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Fungal community composition and metabolism under elevated CO_2 and O_3

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Abstract Atmospheric CO_2 and O_3 concentrations are increasing due to human activity and both trace gases have the potential to alter C cycling in forest ecosystems. Because soil microorganisms depend on plant litter as a source of energy for metabolism, changes in the amount or the biochemistry of plant litter produced under elevated CO_2 and O_3 could alter microbial community function and composition. Previously, we have observed that elevated CO_2 increased the microbial metabolism of cellulose and chitin, whereas elevated O_3 dampened this response. We hypothesized that this change in metabolism under CO_2 and O_3 enrichment would be accompanied by a concomitant change in fungal community composition. We tested our hypothesis at the free-air CO_2 and O_3 enrichment (FACE) experiment at Rhinelander, Wisconsin, in which *Populus tremuloides*, *Betula papyrifera*, and *Acer saccharum* were grown under factorial CO_2 and O_3 treatments. We employed extracellular enzyme analysis to assay microbial metabolism, phospholipid fatty acid (PLFA) analysis to determine changes in microbial community composition, and polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE) to analyze the fungal community composition. The activities of 1,4-β-glucosidase (+37%) and 1,4,-β-*N*-acetylglucosaminidase (+84%) were significantly increased under elevated CO_2, whereas 1,4-β-glucosidase activity (−25%) was significantly suppressed by elevated O_3. There was no significant main effect of elevated CO_2 or O_3 on fungal relative abundance, as measured by PLFA. We identified 39 fungal taxonomic units from soil using DGGE, and found that O_3 enrichment significantly altered fungal community composition. We conclude that fungal metabolism is altered under elevated CO_2 and O_3, and that there was a concomitant change in fungal community composition under elevated O_3. Thus, changes in plant inputs to soil under elevated CO_2 and O_3 can propagate through the microbial food web to alter the cycling of C in soil.

Keywords Free-air CO_2 and O_3 enrichment · Extracellular enzymes · Polymerase chain reaction–denaturing gradient gel electrophoresis · Soil microbial community · Fungal metabolism

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Introduction

Fossil fuel burning and land-use conversion have increased atmospheric CO_2, which has the potential to alter rates of C cycling in forest ecosystems (Zak et al. 1993; DeLucia et al. 1999). The major impacts of CO_2 enrichment on plants include stimulated photosynthesis, accumulation of nonstructural carbohydrates, and reduced tissue N concentration (Mooney et al. 1991; Körner 2000). In plants, a substantial portion of photosynthate is allocated to root growth and maintenance, and elevated CO_2 can further stimulate belowground plant growth (Rogers et al. 1994). For example, many studies observed an increase in root biomass and possibly higher rhizodeposition in response to elevated CO_2 (Allen et al. 2000; Matamala and Schlesinger 2000; Mikan et al. 2000; Pregitzer et al. 2000). Much of this

additional belowground photosynthate eventually becomes available to soil microorganisms; hence, atmospheric CO_2 enrichment may have major impact on energy flow through microbial food webs in the soil. Higher plant litter production and a change in litter biochemistry of CO_2-enriched plants could alter soil microbial community function and composition, and this in turn may alter C and N cycling in soil. However, we have an incomplete understanding of how the aforementioned responses will be modified by other climate change factors, like elevated O_3, which could counteract the effect of elevated CO_2.

Ozone is an atmospheric pollutant that has also increased globally over the past century due to fossil fuel burning (Finlayson-Pitts and Pitts 1997). In contrast to CO_2, elevated O_3 has detrimental effects on plant growth, because it can decrease leaf photosynthesis, lower root and stem biomass, and accelerate leaf senescence (Findlay and Jones 1990; Taylor et al. 1994; Karnosky 1996). Moreover, plants typically allocate less to the roots when exposed to sufficiently high doses of O_3 (Coleman et al. 1996; Andersen et al. 1997; Andersen 2003). Reduced allocation of photosynthate to roots under O_3 enrichment has the potential to suppress microbial metabolism, an effect that could counteract that of elevated CO_2. How will changes in plant growth under elevated CO_2 and O_3 alter microbial community function and composition?

We studied microbial community function and composition under elevated CO_2 and O_3 at the free-air CO_2 and O_3 enrichment (FACE) experiment in Rhinelander, Wisconsin. In this experiment, *Populus tremuloides*, *Betula papyrifera*, and *Acer saccharum* have been exposed to factorial elevated CO_2 and O_3 treatments since 1998 (Dickson et al. 2000). Our previous work has demonstrated that fine root biomass increased significantly under elevated CO_2, and decreased under elevated O_3 (King et al. 2001). Also, the C:N ratio in senescing *Populus* and *Betula* leaves increased significantly under elevated CO_2, and this change was carried through litter deposition (Lindroth et al. 2001). Along with these changes in litter production and chemistry, we have observed an increase in the fungal metabolism of cellulose and chitin under CO_2 enrichment; O_3 enrichment dampened this response (Larson et al. 2002; Phillips et al. 2002). We hypothesized that this change in fungal metabolism has arisen from a change in fungal community composition, a result of altered substrate availability. To test this hypothesis, we used microbial extracellular enzyme analysis to assay microbial metabolism in our experiment. Since most of the extracellular enzymes that decompose the plant litter are synthesized based on the concentration of substrates present in soil (Burns 1982), we reasoned that the extracellular enzyme activity would reflect microbial metabolic potential under elevated CO_2 and O_3. We examined the relationship between belowground plant biomass and enzyme activity to confirm if the changes in substrate availability in response to elevated CO_2 and O_3 were responsible for altering the microbial metabolism. In addition, we used PLFA analysis to determine whether elevated CO_2 and O_3 elicited an overall change in microbial community composition. To specifically analyze the fungal community composition, we extracted DNA from soil, and amplified and separated fungal rDNA using polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE). In this analysis, the generated DNA banding pattern shows the major taxonomic units within a microbial community (Fromin et al. 2002). We used this technique to determine if changes in metabolic activity were accompanied by a shift in fungal community composition.

Methods

Experimental design and sampling procedures

Our study was conducted at the FACE experiment in Rhinelander, WI, USA. In this experiment, factorial CO_2 and O_3 treatments are applied in a randomized complete block ($n = 3$) design. There are a total of twelve 30-m-diameter-FACE rings, and within each ring, trembling aspen (*P. tremuloides*), paper birch (*B. papyrifera*), and sugar maple (*A. saccharum*) are planted at a density of 1 stem/m^2. Each ring was split into three sections; half of the ring was planted with aspen; one quarter of the ring was planted with aspen and birch, and aspen and maple were planted in the remaining quarter. The trees were exposed to CO_2 and O_3 treatments beginning in May 1998. The level of elevated CO_2 was 560 µl/l, which is 200 µl/l above ambient CO_2 concentration. The target level of elevated O_3 treatment is determined at the beginning of each day, based on current meteorological conditions. For hot and sunny days, a maximum O_3 concentration of 90–100 nl/l is applied; 50– 60 nl/l is maintained for cool and cloudy days.

