Serpentine soils promote ectomycorrhizal fungal diversity

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Abstract

Serpentine soils impose physiological stresses that limit plant establishment and diversity. The degree to which serpentine soils entail constraints on other organisms is, however, poorly understood. Here, I investigate the effect of serpentine soils on ectomycorrhizal (ECM) fungi by conducting a reciprocal transplant experiment, where serpentine and nonserpentine ECM fungal communities were cultured in both their native and non-native soils. Contrary to expectation, serpentine soils hosted higher fungal richness compared to nonserpentine, and most species were recovered from serpentine soil, suggesting ECM fungi are not overall specialized or strongly affected by serpentine edaphic constraints.

Keywords: communities, ectomycorrhizal fungi, environmental stress, Serpentine soils

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Introduction

In terrestrial environments, soil conditions are important determinants of biodiversity both above and below ground, influencing the ecology and evolution of plants (Kruckeberg 2002), fungi (Schadt et al. 2003), animals (Freckman & Virginia 1997) and other organisms (Dion & Nautyal 2008). Extreme soils tend to host-specialized communities, with organisms specifically adapted to the offered abiotic conditions. The vast majority of organisms are involved in symbiotic associations (Douglas 1994), yet symbiotic partners are rarely considered by studies focusing on adaptation or environmental stress tolerance. Symbiotic species are often able to colonize unsuitable habitats only when associated with their partners (Gross 2001; Marquez et al. 2007), such that symbioses can be instrumental in structuring ecological communities.

Serpentine soils, derived from ultramafic rocks, are typically low in nutrient levels, exhibit toxic concentrations of heavy metals such as nickel, as well as unbalanced calcium-to-magnesium ratios (Brady et al. 2005; Alexander et al. 2007). These hostile conditions impose strong limits on species establishment and diversity, most notably for plants. Serpentine floras are characterized by low productivity and species richness, as well as high endemism and ecotypic specialization (Kruckeberg 1954; Brady et al. 2005; Alexander et al. 2007). Plants specialized on serpentine environments tend to be poor competitors on other substrates (Brady et al. 2005). Although performing better on nonserpentine soils, serpentine specialists experience a trade-off regarding competitive ability, which leads to exclusion from nonserpentine habitats by their native species (Kruckeberg 1954). All plants in natural ecosystems, including serpentine soils, are thought to be symbiotically associated with fungi (Petrini 1996; Brundrett 2006), and mutualistic fungi are known to facilitate plant establishment in stressful environments, including toxic soils (Sharplies et al. 2000; Andriaensen et al. 2003). Nonetheless, for organisms other than plants, the ecological and evolutionary consequences of inhabiting serpentine soils have received little study.

Here, I investigate the ecological and evolutionary effects of serpentine soils on ectomycorrhizal (ECM) fungi, a group of organisms forming symbiotic associations
with vascular plants. In this type of association, the root systems are in physical contact with fungal tissue, which mediates the host plant water and nutrient uptake by the host plant (Smith & Read 2008). By expanding plant absorption area, ECM fungi enhance resource acquisition and can play an important role in plant establishment under stressful conditions (Panaccione et al. 2001). In addition, fungi can reduce the transfer of toxic metals from the soil to their hosts (Hartley et al. 1997; Andrielsen et al. 2003), thereby improving plant tolerance of soils containing these compounds (Wilkinson & Dickinson 1995).

When in excessive quantities, heavy metals are toxic to most organisms (Antonovics et al. 1971). Fungi are susceptible to heavy metals both directly, through enzymatic inhibition and disruption of cellular integrity (Gadd 1993) and indirectly through the production of free radicals (Dowling & Simmons 2009). It has been proposed that at least some fungi have specialized to serpentine soil (Panaccione et al. 2001; Schechter & Bruns 2008); however, this hypothesis has yet to be rigorously tested. Initial evidence indicates that serpentine communities of ECM fungi do not follow the general pattern of low diversity and high specialization expected in extreme environments. Rather, molecular surveys have revealed serpentine communities to be rich in species and phylogenetic diversity, with patterns statistically indistinguishable from proximate nonserpentine sites (Urban et al. 2008; Moser et al. 2009; Branco & Ree 2010). However, because of the difficulty in acquiring saturated sampling of fungal communities (Taylor 2002), these field studies are limited in their ability to ascertain overlap in communities across sites and do not address the question of serpentine tolerance at the level of individual species.

