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Fungal community composition and metabolism under elevated CO₂ and O₃

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Abstract Atmospheric CO₂ and O₃ 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 CO₂ and O₃ could alter microbial community function and composition. Previously, we have observed that elevated CO₂ increased the microbial metabolism of cellulose and chitin, whereas elevated O₃ dampened this response. We hypothesized that this change in metabolism under CO₂ and O₃ enrichment would be accompanied by a concomitant change in fungal community composition. We tested our hypothesis at the free-air CO₂ and O₃ enrichment (FACE) experiment at Rhineland, Wisconsin, in which *Populus tremuloides*, *Betula papyrifera*, and *Acer saccharum* were grown under factorial CO₂ and O₃ 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 CO₂, whereas 1,4-β-glucosidase activity (–25%) was significantly suppressed by elevated O₃. There was no significant main effect of elevated CO₂ or O₃ on fungal relative abundance, as measured by PLFA. We identified 39 fungal taxonomic units from soil using DGGE, and found that O₃ enrichment significantly altered fungal community composition. We conclude that fungal metabolism is altered under elevated CO₂ and O₃, and that there was a concomitant change in fungal community composition under elevated O₃. Thus, changes in plant inputs to soil under elevated CO₂ and O₃ can propagate through the microbial food web to alter the cycling of C in soil.

Keywords Free-air CO₂ and O₃ 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 CO₂, 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 CO₂ 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 CO₂ 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 CO₂ (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 CO₂ 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 CO₂-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 O₃, which could counteract the effect of elevated CO₂.

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 CO₂, elevated O₃ 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 O₃ (Coleman et al. 1996; Andersen et al. 1997; Andersen 2003). Reduced allocation of photosynthate to roots under O₃ enrichment has the potential to suppress microbial metabolism, an effect that could counteract that of elevated CO₂. How will changes in plant growth under elevated CO₂ and O₃ alter microbial community function and composition?

We studied microbial community function and composition under elevated CO₂ and O₃ at the free-air CO₂ and O₃ enrichment (FACE) experiment in Rhinelander, Wisconsin. In this experiment, *Populus tremuloides*, *Betula papyrifera*, and *Acer saccharum* have been exposed to factorial elevated CO₂ and O₃ treatments since 1998 (Dickson et al. 2000). Our previous work has demonstrated that fine root biomass increased significantly under elevated CO₂, and decreased under elevated O₃ (King et al. 2001). Also, the C:N ratio in senescing *Populus* and *Betula* leaves increased significantly under elevated CO₂, 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 CO₂ enrichment; O₃ 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 CO₂ and O₃. We examined the relationship between belowground plant biomass and enzyme activity to confirm if the changes in substrate availability in response to elevated CO₂ and O₃

were responsible for altering the microbial metabolism. In addition, we used PLFA analysis to determine whether elevated CO₂ and O₃ 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 CO₂ and O₃ 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/m². 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 CO₂ and O₃ treatments beginning in May 1998. The level of elevated CO₂ was 560 μl/l, which is 200 μl/l above ambient CO₂ concentration. The target level of elevated O₃ treatment is determined at the beginning of each day, based on current meteorological conditions. For hot and sunny days, a maximum O₃ 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 non-structural 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 $\text{g}^{-1} \text{h}^{-1}$.

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 $\text{g}^{-1} \text{h}^{-1}$. 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 18:2 ω 6 and 18:1 ω 9c 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.