Seven soil cores, 2 cm in diameter and 15 cm in depth, were randomly collected from each ring section. Samples were collected in July 2001 (summer), November 2001 (autumn), and May 2002 (spring). Cores were composited by ring section and immediately frozen. Soil samples were kept at −80°C prior to enzymatic and molecular analysis.

Microbial community function

To determine microbial community metabolism, we measured the activities of enzymes that degrade nonstructural carbohydrate, cellulose, hemicellulose, chitin, and organic P substrate in soil. We analyzed the activities 1,4-α-glucosidase, 1,4-β-glucosidase, cellobiohydrolase, 1,4-β-xylosidase, 1,4,-β-*N*-acetylglucosaminidase, and phosphatase using methylumbelliferone (MUB) linked substrates (after Saiya-Cork et al. 2002). One gram of soil from each composite was thawed, and then placed in 125 ml of sodium acetate buffer (pH 5.0). The

solution was transferred to a 96-well microplate that contained eight analytical replicates of each enzyme assay. For each enzyme assay, 200 µl of soil-buffer solution and 50 µl of substrate were combined. Plates were incubated at 21°C for all enzyme assays. Phosphatase and 1,4,-β-*N*-acetylglucosaminidase assays were incubated for 0.5 h and 1,4-α-glucosidase, 1,4-β-glucosidase, cellobiohydrolase, and 1,4-β-xylosidase assays were incubated for 2 h. Fluorescence was analyzed using a f-Max fluorometer (Molecular Devices Corp., Sunnydale, CA, USA), in which the excitation energy was set at 355 nm and emission was measured at 460 nm. Enzyme activities were expressed as nmol 4-MUB g^{-1} h^{-1}.

The activities of lignin-degrading enzymes, phenol oxidase and peroxidase, were determined by colorimetric assay using 25 mm L-3,4-dihydroxy-phenylalanine (L-DOPA) as the substrate (Saiya-Cork et al. 2002). The procedure for measuring the activity of these enzymes was similar to that described above. There were 16 analytical replicates for each enzyme assay. Following a 24-h incubation at 21°C, absorbance was read at 450 nm on EL-800 plate reader (Biotek Instruments, Inc., Winooski, VT, USA). Activity was reported as nmol L-DOPA oxidized g^{-1} h^{-1}. The results of all enzymatic assays are expressed on a dry soil weight basis.

Belowground plant biomass of 2001 was reported by King et al. (2005). We used these data to explore the relationship between the belowground plant biomass and extracellular enzyme activity.

Microbial community composition

Microbial lipids were extracted from 5 g of freeze-dried soil collected in each ring section. We used a solvent system that included phosphate buffer to extract total lipids, and silicic acid chromatography to separate the total lipids into neutral, glyco-, and polar lipids (White et al. 1979; Guckert et al. 1985). Polar lipids were methylated with 0.2 M methanolic KOH to form fatty acid methyl esters (FAMEs). FAMEs were identified and quantified using a Finnigan Delta plus mass spectrometer with a GC/C III interface (ThermoElectron, Austin, TX, USA) coupled to a HP 5973 GC (Agilent Technologies, Palo Alto, CA). Fatty acids 18:2ω6 and 18:1ω9c were considered as fungal biomarkers (Bardgett et al. 1996; Bååth 2003).

Fungal community composition

DNA extraction

Total soil DNA was extracted using UltraClean™ soil DNA isolation kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA). One gram from each composite soil sample was placed in a 2-ml tube with glass beads and a buffer solution. The tubes were agitated horizontally for 10 min, allowing DNA from ruptured cells to attach to the glass beads. DNA was then precipitated by adding Solution S2 and incubating at 4°C for 5 min. DNA was purified by diluting ten times with 10 mm Tris–HCl buffer, transferring it to a spin-filter, and centrifuging at 10,000 *g* for 1 min. A tube without soil was subjected to our DNA extraction procedure, and it served as a negative control.

PCR, DGGE, and DNA sequence analysis

The extracted DNA was amplified using fungal specific primers FF390 and FR1 that amplify a 390-base pair region of 18S rDNA (Vainio and Hantula 2000). The reaction mixture was 50 µl in volume, and it contained template DNA, 5 µl of 10× reaction buffer, 1 µl of 10 mm dNTP mixture, 1.75 units of Expand™ High Fidelity PCR system (Roche Diagnostics, Germany), 0.5 µm of forward primer FF390 and 0.5 µm of reverse primer FR1. In the negative control, 1 µl of sterilized water was used as the DNA template. The DNA was amplified according to the following program using a Robocycler temperature cycler (Stratagene, La Jolla, CA): (1) 8 min at 95°C, (2) 30 s at 95°C, (3) 45 s at 50°C, (4) 2 min at 72°C, (5) 29 more times of step (2)–(4), and (6) 10 min at 72°C. We subsequently separated the PCR products on 1.6% agarose gel to determine if the PCR was successful.

For DGGE analysis, the PCR products of each soil sample were loaded on 7.5% (w/v) acrylamide/ bis-acrylamide (37.5:1) parallel gradient gel, which was cast using a Model 475 Gradient Delivery System (Bio-Rad, Hercules, CA, USA). In each gel, we loaded twelve PCR products from one block and EZ Load 100 bp Molecular Ruler (Bio-Rad) (Fig 1). Electrophoresis was conducted on Bio-Rad DCode™ Universal Mutation Detection System (Bio-Rad, Hercules, CA) for 18 h at 50 V and 58°C (after Vainio and Hantula 2000). The gel was stained with ethidium bromide, and the gel image was documented and analyzed by EPI-Chemi Darkroom System (UVP Lab Products, Upland, CA). The size of the bands was assigned by using the software LabWorks (UVP Lab Products, Upland, CA, USA) according to the position of each band in relation to molecular size standards. Since the band size was standardized to molecular size markers, we were able to compare bands across multiple gels.

