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Original Citation:
Specific regions in the Sod1 locus of the ericoid mycorrhizal fungus Oidiodendron maius from metal-enriched soils show different sequence polymorphism / M. Vallino; E. Zampieri; C. Murat; M. Girlanda; S. Picarella; M. Pitet; E. Portis; E. Martino; S. Perotto. - In: FEMS MICROBIOLOGY ECOLOGY. - ISSN 0168-6496. - 75(2)(2011), pp. 321-331.

Availability:
This version is available http://hdl.handle.net/2318/82818 since 2016-08-05T10:18:09Z

Published version:
DOI:10.1111/j.1574-6941.2010.01003.x

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This is an author version of the contribution published on:
Questa è la versione dell’autore dell’opera:

Vallino, M., Zampieri, E., Murat, C., Girlanda, M., Picarella, S., Pitet, M., Portis, E.,
Martino, E. and Perotto, S. (2011), Specific regions in the Sod1 locus of the ericoid
mycorrhizal fungus Oidiodendron maius from metal-enriched soils show a different
doi: 10.1111/j.1574-6941.2010.01003.x

The definitive version is available at:
La versione definitiva è disponibile alla URL:
Specific regions in the Sod1 locus of the ericoid mycorrhizal fungus Oidiodendron maius from metal enriched soils show different sequence polymorphism

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Keywords: ericoid fungi, serpentine, heavy metal toxicity, stress-induced mutagenesis, superoxide dismutase, sequence polymorphism.

Abstract

The huge diversity of fungi may reflect both the heterogeneity of the niches they occupy and the diverse stresses they must cope with. In order to investigate the genetic and functional diversity in the ericoid mycorrhizal fungus Oidiodendron maius subjected to heavy metal stress, we isolated O. maius strains from a serpentine site naturally enriched by heavy metals. Despite the high Cr and Ni soil concentrations, a high level of diversity was found in the serpentine fungal community. The growth of these isolates in the presence of different metal contaminants identified some tolerant strains, suggesting a site-specific adaptation. To investigate within-species gene divergence in stressful environments, we then compared the sequence polymorphism of a neutral (internal transcribed spacer) and a functional (Cu,ZnSOD) gene in O. maius isolates derived from the serpentine site, from a site heavily polluted with industrial wastes and from unpolluted sites. For all isolates tested, the polymorphism was higher in the nucleotide sequence of the functional gene. However, when compared with isolates from the serpentine area, isolates from industrially polluted sites showed a significantly higher polymorphism in the Cu,ZnSOD promoter region, suggesting that environmental stress may influence the rate of mutations in specific regions of the Sod1 locus.

Introduction

Environmental stress exerts positive selection pressures that influence the macro- and microevolution of fungi. The increasing number of genome-sequencing projects on diverse fungal species is providing a rich set of data that allows bioinformatics comparison. Recent findings suggest that fungal stress-signalling pathways evolve rapidly and in a niche-specific way, allowing different species to be protected against the diverse environmental stresses that might be present in their habitats (Nikolaou et al., 2009). Experimental data also reinforce the view that environmental stress exerts positive selection pressures that influence the macro- and microevolution of fungi.
stress can drive gene evolution and speciation. Heavy metals introduced in terrestrial ecosystems through human activities are one of the main causes of pollution and environmental stress, although they may also occur in naturally metalliferous soils. Among fungi, mycorrhizal fungi constitute an important community in metal-polluted soils, as they form mutualistic symbioses that can effectively alleviate the effects of heavy metal toxicity in their host plant (Adriaensen et al., 2005, 2006) and provide a more balanced access to mineral elements, either by improving the supply of essential elements or by reducing the relative uptake of toxic elements (Marschner & Dell, 1994).

Heavy metal toxicity represents a strong selection pressure leading to the evolution of specialized mycorrhizal genotypes (Hartley et al., 1997; Leyval et al., 1997; Martino et al., 2000; Markkola et al., 2002; Colpaert et al., 2004; Adriaensen et al., 2005). The high diversity and presumably shorter lifecycle of the fungal symbionts and their capability for long-distance spore transport are thought to increase their potential for genetic adaptation to heavy metal toxicity compared with tree species (Meharg & Cairney, 2000; Markkola et al., 2002).