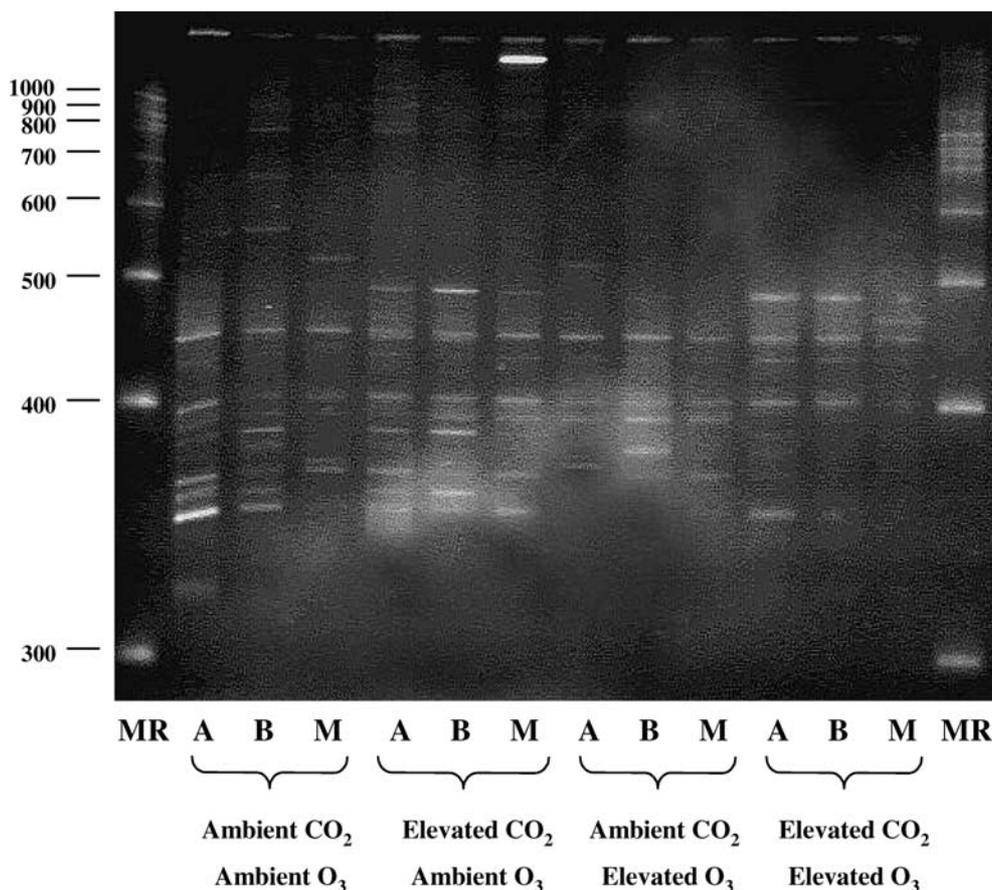
PCR, DGGE, and DNA sequence analysis

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 \times 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, Inc., 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, CO₂, O₃, and species were fixed effects in this model. Carbon dioxide and O₃ treatment combinations were main effects, and they were split by species. Significance of main effects (CO₂ and O₃), 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 CO₂ are not different, and (2) fungal communities under ambient and elevated O₃ 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 CO₂ and O₃ 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 CO₂ 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 CO₂ (Fig 2a), and *N*-acetylglucosaminidase activity increased under elevated CO₂ by 84% when compared to ambient CO₂ (Fig 2b). Elevated CO₂ 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 CO₂ had no effect on peroxidase activity (Table 1, Fig 2g).

Elevated O₃ significantly (main effect) reduced the activity of 1,4- β -glucosidase by 25% relative to the activity of this enzyme at ambient O₃ (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 O₃, but these reductions were not statistically significant (Table 1, Fig 3).

Although there was no significant interaction between elevated CO₂ and O₃ (Table 1), the activity of cellulose-degrading enzymes under elevated CO₂ showed a tendency to be dampened by elevated O₃. For example, 1,4- β -glucosidase and cellobiohydrolase activities, both

under elevated CO₂ and O₃ were lower than the activities under elevated CO₂ and ambient O₃, and were not different from those under ambient CO₂ and O₃ (Fig 4a,b). However, *N*-acetylglucosaminidase activity under elevated CO₂ and O₃ was not different from that under elevated CO₂ 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 CO₂ had a significant interaction effect on 1,4- β -glucosidase activity (Table 1). 1,4- β -glucosidase activity in aspen-maple section under ambient CO₂ was lower than that under aspen and aspen-birch section, but 1,4- β -glucosidase activity under elevated CO₂ in all the three tree compositions were not different (data not shown).

