Interactive effects of preindustrial, current and future atmospheric CO$_2$ concentrations and temperature on soil fungi associated with two Eucalyptus species

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Abstract
Soil microbial processes have a central role in global fluxes of the key biogenic greenhouse gases and are likely to respond rapidly to climate change. Whether climate change effects on microbial processes lead to a positive or negative feedback for terrestrial ecosystem resilience is unclear. In this study, we investigated the interactive effects of [CO$_2$] and temperature on soil fungi associated with faster-growing *Eucalyptus saligna* and slower-growing *Eucalyptus sideroxylon*, and fungi that colonised hyphal in-growth bags. Plants were grown in native soil under controlled soil moisture conditions, while subjecting the above-ground compartment to defined atmospheric conditions differing in CO$_2$ concentrations (290, 400, 650 µL L$^{-1}$) and temperature (26 and 30 °C). Terminal restriction fragment length polymorphism and sequencing methods were used to examine effects on the structure of the soil fungal communities. There was no significant effect of host plant or [CO$_2$]/temperature treatment on fungal species richness (α diversity); however, there was a significant effect on soil fungal community composition (β diversity) which was strongly influenced by eucalypt species. Interestingly, β diversity of soil fungi associated with both eucalypt species was significantly influenced by the elevated [CO$_2$]/high temperature treatment, suggesting that the combination of future predicted levels of atmospheric [CO$_2$] and projected increases in global temperature will significantly alter soil fungal community composition in eucalypt forest ecosystems, independent of eucalypt species composition. These changes may arise through direct effects of changes in [CO$_2$] and temperature on soil fungi or through indirect effects, which is likely the case in this study given the plant-dependent nature of our observations. This study highlights the role of plant species in moderating below-ground responses to future predicted changes to [CO$_2$] and temperature and the importance of considering integrated plant–soil system responses.

Introduction

Atmospheric carbon dioxide concentrations [CO$_2$] have varied considerably over geological time, reaching a minimum of ca. 180 µL L$^{-1}$ in the Last Glacial Maximum (18 000–20 000 years ago) and ca. 280 µL L$^{-1}$ at the beginning of the industrial revolution (200 years ago; Luthi et al., 2008). Over the next century, [CO$_2$] is predicted to exceed 550 µL L$^{-1}$, accompanied by an increase in global mean surface temperature of up to 4.4 °C (Solomon et al., 2007). Over the past two decades, experiments in pots, growth chambers, open-top chambers and free air carbon dioxide enrichment (FACE) have demonstrated that enrichment of atmospheric CO$_2$ can have direct and indirect effects on terrestrial ecosystems and can interact with below-ground carbon (C) cycling (Pritchard, 2011). The direct effect of elevated [CO$_2$] is an increase in net primary production, that is, 'CO$_2$...
fertilisation'. Increased photosynthesis under elevated atmospheric CO₂ concentrations stimulates the production of the above-ground biomass and has a strong effect on C fluxes from above-ground parts into soil. C input to soil generally increases in response to elevated [CO₂], owing to improved plant carbohydrate status, even if there is no significant CO₂ stimulation of above-ground growth (Körner & Arnone, 1992). Thus, rising atmospheric [CO₂] has the potential to impact the quantity and quality of C inputs into soil, a compartment of great ecological importance in terms of plant–soil interactions and terrestrial ecosystem functioning.

While plant responses to elevated [CO₂] (see review by Ainsworth & Rogers, 2007) are relatively well documented, little is known about plant response to the interactive effects of elevated [CO₂] and temperature (Lloyd & Farquhar, 2008; Ghannoum et al., 2010a) and preindustrial [CO₂] (Tissue & Lewis, 2010). Importantly, we know very little about the interactive effects of [CO₂] and temperature on soil fungi. Given the essential roles played by soil fungi in forest carbon and nutrient cycles (Dighton, 2003), the fact that fungal mycelia represent a significant component of the soil carbon pool, and the likelihood that they comprise a considerable belowground carbon sink (Treseder & Allen, 2000), this represents a gap in our current knowledge.

Soil microbial communities are known to change in response to elevated [CO₂] (reviewed in: Pritchard, 2011; King, 2011; Singh et al., 2010; Drigo et al., 2008), with fungal biomass and abundance frequently reported to increase under these conditions (Klironomos et al., 1996; Lipson et al., 2005; Carney et al., 2007; Drigo et al., 2007, 2008, 2009, 2010). The percentage of tree root tips infected by ectomycorrhizal (ECM) fungi has also frequently been shown to increase under elevated [CO₂] (Norby et al., 1987; Ineichen et al., 1995; Berntson et al., 1997; Tingey et al., 1997; Rouhier & Read, 1998; Walker et al., 1998; Kasurinen et al., 2005; Garcia et al., 2008; Pritchard et al., 2008), while arbuscular mycorrhizal (AM) colonisation is generally not affected by elevated [CO₂] (Staddon et al., 1999, 2004; Staddon, 2005; Cavagnaro et al., 2007) or varies with the duration of exposure or plant species (Chen et al., 2007; Garcia et al., 2008).

