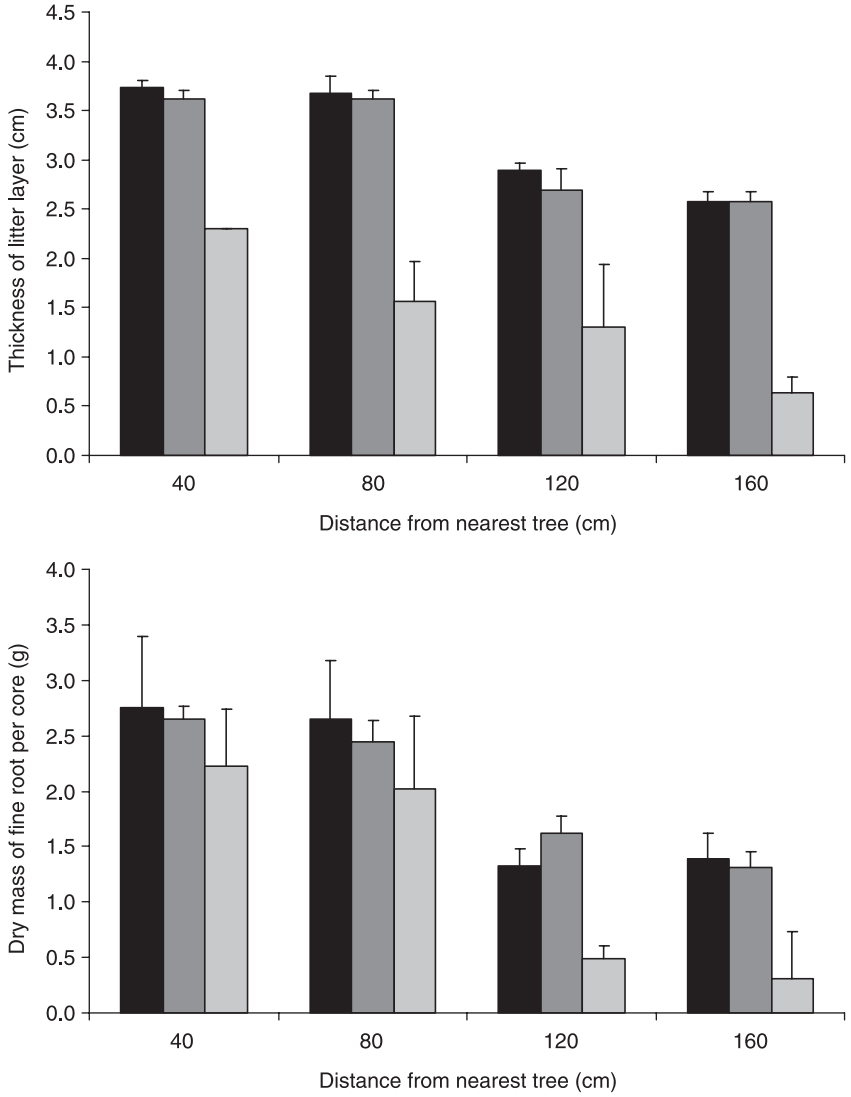

Fig. 1 Mean values and SD (n = 4) of litter/forest floor (top) and fine roots (below) as a function of distance from tree boles in the three treatment types at the SETRES site (NC, USA). Grey bars, unfertilized; black bars, fertilized; stippled, fertilized + irrigated.

For each environmental parameter the coefficient of variation [(SD/mean) × 100] was used as a measure of heterogeneity. All environmental parameters showed skewed distributions, and for many parameters, including forest floor depth, fine root mass and chemical nutrient concentrations, coefficients of variation were 50–135% (Table 1), indicating a considerable degree of within-plot environmental heterogeneity. The transect samples revealed that some intraplot environmental heterogeneity was the result of a radial pattern of forest floor development centered at the bole of growing loblolly pine trees (Fig. 1). Repeated measures indicated that both the depth of the forest floor, and the density of fine roots, were significantly higher 40 and 80 cm from the nearest tree than 120 and 160 cm from the trees (litter, $F = 53$, $P < 0.0001$, 3 df; root density, $F = 9.65$, $P = 0.004$, 3 df). Figure 1 shows that fine root mass in samples proximal to loblolly stems is similar in all treatments, but drops dramatically in the more distal samples of the control transects.

TRFLP resolution

Species belonging to 19 genera were found and identified, 13 of which (*Amanita*, *Cantharellus*, *Clavulina*, *Craterellus*, *Inocybe*, *Laccaria*, *Lactarius*, *Russula*, *Phylloporus*, *Tylopilus*, *Xerocomus* and *Thelephora*) are known or presumed to be ectomycorrhizal, all others (*Agaricus*, *Armillaria*, *Astraeus*, *Geastrum*, *Clavaria*, *Ganoderma* and *Marasimus*) are saprotrophs.

TRFLP divided the sporocarp collection into 27 TRFLP types, and generally each type was defined by a single dominant *Hin*fI, *Taq*I and *Hae*III nrDNA 5′-TRF (Table 2). Exceptions were *Tylopilus* sp. 2, which consistently produced two *Hin*fI TRF of similar intensity at 127.4 and 134.2 bp, and *Russula* sp. 5, which produced two *Taq*I TRF of approximately equal intensity 110.2 and 200.3 bp long. No *Hae*III TRF were observed for *Xerocomus* sp., *Russula* sp. 4, *Russula* sp. 2, or *Craterellus* sp., suggesting that these either lack a *Hae*III restriction site or produce a fragment longer

Table 2 SETRES reference ITS TRFLP types and closest BLAST matches between sporocarp ITS sequences and sequences from the GenBank database