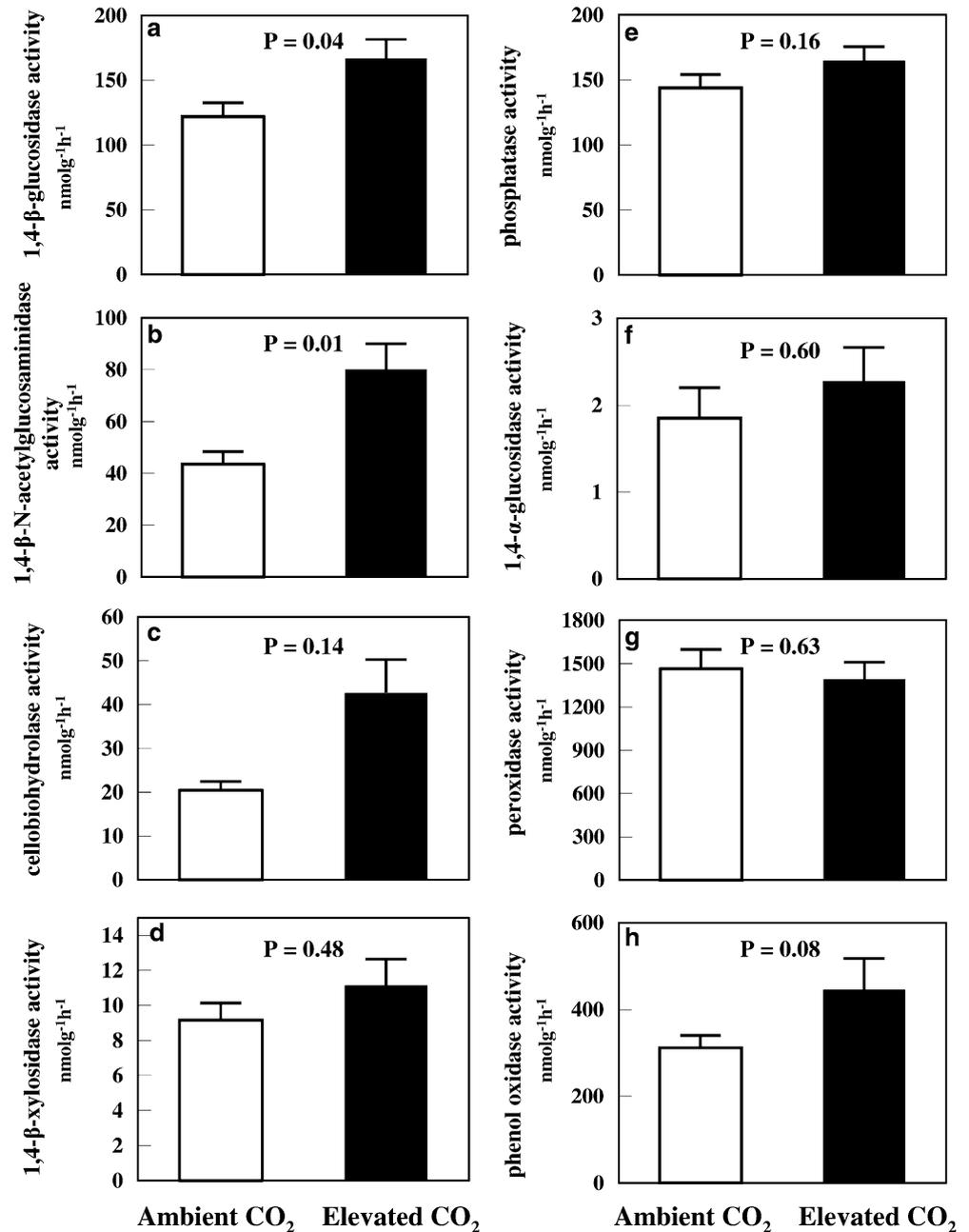
Microbial community composition

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 - <i>N</i> -acetylglucose-aminidase	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 ×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 CO₂ on extracellular enzyme activity. Enzyme activity was averaged across three sampling seasons. Error bars indicate standard error of the mean



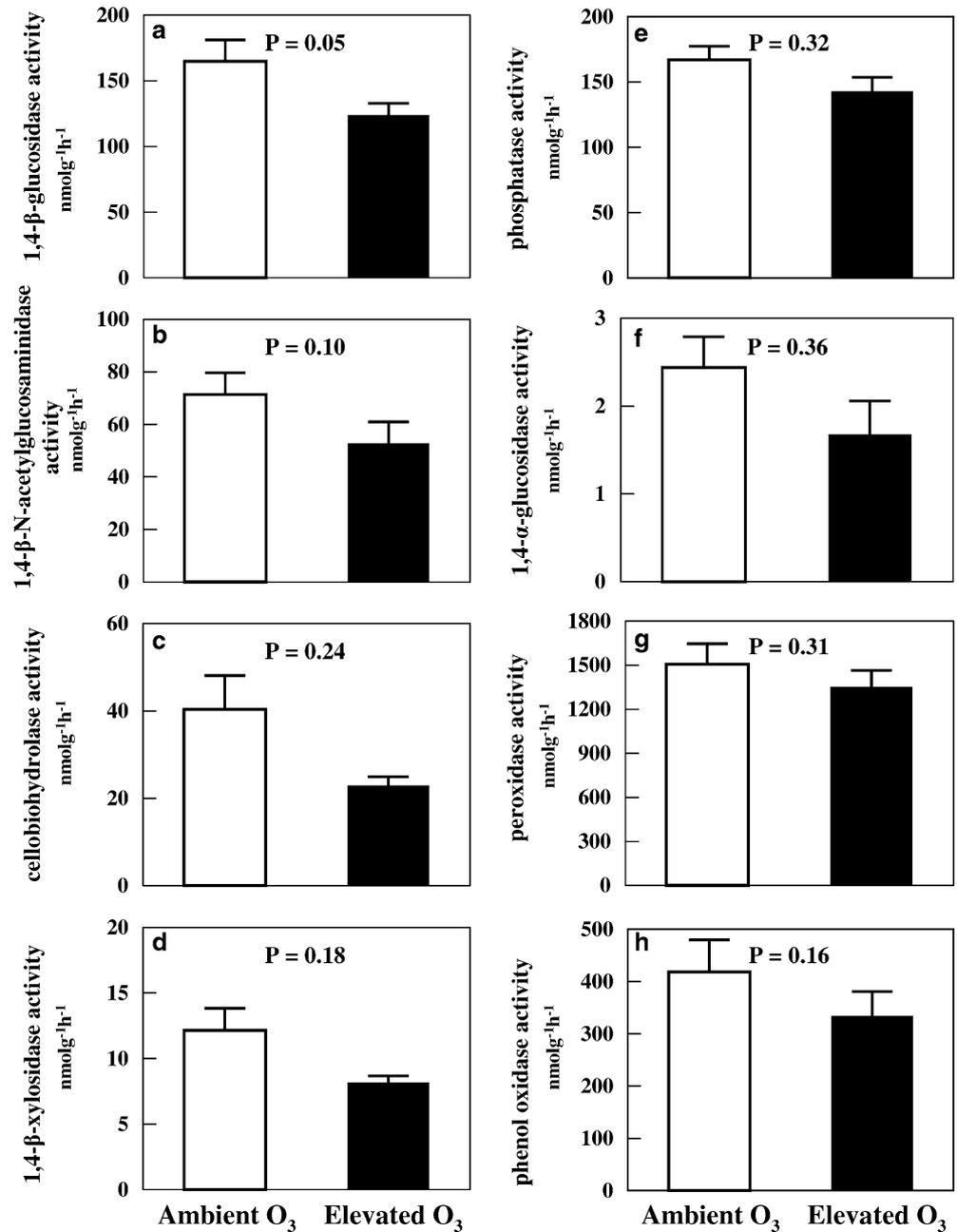
comprising total biomass was considered as the relative abundance of fungi in the soil microbial community. Fungal relative abundance in July 2001 was 30% higher under elevated CO₂, but this was only marginally significant ($P = 0.10$). Fungal relative abundance under elevated O₃ was 17% higher than that under ambient O₃, but this result also was not statistically significant. There was no significant interaction between elevated CO₂ and O₃ 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 O₃ 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 *Verticillium* 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 CO₂ was not different across all the three sampling seasons ($P = 0.85$). No change in fungal community composition under ambient and elevated CO₂ 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 CO₂ treatment, we performed indicator species analysis. Presence/absence of data for each fungal taxonomic unit were analyzed for