I tested a series of hypotheses to address the effects of serpentine soils on ECM fungi and reveal potential fungal specialization to this edaphic environment. More specifically, I tested whether (i) serpentine ECM fungal communities are depauperate and composed by specialists, (ii) serpentine soils offer physiological barriers that prevent nonspecialized fungi to thrive and (iii) serpentine-specialized fungi lack competitive ability and are excluded from nonserpentine soil by the native community. To test these hypotheses, I conducted a fungal community greenhouse reciprocal transplant experiment using natural fungal communities.

I predicted two major possible outcomes from this transplant experiment. In the first, serpentine soil acts as a strong barrier for ECM fungi, imposing physiological constraints and leading to fungal specialization, with fungal species restricted to their native soil. Serpentine communities in this scenario are expected to be distinct and less diverse compared to nonserpentine communities, serpentine specialists should have experienced a trade-off regarding competitive ability and be outcompeted in nonserpentine soils, and nonspecialized fungi should be unable to establish in serpentine soil. Such patterns would provide evidence for serpentine soils as an environmental filter and a strong selective agent for ECM fungi. Assuming the functional traits conferring serpentine tolerance are phylogenetically conserved, one would expect a pattern of phylogenetic clustering on serpentine soil (species more closely related than expected by chance, Webb et al. 2002), suggesting that particular fungal clades have specialized onto this environment. Alternatively, a second scenario predicts that serpentine conditions do not provide a physiological barrier for fungi. In this case, serpentine and nonserpentine fungal communities are not expected to be distinct, with the same fungi detected in both soil types rendering no evidence for fungal specialization. In this case, ECM fungi are able to tolerate the chemical particularities offered by serpentine soils, indicating this environment may not serve as a precursor for fungal adaptation as previously hypothesized.

Materials and methods

Fungal reciprocal transplant experiment

To test the effects of serpentine soils on ectomycorrhizal (ECM) fungi, I conducted a greenhouse fungal reciprocal experiment, where fungal communities were grown in their native and non-native soils. Because ECM fungi are difficult to culture in a laboratory setting, I potted natural fungal communities collected from the field with their plant host seedlings, allowed for fungal root colonization and manipulated the seedlings into the different soil treatments (Fig. 1). ECM fungi were retrieved from the seedlings’ root systems in the end of the experiment.

Soil collection. Serpentine and nonserpentine soils were collected from Quercus ilex subsp. ballota forests in Bragança (Trás-os-Montes, Portugal), where ECM fungal communities have been previously described (Branco & Ree 2010). This oak is the only tree colonizing serpentine soils in the region, occurring in monospecific forests both in serpentine and nonserpentine soils. Cistus ladanifer is the only other ECM plant host present in the sampled sites and occurs evenly across the sampled plots. I collected serpentine and nonserpentine soil from Serra da Nogueira (41°47.965 N, 6°53.924 W) and Rabal (N 41°52.262, W 6°44.682), respectively. Soil was collected 1 m away from at least 7 Q. ilex subsp. ballota trees in each site. To guarantee homogenous fungal inoculum, serpentine and nonserpentine soils were
mechanically homogenized separately using a concrete mixer and potted within 24 h of collection.

Oak seedlings. I used Q. ilex subsp. ballota seedlings as bait for manipulating the fungi present in the soil. I collected oak acorns in serpentine and nonserpentine forests from the same region (Rica Fo, N 41°49.66 W 006°45.43 and Quintas de Seara, N 41°45.45 W 6°43.27, respectively) and grew them to seedlings following germination in sand. At this stage, root tips were not colonized by ECM fungi, showing a very homogenous morphology, with abundant root hairs and no Hartig nets, indicating absence of fungal infection. Furthermore, I did not find ECM fungal DNA in six random root tips (details on molecular protocols below).

Experimental design. Seedlings were grown in 1-L pots with serpentine and nonserpentine soil for 5 months (from May to September 2007) under greenhouse conditions (26 °C, 75% humidity) and watered weekly manually with no fertilization, allowing the ECM fungi present in the soil to colonize the root systems. In September 2007, seedlings (and fungi) were transplanted to a second soil treatment, consisting of serpentine and nonserpentine soil (Fig. 1). I also transplanted seedlings to sterile soil (both serpentine and nonserpentine) to test whether native communities prevent foreign fungi from establishing. The soil used in the transplants was collected from the same forests as before, following the same methodology, and sterile soil was obtained by autoclaving soil for 45 min at 121 °C and 1 kg/cm². As a control, 20 seedlings were each planted only on sterile serpentine and nonserpentine soil (autoclaved as above). The presence of ECM fungi on the root systems before the transplant was confirmed by visual inspection (presence of fungal tissue covering the root tips and, in some cases, abundant mycelium in the soil). Upon transplant, root systems were cleaned of soil remnants, with minimal disruption of root tips. The controls were transplanted to the same type of sterile soil. After the transplant, seedlings were kept in the greenhouse for 10 more months and harvested in July 2008. At that time, all the root systems of surviving seedlings were screened under a dissecting microscope, and five ECM root tips belonging to different root morphotypes present in each pot were collected. There were never more than five distinct root morphologies in each pot, and 985 root tips were collected (five each pot), dried in silica gel and used for molecular analyses. The soil treatments will be referred to as SS (serpentine soil transplanted to serpentine soil), NSNS (nonserpentine soil transplanted to nonserpentine soil), SNS (serpentine soil transplanted to nonserpentine soil) and NSS (nonserpentine soil transplanted to serpentine soil). Sterile soil will be pointed out as ‘st’.