TRFLP type†	5′-TRFLP – pattern (base – pairs ± 1) *Hin*fI–*Taq*I–*Hae*III	GenBank accession	Closest species match (BLAST)	Identity %	Overlap (bases)
Agaricus sp. 1 (1)	264–306–256	AY456333	[AF482832] *Agaricus subrutilescens*	97	678
Amanita sp. 1 (2)	328–284–104	AY456334	[AY228351] *Amanita constricta*	89	508
Amanita sp. 2 (4)	395–342–094	AY456336	[APH308097] *Amanita phalloides*	90	368
Armillaria sp. (2)	452–389–468	AY456338	[AF163558] *Armillaria mellea*	77	419
Geastrum sp. (3)	327–273–>600	AY456375	None		
Clavulina cinerea (2)	364–171–263	AY456339	[AF335456] *Clavulina cinerea*	97	637
Craterellus sp. (1)	322–416–>500	AY456340	[AF385632] *Craterellus tubaeformis*	97	227
Ganoderma lucidum (1)	354–301–100	AY456341	[AF506371] *Ganoderma lucidum*	97	528
Inocybe sp. (1)	381–328–234	AY456377	[IN534934] *Inocybe nitidiuscula*	95	173
Laccaria sp. (1)	093–289–523	AY456342	[AF006595] *Laccaria bicolor*	82	371
Russula sp. 1 (1)	341–121–172	AY456349	[AY061730] *Russula cessans*	99	172
Russula sp. 2 (2)	343–290–>600	AY456357	[AF349710] *Russula* sp. NC2172	98	634
Russula sp. 3 (2)	369–411–174	AY456361	[AY061655] *Russula amoenicolor*	86	352
Russula sp. 4 (3)	369–312–>600	AY456358	[AF418614] *Russula laurocerasi*	96	598
Russula sp. 5 (3)	372–200–505	AY456364	[AF440672] Uncultured Russulales	96	361
Russula sp. 5 (2)	372–110–505	AY456367	[AF440672] Uncultured Russulales		
Russula sp. 6 (1)	360–306–493	AY456077	[AF418616] *Russula fellea*	97	432
Lactarius sp. 1a (2)	367–314–207	AY456350	[AF104254] *Lactarius subsericatus*	92	707
Lactarius sp. 1b (3)	106–314–494	AY456343	[AF104254] *Lactarius subsericatus*	92	658
Lactarius sp. 2 (3)	219–318–499	AY456346	[LQU272247] *Lactarius quietus*	90	582
Phylloporus sp. (2)	375–068–121	AY456356	[AY372287] *Xerocomus* sp. GD – 2003	92	284
Tylopilus sp. 1 (2)	115–068–598	AY456371	[AF402140] *Xerocomus pruinatus*	77	662
Tylopilus sp. 2 (3)	129–069–124	AY456372	[AF402140] *Xerocomus pruinatus*	72	700
Xerocomus sp. (1)	071–068–545	AY456374	[AY372287] *Xerocomus* sp. GD – 2003	90	543
Thelephora terrestris (3)	352–299–095	AY456370	[TTU83486] *Thelephora terrestris*	98	553
Clavaria sp. (2)	180–167–247	AY456373	None		
Marasmius sp. (3)	380–329–101	AY456352	[AF505784] *Gymnopus spongiosus*	86	380

†Number in parentheses indicates number of subsamples sequenced.

than the 500 bp range we examined. Generally, for this collection, *Hin*fI and *Taq*I TRF together were sufficient to distinguish all species, except *Thelephora terrestris* from *Ganoderma* sp. and *Russula* sp. 3 from *Lactarius* sp. 1. Addition of *Hae*III TRF enables *Russula* sp. 3 to be distinguished from *Lactarius* sp. 1, and *Ganoderma lucidum* from *T. terrestris*. Sporocarps of *Astreaus* cf. *hygrometricus*, *Cantharellus* sp. and *Trametes* sp. produced either no, or very faint, PCR products with the basidiomycete primer set, and it was not possible to obtain TRFLP types for these species. No product was observed when loblolly pine DNA was used as the template. Within-gel measurement precision was high, 95% confidence intervals for individual fragments ranged from 0.1 to 0.4 bp, and intergel precision was estimated as 0.75 ± 0.35 bp.

Sequencing produced between 400 and 700 high-quality bases for 46 specimens representing the 27 TRFLP types. Sequence alignments (Fig. 2) showed that specimens grouped together within a single TRFLP type exhibited a high degree of sequence similarity (mean 97.7%), and that unique TRFLP types were created by specimens with < 95% ITS sequence similarity. BLAST searches generally confirmed generic placement of the species and provided an indication of closest species group: *Agaricus* sp. showed a strong overlap (678 bases) and high identity (97.5%) with *Agaricus subrutilescens*, with which it shares also shares an indistinguishable TRFLP type. Similarly, the SETRES isolates of *G. lucidum* and *T. terrestris* showed strong overlap, high identity and indistinguishable TRFLP types with GenBank reference sequences. A SETRES *Clavulina* specimen exhibited high sequence similarity with *Clavulina cinerea* [AF335456], but shares only two of the three TRF with the reference sequence. Sequence analysis shows the observed site for the SETRES *Clavulina* specimen is the second 5′ site in *C. cinerea* AF335456. *Lactarius* species 1a and 1b, which have 95% sequence identity with each other, also share 95% identity with *Lactarius subsericatus* [AF204678]. The TRFLP type for *Lactarius* species 1b is, in addition, identical to that of the reference sequence, suggesting a close relationship between these species. For most SETRES isolates, however, similarity matches with species currently in the GenBank system are often low (Table 2), and further morphological and sequence analysis will be required to improve the SETRES species list.

Basidiomycete communities

Overall, DNA was extracted from 210 samples (162 for the main survey and 48 for the transect survey) and PCR amplification of basidiomycete rDNA ITS was successful from 85% of these. Between one and five TRFLP types were observed per core (mean 3.27), although the mean number of TRFLP types per soil sample was significantly higher in the two types of fertilized plots than in the controls (2.9 in control, 3.5 in fertilized and fertilized–irrigated, $F = 4.59$, $P = 0.012$, 2 and 6 df).