Studies on the biomass of soilborne mycelia of ECM and AM fungi under elevated [CO₂] produced mixed results, namely they have shown either an increase (Ineichen et al., 1995; Rouhier & Read, 1998, 1999; Rillig & Allen, 1999; Treseder & Allen, 2000; Fransson et al., 2005; Pritchard et al., 2008) or decrease in mycelial biomass (Staddon et al., 1999; Parrent & Vilgalys, 2007; Garcia et al., 2008).

At the community level, there is strong evidence for changes in relative abundance of ECM fungi in root tip communities as a result of elevated [CO₂] (Godbold & Berntson, 1997; Godbold et al., 1997; Rey & Jarvis, 1997; Rygiewicz et al., 2000; Fransson et al., 2001; Kasurinen et al., 2005, 2010; Parrent et al., 2006), although this is not universally observed (Garcia et al., 2008). In the only investigation of soilborne ECM mycelial communities to date, species richness was unaffected, while specific thelephoroid and athelioid taxa were both frequent and abundant as extramatrical mycelium, and thelephoroid richness was extremely high under elevated [CO₂] (Parrent & Vilgalys, 2007). Fewer investigations have focused on general soil fungal communities, and varying responses have been noted. While no change in Populus spp. rhizosphere and bulk soil fungal community structure was observed (Laggomarsino et al., 2007), elevated [CO₂] significantly altered the Populus tremuloides, Betula papyrifera, Acer saccharum rhizosphere and bulk soil fungal community structure (Lesaulnier et al., 2008; Edwards & Zak, 2011). In particular, these authors noted increased basidiomycete abundance, accompanied by decreased taxonomic richness of basidiomycetes and zygomycetes. The structure of the soil basidiomycete community in a scrub oak forest was also observed to change as a result of elevated [CO₂] (Klamer et al., 2002).

Although most ecosystems are likely to face other environmental perturbations, such as increased temperature, in addition to elevated [CO₂] as a result of climate change (Solomon et al., 2007), little information on responses of soil fungal communities to increased temperature is available and markedly different outcomes have been reported (Bardgett et al., 1999). ECM and AM fungal mycelium increased with experimental warming, as did species richness and colonisation of understory plants (Clemmensen et al., 2006; Heinemeyer et al., 2006; Hawkes et al., 2008; Olrusd et al., 2010; Deslippe et al., 2011). The abundance of fungi relative to bacteria may increase (Zhang et al., 2005) or decrease (Rinnan et al., 2007; Frey et al., 2008) under similar conditions. Warming has also been shown to both increase soil fungal diversity and alter the relative abundance of fungal taxa in a boreal forest (Allison & Treseder, 2008), but did not significantly alter fungal diversity associated with decomposing litter in a low alpine heathland (Papanikolaou et al., 2010). In a study on the combined effects of elevated [CO₂] and increased temperature on soil fungi, Rygiewicz et al. (2000) showed that elevated temperature partially modulated the effects of elevated [CO₂].
Effects of CO₂ and temperature on soil fungi

Effects of CO₂ and temperature on soil fungi

Materials and methods

Growth conditions and soil sampling

Eucalyptus saligna Sm. and E. sideroxylon A. Cunn, ex Woolls were grown in 10-L cylindrical pots (PVC pipes, 15 cm diameter × 40 cm length) in glasshouse compartments under different temperature and atmospheric CO₂ regimes as fully described by Ghannoum et al. (2010a). Nine kilograms of air-dried, native and locally sourced soil was added to each pot (adjusted to the same mass by the addition of pebbles). The soil was a loamy-sand with low organic matter content (0.7%), fertility [pH 5.5, N (< 1 mg kg⁻¹), P (8 mg kg⁻¹), K (0.23 meq 100 g⁻¹), Ca (1.2 meq 100 g⁻¹), Mg (0.34 meq 100 g⁻¹), S (5 mg kg⁻¹), B (0.2 mg kg⁻¹), Zn (0.9 mg kg⁻¹), Cu (0.2 mg kg⁻¹), Fe (24 mg kg⁻¹), Mn (9.1 mg kg⁻¹), Al (0.14 meq 100 g⁻¹), Na (0.1 meq 100 g⁻¹) and Cl (13 mg kg⁻¹)] and low water holding capacity, collected from the top 50 cm of the Hawkesbury Forest Experiment site (Barton et al., 2010) at the University of Western Sydney, Richmond, NSW, Australia (33°36′40″ S, 150°44′26.5″ E).

