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Susceptibility of ectomycorrhizal fungi to soil heating

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ABSTRACT

Ectomycorrhizal (EcM) fungi are an important biotic factor for successful tree recruitment because they enhance plant growth and alleviate drought stress of their hosts. Thus, EcM propagules are expected to be a key factor for forest regeneration after major disturbance events such as stand-replacing forest fires. Yet the susceptibility of soil-borne EcM fungi to heat is unclear. In this study, we investigated the heat tolerance of EcM fungi of Scots pine (*Pinus sylvestris* L., Pinaceae). Soil samples of three soil depths were heated to the temperature of 45, 60 and 70 °C, respectively, and surviving EcM fungi were assessed by a bioassay using Scots pine as an experimental host plant. EcM species were identified by a combination of morphotyping and sequencing of the ITS region. We found that mean number of species per sample was reduced by the 60 and 70 °C treatment, but not by the 45 °C treatment. Species composition changed due to heat. While some EcM fungi species did not survive heating, the majority of species was also found in the heated samples. The most frequent species in the heat treatment were *Rhizopogon roseolus*, *Cenococcum geophilum* and several unidentified species.

Introduction

Ectomycorrhizal (EcM) fungi associate with a wide range of woody plants, enhancing growth (Smith & Read 2008) and alleviating drought stress of their hosts (Parke *et al.* 1983). The availability of EcM inoculum has been shown to improve tree recruitment (Mikola 1970), particularly in harsh environments (Horton *et al.* 1999; Nara & Hogetsu 2004). Therefore, EcM fungi are thought to be a key factor for successful tree recruitment after major disturbance events such as forest fires. The presence of EcM fungi may be especially important for post-fire recruitment of Scots pine (*Pinus sylvestris* L., Pinaceae) in the Central Alps, where precipitation levels are low and seedling establishment is known to be limited by drought.

Assessment of EcM fungi after fire has been performed over a wide range of forest ecosystems (Cairney & Bastias 2007). It is, however, difficult to draw general conclusions on the impact of forest fire on EcM fungi because results of field surveys are contradictory. Species composition has been demonstrated to change due to forest fire (de Román & de Miguel 2005; Jonsson *et al.* 1999), with some studies that have found a decrease in the number of EcM taxa, i.e. morphotypes or RFLP-taxa (Dahlberg *et al.* 2001; Smith *et al.* 2004; Visser 1995), whereas others have not (de Román & de Miguel 2005; Jonsson *et al.* 1999). This discrepancy may be due to differences in fire intensity as discussed in Jonsson *et al.* (1999) and Erland & Taylor (2002). Furthermore, a higher number of surviving propagules were found in deeper soil layers (Baar

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et al. 1999), which is attributed to decreasing heat penetration with increasing soil depth. Measurements of soil temperature profiles during fire show that, although temperatures at the surface can be very high, forest soil is insulating and temperatures decrease rapidly down the soil profile, with temperature declining 2–20 times within the uppermost 5 cm (DeBano et al. 1998; Whelan 1995; Wüthrich et al. 2002). Temperatures reached in soils during fires can vary widely. Maximum soil surface temperatures are typically in the range of 200–300 °C (Mataix-Solera et al. 2009), but often peak in a very narrow time span (some minutes only) with the cooling process lasting for more than one hour (DeBano et al. 1998).