Soil chemical analyses

Soil chemistry was determined by analysing four soil samples per sampled forest (serpentine and nonserpentine), each consisting of the combination of five subsamples collected 5 m apart. Standard soil parameters, macro- and micronutrients, and heavy metal contents
were analysed (pH, N, C, Al, P, K, Ca, Mg, B, Mn, Zn, Cu, Fe, Pb, Ni, Cr, Cd, NO₃-N, cation-exchange capacity, per cent base saturation for K, Mg and Ca). Analyses were conducted at the University of Massachusetts Soil and Plant Tissue Testing Laboratory (Amherst, NY, USA), except for C and N that were performed at Argonne National Laboratory and pH, which was measured in the Soil Laboratory of Escola Superior Agrária de Bragança (Portugal). Soil nitrogen and carbon analyses were performed using a LECO CN-2000 analyzer (LECO Corporation, St. Joseph, MI, USA); all remaining elements were analysed using a modified Morgan extraction and ICP (Spectro Analytical Instruments, Fitchburg, MA, USA). Serpentine and nonserpentine soils were compared using a standard one-way ANOVA.

Molecular analyses

I generated DNA sequence data to distinguish species and characterize the diversity of ECM communities present in all four treatments (species richness and frequency, i.e. the number of root tips per species). First, I extracted DNA from the 985 collected root tips using a REDExtract-N-Amp Plant PCR kit (Sigma, St. Louis, Missouri, MO, USA). For each sample, I amplified the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA repeat using the primers ITS1-F and ITS4 (White et al. 1990; Garde & Bruns 1996) and directly sequenced PCR products on an ABI 3730 DNA analyser. Of them, 569 sequences showed clean and unambiguous base calls. These sequences were recovered evenly across treatments and were clustered by 95% similarity (Peay et al. 2007; Jumpponen & Jones 2009, Branco & Ree 2010). Each cluster was assumed to represent a distinct species. Species were identified to the level of genus by comparing their consensus sequences against the NCBI nucleotide database using BLAST (see Table S1 and S2 in Supporting information for GenBank accession numbers).

Statistical analyses

I conducted a series of statistical analyses to test the hypotheses investigated in this study. To evaluate whether there were differences in fungal diversity across serpentine and nonserpentine soil (hypothesis 1), I compared the species richness, frequency and overlap across the SS and NSNS treatments, conducted a multidimensional scaling analysis with species frequencies (using shortest Bray–Curtis dissimilarity and the R vegan package (Okanen et al. 2005) and compared the mean number of species per treatment using Kruskal–Wallis tests (Zar 1999) followed by Nemenyi–Damico–Wolfe–Dunn (NDWD) multiple-comparison tests (Hollander & Wolfe 1999) with sequential Bonferroni corrections (Sokal & Rohlf 1995). An extended analysis including all the treatments in the experiment showed no differences between treatments involving sterile and nonsterile soil (Kruskal–Wallis \( \chi^2 = 33.8598, \) d.f. = 7, \( P < 0.05; \) multiple-comparison tests: \( P \) (NSNS-NSstNS) = 0.998, \( P \) (NS-SSstNS) = 0.662, \( P \) (NS-SSstNS) = 0.999, \( P \) (SS-SSstS) = 0.819). For this reason, I combined the data from transplants involving the same type of nonsterile and sterile soil (except controls) in a single matrix for use in subsequent analyses. I used a nonparametric approach for the univariate analyses because the data violated assumptions of normality and homoscedasticity. Comparing the frequencies of the two most frequent species (Tomentella sp. 24 and Laccaria sp.) using the same nonparametric approach described above further tested hypothesis 1.