Seedlings were established as described by Ghannoum et al. (2010a) before being transferred to six adjacent, naturally lit and temperature-controlled glasshouse compartments (3 m wide × 5 m long × 3.5 m tall). Seedlings were watered daily, and pots were irrigated with a nutrient solution containing a commercial fertiliser (General Purpose; Thrive Professional, Yates, NSW, Australia) 30, 120 and 135 days after planting, as described by Ghannoum et al. (2010a). Pots were routinely moved within the glasshouses during the experimental period. At planting, hyphal in-growth bags were buried at a depth of ca. 20 cm in five pots for each tree species and each treatment outlined below. The bags were constructed by sealing 10 g oven-dried, acid-washed, quartz-propagating sand (median particle size ca. 0.5 mm) into 50-μm nylon mesh bags (ca. 5 × 3 cm) using a plastic bag sealer (Wallander et al., 2001).

Three glasshouse compartments were programmed to day/night temperatures of 26/18 °C (ambient temperature treatment) and three compartments to 30/22 °C (high temperature treatment), with temperature monitored by thermocouples and adjusted by the temperature control system. One compartment in each temperature treatment was supplied with preindustrial CO₂ (target 290 μL L⁻¹), ambient CO₂ (target 400 μL L⁻¹) and elevated CO₂ (target 650 μL L⁻¹). Preindustrial CO₂ was achieved by continuously passing glasshouse compartment air over trays filled with calcium hydroxide (Schaefer Kalk GmbH & Co KG, Diez, Germany), and elevated CO₂ was maintained by injecting CO₂ gas (Food grade; Air Liquide, Australia) from pressurised cylinders through solenoid valves connected to a CO₂ monitor/controller (Lambda T; ADC BioScientific Ltd., Hoddesdon, Herts, UK) as described by Ghannoum et al. (2010a).

The complete experimental design (for measurement of plant growth and physiology) comprised 50 pots of each species and treatment, for a total of 600 pots (Ghannoum et al., 2010a). However, we sampled nine pots from each treatment and species combination (except eight pots for E. sideroxylon in ambient CO₂ and ambient temperature) after 150 days and collected a single soil core (2 × 15 cm, w × l) from each pot. Soil was sieved (2 mm) and stored at −20 °C until DNA extraction. Hyphal in-growth bags were harvested at the same time.

DNA extraction and PCR amplification

DNA was extracted from 0.5 g sieved soil or sand from each core or hyphal in-growth bag using Ultra Clean Soil DNA Isolation Kit (MoBio Laboratories, CA) according
to the manufacturer’s instructions, except that DNA was eluted in 50 µL Tris-EDTA pH 8.0 using bead beating for 30 s at a speed of 5.0 m s⁻¹ in a FastPrep (FP120) Thermos Savant bead beating system (Bio-101; Vista, CA). DNA extracts were purified using the Wizard DNA Clean-Up System (Promega, Sydney) following the manufacturer’s instructions.

Fungal rDNA ITS regions were PCR-amplified using the fluorescently labelled primers (5'-6-FAM) ITS1F (Gardes & Bruns, 1993) and (5'-HEX) ITS4 (White et al., 1990). PCR amplifications were performed in 50 µL reactions containing 1.0 µL DNA; 5× Green GoTaq® Flexi buffer (Promega); 200 µM dNTPs; 2.5 mM MgCl₂; 20 pmol of both primers; 0.3 µL bovine serum albumin (BSA) solution (10 mg mL⁻¹) and 2.5 units GoTaq Flexi DNA polymerase (Promega). Cycling parameters were based on those described by Anderson et al. (2007) and consisted of 5 min at 95 °C followed by 29 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, with a final extension step at 72 °C for 10 min. PCR products were visualised using standard electrophoresis.

TRFLP analysis of fungal community composition

Fluorescently labelled PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega) using the manufacturer’s instructions. Restriction digestes were performed in 10 µL volumes containing 8.0 µL purified PCR product, 5 units of the restriction endonucleases HinfI or TaqI (Promega), 0.1 µL BSA solution and 0.5 µL of the appropriate 10× buffer. Digests were incubated at 37 °C (HinfI) or 65 °C (TaqI) for 3 h. Terminally labelled restriction fragments were separated along a 50-cm column, using POP 4 polymer, on an ABI PRISM 3700 DNA sequencer (Applied Biosystems Inc, CA). A GeneScan 500 ROX internal size standard (Applied Biosystems Inc) was applied to each sample. TRFLP profiles were analysed using a second-order least squares size calling method (peaks between 50 and 500 bp in size) and a peak amplitude threshold setting of 50, using GENEMAPPER version 4.0 software (Applied Biosystems Inc).

Statistical analysis

The fungal TRFLP fingerprints obtained were binary-coded and used in statistical analysis as ‘species’ presence—absence matrices. Main effects of tree species (E. sideroxylon vs. E. saligna), temperature, [CO₂] and two-way and three-way interactions between these three factors on fungal community structure, as examined by TRFLP, were tested by distance-based redundancy analysis (db-RDA, Legendre & Anderson, 1999). Jacquard’s coefficients of similarity were first calculated between samples and used to compute principal coordinates [principal coordinates analysis (PCoA)] in the R-package (Casgrain & Legendre, 2001). When necessary, eigenvectors were corrected for negative eigenvalues using the procedure of Lingoes (1971), and then all the PCoA axes were exported to CANOCO version 4.5 (Ter Braak & Šmilauer, 2002) and treated as ‘species’ data. To test the effects of the three groups of factors (plants, temperature and CO₂), they were entered as dummy binary variables. In CANOCO, one group of factors was entered as the explaining variables in the model, while the other two groups of factors were entered as covariables. The significance of such models was tested with a Monte Carlo test based on 999 permutations restricted for split-plot design, with glasshouse compartments designated as whole plots. Further db-RDA analyses were conducted as described previously, but on subsets of the whole data set, by analysing each tree species separately. The percentage of variation in the data set that was related to the different factors was determined by variance partitioning (Borcard et al., 1992) using the same strategy as for db-RDA analyses.