Soil temperatures during fire depend on various interrelated factors such as weather conditions (relative humidity, antecedent rainfall, wind speed and direction), topography, microrelief, soil characteristics (organic matter content, soil moisture, soil texture) and fuel characteristics (vegetation type, amount of fuel, moisture content; DeBano et al. 1998; Neary et al. 1999). The spatial variation of these factors implies that fire behaviour and thus burn severity can vary widely even at small scales. Burned soils often appear as chaotic mosaics of areas with minor fire impacts intermingled with others that have been strongly affected (Hiers et al. 2009; Mataix-Solera et al. 2009; Thaxton & Platt 2006). While the EcM fungal community may be destroyed in severely burnt areas, less heated patches may serve as refuges and contain inoculum for fungal recolonization after fire. Temperatures lethal for soil inhabiting fungi in general are estimated to range between 60 and 80 °C (reviewed by Neary et al. 1999). For EcM fungi, lethal temperatures were found to be between 30 and 40 °C (Cline et al. 1987; Laiho 1970; Sánchez et al. 2001). However, the above-mentioned studies were performed with different isolated strains *in vitro*, investigating only growth of mycelium. Knowledge on survival of other life forms (e.g. spores and sclerotia) is scarce, but species-specific differences in heat tolerance can be assumed. For four selected species, differences in their resistance to heat (75 °C) have been shown (Peay et al. 2009). To our knowledge, only Izzo et al. (2006) analyzed the effect of different temperature levels on soil-borne EcM species, with soil samples from a forest dominated by white fir (*Abies concolor* Lindl. ex Hildebr.) and red fir (*Abies magnifica* A. Murr), both Pinaceae. Our study focuses on Scots pine because failure of post-fire tree recruitment of this species in the Central Alps has been reported (Delarze & Werner 1985; Moser et al. 2010). To determine the influence of heat on EcM fungal diversity, we assessed the inoculum potential of experimentally heated soil samples from a Scots pine stand in Valais, Switzerland, by means of a bioassay. An experimental approach has been chosen in order to heat the soil samples homogenously and to control the achieved temperature, so that the presence of a certain species could undoubtedly be attributed to its resistance to heat.

Materials and methods

Soil sampling and heating

Soil samples were collected in a 180-year-old Scots pine stand in Leuk, Valais (46° 19′ 37.7″ N, 7° 38′ 1.0″ E, 1020 m a.s.l.). Five parallel transects, each 40 m long and separated by 10 m, were established, and cubic soil samples (10 × 10 × 9 cm) were dug out every 10 m and stored en bloc in plastic boxes. Six transect positions where soils were too stony for sampling were excluded, giving a total of 19 samples. In the lab, each cube was divided vertically into two pieces for heat treatment and control, and horizontally into three layers, 0–3 cm, 3–6 cm and 6–9 cm, respectively, giving 6 subsamples. In order to ensure that temperatures reached target levels in all parts of the soil samples and survival of EcM fungi was not a result of less heated refuges, each subsample was crumbled to allow the heat to penetrate evenly and thoroughly. Coarse wood and stones were removed, but roots were left. Subsamples for the heat treatment were put on flat, heat-resistant trays which were placed in a drying oven and were heated to the temperature of 45 °C (6–9 cm), 60 °C (3–6 cm) and 70 °C (0–3 cm), respectively (Fig 1). The purpose of choosing three temperatures for the three soil depths was to mimic the insulating property of the forest soil. We choose temperatures between 45 and 70 °C because they have already been shown to affect EcM fungi (Izzo et al. 2006) as well as AM fungi (Klopatek et al. 1988; Pattinson et al. 1999). The temperature levels chosen exceed those from *in vitro* studies with EcM fungi, but are still low enough that survival of some fungal life forms is probable. Maximum temperatures reached on the soil surface during a fire may well exceed 70 °C, but we did not aim to test temperatures that are lethal for the entire EcM community. This would be likely at around 100 °C, which is regarded to be the uppermost threshold for most soil microorganisms to survive (Hartford & Frandsen 1992), with eukaryotes being generally more sensitive than bacteria (Mataix-Solera et al. 2009). Soil temperature during heating in the oven was measured with a thermocouple which had been stuck into one of the samples. When the target temperature was reached, the oven was switched off, and subsamples were allowed to cool down to room temperature. Due to the thermal inertia of the oven, temperature still increased for 1–2 °C before decreasing. Fig 1 shows the soil temperatures measured with