Oidiodendron maius Barron (1962) is an ascomycete (Leotiomycetes) widely distributed in temperate and tropical and subtropical areas (Hambleton et al., 1998). Oidiodendron maius can establish an endomycorrhizal symbiosis with the roots of ericaceous plants (Perotto et al., 1996; Hambleton et al., 1998; Chambers et al., 2000), and fungal strains have been isolated from plants growing in soils polluted by heavy metals of anthropic origin. In particular, several O. maius strains were isolated in the Niepolomice forest (Poland) from the roots of Vaccinium myrtillus growing in plots that were heavily contaminated with industrial dusts containing different proportions of Zn, Cd, Al and Fe (Turnau, 1988). Recolonization of these plots by ericoid mycorrhizal plants suggests that O. maius can confer to the host plant the ability to survive in metal-polluted environments (Turnau, 1988). Metal tolerance and the genetic diversity of these O. maius isolates have been investigated and described in previous papers (Lacourt et al., 2000; Martino et al., 2000).

Whereas adaptation of ecto- and endomycorrhizal fungi to soil pollution derived from human activities has been reported (Leyval et al., 1997; Martino et al., 2000; Meharg & Cairney, 2000; Colpaert et al., 2004; Adriaensen et al., 2005, 2006; Zarei et al., 2008; Gamalero et al., 2009; Krznaric et al., 2009), more scanty information is available on the mycorrhizal fungal community in naturally polluted soils, such as serpentine soils derived from ultramafic rocks (Gonçalves et al., 2007, 2009; Urban et al., 2008; Jourand et al., 2010). Serpentine soils are low in plant nutrients such as K⁺ and Ca²⁺, but contain high levels of potentially toxic elements such as Ni²⁺ and Cr³⁺. The characteristic of serpentine soils are an unfavourable Ca/Mg ratio and phenomena associated with poor soil development and low phosphorus availability (Kazakou et al., 2008). Altogether, these factors severely restrict plant and microbial growth and select for metal tolerance (Amir & Pineau, 1998). In these soils, some authors have described the occurrence of ectomycorrhizal (Gonçalves et al., 2007, 2009; Urban et al., 2008) and arbuscular mycorrhizal fungi (Castelli & Casper, 2003; Boulet & Lambers, 2005; Cumming & Kelly, 2007; Schechter & Bruns, 2008), and specific ectomycorrhizal communities have been found (Panaccione et al., 2001; Moser et al., 2005).

The aims of this work were: (1) to investigate the genetic and functional diversity of O. maius isolates from serpentine soils and compare them with the behaviour of strains isolated previously from an industrially polluted site and (2) to study within-species genes diversity, which could provide useful insights into the evolution of genes involved in fungal survival.

For these purposes, we first isolated some new O. maius strains from mycorrhizal V. myrtillus growing on serpentine soils and characterized their genetic diversity and metal tolerance in vitro. The sampling site was the Mont Avic Park (Northern Italy), established in 1989 and declared a Site of Community Importance and a Special Protection Zone included in ‘Natura 2000’ (EU directives
Serpentine rocks (hydrous magnesium silicate) can be found almost everywhere in the Park and characterize its landscape and biological aspects (Calipari, 2000).

We have then analysed, in representatives of the *O. maius* genets occurring at the different polluted sites, the sequence polymorphism in a neutral gene [the internal transcribed spacer (ITS) of the nuclear ribosomal gene] and in the gene coding for the Cu,Zn superoxide dismutase (SOD). The sequence coding for the functional Cu,ZnSOD polypeptide was used recently to investigate gene divergence in different species and isolates of arbuscular mycorrhizal fungi (Corradi *et al.*, 2009). Furthermore, the *Sod1* locus was identified recently in a metal-tolerant *O. maius* isolate (Vallino *et al.*, 2009), where it is present as a single copy and encodes for a functional Cu,ZnSOD. A general major role of this enzyme is in the cell defence against toxic reactive oxygen species (ROS), which are generated as byproducts of many biological oxidations. Environmental stress, including heavy metals, can cause an increase in the generation of ROS (Schützendübel & Polle, 2002), and an important role of the *O. maius Sod1* gene in metal tolerance and oxidative stress was demonstrated recently by complementation of yeast mutants and targeted gene disruption in *O. maius* (Abbà *et al.*, 2009; Vallino *et al.*, 2009).