Relationships between fungal community structure in soil samples, [CO₂] (preindustrial, ambient, elevated) and temperature (ambient and high) treatments were determined with correspondence analyses (CA) at the plant species level. Separate analyses were carried out for E. sideroxylon and E. saligna to distinguish key environmental parameters influencing fungal community structure. Calculations were undertaken using CANOCO version 4.5 (Ter Braak & Šmilauer, 2002).

ITS amplification, cloning, RFLP analysis and sequence analysis

PCR products from hyphal in-growth bags were cloned. Fungal ITS regions were amplified in 50 µL reactions using 25 pmol each of the primers ITS1F and ITS4 as described previously and cloned using the pGEM-T easy vector system (Promega) following the manufacturer’s instructions. A total of 100 white clones were screened for each ligation using the SP6 and T7 primer pair with an initial denaturation step of 94 °C for five min, followed by 30 cycles of 95 °C for 1 min, 50 °C for one min and 72 °C for one min and a final extension step of 72 °C for 10 min. PCR products were electrophoresed in
2% (w/v) gels, stained with ethidium bromide and visualised under UV light to check for positive clone inserts.

PCR products from each clone (ca. 100 ng) were individually digested with five units of the restriction endonuclease HinfI (Promega) for 3 h at 37 °C. Restriction fragments were electrophoresed in 3.0% (w/v) gels with phiX174 DNA/HinfI markers (Promega) for 1 h at 110 V. RFLP banding patterns were used to sort clones into RFLP types, with clones that had identical banding patterns considered to be the same RFLP type. Prior to sequencing, representative clones from each RFLP type were purified using the Wizard SV gel and PCR clean-up system (Promega) and sequenced using an ABI 3730xl sequencer (Applied Biosystems). Sequences were analysed using SEQUENCHER version 4.8 (Gene Codes Corporation, Ann Arbor, MI), aligned with CLUSTAL W and edited manually to include the ITS1, 5.8S subunit and ITS2 regions. A similarity matrix was generated using BIOEDIT (version 7), and clone sequences that had > 98% sequence similarity were regarded as duplicates and discarded accordingly. To determine the closest sequence matches in the GenBank database, ITS sequences were analysed using BLAST and screened for likely chimeric sequences by comparing BLAST matches. Taxonomic affinities were assigned conservatively based on the closest several database matches and the ‘distance tree’ feature in BLAST.

TRFLP clone database

Representative clones (3) from each RFLP type were amplified using the primers ITS1F-FAM and ITS4-HEX and cycling parameters described previously. Labelled PCR products were purified using the Wizard SV gel and PCR clean-up system (Promega) and digested using five units of HinfI and TaqI as described previously. Clone TRFs were sized with GeneScan 500 ROX (Applied Biosystems) and run on the same DNA sequencer using the same conditions as the community samples. Clone TRFLP profiles were analysed using GENEMAPPER version 3.7 (Applied Biosystems), with peaks below 50 or above 500 bp removed from the analysis. A database of clone TRFs was constructed and compared to community TRFLP profiles for both soil and hyphal in-growth bags using FRAGMATCH (Saari et al., 2007).

Results

Fungal species richness

A total of 212 FAM and 226 HEX TRFs were obtained using TaqI, with ≤ 39 FAM and ≤ 40 HEX fragments obtained for individual soil samples. HinfI yielded similar total TRF numbers (222 FAM and 206 HEX), while ≤ 45 FAM and ≤ 42 HEX TRFs were obtained for individual soil samples. A total of 167 FAM and 179 HEX TRFs were obtained using TaqI, with ≤ 43 FAM and ≤ 39 HEX fragments obtained for individual hyphal in-growth bag samples. Total TRF numbers were similar with HinfI (199 FAM and 199 HEX), with ≤ 50 FAM and ≤ 48 HEX TRFs obtained for individual hyphal in-growth bags. Overall, a total of 866 and 744 unique TRFs (two enzymes x two fragments) were identified in the preindustrial [CO2] × high temperature samples across all treatments, with the exception of the elevated [CO2] × high temperature conditions where the mean TRF number was significantly (P = 0.01) lower (Table 1). In the hyphal in-growth bags, significantly (P = 0.038) higher TRF numbers were detected in the preindustrial [CO2] × high temperature conditions compared to soil (Table 1). There were no significant differences in mean TRF numbers in soil samples across all treatments, with the exception of the elevated [CO2] × high temperature conditions for which the mean TRF number was significantly (0.01) lower (Table 1). In the hyphal in-growth bags, significantly (P = 0.038) higher TRF numbers were detected in the preindustrial [CO2] × high temperature conditions compared to soil (Table 1).