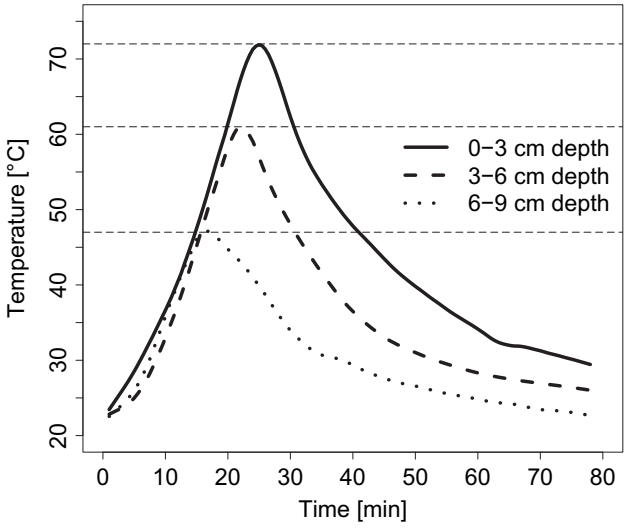

Fig 1 – Soil temperatures in the drying oven during the heating and cooling process.

the thermocouple during the heating and cooling process. Subsequently, the soil from both the heated and the control subsamples (2 heat treatments × 3 soil depths × 19 replicates) was filled in plastic pots and sown with seeds of Scots pine. Four additional pots were filled with autoclaved (120 °C, 20 min) soil to assay for possible contaminants in the growth chamber.

Bioassay and morphotyping

The pots were kept in a growth chamber (day/night cycles of 16 h/8 h, day temperature 24 °C, night temperature 20 °C, relative humidity 50 %, mean irradiance 746 lux) and were watered with distilled water every 3–4 d. After 6 months, the seedlings were harvested and the roots were carefully rinsed under tap water. The root tips were examined under a dissecting microscope and classified into ectomycorrhizal morphotypes according to Agerer (1987–97). Additionally, one representative of each morphotype was sampled for molecular identification. Mycorrhizae formed by *Cenococcum geophilum* were regarded as sufficiently characteristic to be identified with certainty without molecular analysis.

DNA extraction, amplification, and sequencing

DNA was extracted from the root tips using a DNeasy Plant Mini Kit according to the manufacturer's protocol (Qiagen 2006), and amplified following the PCR protocol of Gardes & Bruns (1993) using the primer pair ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990). 5.5 μl PCR product were used together with 1 μl of the Terminator Ready Reaction Mix for cycle sequencing with the ITS1F primer according to the protocol of Applied Biosystems (Applied Biosystems 2002), and subsequent electrophoreses were performed on an ABI 3130 Genetic Analyzer. Obtained sequences with a high degree of similarity were aligned together and were visually optimized using the programs Sequencing Analysis 5.1 (Applied Biosystems, Foster City, USA) and DNADynamo (Blue Tractor Software Ltd., North Wales, UK). The range in size of the sequences was between 380 and 726 bp. To search for sequence identity the BLASTn algorithm (Altschul *et al.* 1997) in the UNITE sequence database (Kõljalg *et al.* 2005) and the NCBI GenBank was used. Sequences showing <95 % identity with a relevant database entry were identified at family or ordinal level, 95–97 % at genus level, and >97 % at species level. Where two or more sequences matched the same database entry, but differed between each other (<98 % identity), a number was added to the name to mark the difference between the two, e.g. *Rhizopogon* sp. 1 and *Rhizopogon* sp. 2. A list with the species names and the accession numbers of the corresponding database entry is given in Appendix A. Root tips belonging to 17 different morphotypes remained unidentified. They were named Unidentified 1, Unidentified 2, etc. and their morphotypes are described in Appendix B. The descriptions of the morphotypes of successfully identified species can be found in Agerer (1987–97). One species identified as member of the Pyronemataceae was excluded from further analysis because it also occurred in one of the pots with autoclaved soil, and was therefore considered a contaminant in the growth chamber.

Statistical analyses

Differences in number of species per sample between the three soil depths and between the heat treatment and control were tested with a two-way ANOVA. Soil depth and temperature level were treated as one factor with the 3 levels 0–3 cm and 70 °C, 3–6 cm and 60 °C, 6–9 cm and 45 °C. *A posteriori* differences between treatment means were assessed using Tukey's HSD tests. Calculations were performed using the statistical computing system R version 2.6.2 (R Development Core Team 2008).