their occurrence under ambient and elevated CO₂ treatment. In summer, one operational taxonomic unit (Unknown 2 in Fig 6a) occurred in 11% of the soil samples under ambient CO₂, whereas it was present in 67% of the soil samples under elevated CO₂. This species was a significant indicator of elevated CO₂ treatment ($P = 0.003$).

Elevated O₃ significantly altered fungal community composition across all the three sampling seasons ($P = 0.02$). Fungal community composition under ambient and elevated O₃ 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

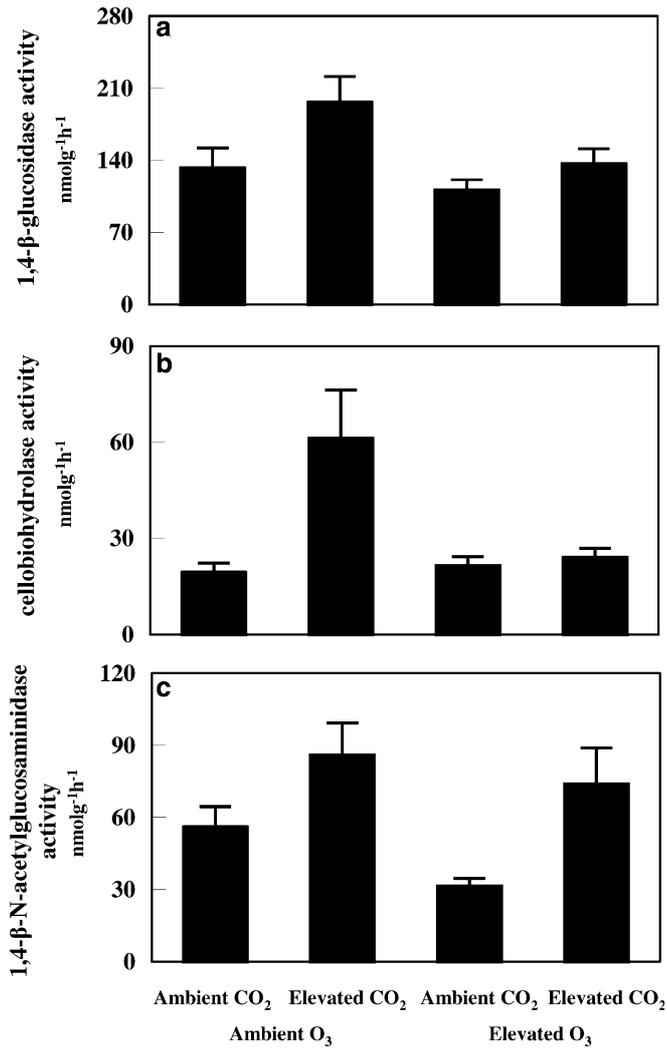


Fig. 4 Effect of CO₂ and O₃ on activities of 1,4-β-glucosidase, cellobiohydrolase, and 1,4-β-N-acetylglucosaminidase. Error bars indicate standard error of the mean

elevated O₃ treatment in spring ($P = 0.02$). This OTU was present in 6 % of the soil samples under ambient O₃, whereas it occurred in 44 % of the soil samples under elevated O₃ in spring.

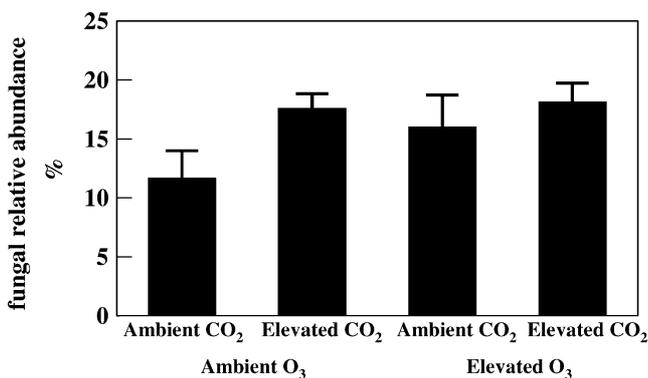


Fig. 5 Fungal relative abundance under elevated CO₂ and O₃ in July 2001. Error bars indicate standard error of the mean

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 CO₂ and O₃ 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 CO₂, whereas plant production was suppressed by O₃ enrichment (Kull et al. 1996; Lindroth et al. 2001). Here, we demonstrate that 1,4-β-glucosidase and *N*-acetylglucosaminidase activities are enhanced under CO₂ enrichment, and 1,4-β-glucosidase activity was suppressed under O₃ enrichment. These changes in the extracellular enzyme activity were accompanied by alteration in fungal community composition under elevated O₃. Our results indicate that increases in atmospheric CO₂ and O₃ can induce changes in plant growth that cascade into the soil food web to modify the fungal community composition, and that O₃ enrichment can concurrently alter fungal community composition.