Table 1. Data are presented for TRFs [mean ± standard error (SE)] in soil and hyphal in-growth bags for Eucalyptus saligna and Eucalyptus sideroxylon in the three [CO2] and two temperature treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Soil</th>
<th>Hyphal in-growth bags</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. saligna</td>
<td></td>
<td></td>
</tr>
<tr>
<td>290 µL L⁻¹ CO2 ambient temperature</td>
<td>114.8 ± 8.6b</td>
<td>70.6 ± 16.6ab</td>
</tr>
<tr>
<td>400 µL L⁻¹ CO2 ambient temperature</td>
<td>114.3 ± 7.6b</td>
<td>105.6 ± 3.1c</td>
</tr>
<tr>
<td>650 µL L⁻¹ CO2 ambient temperature</td>
<td>111.1 ± 4.4b</td>
<td>82.5 ± 6.7b</td>
</tr>
<tr>
<td>290 µL L⁻¹ CO2 high temperature</td>
<td>114.5 ± 4.4b</td>
<td>102.6 ± 8.0c</td>
</tr>
<tr>
<td>400 µL L⁻¹ CO2 high temperature</td>
<td>107.5 ± 6.0b</td>
<td>60.0 ± 13.9a</td>
</tr>
<tr>
<td>650 µL L⁻¹ CO2 high temperature</td>
<td>90.3 ± 4.7a</td>
<td>63.4 ± 8.5a</td>
</tr>
<tr>
<td>E. sideroxylon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>290 µL L⁻¹ CO2 ambient temperature</td>
<td>108.8 ± 6.1b</td>
<td>63.2 ± 26.2a</td>
</tr>
<tr>
<td>400 µL L⁻¹ CO2 ambient temperature</td>
<td>113.6 ± 6.5b</td>
<td>79.4 ± 12.1ab</td>
</tr>
<tr>
<td>650 µL L⁻¹ CO2 ambient temperature</td>
<td>114.6 ± 7.8b</td>
<td>75.4 ± 7.2ab</td>
</tr>
<tr>
<td>290 µL L⁻¹ CO2 high temperature</td>
<td>113.3 ± 6.3b</td>
<td>103.8 ± 10.2c</td>
</tr>
<tr>
<td>400 µL L⁻¹ CO2 high temperature</td>
<td>112.3 ± 5.3b</td>
<td>84.2 ± 8.3b</td>
</tr>
<tr>
<td>650 µL L⁻¹ CO2 high temperature</td>
<td>107.6 ± 8.7b</td>
<td>62.0 ± 6.2a</td>
</tr>
</tbody>
</table>

Different letters indicate significant differences (P < 0.05) between mean TRF numbers (as determined by Tukey’s HSD test) within either the soil or hyphal in-growth bag data across both plant species.
treatment for both eucalypt species and in the ambient [CO₂] × ambient temperature treatment for *E. saligna* (*P* = 0.039). Significantly (*P* = 0.027) lower TRF numbers were detected at elevated [CO₂] × high temperature (both eucalypt species), preindustrial [CO₂] × ambient temperature (*E. sideroxylon*) and in the ambient [CO₂] × high temperature (*E. saligna*) treatments than in the other treatment combinations (*P* = 0.015; Table 1).

**Fungal community composition**

db-RDA of the TRFLP data showed that plant species (*P* < 0.001, soil cores; *P* = 0.018, hyphal in-growth bags), temperature (*P* < 0.001, soil cores; *P* = 0.021, hyphal in-growth bags) and CO₂ treatments (*P* = 0.016, soil cores; *P* = 0.035, hyphal in-growth bags), along with the interactions between these factors, explained a significant part of the shifts in fungal community composition (Table 2).

PCoA of the TRFLP data indicated that tree species had a highly significant effect on fungal community structure in soil cores as evidenced by the separation of *E. saligna* from *E. sideroxylon* along axis 1 (*P* < 0.001; Fig. 1a). There was no further separation based on either temperature or [CO₂] alone (Fig. 1a). There was, however, a clear interactive effect between preindustrial [CO₂] and high temperature for both *E. saligna* and *E. sideroxylon*, and a further separation along axis 2 (*P* = 0.012). High temperature coupled with elevated [CO₂] significantly (*P* = 0.005) influenced the fungal community structure in soil cores. Samples from both eucalypt species clustered tightly together (Fig. 1a), suggesting that this treatment

### Table 2. db-RDA results for sample type (soil cores and hyphal in-growth bags), plant species (*Eucalyptus saligna* and *Eucalyptus sideroxylon*), temperature (ambient and high) and atmospheric [CO₂] (preindustrial, ambient and elevated) on fungal community TRFLP profiles based on 999 Monte Carlo permutations test.