Results

The heat treatment had a significant effect on species number (two-way ANOVA, p value < 0.01). The mean number of species per sample was reduced by the 60 °C and 70 °C treatment (Tukey's HSD tests, both p values < 0.01), but not by the 45 °C treatment (p value of 0.089; Fig 2). There were no significant differences in number of species between the three heat levels (pairwise Tukey's HSD tests, p values > 0.1).

Species composition changed due to the heat treatment, with the overall species pool being reduced from 46 species in the control samples to 32 species in the heated samples. A total of 22 species were found in both the heated and the control samples, whereas 10 and 24 species occurred only in the heated and only in the control samples, respectively (Table 1). The most frequent species in the heated samples were *Rhizopogon roseolus*, *Cenococcum geophilum* and several unidentified species, whereas many of the species restricted to the control samples belong to resupinate EcM fungi (sensu Erland & Taylor 1999), e.g. *Amphinema byssoides*, *Tomentella badia*, *Tomentella pilosa*, *Tomentella stuposa*, *Tomentella* sp. 1, *Tomentella* sp. 2, *Tomentella* sp. 3 and *Tylospora asterophora*.

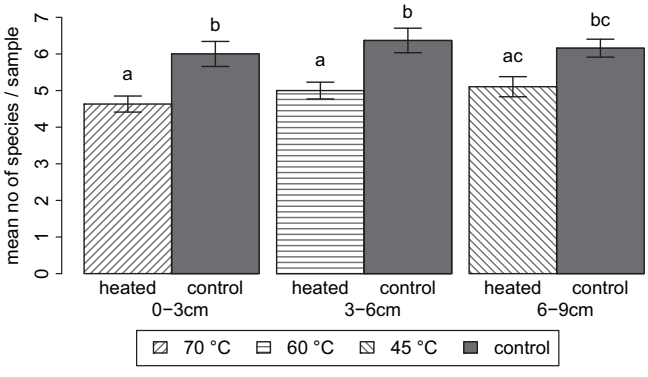

Fig 2 – Mean number of species per sample for the two treatments (heated/control) and the three soil depths. Different letters above the bars indicate significant differences between means (Tukey's HSD, $p < 0.05$). Whiskers indicate standard errors. N = 19.

Table 1 – List of species and their occurrence in different soil layers in untreated and heated soil samples. Species occurring in 5 or more samples are written in bold. For details of species identification see Appendices A and B.

Species name	Treatment					
	0–3 cm	3–6 cm	6–9 cm	0–3 cm	3–6 cm	6–9 cm
	Control	Control	Control	70 °C	60 °C	45 °C
Amphinema byssoides	1					
Astrosporina sp.	1	1				
Clavulinaceae sp.		1	1			
Craterellus lutescens	1		1			
Geopora sp.			1			
Humaria hemisphaerica	**4**	**1**				
Phialocephala fortinii	1					
Sebacinaceae sp. 1	1					
Telephoraceae sp. 1			1			
Telephoraceae sp. 2	1					
Tomentella badia	1	2	1			
Tomentella pilosa	1	1				
Tomentella sp. 1	1					
Tomentella sp. 2	1					
Tomentella sp. 3	1		1			
Tomentella stuposa		1				
Tylospora asterophora	1					
Uncultured Pyronemataceae sp. 4			1			
Unidentified 1		1				
Unidentified 2	2	2				
Unidentified 3			1			
Unidentified 4	**1**	**5**				
Unidentified 5		1				
Unidentified 6	**2**	**3**	**1**			
Cenococcum geophilum	**15**	**10**	**7**	**4**	**6**	**12**
Phialocephala europaea	**2**		**2**	**1**	**1**	**1**
Phialophora sp.		1	1	1		
Rhizopogon roseolus	**15**	**10**	**12**	**13**	**10**	**8**
Rhizopogon sp. 2			**2**	**3**	**1**	
Telephoraceae sp. 3		1			1	
Trichophaea gregaria	**2**	**3**	**1**			**1**
Uncultured agaricomycete sp.		**2**	**2**		**1**	**4**
Uncultured Pyronemataceae sp. 1	**2**	**1**	**4**	**1**		**2**
Uncultured Pyronemataceae sp. 2	1			1	1	
Uncultured Rhizopogonaceae sp.	**2**	**1**	**2**	**1**	**1**	**3**
Uncultured Sebacinaceae sp. 2	**2**				**2**	**2**
Unidentified 7	**5**	**4**	**7**	**4**	**4**	**3**
Unidentified 8	**2**	**1**	**2**		**1**	**1**
Unidentified 9	**1**	**5**	**6**		**1**	
Unidentified 10	1					1
Unidentified 11	**2**	**3**	**1**		**1**	
Unidentified 12	**1**	**7**	**3**	**5**	**4**	**4**
Unidentified 13	**1**	**3**	**1**			**2**
Unidentified 14	**4**	**8**	**8**	**13**	**12**	**7**
Unidentified 15		**7**	**8**	**7**	**7**	**3**
Wilcoxina rehmii		**1**	**1**		**2**	**1**
Ericoid mycorrhizal sp.				1		
Meliniomyces bicolor					1	
Rhizopogon sp. 1					1	
Sphaerosporella brunnea				1		
Tuberaceae sp.						1
Uncultured ectomycorrhiza (Cortinarius)				1		
Uncultured Pyronemataceae sp. 3						1
Uncultured Tricholomataceae						1
Unidentified 16					1	
Unidentified 17				1	2	
Total number of species	32	28	27	16	21	19
Total number of species in control/heat treatment	–	46	–	–	32	–