To elute the PCR products for sequencing, DNA bands were cut from the DGGE gel and were kept at −20°C for 12 h, and then at 4°C for another 12 h in Tris–EDTA buffer. The eluted DNA was re-amplified through PCR, and the PCR product was subject to sequence analysis at the Sequencing Core Facility at University of Michigan (Ann Arbor, MI). Since the DNA fragments that have different base composition may migrate at identical rates in a DGGE gel (Sekiguchi et al. 2001), we eluted two bands that were at the same position in a gel and sequenced them. We also eluted two bands of same base pair size from two different gels and

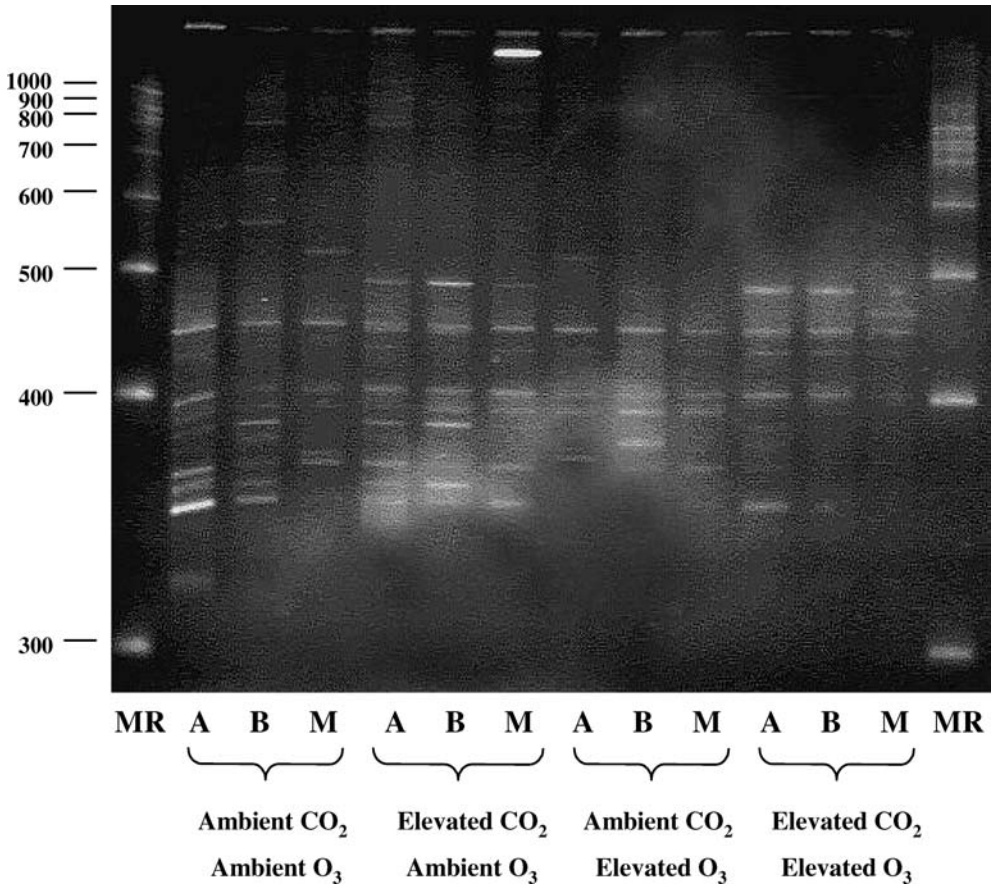

Fig. 1 **A** PCR-DGGE gel image. PCR products were amplified from the soil samples collected in May 2002 (spring) from block 3. MR: EZ Load 100 bp Molecular Ruler (The size of each band is shown in base pairs), **A** aspen section, **B** aspen-birch section, **M** aspen-maple section

compared the DNA sequence. The DNA sequences were identical for bands that were of equal molecular size.

We performed PCR–DGGE on five analytical replicates to determine whether microbial community composition in 1 g of soil was representative of the microbial community in the composite soil sample from each ring section. DNA was extracted from five 1-gram subsamples of one composite soil sample, and each DNA extract was amplified using primers described above. DGGE analysis confirmed that the banding pattern of five replicates was identical (data not shown).

To determine the phylogenetic affinity of the sequenced operational taxonomic units (OTU), related sequences were obtained using BLAST (NCBI, Bethesda, MD) searches. All sequences were imported into Bioedit Sequence Alignment Editor version 6.0.7 (1997–2004, Tom Hall, Isis Pharmaceuticals, Inc7., Carlsbad, CA) and aligned using the Clustal W accessory application. Alignments were checked and adjusted manually where needed. Phylogenetic trees were generated using PAUP* version 4.0 b10 for Windows (Sinauer Associates, Inc., Sunderland, MA). A heuristic search was carried out using maximum parsimony, with gaps treated as missing data, ten replicates, and no more than 100 trees saved in each replicate. After an initial analysis, we performed a bootstrap analysis using 1,000 replicates to generate probability estimates for the branches.

Statistical analyses

Enzyme activities were analyzed using repeated-measures ANOVA for a split-plot randomized complete block design. Block, CO_2, O_3, and species were fixed effects in this model. Carbon dioxide and O_3 treatment combinations were main effects, and they were split by species. Significance of main effects (CO_2 and O_3), split-plot effects (species), time and their interaction was accepted at $\alpha = 0.05$. We performed linear regression analysis to determine the relationship between the belowground plant biomass and enzyme activity.

For the analysis of fungal community composition, multi-response permutation procedures (MRPP) were conducted using PC-ORD (Mjm Software Design, Gleneden Beach, OR, USA). MRPP is a non-parametric method for testing the hypothesis of no difference between two or more communities, and this method does not require distributional assumptions such as multivariate normality and homogeneity of variance (McCune and Grace 2002). The null hypotheses tested through blocked MRPP were as follows: (1) fungal communities under ambient and elevated CO_2 are not different, and (2) fungal communities under ambient and elevated O_3 are not different. Euclidian distance was measured and compared for each fungal community. We report the level of significance for each comparison procedure; significance between any two groups was accepted at $\alpha = 0.05$.

We performed indicator species analysis with PC-ORD to determine if any taxonomic unit was specific to the elevated CO_2 and O_3 treatments. Through this method, we determined how faithfully a taxonomic unit occurs in a particular treatment. The significance of the resulting indicator value was tested through Monte Carlo test and was accepted at $\alpha = 0.05$.

Results

Extracellular enzyme activity

As a main effect, elevated CO_2 significantly increased the activities of 1,4-β-glucosidase and *N*-acetylglucosaminidase (Table 1, Fig 2). 1,4-β-glucosidase activity was 37% higher under elevated CO_2 (Fig 2a), and *N*-acetylglucosaminidase activity increased under elevated CO_2 by 84% when compared to ambient CO_2 (Fig 2b). Elevated CO_2 also enhanced the activities of cellobiohydrolase, 1,4-β-xylosidase, phosphatase, 1,4-α-glucosidase, and phenol oxidase, but this effect was not statistically significant (Table 1, Fig 2). Elevated CO_2 had no effect on peroxidase activity (Table 1, Fig 2g).