<table>
<thead>
<tr>
<th>Location</th>
<th>Plant species</th>
<th>Variables</th>
<th>Co-variables</th>
<th>F</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil cores</td>
<td><em>E. saligna</em> + <em>E. sideroxylon</em></td>
<td>Plants × temperature × CO₂</td>
<td>–</td>
<td>3.778</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plants</td>
<td>–</td>
<td>8.894</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Temperature</td>
<td>–</td>
<td>3.068</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO₂</td>
<td>–</td>
<td>1.371</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Temperature × CO₂</td>
<td>–</td>
<td>8.954</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plants × CO₂</td>
<td>–</td>
<td>3.229</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plants × temperature</td>
<td>–</td>
<td>1.463</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td><em>E. saligna</em></td>
<td>Temperature × CO₂</td>
<td>–</td>
<td>1.964</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Temperature</td>
<td>–</td>
<td>3.136</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO₂</td>
<td>–</td>
<td>1.304</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Temperature × CO₂</td>
<td>–</td>
<td>3.224</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Temperature</td>
<td>–</td>
<td>1.366</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td><em>E. sideroxylon</em></td>
<td>Temperature × CO₂</td>
<td>–</td>
<td>1.375</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Temperature</td>
<td>–</td>
<td>1.447</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO₂</td>
<td>–</td>
<td>1.315</td>
<td>0.013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Temperature × CO₂</td>
<td>–</td>
<td>1.484</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO₂</td>
<td>–</td>
<td>1.335</td>
<td>0.021</td>
</tr>
<tr>
<td>Hyphal in-growth bags</td>
<td><em>E. saligna</em> + <em>E. sideroxylon</em></td>
<td>Plants × temperature × CO₂</td>
<td>–</td>
<td>1.505</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plants</td>
<td>–</td>
<td>1.542</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Temperature</td>
<td>–</td>
<td>1.678</td>
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<tr>
<td></td>
<td></td>
<td>CO₂</td>
<td>–</td>
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<td>Plants × temperature × CO₂</td>
<td>–</td>
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<td>Plants × temperature</td>
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<tr>
<td></td>
<td><em>E. saligna</em></td>
<td>Temperature × CO₂</td>
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<td>1.227</td>
<td>0.027</td>
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<td></td>
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<td></td>
<td></td>
<td>CO₂</td>
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<td>0.115</td>
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<td>1.493</td>
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<tr>
<td></td>
<td></td>
<td>CO₂</td>
<td>–</td>
<td>1.237</td>
<td>0.094</td>
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<td><em>E. sideroxylon</em></td>
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<td>0.027</td>
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<td></td>
<td></td>
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<td>1.223</td>
<td>0.061</td>
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<tr>
<td></td>
<td></td>
<td>CO₂</td>
<td>–</td>
<td>1.241</td>
<td>0.059</td>
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***P < 0.001.
combination (elevated [CO₂] plus high temperature) had a greater effect on soil fungal community structure than tree species. CA of the *E. sideroxylon* soil TRFLP data showed preindustrial and elevated [CO₂] coupled with high temperature as the main factors influencing soil fungal communities (*P* = 0.005; Fig. 2a). The same analysis of the *E. saligna* soil TRFLP data showed high temperature as the main driving factor with an interactive effect between preindustrial [CO₂] and high temperature (*P* = 0.006; Fig. 2b). There was no obvious separation of the hyphal in-growth bag data based on the independent, main effects of tree species, temperature or [CO₂] (Fig. 1b). The only separation observed was between the high and low temperature samples at elevated [CO₂] (Fig. 1b).

Variance partitioning analyses of soil and hyphal in-growth bag data for each tree species (*E. sideroxylon* and *E. saligna*) revealed similar trends (Fig. 3). In the soil cores and in both plant species, [CO₂] significantly explained the greatest amount of variation in fungal community composition (43%; *P* = 0.021 for *E. sideroxylon*; 41.5%; *P* = 0.006 for *E. saligna*) (Table 2; Fig. 3a and b). In the hyphal in-growth bags, the variation in the *E. sideroxylon* and *E. saligna* fungal community composition was not significantly explained by either [CO₂] or temperature (Table 2; Fig. 3c and d).
Taxonomic affinities of fungal clones

In total, 38 OTUs were detected in the hyphal in-growth bag clone libraries. Of these, 10 were putatively identified as ECM basidiomycetes, 22 were a range of ascomycete and basidiomycete taxa and six remained unidentified (Supporting Information, Tables S1 and S2). TRFs produced for each of the 38 OTUs were included in the database for comparisons with the soil and hyphal in-growth bag TRFLP data.