Hypothesis 2, which states that serpentine soil constitutes an environmental filter for ECM fungi, was tested by comparing the phylogenetic community structure of the SS and NSNS treatments. This hypothesis was further tested by comparing the species present in each soil treatment.

To assess whether serpentine fungi are outcompeted from nonserpentine soil by its native communities because of a tolerance/competition trade-off (hypothesis 3), I compared the species frequencies of NSS with NSstS and SNS with SstNS. These comparisons were also performed using the Kruskal–Wallis tests described previously. All analyses were implemented in R (R Development Core Team, version 2.8.1).

Surveys of root tips from the control seedlings revealed a low level of contamination, as the species Tomentella sp. 24, Hebeloma sp. a and Hebeloma sp. b were recovered from 24 root tips from eight of the control pots. Although Tomentella sp. 24 was the most frequent fungus in the experiment, it was absent from the SS treatment and is a member of the ECM community described from the nonserpentine site where the soil was collected (Branco & Ree 2010). This indicates that the contamination was likely due to incomplete sterilization of the nonserpentine soil used in the transplant. I corrected the data by subtracting the mean number of root tips per control pot from each contaminant species in the treatments involving sterile soil. This procedure reduced the abundance of Tomentella sp. 24 and did not affect comparisons of overall diversity across treatments.

Fungal phylogenetic tree

I used a hierarchical approach to construct a species-level phylogenetic tree and estimate the phylogenetic
diversity of fungal communities in the different soil treatments. The fungal species identified from root tips belonged to a wide range of taxa within Ascomycota and Basidiomycota (Fig. 2), making it difficult to accurately reconstruct phylogenetic distances. I constructed a community tree including all species recovered in this study as well as sequences from ECM fungi obtained from the sites where the soil was collected (Branco & Ree 2010), allowing for a better estimate of the phylogenetic distances across species.

Constructing the tree involved three steps: (i) compilation of a genus-level tree, (ii) compilation of a species-level tree for each of the included genera, and (iii) grafting the species-level trees onto the genus-level tree.

Genus-level tree. I defined the phylogenetic relationships between the detected genera based on recent published studies on the phylogenetic systematics of fungi, including the Assembling the Fungal Tree of life project (Binder & Hibbett 2006; Hansen & Pfister 2006; James et al. 2006; Matheny et al. 2006a,b; Miller et al. 2006; Moncalvo et al. 2006; Spatafora et al. 2006; Sugiyama et al. 2006; Zhang et al. 2006). To estimate branch lengths on this topology, I assembled a molecular data set for divergence time analysis. Sequences of nuclear ribosomal large subunit (LSU) DNA were retrieved from GenBank for each genus found in our survey, and for an outgroup, *Glomus intraradices* (Table S1, Supporting Information). Genera were assumed to be monophyletic, except for *Russula* and *Lactarius* (Miller et al. 2006), which were grouped together, and one *Russula* LSU sequence was included in the genus-level tree. As *Cenococcum* is a complex genus that encompasses much genetic diversity and is suspected to include several distinct lineages (Douhan et al. 2007), LSU sequences for each species of *Cenococcum* in our sample were generated using the LOR, LR6 and LR3 primers (Vilgalys & Hester 1990) and included in a Dothidiomycete phylogeny (G. Mugambi and S. Hundorf, unpublished data). These species formed a monophyletic group (data not shown), and one sequence (FJ897251) was selected and used in the genus-level tree. Branch lengths for the genus-level topology were first estimated from the LSU data set using *pala* (Swofford 2002) by maximum-likelihood, under the HKY85 model of nucleotide evolution. Next, they were adjusted by nonparametric rate smoothing (Sanderson 2002) using the software package APE (Paradis et al. 2004).

Species-level trees. For each genus, a data set of nuclear ribosomal ITS sequences (obtained both from this experiment and field surveys) was assembled, including

```
  S    NS    SNS    NSS
Sebacina sp. a 0.01
Thelephora sp. a 0.02
  0.03
Tomentella sp. d 0.01 0.01
  0.01
  0.61
  0.84
  0.24
Tomentella sp. 24* 0.11
  0.03
Tomentella sp. b 0.01
  0.01
Tomentella sp. 28* 0.03
  0.03
Tomentella sp. 8* 0.01 0.01
Tomentella sp. g 0.01 0.01
  0.01
  0.01
  0.01
Tomentella sp. c 0.01
  0.01
Tomentella sp. k 0.01
Hebeloma sp. b 0.12 0.07
Hebeloma sp. a 0.01
  0.01
Cortinarius sp. a 0.01
Cortinarius sp. b 0.01
  0.01
Laccaria sp. * 0.53 0.13 0.48 0.29
  0.01
Scleroderma sp. a 0.01
Scleroderma sp. b 0.01
Russula sp. 2* 0.06 0.04
  0.01
Russula sp. 2* 0.03 0.01
  0.01
Russula sp. 5* 0.06 0.04
  0.01
Phialophora sp. 1* 0.01
Phialophora sp. 1 0.01
  0.01
Cenococcum sp. a 0.01
Cenococcum sp. 1* 0.01
  0.01
  0.01
  0.01
  0.01
Tuber sp. c 0.02
Tuber sp. 2* 0.03 0.03 0.01
Tuber sp. 1* 0.15 0.01 0.1
Humaria sp. 1 0.01
  0.01
```