Elevated O_3 significantly (main effect) reduced the activity of 1,4-β-glucosidase by 25% relative to the activity of this enzyme at ambient O_3 (Table 1, Fig 3a). *N*-acetylglucosaminidase, cellobiohydrolase, 1,4-β-xylosidase, phosphatase, 1,4-α-glucosidase, peroxidase, and phenol oxidase activities were suppressed under elevated O_3, but these reductions were not statistically significant (Table 1, Fig 3).

Although there was no significant interaction between elevated CO_2 and O_3 (Table 1), the activity of cellulose-degrading enzymes under elevated CO_2 showed a tendency to be dampened by elevated O_3. For example, 1,4-β-glucosidase and cellobiohydrolase activities, both under elevated CO_2 and O_3 were lower than the activities under elevated CO_2 and ambient O_3, and were not different from those under ambient CO_2 and O_3 (Fig 4a,b). However, *N*-acetylglucosaminidase activity under elevated CO_2 and O_3 was not different from that under elevated CO_2 alone (Fig 4c). 1,4-β-glucosidase, cellobiohydrolase and 1,4,-β-*N*-acetylglucosaminidase activity in July 2001 showed significant positive correlation with total root biomass [1,4-β-glucosidase activity (nmol g^{-1} h^{-1}) = 0.4(root biomass (gm^{-2})) + 45.6, $n = 36$, $r^2 = 0.20$, $P = 0.01$]; [Cellobiohydrolase activity (nmol g^{-1} h^{-1}) = 0.21(root biomass (gm^{-2})) − 41.6, $n = 33$, $r^2 = 0.15$, $P = 0.02$]; [*N*-acetylglucosaminidase activity (nmol g^{-1} h^{-1}) = 0.15(root biomass(gm^{-2})) + 24.4, $n = 36$, $r^2 = 0.20$, $P = 0.01$].

1,4-β-glucosidase and 1,4,-β-*N*-acetylglucosaminidase displayed a strong seasonal pattern (Table 1), wherein the activities were highest in July compared to other sampling dates; peroxidase activity was highest in November (data not shown). 1,4,-α-glucosidase, cellobiohydrolase, 1,4-β-xylosidase, phosphatase, and phenol oxidase did not show a significant temporal pattern (Table 1). There was a significant effect of tree species on peroxidase activity, and it was highest under aspen. Tree species composition and elevated CO_2 had a significant interaction effect on 1,4-β-glucosidase activity (Table 1). 1,4-β-glucosidase activity in aspen–maple section under ambient CO_2 was lower than that under aspen and aspen–birch section, but 1,4-β-glucosidase activity under elevated CO_2 in all the three tree compositions were not different (data not shown).

Microbial community composition

Biomass of each microbial group was determined by PLFA analysis, and the percentage of fungal biomass

Table 1 *P*-values for microbial extracellular enzyme activities analyzed by repeated-measures analysis of variance (ANOVA). *P*-values equal to or lower than 0.05 are in bold face print

	1,4-α-glucosidase	1,4-β-glucosidase	Cellobiohydrolase	1,4-β-xylosidase	1,4,-β-*N*-acetylglucosaminidase	Phosphatase	Peroxidase	Phenol oxidase
Whole plot								
CO_2	0.60	**0.04**	0.14	0.48	**0.01**	0.16	0.63	0.08
O_3	0.36	**0.05**	0.24	0.18	0.10	0.32	0.31	0.16
$CO_2 \times O_3$	0.34	0.30	0.09	0.66	0.56	0.10	0.11	0.65
Split-plot								
Species	0.87	0.26	0.99	0.06	0.72	0.20	**0.02**	0.78
Species×CO_2	0.34	**0.04**	0.43	0.10	0.46	0.85	0.31	0.17
Species×O_3	0.50	0.85	0.18	0.21	0.18	0.66	0.08	0.35
Species×CO_2×O_3	0.87	0.20	0.57	0.12	0.45	0.50	0.79	0.50
Time	0.45	**0.01**	0.22	0.10	<0.001	0.30	**0.01**	0.17
Time×CO_2	0.98	0.22	0.72	0.48	0.45	0.63	0.27	0.42
Time×O_3	0.34	0.20	0.46	0.07	0.66	0.63	0.42	0.23
Time×CO_2×O_3	0.70	0.89	0.29	0.75	0.83	0.87	0.32	0.38
Time×Species	0.38	0.67	0.84	0.93	0.66	0.30	0.26	0.84
Time×CO_2×Species	0.47	0.75	0.29	0.41	0.68	0.46	0.68	0.86
Time×O_3×Species	0.15	0.78	0.75	0.64	0.42	0.26	0.58	0.54
Time×CO_2×O_3×Species	0.39	0.40	0.27	0.43	0.79	0.78	0.97	0.52