Activity of the cellulose-degrading enzyme 1,4-β-glucosidase significantly increased beneath plants exposed to elevated CO₂ alone, but this response showed a tendency to be dampened by O₃ (i.e., in elevated CO₂ and elevated O₃ treatment), suggesting that elevated O₃ may counteract the effect of elevated CO₂. 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 CO₂ increased the production of fine roots, whereas elevated O₃ 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 CO₂ and O₃. 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 CO₂, and this could indicate that there is a higher input of fungal litter under elevated CO₂. 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 ¹³C-labelled *N*-acetylglucosamine into fungal biomass under elevated CO₂ (Phillips et al. 2002), and taken together this suggests that there may be higher fungal biomass under CO₂ enrichment. Phospholipid fatty acid analysis of soil samples collected in July 2001 showed that fungal biomass significantly increased under

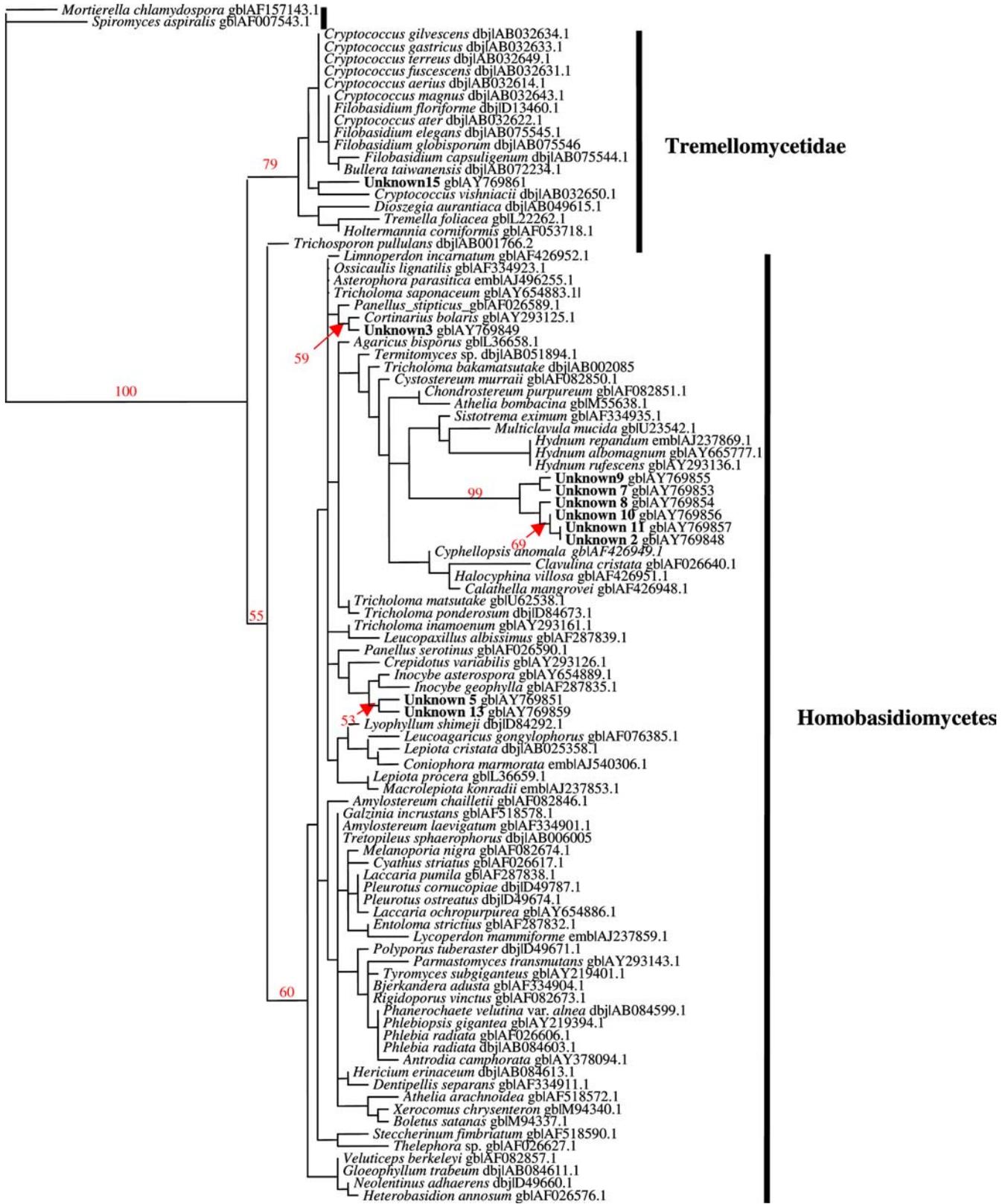


Fig. 6 Phylograms showing the placement of the sequenced OTUs amplified from soils at the Rhinelander FACE site for **a** Basidiomycota, and **b** Ascomycota and Zygomycota. Numbers above the clades indicate bootstrap support (%), shown only for the clades containing the 16 unknowns