For the transect study 23 TRFLP types were observed, 18 of which could be matched to species collected as sporocarps at the site. Sixteen of the 18 are ectomycorrhizal, one a root-infecting pathogen (*Armillaria* sp.), and one a litter-decomposing saprotroph (*Marasmius* sp.). Between five and nine TRFLP types were observed per transect, and no TRFLP type was observed in all six transects, although *Lactarius* sp. 2 was observed in five. Cluster analysis of TRFLP presence–absence data (not shown) showed no clear treatment-related pattern and a high degree of sample-to-sample variation in TRFLP assemblage, indicating a discontinuous distribution of basidiomycete species below ground. Despite this, within a transect the same TRFLP type was often sampled in several adjacent cores, suggesting small-scale patches of distribution. Patches of *T. terrestris* were observed on three of the six transects and account for 11/12 of all *T. terrestris* observations. All *Geastrum* sp. and TRFLP type 34 observations were restricted to adjacent cores, as were 90% of *C. cinerea*, > 70% of all *Lactarius* sp. 2, and 30–50% of all other observations. The patch size (estimated as the number of adjacent cores) of several species tended to be larger in optimally fertilized plots (Fig. 2), and the within-transect Dice similarity coefficient was also

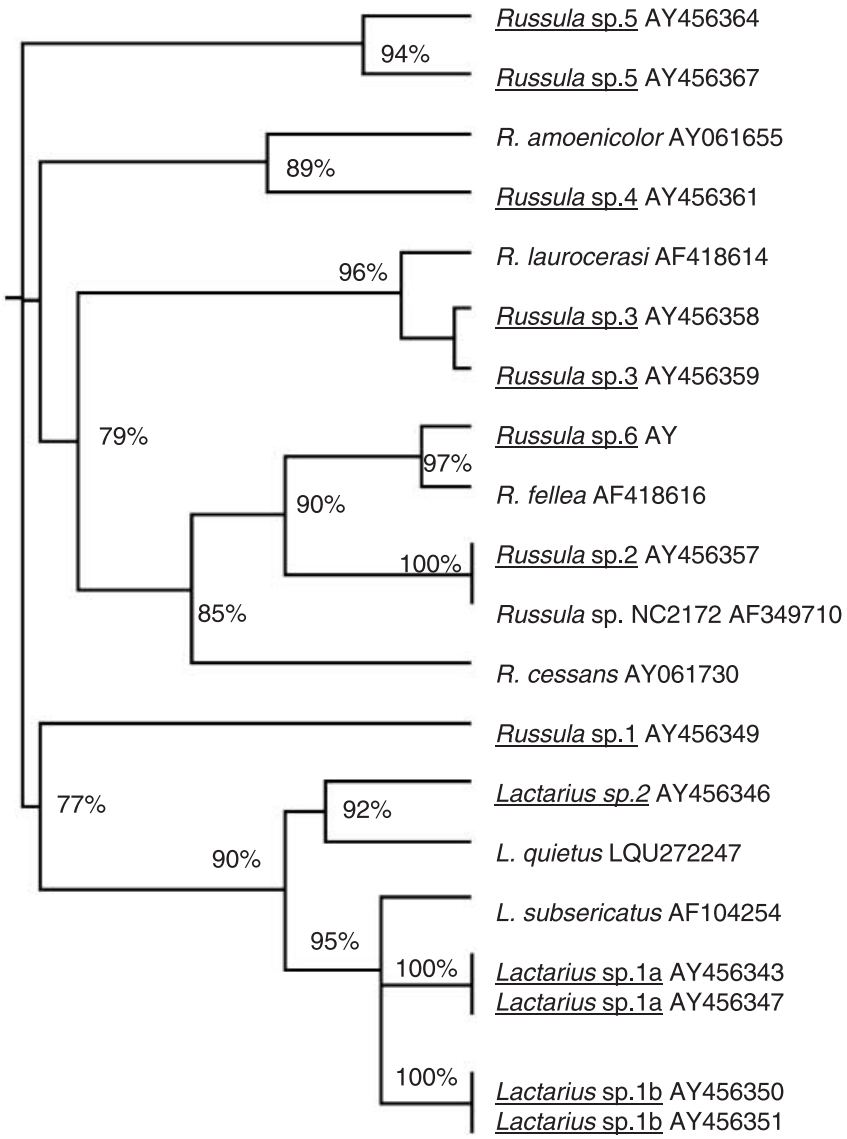

Fig. 2 rDNA ITS sequence alignment for SETRES site (NC, USA). *Russulaceae* and best BLAST matches indicating percentage sequence identity. SETRES sequences are underlined.

Table 3 Mean relative abundance (%) of basidiomycete species at the SETRES site (North Carolina, USA), on control (NP), optimally fertilized (NR) and optimally fertilized and irrigated (NR-w) treatments