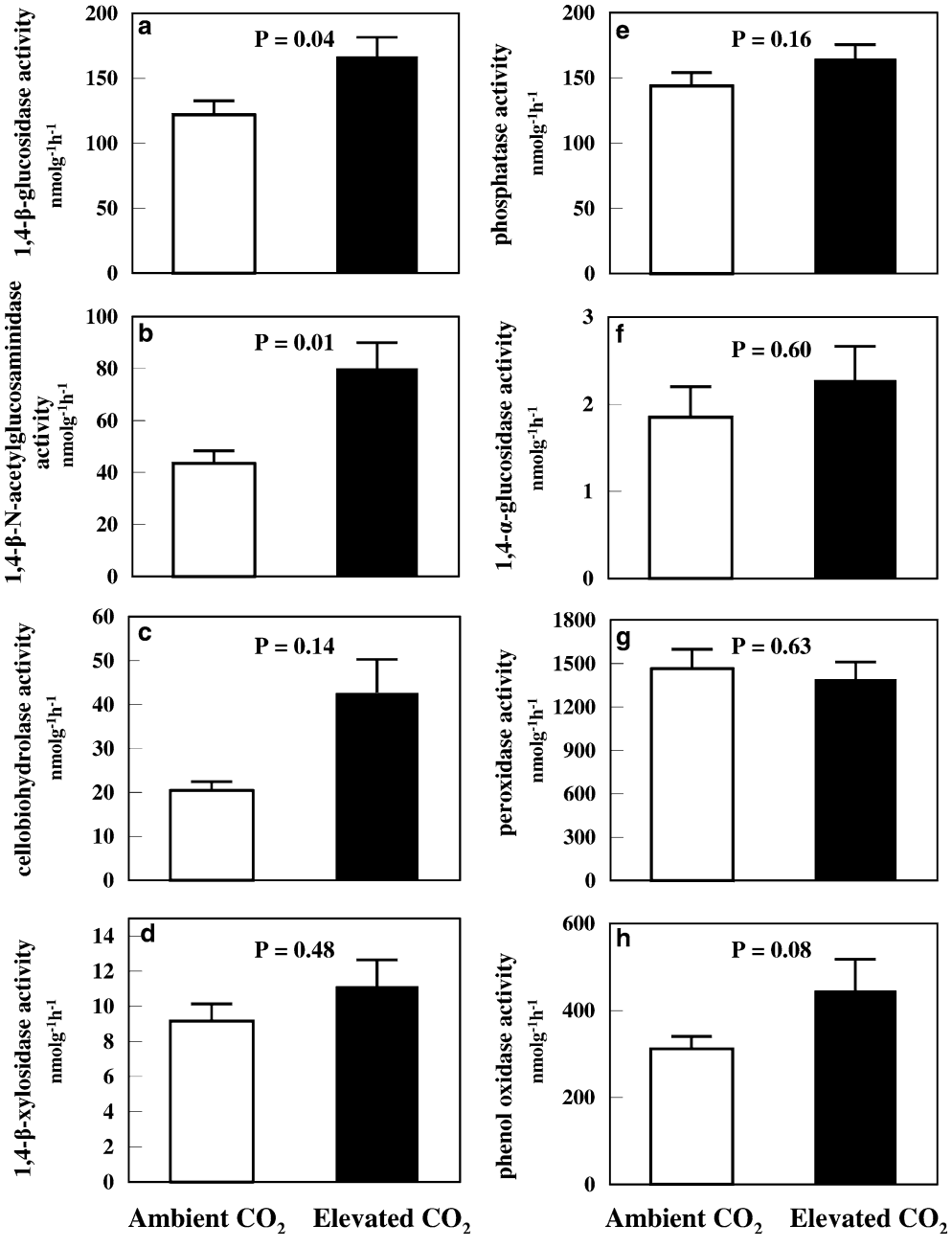

Fig. 2 Main effect of CO_2 on extracellular enzyme activity. Enzyme activity was averaged across three sampling seasons. *Error bars* indicate standard error of the mean

comprising total biomass was considered as the relative abundance of fungi in the soil microbial community. Fungal relative abundance in July 2001 was 30% higher under elevated CO_2, but this was only marginally significant ($P = 0.10$). Fungal relative abundance under elevated O_3 was 17% higher than that under ambient O_3, but this result also was not statistically significant. There was no significant interaction between elevated CO_2 and O_3 on fungal abundance (Fig 5). Tree species composition had no significant effect on fungal relative abundance.

Fungal community composition

A total of 39 operational taxonomic units (OTU) were identified using DGGE. Sixteen prominent OTUs were sequenced; ten OTUs were in the Basidiomycota, four in the Ascomycota and two in the Zygomycota (Fig 6). Of the ten OTUs in the Basidiomycota, one was in the Tremellomycetidae (Unknown 15), a clade containing many mycoparasites. All others were in the Homobasidiomycetes. One cluster in the Homobasidiomycetes that includes six OTUs (Unknown 2, 7, 8, 9, 10, and 11), resided on a long branch with unknown affinity. Two OTUs in the Basidiomycota (Unknown 5 and 13) may be related to *Inocybe*, an ectomycorrhizal genus in the Cortinariaceae that fruits commonly in these plots, although bootstrap support for this grouping was less than 50% (Fig 6a). One OTU (Unknown 3) appears to be related to the genus *Cortinarius*, also in the Cortinariaceae. Two OTUs in the Ascomycota (Unknown