Fig. 2 Phylogenetic relationships of the ectomycorrhizal fungal species detected in the experiment, respective root tip per cent frequencies for each treatment, and total number of species detected in the different treatments. Grey taxa belong to the Ascomycota and black taxa to the Basidiomycota; *species also detected in the field community descriptions (Branco & Ree 2010). S, serpentine soil; NS, nonserpentine soil; SNS, serpentine followed by nonserpentine soil; NSS, nonserpentine followed by serpentine soil.

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outgroup sequences from its sister group in the genus-level tree. Data sets were aligned using ClustalX (Larkin et al. 2007). For each data set, a maximum-likelihood tree was computed using Garli (Zwickl 2006) with the default settings. Table S2 (Supporting information) shows which sequences were used in each phylogeny, including ITS sequences of congeneric species from GenBank. The species-level trees were rooted in Mesquite (Maddison & Maddison 2008) and made ultrametric as above.

Assembling the final fungal community tree, for each species-level tree, branch lengths were scaled to be common with the LSU genus-level tree and outgroups and GenBank sequences were pruned. To account for differences in molecular rates of evolution between ITS and LSU, genus-specific scaling factors were estimated by comparing branch lengths separating ingroup species from a common outgroup. Species-level trees were then grafted on the genus-level tree (scaling and grafting script available from the author upon request). Fig. S1 (Supporting information) shows the complete tree used to measure the phylogenetic community structure, while Fig. 2 is a pruned tree, including only the species relevant for this study (all fungi recovered from the greenhouse experiment).

Community phylogenetic structure

I compared the phylogenetic structure of ECM communities across treatments to test the hypothesis that serpentine soils act as an environmental filter (hypothesis 2; Cavender-Bares et al. 2004). Assuming the traits concerning serpentine tolerance are conserved across the phylogenetic tree, a pattern of phylogenetic clustering (species more closely related than expected by chance) on serpentine soil would be consistent with this hypothesis, while phylogenetic overdispersion (species less closely related than expected by chance) would run counter to it. I measured phylogenetic structure with the net relatedness index (NRI) and the nearest taxon index (NTI) (Webb et al. 2002) implemented in Phylocom (Webb et al. 2008), using a phylogenetic tree reconstructed from the regional species pool (all species detected in this experiment and recovered from the sites where soil was collected) and a community matrix (species by pot) for each of the soil treatments. NRI and NTI indicate the degree to which species in a community are phylogenetically clustered or overdispersed by their deviation from zero, the expectation of random assembly.

Results

Soil analyses revealed profound chemical differences across soil types, with serpentine soil showing high levels of heavy metals and unbalanced calcium-to-magnesium ratio (Table 1).

A total of 26 ECM fungal species were recovered in this experiment (Fig. 2), with treatments involving serpentine soil hosting more fungal species. Half of the fungi of nonserpentine origin were found in treatments involving serpentine soil, suggesting a general pattern of fungal tolerance to both soil types. Furthermore, only two of ten species of serpentine origin were not recovered from treatments involving nonserpentine soil, corroborating this idea. Some species matched those collected through sampling in the field (Fig. 2; Branco & Ree 2010). The SS treatment showed higher species richness than NSNS, contradicting hypothesis 1, and SNS showed the highest species richness (Fig. 2). Most species were found at low frequencies; however, communities were highly uneven, with the SS and SNS treatments clearly dominated by Laccaria sp., while Tomentella sp. 24 was most frequent in NSNS and NSS (Fig. 2). These two species colonized the vast majority of all roots. A closer look at the frequencies of Tomentella sp. 24 and Laccaria sp. (Fig. 3a,b) reveals contrasting edaphic preferences.