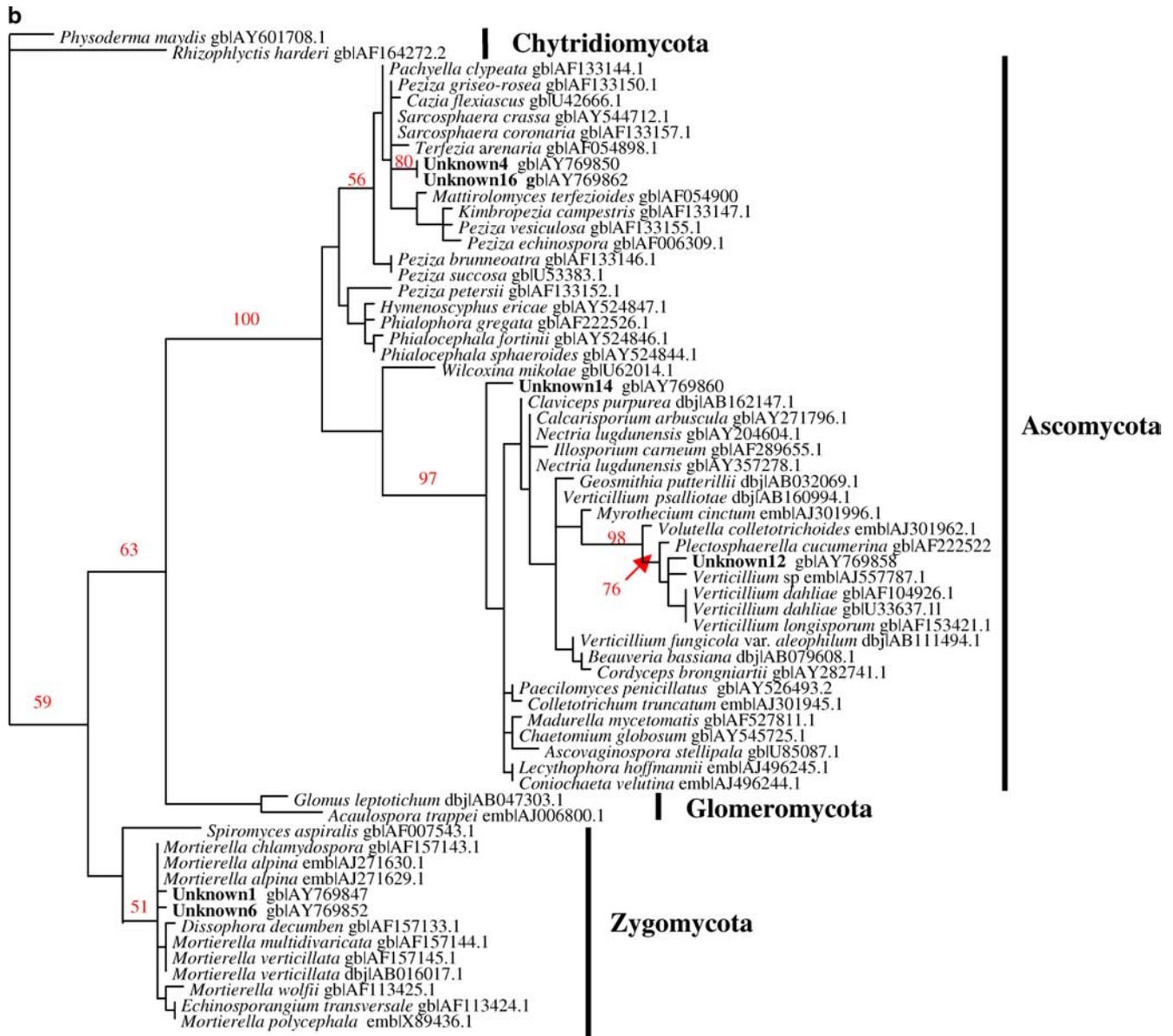


Fig. 6 (Contd.)

elevated CO₂ in the aspen section, but there was no difference in the fungal biomass under ambient and elevated CO₂ 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 CO₂ 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 CO₂ (Rillig et al. 1999). Jones et al. (1998) also demonstrated that cellulose-decomposing fungi had higher biomass under elevated CO₂, probably account-

ing for the increased decomposition rates of cotton strips. Because we have observed higher 1,4- β -glucosidase and *N*-acetylglucosaminidase activity under elevated CO₂ and dampened 1,4- β -glucosidase activity under elevated O₃, 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 CO₂ did not differ, but there was one indicator OTU (Unknown 2 in Fig 6a) that occurred more frequently under elevated CO₂ in summer. Elevated O₃ significantly altered fungal community composition from that under ambient O₃, and one OTU

(Unknown 1 in Fig 6b) was an indicator of elevated O₃ treatment in spring. This OTU was closely related to the genus *Mortierella*, a common group of saprophytic fungi capable of producing chitinolytic, proteolytic, and cellulolytic enzymes (De Boer et al. 1999; Lähn et al. 2002). We cannot infer whether the biomass of this OTU changed under elevated O₃ treatment because PCR-DGGE is not quantitative, but this OTU occurred significantly more frequently in soils under elevated O₃. This fungus may have an advantage over other fungi when there is less belowground production under O₃ enrichment.