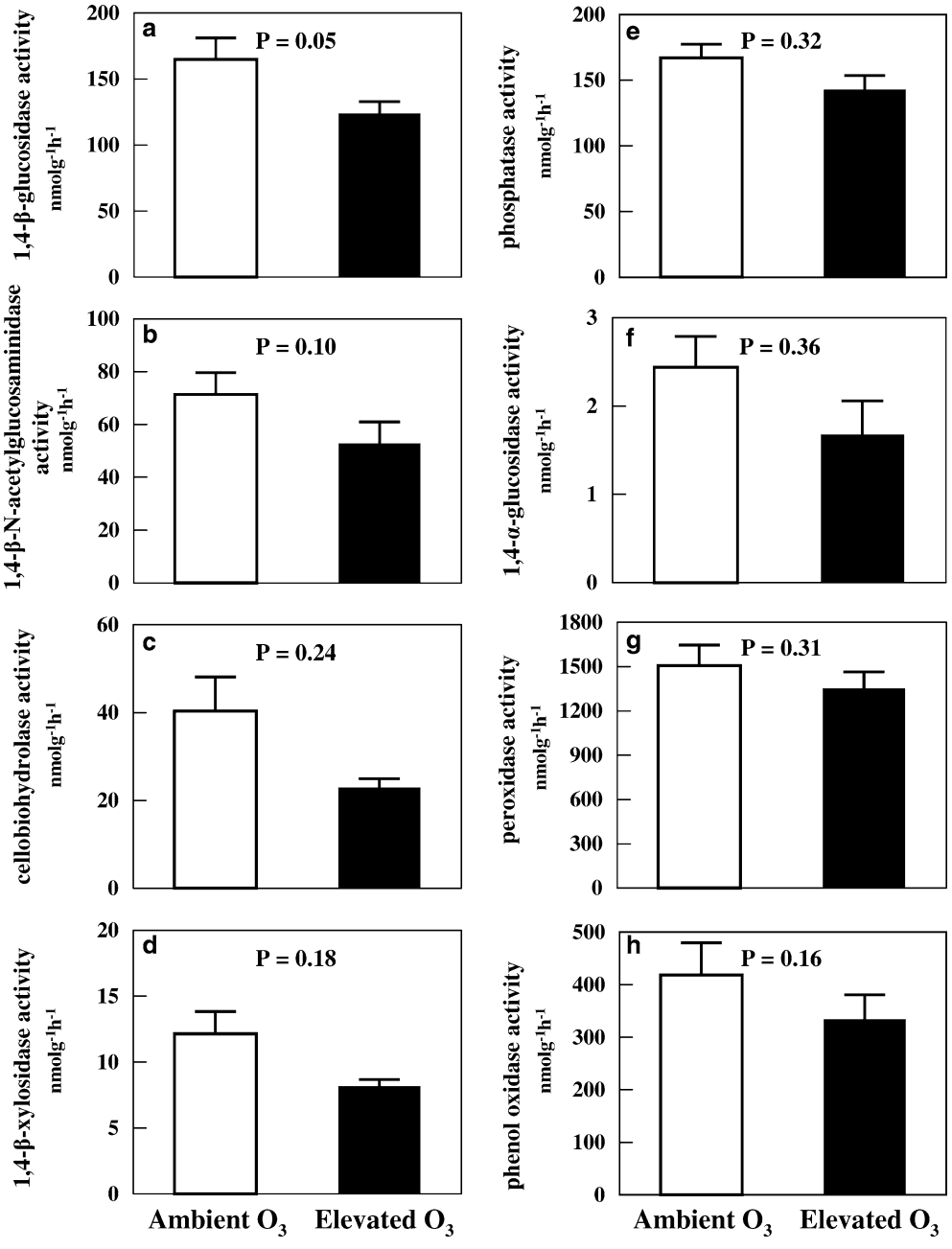

Fig. 3 Main effect of O_3 on extracellular enzyme activity. Enzyme activity was averaged across three sampling seasons. *Error bars* indicate standard error of the mean

4 and 16) clustered with the Pezizaceae. One OTU (Unknown 12) was affiliated with *Verticillum* spp., which are soil-borne pathogens. The two OTUs (Unknown 1 and 6) in the Zygomycota clustered with the Mortierellaceae (Fig 6b).

Fungal community composition under ambient and elevated CO_2 was not different across all the three sampling seasons ($P = 0.85$). No change in fungal community composition under ambient and elevated CO_2 was detected in spring ($P = 0.23$), summer ($P = 0.74$) or fall ($P = 0.63$). To determine if any taxonomic unit was an indicator of the elevated CO_2 treatment, we performed indicator species analysis. Presence/absence of data for each fungal taxonomic unit were analyzed for their occurrence under ambient and elevated CO_2 treatment. In summer, one operational taxonomic unit (Unknown 2 in Fig 6a) occurred in 11% of the soil samples under ambient CO_2, whereas it was present in 67% of the soil samples under elevated CO_2. This species was a significant indicator of elevated CO_2 treatment ($P = 0.003$).

Elevated O_3 significantly altered fungal community composition across all the three sampling seasons ($P = 0.02$). Fungal community composition under ambient and elevated O_3 was different in spring ($P = 0.04$) and summer ($P = 0.02$), but no change was detected in fall ($P = 0.57$). Indicator analysis showed that one OTU (Unknown 1 in Fig 6b) was a significant indicator of

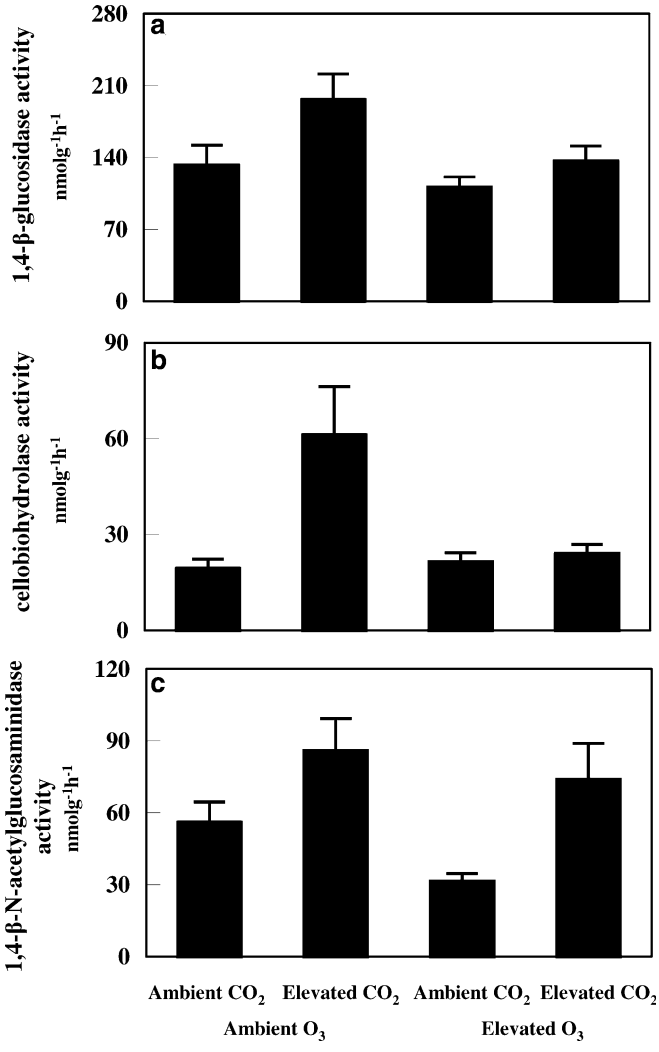

Fig. 4 Effect of CO_2 and O_3 on activities of 1,4-β-glucosidase, cellobiohydrolase, and 1,4,-β-*N*-acetylglucosaminidase. *Error bars* indicate standard error of the mean

elevated O_3 treatment in spring ($P = 0.02$). This OTU was present in 6 % of the soil samples under ambient O_3, whereas it occurred in 44 % of the soil samples under elevated O_3 in spring.

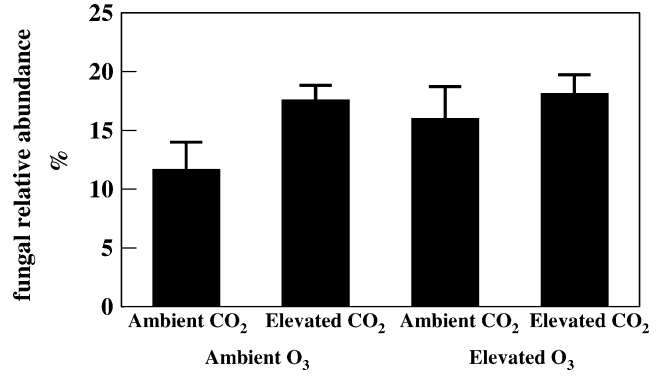

Fig. 5 Fungal relative abundance under elevated CO_2 and O_3 in July 2001. *Error bars* indicate standard error of the mean

Discussion

Since soil microbial communities carry out key processes in soil C and N cycling, determining how microbial community composition and function may change under CO_2 and O_3 enrichment is central in predicting how ecosystem function will be altered by these rising trace gases. In our experiment, plant production increased and the N content of the litter was lower under elevated CO_2, whereas plant production was suppressed by O_3 enrichment (Kull et al. 1996; Lindroth et al. 2001). Here, we demonstrate that 1,4-β-glucosidase and *N*-acetylglucosaminidase activities are enhanced under CO_2 enrichment, and 1,4-β-glucosidase activity was suppressed under O_3 enrichment. These changes in the extracellular enzyme activity were accompanied by alteration in fungal community composition under elevated O_3. Our results indicate that increases in atmospheric CO_2 and O_3 can induce changes in plant growth that cascade into the soil food web to modify the fungal community composition, and that O_3 enrichment can concurrently alter fungal community composition.