<table>
<thead>
<tr>
<th>Soil parameter</th>
<th>Serpentine soil</th>
<th>Nonserpentine soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al (ppm)</td>
<td>12.3 (±5.4)</td>
<td>28.8 (±0.7)</td>
</tr>
<tr>
<td>B (ppm)*</td>
<td>1.1 (±0.1)</td>
<td>0.3 (±0.0)</td>
</tr>
<tr>
<td>C (%)*</td>
<td>10.7 (±1.6)</td>
<td>1.6 (±0.2)</td>
</tr>
<tr>
<td>Ca (%BS)</td>
<td>15.9 (±2.0)</td>
<td>42.9 (±3.3)</td>
</tr>
<tr>
<td>Ca (ppm)*</td>
<td>1014.3 (±89.5)</td>
<td>1349.8 (±83.4)</td>
</tr>
<tr>
<td>Ca/Mg*</td>
<td>0.4 (±0.0)</td>
<td>2.0 (±0.0)</td>
</tr>
<tr>
<td>Cation-exchange capacity*</td>
<td>32.3 (±2.1)</td>
<td>16.6 (±0.9)</td>
</tr>
<tr>
<td>Cd (ppm)*</td>
<td>0.38 (±0.1)</td>
<td>0 (±0.0)</td>
</tr>
<tr>
<td>Cr (ppm)*</td>
<td>0.5 (±0.1)</td>
<td>0.2 (±0.1)</td>
</tr>
<tr>
<td>Cu (ppm)</td>
<td>0.1 (±0.1)</td>
<td>0.4 (±0.1)</td>
</tr>
<tr>
<td>Fe (ppm)*</td>
<td>23.3 (±4.8)</td>
<td>6.9 (±0.8)</td>
</tr>
<tr>
<td>K (%BS)*</td>
<td>0.7 (±0.2)</td>
<td>2.6 (±0.4)</td>
</tr>
<tr>
<td>K (ppm)*</td>
<td>81.3 (±21.7)</td>
<td>154.5 (±26.2)</td>
</tr>
<tr>
<td>Mg (%BS)*</td>
<td>60.4 (±5.7)</td>
<td>22.4 (±8.6)</td>
</tr>
<tr>
<td>Mg (ppm)*</td>
<td>2378.8 (±327.6)</td>
<td>430.5 (±171.8)</td>
</tr>
<tr>
<td>Mn (ppm)</td>
<td>180.6 (±44.0)</td>
<td>117.8 (±7.1)</td>
</tr>
<tr>
<td>N (%)</td>
<td>0.5 (±0.1)</td>
<td>0.2 (±0.0)</td>
</tr>
<tr>
<td>Ni (ppm)*</td>
<td>29.8 (±8.1)</td>
<td>1.0 (±0.4)</td>
</tr>
<tr>
<td>NO3-N (ppm)</td>
<td>7.8 (±3.3)</td>
<td>1.3 (±0.0)</td>
</tr>
<tr>
<td>P (ppm)*</td>
<td>27.3 (±3.9)</td>
<td>7.5 (±2.1)</td>
</tr>
<tr>
<td>Pb (ppm)</td>
<td>31.7 (±0.3)</td>
<td>30.5 (±0.0)</td>
</tr>
<tr>
<td>pH*</td>
<td>6.1 (±0.1)</td>
<td>5.3 (±0.1)</td>
</tr>
<tr>
<td>Zn (ppm)</td>
<td>1.9 (±0.9)</td>
<td>1.1 (±0.5)</td>
</tr>
</tbody>
</table>

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**Tomentella** sp. 24 is a nonserpentine species, absent from the SS treatment and decreasing in frequency when exposed to serpentine soil [Kruskal–Wallis \( \chi^2 = 73.882 \), d.f. = 3, \( P < 0.05 \); NDWD test: \( P \) (SS-NSS) < 0.008, \( P \) (SS-NSNS) < 0.01, \( P \) (SNS-NSNS) < 0.013, \( P \) (SNS-SS) < 0.017]. **Laccaria** sp., on the other hand, dominated in treatments where seedlings were exposed first to serpentine soil and less so when in the NSNS or NSS treatments [Kruskal–Wallis \( \chi^2 = 19.184 \), d.f. = 3, \( P < 0.05 \); NDWD test: \( P \) (SS-NSNS) < 0.009, \( P \) (SS-SS) < 0.010, \( P \) (SNS-NSNS) < 0.013, \( P \) (SNS-SS) < 0.05].