We have demonstrated that the activity of cellulose- and chitin-decomposing enzyme was significantly higher under CO₂ enrichment. Moreover, we observed that elevated O₃ 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 CO₂ and O₃ enrichment, further supporting our contention that microbial metabolism is enhanced under elevated CO₂ and suppressed under elevated O₃. We conclude that the change in substrate availability under CO₂ and O₃ enrichment altered microbial community function, and this was accompanied by a change in fungal community composition, at least in response to elevated O₃. This indicates that a change in plant production and litter biochemistry under elevated CO₂ and O₃ may alter the metabolism of fungal communities, and that elevated O₃ can concurrently modify fungal community composition. Taken together, our results imply that changes in fungal community function under elevated CO₂ and O₃ and alterations in fungal community composition under elevated O₃ are driven by changing substrate quantity and quality, and this may in turn alter soil C cycling as CO₂ and O₃ accumulate in the atmosphere.

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References

- Andersen CP (2003) Source-sink balance and carbon allocation belowground in plants exposed to ozone. *New Phytol* 157:213–228
- Andersen CP, Wilson R, Plocher M, Hogsett WE (1997) Carry-over effects of ozone on root growth and carbohydrate concentrations of ponderosa pine seedlings. *Tree Physiol* 17:805–811
- Allen AS, Andrews JA, Finzi AC, Matamala R, Richter DD, Schlesinger WH (2000) Effects of free-air CO₂ enrichment (FACE) on belowground processes in a *Pinus taeda* forest. *Ecol Appl* 10:437–448
- Bååth E (2003) The use of neutral lipid fatty acids to indicate the physiological conditions of soil fungi. *Microb Ecol* 45:373–383
- Bardgett RD, Hobbs PJ, Frostegård Å (1996) Changes in soil fungal:bacterial biomass ratios following reductions in the intensity of management of an upland grassland. *Biol Fertility Soils* 22:261–264
- Burns RG (1982) Enzyme activity in soil: location and a possible role in microbial ecology. *Soil Biol Biochem* 14:423–427
- Coleman MD, Dickson RE, Isebrands JG, Karnosky DF (1996) Root growth and physiology of potted and field-grown trembling aspen exposed to tropospheric ozone. *Tree Physiol* 16:145–152
- De Boer W, Gerards S, Gunnewiek PJA, Modderman R (1999) Response of the chitinolytic microbial community to chitin amendments of dune soils. *Biol Fertil Soils* 29:170–177
- DeLucia EH, Hamilton JG, Naidu SL, Thomas RB, Andrews JA, Finzi A, Lavine M, Matamala R, Mohan JE, Hendrey GR, Schlesinger WH (1999) Net primary production of a forest ecosystem with experimental CO₂ enrichment. *Science* 284:1177–1179
- Dickson RE, Lewin KF, Isebrands JG, Coleman MD, Heilman WE, Riemenschneider DE, Sober J, Host, GE, Zak DR, Hendrey GR, Pregitzer KS, Karnosky DF (2000) Forest atmosphere carbon transfer and storage (FACTS-II) the aspen Free-air CO₂ and O₃ Enrichment (FACE) Project: an overview. Gen Tech Rep NC-214. St. Paul, MN: US Department of Agriculture, Forest Service, North Central Research Station
- Findlay S, Jones CG (1990) Exposure of cottonwood plants to ozone alters the subsequent leaf decomposition. *Oecologia* 82:248–250
- Finlayson-Pitts BJ, Pitts JN Jr (1997) Tropospheric air pollution: ozone, airborne toxics, polycyclic aromatic hydrocarbons, and particles. *Science* 276:1045–1052
- Fromin N, Hamelin J, Tarnawski S, Roesti D, Jourdain-Miserez K, Forestier N, Teyssier-Cuvellé S, Gillet F, Aragno M, Rossi P (2002) Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. *Environ Microbiol* 4:634–643
- Guckert JB, Antworth CP, Nichols PD, White DC (1985) Phospholipid, ester-linked fatty acid profiles as reproducible assays for change in prokaryotic community structure of estuarine sediments. *FEMS Microbiol Ecol* 31:147–158
- Hayano K, Katami A (1977) Extraction of β-glucosidase activity from pea field soil. *Soil Biol Biochem* 9:349–351
- Jones TH, Thompson LJ, Lawton JH, Bezemer TM, Bardgett RD, Blackburn TM, Bruce KD, Cannon PF, Hall GS, Hartley SE, Howson G, Jones CG, Kampichler C, Kandler E, Ritchie DA (1998) Impacts of rising atmospheric carbon dioxide on model terrestrial ecosystems. *Science* 280:441–443
- Karnosky DF (1996) Photosynthetic responses of aspen clones to simultaneous exposures of ozone and CO₂. *Can J For Res* 26: 639–648
- King JS, Pregitzer KS, Zak DR, Sober J, Isebrands JG, Dickson RE, Hendrey GR, Karnosky DF (2001) Fine-root biomass and fluxes of soil carbon in young stands of paper birch and trembling aspen as affected by elevated atmospheric CO₂ and tropospheric O₃. *Oecologia* 128: 237–250
- King JS, Kubiske ME, Pregitzer KS, Hendrey GR, McDonald EP, Giardina EP, Quinn VS, Karnosky DF (2005) Tropospheric O₃ compromises net primary production in young stands of trembling aspen, paper birch and sugar maple in response to elevated atmospheric CO₂. *New Phytol* DOI 10.1111/j.1469-8137.2005.01557.X
- Körner C (2000) Biosphere responses to CO₂ enrichment. *Ecol Appl* 10:1590–1619
- Kull O, Sober A, Coleman MD, Dickson RE, Isebrands JG, Gagnon Z, Karnosky DF (1996) Photosynthetic responses of aspen clones to simultaneous exposures of ozone and CO₂. *Can J For Res* 26:639–648
- Lähn K, Wolf G, Ulrich-Eberius C, Koch E (2002) Cultural characteristics and in vitro antagonistic activity of two isolates of *Mortierella alpina* Peyronel. *Z Pflanzenkr Pflanzenschutz* 109: 166–179