Species	Species code†	NP	NR	NR-w
Lactarius sp. 2	Lact2	21.3 ± 6.50 a‡	15.7 ± 1.25 a	12.6 ± 6.28 a
Thelephora terrestris	Theter	8.82 ± 1.02 b	15.7 ± 1.26 a	14.5 ± 3.37 a
Russula sp. 6	Russ6	5.71 ± 0.97 a	4.19 ± 3.03 a	3.21 ± 2.90 a
TRFLP type 4	TRF4	4.04 ± 7.00	0.00 ± 0.00	0.00 ± 0.00
TRFLP type 10	TRF10	3.74 ± 5.20 a	0.64 ± 1.11 a	0.00 ± 0.00 a
TRFLP type 2	TRF2	3.54 ± 4.87	0.00 ± 0.00	0.95 ± 1.65
TRFLP type 9	TRF9	3.11 ± 0.10 a	0.81 ± 1.41 b	2.56 ± 0.48 a
Russula sp. 5	Russ5	3.10 ± 3.03	0.00 ± 0.00	1.61 ± 1.45
TRFLP type 18	TRF18	3.08 ± 5.33 a	11.0 ± 6.23 a	5.69 ± 3.79 a
Inocybe sp.	Inoe	2.56 ± 4.44	0.00 ± 0.00	1.62 ± 1.47
Clavulina cinerea	Clacri	2.55 ± 2.35 a	2.74 ± 2.90 a	3.97 ± 2.70 a
Clavaria sp.	Claa	2.15 ± 3.72 a	3.25 ± 3.73 a	3.89 ± 1.83 a
TRFLP type 17	TRF17	2.10 ± 1.82 a	0.64 ± 1.11 a	0.95 ± 1.65 a
TRFLP type 21	TRF21	2.29 ± 2.66 a	0.00 ± 0.00 a	1.82 ± 2.90 a
Tylopilus sp. 2	Tylo2	2.09 ± 1.81 c	14.7 ± 2.56 a	6.34 ± 3.76 b
TRFLP type 7	TRF7	2.09 ± 1.81	0.00 ± 0.00	0.00 ± 0.00
Amanita sp. 2	Amat3	2.05 ± 3.55	0.00 ± 0.00	0.00 ± 0.00
Ganoderma lucidum	Ganluc	2.02 ± 3.50 a	0.00 ± 0.00 a	4.74 ± 5.02 a
Lactarius sp. 1a	Lact 1a	1.59 ± 1.61 a	3.55 ± 3.11 a	1.89 ± 1.64 a
TRFLP type 21	TRF21	1.53 ± 2.66	0.00 ± 0.00	2.37 ± 2.98
Russula sp. 1	Russ 1	1.54 ± 2.66	0.00 ± 0.00	0.00 ± 0.00
Xerocomus sp.	Xers	1.08 ± 1.86 a	2.27 ± 2.46 a	4.54 ± 4.11 a
Russula sp. 4	Russ4	1.08 ± 1.86 a	0.64 ± 1.11 a	0.94 ± 1.63 a
TRFLP type 16	TRF16	1.08 ± 1.86 a	0.00 ± 0.00 a	0.95 ± 1.65 a
TRFLP type 15	TRF15	1.08 ± 1.86	0.00 ± 0.00	0.47 ± 0.81
Marasmius sp.	Mars	1.03 ± 1.78 b	0.00 ± 0.00 b	2.56 ± 0.48 a
TRFLP type 19	TRF19	1.03 ± 1.78 a	0.81 ± 1.41 a	0.95 ± 1.65 a
Lactarius sp. 1b	Lact1b	1.01 ± 1.75 a	0.81 ± 1.41 a	1.62 ± 1.47 a
Russula sp. 2	Russ2	0.51 ± 0.89 b	0.00 ± 0.00 b	2.09 ± 0.73 a
TRFLP type 26	TRF26	0.51 ± 0.89	0.64 ± 1.11	0.95 ± 1.65
TRFLP type 34	TRF34	0.00 ± 0.00 a	5.52 ± 3.96 a	3.31 ± 2.96 a
Phylloporus sp.	Phys	0.00 ± 0.00 a	2.44 ± 4.22 a	0.67 ± 1.15 a
TRFLP type 35	TRF 35	0.00 ± 0.00	0.64 ± 1.11	0.95 ± 1.65
Geastrum sp.	Geas	0.00 ± 0.00	1.28 ± 2.22	0.67 ± 1.15
TRFLP type richness		19.7 ± 5.7 a	15.7 ± 4.0 a	23.3 ± 3.1 a
Hurlbert's PIE		0.93 ± 0.03 a	0.91 ± 0.02 a	0.94 ± 0.03 a

†TRFLP type abbreviation used in CCA biplot. ‡Values followed by a different letter are significantly different at alpha = 0.05. TRFLP types with no letter were not assessed with ANOVA because of highly skewed distributions.

significantly higher in optimally fertilized plots (NP mean 0.25 ± 0.31; NR-w mean 0.46 ± 0.34; NR mean 0.68 ± 0.28; $F = 3.913$, $P = 0.03$, 2 and 3 df).

For the main study, PCR products produced a total of 444 *Hin*fI TRF, 422 *Taq*I TRF and 225 *Hae*III TRF of = 2% relative signal intensity, and the TRF-matching procedure defined 64 TRFLP types, 22 of which could be matched with those produced by the sporocarps at the site. Thirty TRFLP types were observed only once, in a single soil sample. Table 3 lists the relative abundance of the 34 TRFLP types found at least twice. Nineteen of these 34 TRFLP types could be matched to sporocarp TRFLP types, and 20 were common to both main and transect surveys. The data in Table 3 show a high degree of compositional similarity between control and optimally fertilized treatments, with Dice similarity values ranging from 60 to 77%. Both *Tylopilus* sp. 2 and *T. terrestris* were significantly more abundant on the optimally fertilized plots, and the unidentified TRFLP type 9 was significantly less abundant on the fertilized-only plots. Russula sp. 1, TRFLP type 7 and TRFLP type 4 were observed solely on NP plots, and *Phylloporus*, *Geastrum*, TRFLP type 34 and TRFLP type 35 solely on optimally fertilized plots. Interestingly, *Russula* sp. 2 and the litter-decomposing *Marasmius* species were not observed on optimally fertilized (NR) plots, but were significantly more abundant on NR-w than on NP, suggesting a differential response to nutrient addition and irrigation. The most frequently encountered TRFLP type (*Lactarius* sp. 2) showed a trend towards decreasing relative abundance on optimally fertilized treatment plots, but this was not significant.