The mean number of species per pot was significantly different across some treatments (Fig. 3c, meanNSNS = 1.28, sdNSNS = 0.84, meanNS = 1.37, sdNS = 0.56, meanSN = 2.08, sdSN = 0.82, meanSS = 1.83, sdSS = 0.69), with a tendency for more species to be associated with treatments starting with serpentine soil and contradicting hypothesis 1 [mean Kruskal–Wallis \( \chi^2 = 49.9384 \), d.f. = 10, \( P < 0.05 \); NDWD test: \( P \) (SNS-NSNS) < 0.009, \( P \) (SNS-SS) < 0.01]. The ordination analysis did not clearly differentiated communities from different treatments into separate clusters (Fig. 4; stress = 20.06). However, NMDS axis 1 separated NSNS (black squares) from SS (white circles). And although SNS (light grey diamonds) was widespread across the plot, it overlapped mostly with SS, indicating the similarity between those two treatments. Likewise, NSS (dark grey triangles) was more similar to NSNS.
Analyses of phylogenetic community structure (Table 2) contradicted hypothesis 2. NRI values, a measure of the overall clustering on the phylogenetic tree of species found across soil treatments, were nonsignificant, suggesting that basal fungal relationships do not contribute significantly to community structure. However, there was a tendency for positive NTI values, which measures the level of terminal clustering independent of the deeper branches, suggesting promoness for phylogenetic clustering of species within SS and SNS treatments with respect to relationships near the tips of the tree.

There were no significant differences between the number of species per pot across soil transplants involving sterile and nonsterile soil [Fig. 3d; Kruskal–Wallis $\chi^2 = 11.8273$, d.f. = 10, $P < 0.005$; NDWD test: $P$ (SSstS-NSS) > 0.03, $P$ (SstNS-SNS) > 0.05], indicating native fungal communities do not impede establishment of foreign fungi and contradicting hypothesis 3.

### Discussion

Serpentine soils do not represent a strong physiological barrier requiring ecological specialization for ectomycorrhizal (ECM) fungi. I found no evidence to support any of the hypotheses being tested or the two initially theorized scenarios. I found different fungal communities in serpentine and nonserpentine soils; however, serpentine communities are not depauperate and not characteristically composed by specialized species. Furthermore, serpentine soil was not found to be a strong physiological barrier for the majority of fungi, and no overall strong trade-offs between serpentine specialization and competitive ability were uncovered.

Fungal diversity is not limited by the chemical particularities of serpentine soils, including low calcium-to-magnesium ratio and high levels of nickel and other heavy metals. Corroborating results reported from the field (Branco & Ree 2010), serpentine fungi in this experiment ranged across a wide variety of taxa, including several groups within the Ascomycota and Basidiomycota, with no strong evidence for phylogenetic clustering (Fig. 2, Table 2). These results suggest a general pattern of serpentine tolerance in ECM fungi, with no evidence for serpentine soil as an environmental barrier, corroborating previous findings of phylogenetically diverse ECM communities associated with serpentine soils (Urban et al. 2008; Moser et al. 2009; Branco & Ree 2010). These previous field-based studies were unable to detect differences in the structure of ECM communities from serpentine and nonserpentine soil (Moser et al. 2009; Branco & Ree 2010). Here, a controlled transplant approach revealed lower species richness on nonserpentine soil treatments, high overlap between serpentine and nonserpentine communities, and two dominating species, *Laccaria* sp. and *Tomentella* sp. 24 (Fig. 2). These species are able to grow in serpentine and nonserpentine soil; however, they appear to be better colonizers in their preferred environment and may competitively exclude other species once established. The NSNS treatment is the most extreme case, with *Tomentella* sp. 24 occurring at a frequency of 84% in a community of only four species (Fig. 2). Although *Tomentella* sp. 24 was not found on serpentine soil alone, it was present in the NSS treatment, indicating it is able to tolerate serpentine conditions at least temporarily. It is possible that this species is more vulnerable to serpentine soil when it is not connected to the oak host, suggesting its dependency on colonizing the roots early before competitors do so. As its frequency tended to decrease when exposed to serpentine, it is possibly absent from the SS treatment because of a combination of lower serpentine tolerance and competitive exclusion. *Laccaria* sp., on the other hand, occurs in both soil types but shows higher frequency in serpentine soil.