Comparison of the TRFLP data for the soil and hyphal in-growth bags against the database showed that while most OTUs were equally abundant in both the *E. saligna* and *E. sideroxylon* samples, several were ≥ 2 times more abundant under either the *E. saligna* or *E. sideroxylon*. For example, HQ829324 (*Laccaria* sp.) and HQ829334 (*Pezizomycotina*) were more abundant under *E. saligna* than *E. sideroxylon* (both soil and hyphal in-growth bags) (Tables S1 and S2). In contrast, several OTUs such as HQ829342 (*Ascomycoota*) were more abundant in *E. sideroxylon* samples than for *E. saligna* (hyphal in-growth bags) irrespective of temperature or [CO2] treatment (Table S2). While 10 OTUs (including HQ829324, *Laccaria* sp. and HQ829334, *Pezizomycotina*) were more abundant in the high temperature samples (both soil and hyphal in-growth bags) regardless of [CO2] treatment or eucalypt species, only a single OTU in soil (HQ829332; *Penicillium canescens*) and two in hyphal in-growth bags (HQ829331, *Sclerodermia* sp. and HQ829341, *Capnodiales*) were more abundant in the ambient temperature samples (Tables S1 and S2). While differences were detected in some hyphal in-growth bags, these were not consistent across treatments or [CO2] treatments.

Discussion

db-RDA of the fungal community TRFLP profiles showed that increased atmospheric [CO2] combined with elevated temperature exerted differential influences on the fungal communities associated with *E. sideroxylon* and *E. saligna*. Large effects of [CO2] and temperature treatments were observed on soil fungal community composition (β diversity), but there were few significant effects on mean OTUs and therefore on fungal species richness (i.e. α diversity). While differences were detected in some hyphal in-growth bags, these were not consistent across treatments or [CO2] treatments.
Eucalypt species. These observations are consistent with previous laboratory and field-based investigations of [CO₂] and temperature effects on soil fungal communities (Klamer et al., 2002; Parrent & Vilgalys, 2007; Pap nikolaou et al., 2010; Edwards & Zak, 2011). However, they contrast with the observations of Allison & Treseder (2008) who showed that warming had a significant positive effect on the richness of active fungi (determined by bromodeoxyuridine incorporation) in a boreal forest. Although we did not specifically target active fungi in our study, hyphal in-growth bags contain mycelia of fungi that actively colonise the bags during the incubation period.

The structure of fungal communities (i.e. β diversity) was largely dependent on the eucalypt species growing in the soil with the exception of the elevated CO₂ (650 μL L⁻¹) × high temperature (30 °C) treatment combination where the data from both species clustered together in the ordination, suggesting this treatment combination overrode any effect of plant species. There are some reports that plant species, including those in the same genus, can alter soil fungal community structure in the field (Bastias et al., 2007; Curlevski et al., 2010). For example, Edwards & Zak (2011) compared soil fungal communities under birch and birch/aspen grown in elevated [CO₂] in a FACE experiment and found that plant community composition was the most significant determinant of soil fungal community composition changes, with elevated [CO₂] having a secondary effect. While the observed differences in soil fungal communities under birch and birch/aspen (Edwards & Zak, 2011) and Araucariaceae species (Curlevski et al., 2010) may reflect differences in litter inputs from different plant species over 10 and 80 years, respectively, this was not the case with our shorter-term experiment in the glasshouse. Subsequently, a more direct and fundamental plant host-driven effect must have affected our soil fungal community.

The two eucalypt species used in this work have been shown to differ significantly in their growth rates, with E. saligna accumulating threefold more biomass than E. sideroxylon when grown at ambient [CO₂] and temperature (Ghannoum et al., 2010a). Although the root/shoot ratio did not significantly differ between the two species, root biomass was greater in the larger E. saligna plants under all treatments (Ghannoum et al., 2010a). This suggests that a greater amount of C was supplied to the E. saligna rhizosphere and that this might have affected the soil fungal community. In addition, larger E. saligna plants with greater leaf area transpired significantly higher amounts of water than E. sideroxylon (Ghannoum et al., 2010b), which is likely to have led to more rapid depletion of soil water in E. saligna pots. Communities of soil fungi are influenced by soil moisture depletion (Toberman et al., 2008; Bell et al., 2009; Castro et al., 2010; Schmitt & Glaser, 2011), and concomitant alterations in soil carbon availability may partially explain the observed differences in fungal community composition under the two eucalypt species.

Eucalypt species did not affect the overall structure of fungal communities in hyphal in-growth bags. This is consistent with the observations of Edwards & Zak (2011) that saprotrophic and ECM fungi respond differently to treatments such as elevated [CO₂]. As hyphal in-growth bags are known to select for ECM fungi in many forest soils (Bastias et al., 2006; Kjøller, 2006; Wallander et al., 2010), and there is no evidence that Eucalyptus species differ in their ability to form ECM associations with particular fungal taxa (Malajczuk et al., 1982), these results are not surprising. Putative ECM OTUs represented only 26% of the 38 OTUs generated from hyphal in-growth bags (i.e. TRFLP database OTUs), but some were detected in multiple hyphal in-growth bags across all treatments. While our data show the number of individual hyphal in-growth bags within which each OTU was detected (i.e. frequency), they give no indication of the relative abundance of each OTU in the mycelial communities within individual bags. It is thus possible that these ECM OTUs represented a significant proportion of the overall fungal mycelial biomass in the hyphal in-growth bags; however, we did not test this directly.