Activity of the cellulose-degrading enzyme 1,4-β-glucosidase significantly increased beneath plants exposed to elevated CO_2 alone, but this response showed a tendency to be dampened by O_3 (i.e., in elevated CO_2 and elevated O_3 treatment), suggesting that elevated O_3 may counteract the effect of elevated CO_2. Cellulose is a major component of the plant tissue, and plant production, especially belowground production will determine cellulose input to soil. King et al. (2001) have found similar pattern in fine root production in our study site; elevated CO_2 increased the production of fine roots, whereas elevated O_3 dampened this response. Total belowground biomass was significantly correlated with 1,4-β-glucosidase and cellobiohydrolase, indicating that the activities of cellulose-degrading enzymes were induced according to the amount of cellulose entering soil, thereby closely reflecting the cellulose availability under elevated CO_2 and O_3. This observation is consistent with the idea that plant growth responses to these trace gases will drive the response of microbial communities in soil.

Activity of chitin-degrading enzyme *N*-acetylglucosaminidase was significantly higher under elevated CO_2, and this could indicate that there is a higher input of fungal litter under elevated CO_2. Chitin is the main component of the fungal cell wall, which is built from *N*-acetylglucosamine subunits (Swift et al. 1979). *N*-acetylglucosaminidase is produced by a diverse group of fungi, and its activity is positively correlated with fungal biomass (Miller et al. 1998). We also have found greater incorporation of ^{13}C-labelled *N*-acetylglucosamine into fungal biomass under elevated CO_2 (Phillips et al. 2002), and taken together this suggests that there may be higher fungal biomass under CO_2 enrichment. Phospholipid fatty acid analysis of soil samples collected in July 2001 showed that fungal biomass significantly increased under

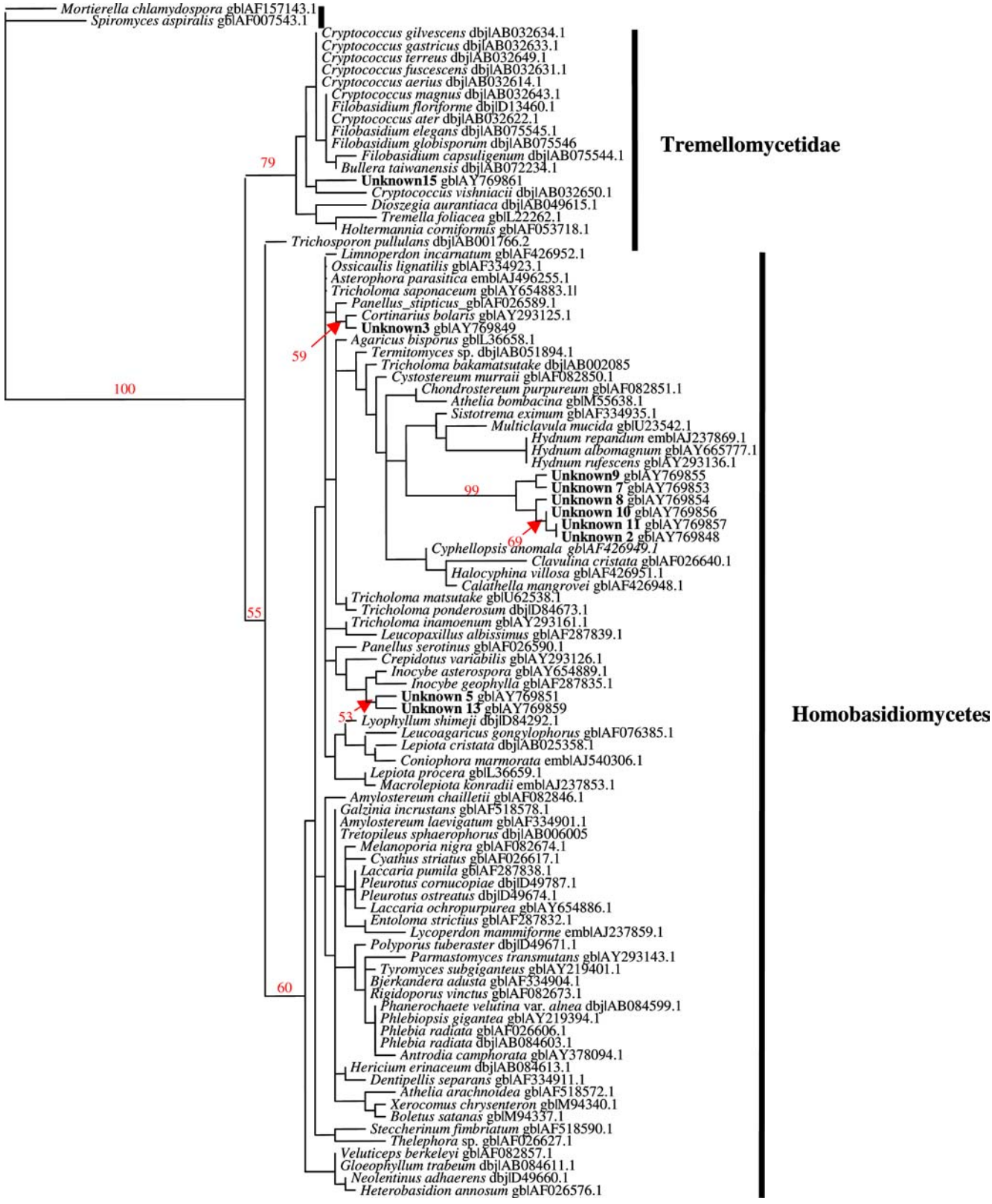

Fig. 6 Phylograms showing the placement of the sequenced OTUs amplified from soils at the Rhinelander FACE site for **a** Basidomycota, and **b** Ascomycota and Zygomycota. Numbers above the clades indicate bootstrap support (%), shown only for the clades containing the 16 unknowns