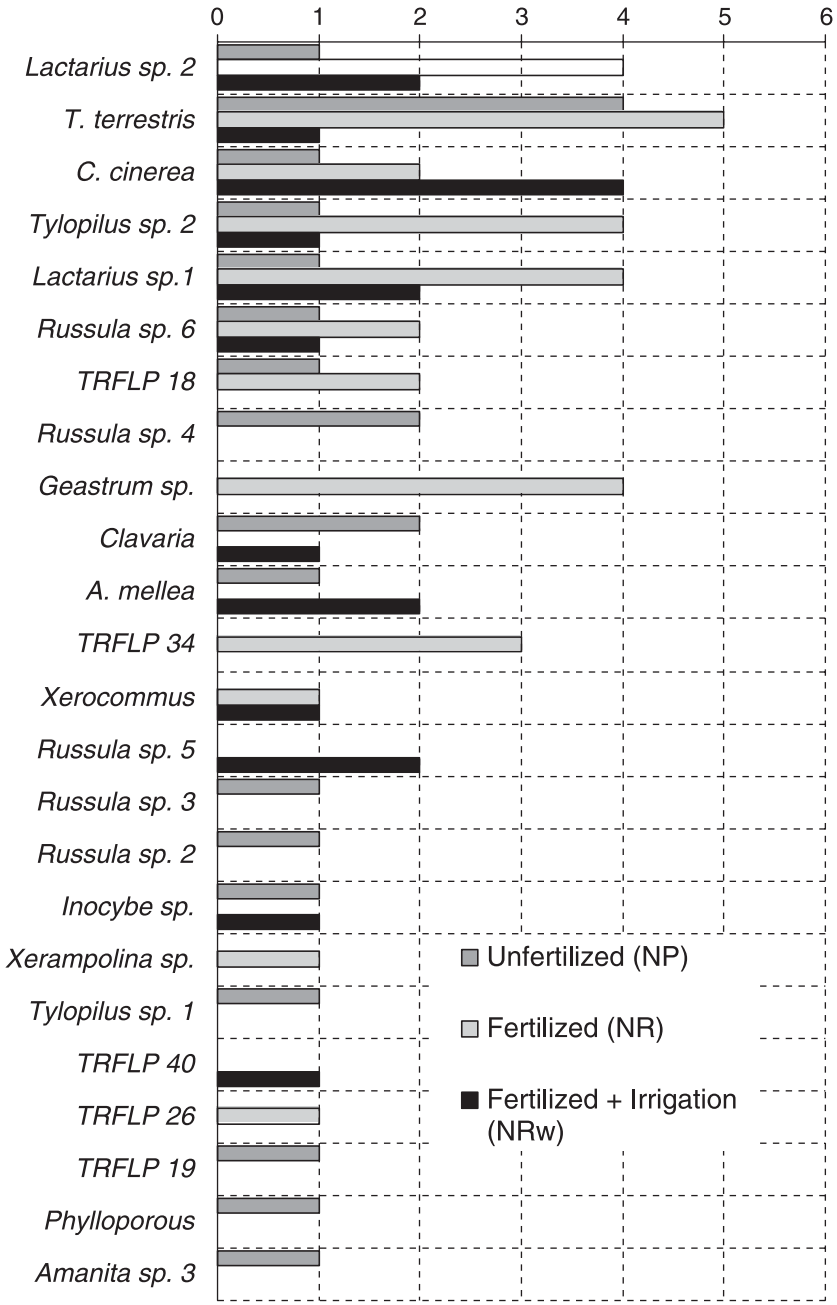

Fig. 3 Comparative maximum number of adjacent cores in which individual TRFLP types were recovered from small-scale transects at the SETRES site (NC, USA). Cores were spaced approx. 40 cm apart on transects linking neighbouring trees.

Canonical correspondence analysis revealed that the optimal nutrition treatments accounted for 32.7% of the variance in TRFLP type relative abundance, and Monte Carlo tests determined that both the first canonical axis and the overall ordination were statistically significant (*P* values 0.002 and 0.03, respectively). In the CCA biplot (Fig. 4) the points for optimal fertilization and irrigation are closer to each other than to the point for no fertilization, indicating that fertilization has a greater effect than irrigation on TRFLP type abundance. The first canonical axis (eigenvalue 0.210) separated TRFLP types with a higher abundance in unfertilized, nutrient-poor plots from those that responded positively to optimal fertilization. Projection showed that approximately half the TRFLP types and TRFLP richness project on the nutrient-poor side of this axis. The second axis (eigenvalue 0.078) represented a weaker gradient determined by TRFLP types with a differential response to irrigation. As expected from the ANOVA, the vectors for litter depth and total N content defined an axis linking the point for optimal fertilization to that for no fertilization. By contrast, the vectors for NO_3^-, Ca^{2+} and Mg^{2+} are strongly correlated to the first axis, while the vectors for NH_4^+ and thickness of the FH layer are

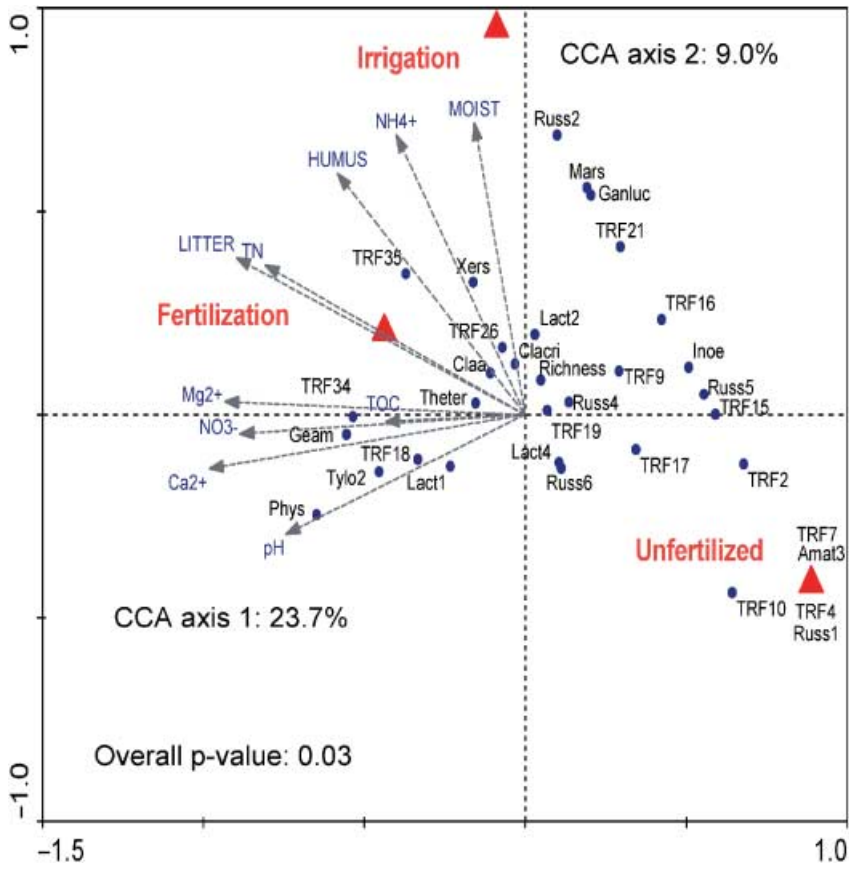

Fig. 4 Species biplot based on a canonical correspondence analysis of SETRES (NC, USA). TRFLP type displaying 32.7% of the variance in TRFLP type abundances explained by the model optimal fertilization + irrigation. Available nutrients, pH, litter depth, moisture content, total organic C and total N are added as supplementary variables (dashed arrows indicate marginal effects). TRFLP types are abbreviated as in Table 2.

strongly correlated to the second axis, reflecting the differential behavior of these variables in NR and NR-w plots (Table 1). General linear models indicated significant relationships between the abundance of *T. terrestris*, *Tylopilus* sp. 2, TRFLP type 34, *Russula* sp. 5, TRFLP type 7 and TRFLP type 9 with the first canonical axis (Fig. 5), and between *Russula* sp. 2 and *Marasmius* sp. with canonical axis 2.