The diversity of ECM fungi in this system may be maintained by a combination of environmental effects and competitive relationships among species, and an environmental gradient hypothesis can therefore help explain the community differences observed across the different soil treatments. Higher biological diversity has been described to occur in the moderate or middle range of a physical gradient (Odum 1963), and if serpentine soils were to represent an extreme environment, one would expect to find low serpentine species richness. While the nonserpentine soil used here is not stress-free (it is affected by seasonal Mediterranean drought), it is reasonable to assume that serpentine soil offers a much harsher environment than nonserpentine (Table 1). One of the effects of severe environmental stress on biological communities is the replacement of species that are otherwise highly competitive by species that are more stress tolerant (Grime 1973, 1977). This type of hypothesis may

### Table 2 Community phylogenetic structure for the different treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NRI</th>
<th>$P$ value</th>
<th>NTI</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSNS</td>
<td>−1.17</td>
<td>0.110</td>
<td>−0.83</td>
<td>0.270</td>
</tr>
<tr>
<td>NSS</td>
<td>−0.61</td>
<td>0.430</td>
<td>−0.71</td>
<td>0.230</td>
</tr>
<tr>
<td>SS</td>
<td>−1.10</td>
<td>0.140</td>
<td>1.57</td>
<td>0.070</td>
</tr>
<tr>
<td>SNS</td>
<td>−0.05</td>
<td>0.160</td>
<td><strong>2.15</strong></td>
<td><strong>0.020</strong></td>
</tr>
</tbody>
</table>

NTI, nearest taxon index; NRI, net relatedness index; NTI, NRI < 0, overdispersion; NTI, NRI > 0, clustering; S, serpentine soil; NS, nonserpentine soil; SNS, serpentine followed by nonserpentine soil; NSS, nonserpentine followed by serpentine soil.
help explain why the Laccaria sp. and Tomentella sp. 24 frequencies appear to be dependent on both the level of environmental stress (soil type) and the establishment of other ECM fungi. Tomentella sp. 24 is competitively dominant on nonserpentine soil, but yields to the more stress-tolerant species Laccaria sp. in serpentine soil. When either species is dominant, species richness is observed to be lower. The soil treatments used in this experiment range along a soil stress gradient, as displayed in the x-axis of Fig. 5, with NSNS on the left and SS on the right. Expectations would suggest low species richness in SS (extreme right on the axis); however, the latter holds the second highest richness level. NS and SNS fall in between NS and S, probably because of lack of opportunity for Tomentella sp. 24 and Laccaria sp. to completely dominate.

The results of this experiment are considerably different from those observed in the field (Branco & Ree 2010), both in terms of species richness and community structure. Here, richness was comparatively lower and two species dominated the communities, while in the field equally rich, fungal communities colonize both habitats and all species are relatively rare. In nature, serpentine soil patches are scattered across a large matrix of nonserpentine soil and there is constant opportunity for propagules to move across soil types. This probably increases inter-specific competition, making it difficult for particular species to become dominant. Under greenhouse conditions, the propagule rain is virtually eliminated, restricting the pool of species to the fungi present in the soil collected from the field and able to persist in a pot environment. Moreover, plant root systems in the wild are never completely mycorrhizal-free, and it is very unlikely that species like Tomentella sp. 24 or Laccaria sp. would have the chance to occupy the majority of root tips in a root system as seen in the experiment. Also, sampling effects and/or methodological constraints might have impeded accurate fungal community descriptions. Given the patchy nature of ECM fungi, the inoculum used in the experiment might have failed to reflect the high levels of fungal diversity in the field, contributing for potential biases. Furthermore, the low sequencing success, probably a reflection of colonization of single root tips by multiple ECM fungal species, might have contributed for the incomplete community descriptions.

There were no differences in the fungal communities from transplants done to sterile and nonsterile soil, indicating that at least in a short-term greenhouse experimental setting, native communities are not preventing non-native fungi from establishing. Assuming there were no chemical differences across sterile and nonsterile soil, this result suggests that if there is some kind of undetected cryptic fungal serpentine specialization, it contrasts with plant patterns, where serpentine specialists grow well on nonserpentine soil but are outcompeted from nonserpentine sites by the native plants (Kruckenberg 1954; Brady et al. 2005). Rather than constituting a barrier for ECM fungi, serpentine soils host diverse fungal communities and under greenhouse conditions promote ECM fungal diversity. Further research is needed to shed light on the physiological mechanisms involved in ECM fungal serpentine tolerance. Linking such information with the well-studied patterns of serpentine plants should help illuminate the role of symbioses in the maintenance of biodiversity in stressful habitats.

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References


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Supporting information

Additional supporting information can be found in the online version of this article.

Table S1 nrDNA Large Subunit sequences used to compile the genus-level fungal phylogeny. *Outgroup

Table S2 nrDNA Internal Transcribed Spacer sequences used to compile each of the species-level fungal phylogenies

Fig. S1 Phylogenetic relationships of all ECM fungal species detected in the sites were soil was collected. All methodological methods can be found in Branco & Ree, 2010.

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