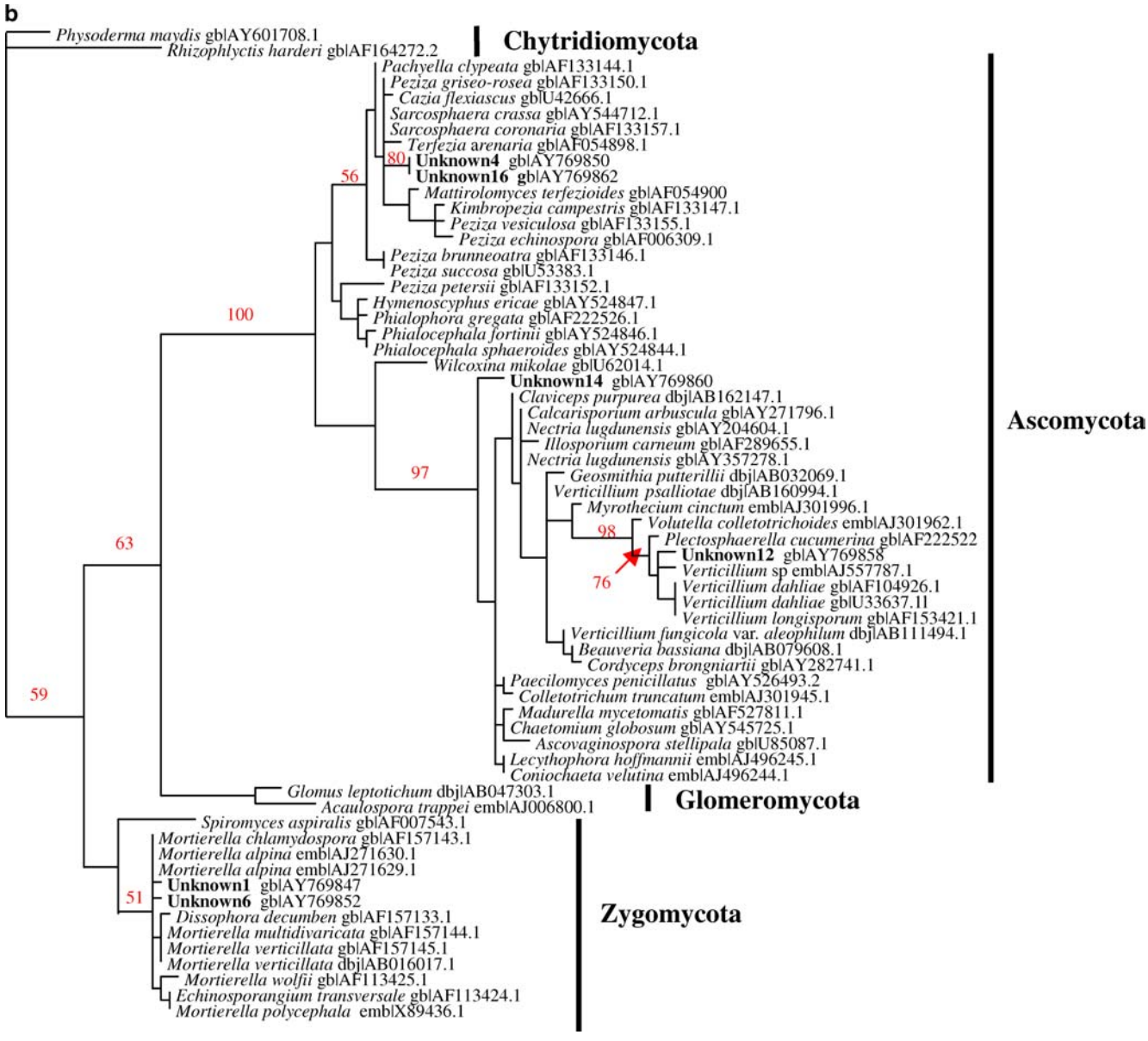

Fig. 6 (Contd.)

elevated CO_2 in the aspen section, but there was no difference in the fungal biomass under ambient and elevated CO_2 when all the three tree sections were included (data not shown). Because we were unable to perform PLFA analysis for soil samples collected in all three seasons, this needs to be investigated further.

Fungi are major producers of 1,4-β-glucosidase and *N*-acetylglucosaminidase in soil (Hayano and Katami 1977; Miller et al. 1998), and greater activities of these two enzymes under elevated CO_2 indicate that fungal metabolism is stimulated by changes in plant growth due to this trace gas. This is consistent with greater total hyphal lengths, culturable and active fungi under elevated CO_2 (Rillig et al. 1999). Jones et al. (1998) also demonstrated that cellulose-decomposing fungi had higher biomass under elevated CO_2, probably accounting for the increased decomposition rates of cotton strips. Because we have observed higher 1,4-β-glucosidase and *N*-acetylglucosaminidase activity under elevated CO_2 and dampened 1,4-β-glucosidase activity under elevated O_3, we then wondered if these physiological responses were accompanied by a change in fungal community composition.

There was no significant difference in fungal relative abundance in the soil microbial community as determined by PLFA analysis. Our amplification of fungal rDNA also suggests that fungal communities under ambient and elevated CO_2 did not differ, but there was one indicator OTU (Unknown 2 in Fig 6a) that occurred more frequently under elevated CO_2 in summer. Elevated O_3 significantly altered fungal community composition from that under ambient O_3, and one OTU

(Unknown 1 in Fig 6b) was an indicator of elevated O_3 treatment in spring. This OTU was closely related to the genus *Mortiellera*, a common group of saprophytic fungi capable of producing chitinolytic, proteolytic, and cellulytic enzymes (De Boer et al. 1999; Lähn et al. 2002). We cannot infer whether the biomass of this OTU changed under elevated O_3 treatment because PCR–DGGE is not quantitative, but this OTU occurred significantly more frequently in soils under elevated O_3. This fungus may have an advantage over other fungi when there is less belowground production under O_3 enrichment.

We have demonstrated that the activity of cellulose- and chitin-decomposing enzyme was significantly higher under CO_2 enrichment. Moreover, we observed that elevated O_3 decreased the activity of cellulose degrading enzymes and it also altered fungal community composition. Although not statistically significant, activities of other enzymes responded in a similar way under CO_2 and O_3 enrichment, further supporting our contention that microbial metabolism is enhanced under elevated CO_2 and suppressed under elevated O_3. We conclude that the change in substrate availability under CO_2 and O_3 enrichment altered microbial community function, and this was accompanied by a change in fungal community composition, at least in response to elevated O_3. This indicates that a change in plant production and litter biochemistry under elevated CO_2 and O_3 may alter the metabolism of fungal communities, and that elevated O_3 can concurrently modify fungal community composition. Taken together, our results imply that changes in fungal community function under elevated CO_2 and O_3 and alterations in fungal community composition under elevated O_3 are driven by changing substrate quantity and quality, and this may in turn alter soil C cycling as CO_2 and O_3 accumulate in the atmosphere.

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