TRFLP type richness showed no significant relationship with the canonical axes (Fig. 5). Rank–abundance curves (Fig. 6) suggested a reduced number of low-abundance 'tail' species in the optimally fertilized community relative to both nutrient-poor and optimally fertilized and irrigated treatments. Mean PIE values ranged from 0.91 to 0.94 (Table 3), supporting this trend, but ANOVA revealed no significant treatment-related difference ($F = 2.26$, $P = 0.158$, 2 and 6 df).

Discussion

TRFLP is a restriction enzyme-based method that samples sequence divergence in PCR-amplified DNA targets, and its primary advantage over the more established RFLP method is that it can be used with complex DNA mixtures extracted directly from soil. Because of this, large numbers of field samples can be processed which may allow for a more effective description of the distribution and relative abundance of the often discontinuously distributed basidiomycete species at a site (Horton & Bruns, 2001). However, comparatively little is known about the level of genetic resolution of the TRFLP technique (Zhou & Hogetsu, 2002). In this study TRFLP of the basidiomycete nrDNA ITS region divided our sporocarp specimens into 27 TRFLP types, and subsequent sequence analysis revealed that identical TRFLP types were produced by specimens with = 95% ITS sequence similarity. Variable and overlapping ranges for inter- and intraspecific ITS variation have been reported in the literature (Aanen *et al.*, 2001; Manian *et al.*, 2001), and it is therefore possible that the TRFLP approach will fail to distinguish closely related species, or alternatively may split a single species into several TRFLP types. Despite this, the level of resolution achieved with our approach compares favorably with RFLP (Glen *et al.*, 2001; Horton, 2002), and our use of TRFLP types as operational taxonomic units for community analysis is consistent with the approach taken by other studies (Horton & Bruns, 2001; Dickie *et al.*, 2002).

We used TRFLP to examine the response of a loblolly pine-associated basidiomycete community to optimal nutrition strategies in North Carolina. In total, we observed 27 TRFLP types above-ground and 64 in the below-ground survey, 22 of which matched to the above-ground collection (30%). In comparison, Peter *et al.* (2001b), using a similar sampling scheme to ours, observed 23–45 RFLP types in Norway

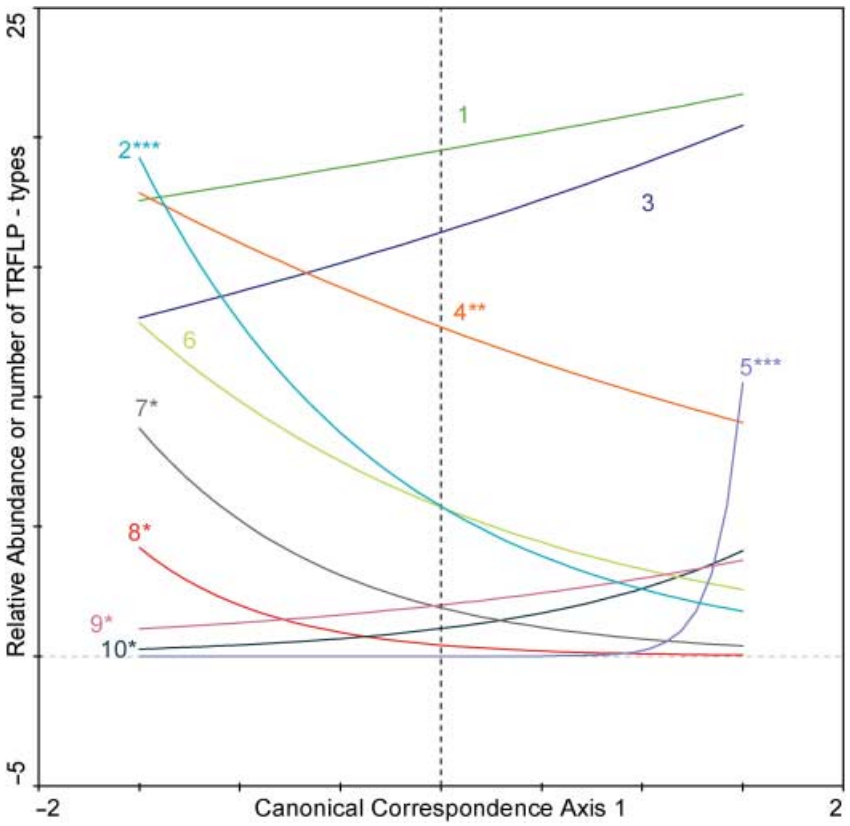

Fig. 5 GLM-fitted response curves for SETRES TRFLP type richness and for individual TRFLP types showing a response to the primary environmental gradient created by optimal fertilization (n = 8).
1, Richness; 2, *Tylopilus* sp. 2; 3, *Lactarius* sp. 2; 4, *Thelephora terrestris*; 5, TRFLP type 7; 6, TRFLP type 18; 7, TRFLP type 34; 8, *Phylloporus* sp.; 9, TRFLP type 9; 10, *Russula* sp. 5. Significant correlations are indicated (*$P \leq 0.10$; **$P < 0.01$; ***$P < 0.001$).

spruce stands 22–48% of which could be matched to species in a sporocarp reference collection. Basidiomycete communities associated with pine are typically dominated by ectomycorrhizal species of the *Russulaceae*, *Thelephoraceae* and *Boletaceae* families (Gardes & Bruns, 1996; Stendell *et al.*, 1999; Bruns *et al.*, 2001), and in this respect our results show that the community at SETRES is typical, with species of *Russula*, *Lactarius*, *Thelephora* and *Tylopilus*/*Xerocomus* well represented both above-(Table 2) and below-ground (Table 3). Currently it is not possible to determine the identity of the unidentified basidiomycete TRFLP types without access to either a large reference collection (Dickie *et al.*, 2002), or through additional sampling of ectomycorrhizae and/or the creation of clone libraries. However, over 80% of the species we collected as sporocarps are ectomycorrhizal, and similar ectomycorrhiza : saprotroph ratios have been recorded in European pine forests (Kost, 1992) so it is likely that most of our unidentified TRFLP types also represent ectomycorrhizal species. Overall, our results suggest that the 3 ha of monoculture loblolly pine at SETRES harbor in excess of 70 species of basidiomycete, the majority of which are ectomycorrhizal.