While there were few significant effects of [CO₂] or temperature on soil fungal species (OTU) richness (i.e. α diversity) when the E. saligna and E. sideroxylon data were analysed independently (to remove the effect of host from the analysis), there were strong and significant effects on soil fungal community structure (i.e. β diversity), although the same effect was not observed for hyphal in-growth bags. While Klamer et al. (2002) reported no effect of elevated atmospheric [CO₂] on soil fungal species richness, Fransson et al. (2001) and Parrent & Vilgalys (2007) both reported significant differences in the abundance of individual ECM taxa under elevated [CO₂]. Interestingly, Parrent & Vilgalys (2007) reported a decline in the frequency of a Thelephoraceae taxon under elevated [CO₂], which we also observed for Thelephoraceae OTU HQ829321 in both the soil and hyphal in-growth bags. In contrast, two Laccaria OTUs (HQ829324 in soil and HQ829322 in hyphal in-growth bags) were found to increase in frequency under elevated [CO₂]. Both Laccaria bicolor and Laccaria laccata have been shown to produce more mycelial biomass when exposed to increasing amounts of C in pure culture (Fransson et al., 2007a), and L. bicolor mycelium has been shown to obtain more host-derived C under elevated [CO₂] than ambient [CO₂] when in symbiosis with Pinus sylvestris (Fransson et al., 2007b). Therefore, increased frequency of the Laccaria sp. OTUs HQ829324
and HQ829322 with rising [CO$_2$] in our study might be a function of increased below-ground plant C inputs. While Ghannoun et al. (2010a) did not measure changes to soil C inputs by E. saligna and E. sideroxylon via exudation, they observed increased root biomass with rising [CO$_2$] in both species, suggesting increased C allocation to soils.

Although our data indicated that increased temperature altered the structure of soil fungal communities, some previous investigations of the effects of elevated temperature on soil fungal communities reported no effect on community structure or diversity (Allison et al., 2010; Papanikolaou et al., 2010). Others, however, have reported that warming increased diversity and altered the relative abundance of individual taxa (Allison & Treseder, 2008; Deslippe et al., 2011). Allison & Treseder (2008) observed that a Thelephoroid taxon decreased in relative abundance with increasing temperature, which contrasts with our observation for Thelephoraceae OTU HQ829321 in both soil and hyphal in-growth bags, while we observed no change in the relative abundance of other Thelephoraceae OTUs. Fungal respiration generally increases with temperature (Hacskaylo et al., 1965; Malcolm et al., 2008), but this has been shown to be readily negated in some ECM fungi if they are acclimated at elevated temperature (Malcolm et al., 2008). Such an ability to metabolically acclimate to higher temperature may explain why the relative abundance of some OTUs increased in the high temperature treatment in the current investigation.

We observed significant interactive effects of [CO$_2$] and temperature on soil fungal communities. Several previous studies have investigated the interactive effects of increased atmospheric [CO$_2$] with other environmental variables on the diversity of both soil fungi and ECM fungi (Tingey et al., 1997; Kasurinen et al., 2005; Lagomarsino et al., 2007; Garcia et al., 2008; Edwards & Zak, 2011). The only study, however, to demonstrate a true interactive effect (between elevated [CO$_2$] and N) was that of Lagomarsino et al. (2007) as the study of Rygiewicz et al. (2000), which investigated the interactive effects of [CO$_2$] and temperature, reported no interactive effects on root tip communities of ECM fungi. Understanding the interactive effects of multiple environmental variables is not straightforward, and the trends in such data sets are often difficult to untangle. Indeed, our data demonstrate that interactive effects of [CO$_2$] and temperature on the diversity of soil fungal communities are complex, and in the case of all but one treatment combination (elevated [CO$_2$] and high temperature), the responses were plant species dependent. Such observations highlight the complex nature of below-ground responses to environmental change and the important role that different plant species have in moderating these responses. In our study, elevated [CO$_2$] (650 µL L$^{-1}$) plus high temperature (30 °C) was the only treatment combination to have a stronger effect on soil fungal community structure than the effect of eucalypt species. This suggests that the combination of future predicted levels of atmospheric [CO$_2$] and projected increases in global temperature might have a significant effect on soil fungi in eucalypt forest ecosystems, independent of eucalypt species composition. Although this study provides new insight into specific below-ground plant–soil system responses under future predicted changes to [CO$_2$] and temperature, knowledge is still rather scarce with respect to relative C flow to different biological groups. Such knowledge is critical for not only advancing our understanding of the soil food web, but also for predicting the future impacts of climate change.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Putative taxonomic affinities of database OTUs and their frequency of detection in soil TRFLP profiles across all experimental treatments (nmax = 9).

Table S2. Putative taxonomic affinities of database OTUs and their frequency of detection in hyphal in-growth bag TRFLP profiles across all experimental treatments (nmax = 5).

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