Discussion

The decrease in number of species as well as the change in species composition due to heat is in accordance with what has been found in field studies (Dahlberg *et al.* 2001; de Román & de Miguel 2005; Jonsson *et al.* 1999; Smith *et al.* 2004). Some species seem to be sensitive to heat, as it was the case for *Humaria hemisphaerica*, Unidentified 4 and Unidentified 9, the three most frequent species in our study (Table 1). Furthermore, seven species belonging to the resupinate EcM fungi were only present in the control samples, which corresponds to the study of Peay *et al.* (2009), who found *Tomentella sublilacina* and *Tylospora* sp. being absent from seedlings grown in 70 °C-heated soil, but abundant in control samples. In contrast, hypogeous species of the genus *Rhizopogon* were found to dominate in a post-fire fungal community on Bishop pine (*Pinus muricata* D. Don., Pinaceae; Baar *et al.* 1999) as well as on bioassay seedlings after experimentally heating to 75 °C and 70 °C, respectively (Izzo *et al.* 2006; Peay *et al.* 2009). Also *Cenococcum geophilum* and the E-strain fungus *Wilcoxina rehmii* were recorded frequently in post-fire fungal communities (Baar *et al.* 1999; de Román & de Miguel 2005; Fujimura *et al.* 2005; Torres & Honrubia 1997; Visser 1995).

Unexpectedly, we found no differences in mean number of species between different temperature levels, indicating that some species were diminished already at moderate heat, while the majority of species in our samples even survived 70 °C. This is surprising since temperatures in the range of 60–80 °C are considered to be lethal for soil inhabiting fungi (Neary *et al.* 1999). Given the fact that no difference between the three temperature levels (45, 60 and 70 °C) could be detected, temperature sensitivity of native EcM fungi in soil should be tested over a broader range of temperatures, focusing especially on temperature levels above 70 °C. Experimental soil heating does not only affect survival of fungal propagules, but also subsequent fungal growth (Bárcenas-Moreno & Bååth 2009), leading to shifts in species abundances. Furthermore, it is obvious that experimental soil heating addresses only one of manifold aspects of fire disturbance. The effects of fire are much more complex, causing changes in soil characteristics such as an increase in pH, the enhancement of hydrophobicity or a change of available nutrients (for a review see Certini 2005).

The bioassay approach has been used in many studies to assess the inoculum potential of a soil (Brundrett *et al.* 1996), even though it cannot detect all fungal species present. But since only infective propagules of the species that form ectomycorrhiza colonize the experimental host species, a bioassay reflects the subset of fungi that potentially have an effect on tree recruitment via symbiosis. Heating of soil samples has lead to a decrease in number of species, and therefore, further studies should investigate whether the lower species number and the probable lack of host-specialist species have an effect on tree performance.

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.funbio.2010.03.008.

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