We examined the distribution of basidiomycetes at two spatial scales: along small transects connecting neighboring trees, and more broadly across the entire SETRES site. Although both surveys demonstrated a patchy distribution of species, the transect survey indicated that adjacent cores in a transect from both optimally fertilized treatments showed a significantly higher degree of similarity than those on control transects. Peter *et al.* (2001b) have also previously observed spatial autocorrelation in basidiomycete species distribution patterns for soil samples taken within 1–5 m of each other, and this may be caused by spatially extensive vegetative growth creating a patch of mycelia. The largest patch at SETRES was encountered on one of the two optimally fertilized transects where *T. terrestris*, occurring in five consecutive adjacent cores, extended for at least 160 cm (Fig. 2). Although our TRFLP approach was not designed to distinguish genets within a species, it is interesting to note that the range of patch sizes is of the same magnitude as many estimates of genet size (Anderson *et al.*, 1998; Gherbi *et al.*, 1999; Redecker *et al.*, 2001; Bergemann & Miller, 2002). The species with the largest patches, *Lactarius* sp. 2, *T. terrestris*, *C. cinerea*, *Tylopilus* sp. 2, *Lactarius* sp. 1b and *Geastrum* sp., are also from genera characterized by Agerer (2001) as medium- or long-distance exploration types, forming rhizomorphs that may extend several dm to meters in soil. The increase in patch size in the optimally fertilized plots suggests a more favorable environment for mycelial growth, consistent with the environmental data that showed a deeper, more extensive forest floor and denser, more extensive fine root distribution on optimally fertilized plots than on controls (Fig. 6). This hypothesis, and its possible implications for

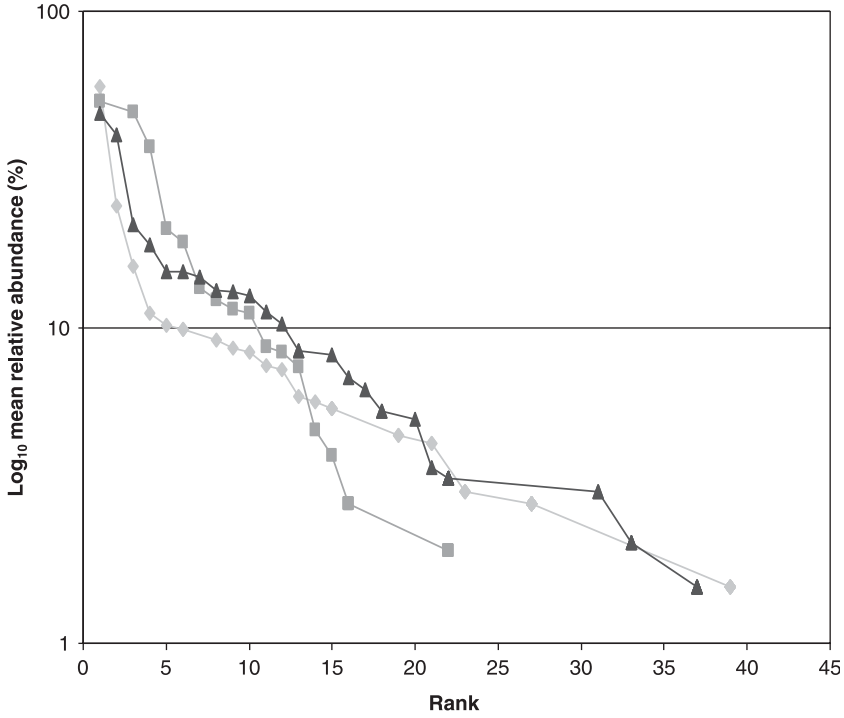

Fig. 6 Mean TRFLP type rank–abundance plots for the three SETRES (NC, USA) treatment types. diamonds, control (NP); squares, optimally fertilized (NR); triangles, optimally fertilized and irrigated (NR-w).

basidiomycete population ecology in optimally fertilized monocultures, requires further investigation.

Our primary hypothesis was that optimal fertilization acts in a manner analogous to increased atmospheric N deposition. Lilleskov *et al.* (2002a) examined the distribution of ectomycorrhizal species in white pine (*Pinus glauca*) forests over a gradient of atmospheric N deposition that spanned approximately an order of magnitude (Lilleskov *et al.*, 2002a). Their results showed that the ectomycorrhizal community at the high end of the gradient was dramatically less species-rich than that at the low end, and was dominated by nitrophilic species (Lilleskov *et al.*, 2002b). In terms of N alone, additions at SETRES since the beginning of optimal fertilization exceed local atmospheric deposition by a factor of approx. 10, and our chemical analyses show that nitrate production, often an indicator of N saturation (Aber *et al.*, 1989), is significantly higher in the fertilized plots. Although there is a similarity in the scale of the gradient, in terms of extractable NH_4^+ and NO_3^- the SETRES site is much closer to the low end of the N gradient examined by Lilleskov *et al.* (2002a), and even with optimal fertilization the soil at SETRES remains extremely N-poor. Despite this, the response of the ectomycorrhizal community at SETRES was similar in nature to that observed by Lilleskov *et al.* (2002a). Several species increased in abundance in the fertilized plots (Table 3; Fig. 4). The most dramatic increases were observed for *Tylopilus* sp. 2, *Phylloporus* sp. and the unidentified TRFLP type 34, which generally were uncommon and of low abundance on the unfertilized plots, but became dominant or subdominant types on the fertilized (NR) plots. Our analysis showed significant positive associations between the relative abundance of these species and increasing nitrate, calcium and magnesium availability. Jonsson *et al.* (1999) have observed previously that the abundance of *Tylopilus felleus* increased in pine forests following liming.