- Larson J, Zak DR, Sinsabaugh RL (2002) Extracellular enzyme activity and metabolism of root-derived substrates beneath temperate trees growing under elevated CO₂ and O₃. *Soil Sci Soc Am J* 66:1848–1856
- Lindroth RL, Kopper BJ, Parsons WFJ, Bockheim JG, Karnosky DF, Hendrey GR, Pregitzer KS, Isebrands JG, Sober J (2001) Consequences of elevated carbon dioxide and ozone for foliar chemical composition and dynamics in trembling aspen (*Populus tremuloides*) and paper birch (*Betula papyrifera*). *Environ Pollut* 115:395–404
- Matamala R, Schlesinger WH (2000) Effects of elevated atmospheric CO₂ on fine root production and activity in an intact temperate forest ecosystem. *Global Change Biol* 6:967–979
- McCune B, Grace JB (2002) Analysis of ecological communities. MjM Software Design, Gleneden Beach, Oregon
- Mikan CJ, Zak DR, Kubiske ME, Pregitzer KS (2000) Combined effects of atmospheric CO₂ and N availability on the below-ground carbon and nitrogen dynamics of aspen mesocosms. *Oecologia* 124:432–445
- Miller M, Palojarvi A, Rangger A, Reeslev M, Kjoller A (1998) The use of fluorogenic substrates to measure fungal presence and activity in soil. *Appl Environ Microbiol* 64:613–617
- Mooney HA, Drake BG, Luxmoore RJ, Oechel WC, Pitelka LF (1991) Predicting ecosystem responses to elevated CO₂ concentrations. *Bioscience* 41:96–104
- Phillips RL, Zak DR, Holmes WE, White DC (2002) Microbial community composition and function beneath temperate trees exposed to elevated atmospheric carbon dioxide and ozone. *Oecologia* 131:236–244
- Pregitzer KS, Zak DR, Maziasz J, DeForest J, Curtis PS, Lusenhop J (2000) Interactive effects of atmospheric CO₂ and soil-N availability on fine roots of *Populus tremuloides*. *Ecol Appl* 10: 18–33
- Rillig MC, Field CB, Allen MF (1999) Soil biota responses to long-term atmospheric CO₂ enrichment in two California annual grasslands. *Oecologia* 119:572–577
- Rogers HH, Prior SA, Krupa SV (1994) Plant responses to atmospheric CO₂ enrichment with emphasis to roots and rhizosphere. *Environ Pollut* 83:155–189
- Saiya-Cork KR, Sinsabaugh RL, Zak DR (2002) The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. *Soil Biol Biochem* 34:1309–1315
- Sekiguchi H, Tomioka N, Nakahara T, Uchiyama H (2001) A single band does not always represent single bacterial strain in denaturing gradient gel electrophoresis. *Biotechnol Lett* 23:1205–1208
- Swift MJ, Heal OW, Anderson JM (1979) Decomposition in terrestrial ecosystems. Blackwell, Oxford
- Taylor GE, Johnson DW, Andersen CP (1994) Air pollution and forest ecosystems: a regional to global perspective. *Ecol Appl* 4:662–689
- Vainio EJ, Hantula J (2000) Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. *Mycol Res* 104:927–936
- White DC, Davis WM, Nickels JS, King JD, Bobbie RJ (1979) Determination of the sedimentary microbial biomass by extractable lipid phosphate. *Oecologia* 40:51–62
- Zak DR, Pregitzer KS, Curtis PS, Teeri JA, Fogel R, Randlett DL (1993) Elevated atmospheric CO₂ and feedback between carbon and nitrogen cycles. *Plant Soil* 151:105–117