The other species clearly advantaged by the optimal fertilization treatments is *T. terrestris*, which showed a significant increase in relative abundance relative to the unfertilized treatment. Unlike *Tylopilus* sp. 2 and *Phylloporus* species, *T. terrestris* is also common on unfertilized sites where it is subdominant to *Lactarius* sp. 2. As a result CCA places both *T. terrestris* and *Lactarius* sp. 2 near the center of the ordination diagram, and as both seem to have similar niches, and may have the same foraging strategy (Agerer, 2001), it is possible that they are in competition for space. *Lactarius* sp. 2 occurred in 67 cores, and *T. terrestris* in 54. The two species were observed together in 24 cores, and a χ^2 test found no evidence for mutual exclusion (χ^2 0.28, $P = 0.59$, 1 df). It is still possible that the proportion of host root tips colonized by the two species when they occur together differs according to local environmental conditions (Jonsson *et al.*, 2001), but generally we feel that these results indicate limited niche overlap between these two species. The dominance of *Lactarius* sp. 2 in the unfertilized treatment plots suggests that it is well adapted to N-deficient conditions. Giltrap (1982) showed *in vitro* that many *Lactarius* species express polyphenol oxidase, and so may be able to access organic forms of nutrients. Despite this, we also found *Lactarius* sp. 2 to be dominant in the communities of both nutrient-enriched treatments, and suggest that this is consistent with the classification of

many *Lactarius* species as 'intermediate-stage' (Last *et al.*, 1987; Kranabetter & Wylie, 1998). By contrast, although *T. terrestris* isolates have been shown to possess the ability to grow on proteins (Abuzinadah & Read, 1986), this species has been shown to lack polyphenol oxidase (Colpaert & Van Tichelin, 1996) and to extract only a limited amount of N directly from organic material (Bending & Read, 1995). Because of their abundance at the SETRES site, we feel that the degree of niche overlap and potential functional complementarity of these two species should be the subject of further study.

Although our survey identified several species that appeared to benefit from the optimal fertilization, it is less clear which species were negatively affected, partly because many of the TRFLP types encountered in the unfertilized treatment were observed too infrequently for clear trends to emerge. Exceptions were TRFLP type 7, which was observed only on unfertilized plots and which showed a significant negative relationship to increased nutrient availability, and *Russula* sp. 5 which, despite high variability in distribution, also showed a significant negative correlation with increased nutrient availability. Species richness and PIE values suggest a trend towards reduced species richness and diversity in optimally fertilized plots, although this was not significant. The trend appears to result from a reduction in the number of rare or infrequent species, i.e. those that were recorded only once in the survey or were restricted to a single plot. The primary colonization of newly established seedlings often shows a random pattern (Grogan *et al.*, 2000), and a high degree of tree-to-tree variability can be maintained in ecosystems where trees are widely dispersed and poorly connected by forest (Gehring *et al.*, 1998). Although within-plot environmental heterogeneity is seen in all three treatments, both transect survey and main survey suggest that forest floor depth, fine root density, total organic C, total N and NO_3^- tend to be more homogeneously distributed on the optimally fertilized plots than on the unfertilized controls. Our results suggest that improved connectivity within the forest floor, and favorable growth conditions for species such as *Tylopilus* sp. 2 and *T. terrestris* in response to fertilization acts to prevent new species from becoming established, or to displace species less well adapted to the nutrient-enriched environment, but which may have benefited from chance early colonization success.

Heterogeneity alone cannot fully explain the trend towards reduced species richness, however, as the decline is observed primarily in the optimally fertilized plots, not in the optimally fertilized and irrigated plots, although these two treatments have very similar distributions of fine roots and mineral nutrients (Table 1). Both NH_4^+ and NO_3^- concentrations are significantly higher on the two types of fertilized plot than on the control plots (Table 1). The mean concentration of NO_3^-, Ca^{2+} and Mg^{2+} tends to be higher on the fertilized plots relative to fertilized and irrigated, suggesting that $Ca(NO_3)_2$ and $Mg(NO_3)_2$ are leaching in the irrigation water. We suspect that nitrate is the critical difference between these two treatment types, as nitrate is generally a poor source of N to ectomycorrhizal species (Keller, 1996). Lilleskov *et al.* (2002a) found that nitrification potential and organic horizon mineral nitrate concentrations were significantly correlated with the decline in species richness they observed across an N-deposition gradient in Alaska, and we observe more and stronger changes in the relative abundance of TRFLP types along the gradient of nitrate, calcium and magnesium availability (Fig. 4).

Overall, our study indicates high species richness in the basidiomycete community, and a patchy distribution of basidiomycete species at SETRES, with approx. 50% of the species observed only once, and only seven species observed on the majority of the nine treatment plots. A comparison of the structure of the communities, as revealed by rank–abundance plots, generally supports the hypothesis that long-term optimal fertilization acts in a manner analogous to increased atmospheric N deposition, and may retard the natural successional development of the ectomycorrhizal community. Our results show that this effect is most pronounced on optimally fertilized, unirrigated plots, and this may be the result of increased nitrate availability on these plots. Although the change in rank–abundance and apparent decline in species richness observed in response to optimal fertilization at SETRES is considerably less severe than that observed by Lilleskov *et al.* (2002a), it has been induced in a considerably shorter time period (10 years as opposed to over 30 years), and further monitoring of this site is therefore warranted. By contrast to the optimal fertilization treatment, optimally fertilized and irrigated treatment plots did not differ significantly from unfertilized plots in species diversity, and may in fact be slightly more diverse; we attribute this to both removal of nitrate and improved host root development. Based on our survey results, and on the complementary study of King *et al.* (2002) which showed increased mycorrhizal root development and longevity in response to fertilization at SETRES, we conclude that generally optimal fertilization of loblolly pine on extremely nutrient-poor soils has a small impact on the basidiomycete community, but that a combination of foliar and soil analysis is required to control nitrate accumulation more effectively if it is desired to allow natural successional development in the basidiomycete community.

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