**Materials and methods**

**Fungal isolates**

The fungal isolates investigated are listed in Table 1. All of them belong to the species *O. maius* and were divided into three groups: group I contains strains isolated in this work (see next paragraph and Results), group II contains strains isolated previously from unpolluted sites and group III contains strains isolated previously from an industrially polluted site. The characteristics of the sites and the identification of group II and III fungal isolates have been described previously (see references in Table 1).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin</th>
<th>Site</th>
<th>Host plant</th>
<th>Reference</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.O. maius Ma1</td>
<td>Italy</td>
<td>Serpentine</td>
<td><em>Vaccinium myrtillus</em></td>
<td>This work</td>
<td>OmMa1</td>
</tr>
<tr>
<td>I.O. maius Ma2</td>
<td>Italy</td>
<td>Serpentine</td>
<td><em>V. myrtillus</em></td>
<td>This work</td>
<td>OmMa2</td>
</tr>
<tr>
<td>I.O. maius Ma3</td>
<td>Italy</td>
<td>Serpentine</td>
<td><em>V. myrtillus</em></td>
<td>This work</td>
<td>OmMa3</td>
</tr>
<tr>
<td>I.O. maius Ma4</td>
<td>Italy</td>
<td>Serpentine</td>
<td><em>V. myrtillus</em></td>
<td>This work</td>
<td>OmMa4</td>
</tr>
<tr>
<td>I.O. maius Ma5</td>
<td>Italy</td>
<td>Serpentine</td>
<td><em>V. myrtillus</em></td>
<td>This work</td>
<td>OmMa5</td>
</tr>
<tr>
<td>I.O. maius Ma6</td>
<td>Italy</td>
<td>Serpentine</td>
<td><em>V. myrtillus</em></td>
<td>This work</td>
<td>OmMa6</td>
</tr>
<tr>
<td>I.O. maius Ma7</td>
<td>Italy</td>
<td>Serpentine</td>
<td><em>V. myrtillus</em></td>
<td>This work</td>
<td>OmMa7</td>
</tr>
<tr>
<td>I.O. maius Ma8</td>
<td>Italy</td>
<td>Serpentine</td>
<td><em>V. myrtillus</em></td>
<td>This work</td>
<td>OmMa8</td>
</tr>
<tr>
<td>I.O. maius Ma9</td>
<td>Italy</td>
<td>Serpentine</td>
<td><em>V. myrtillus</em></td>
<td>This work</td>
<td>OmMa9</td>
</tr>
<tr>
<td>I.O. maius Ma10</td>
<td>Italy</td>
<td>Serpentine</td>
<td><em>V. myrtillus</em></td>
<td>This work</td>
<td>OmMa10</td>
</tr>
<tr>
<td>I.O. maius Ma11</td>
<td>Italy</td>
<td>Serpentine</td>
<td><em>V. myrtillus</em></td>
<td>This work</td>
<td>OmMa11</td>
</tr>
<tr>
<td>I.O. maius Ma12</td>
<td>Italy</td>
<td>Serpentine</td>
<td><em>V. myrtillus</em></td>
<td>This work</td>
<td>OmMa12</td>
</tr>
<tr>
<td>I.O. maius Ma13</td>
<td>Italy</td>
<td>Serpentine</td>
<td><em>V. myrtillus</em></td>
<td>This work</td>
<td>OmMa13</td>
</tr>
<tr>
<td>I.O. maius Ma14</td>
<td>Italy</td>
<td>Serpentine</td>
<td><em>V. myrtillus</em></td>
<td>This work</td>
<td>OmMa14</td>
</tr>
<tr>
<td>I.O. maius Ma15</td>
<td>Italy</td>
<td>Serpentine</td>
<td><em>V. myrtillus</em></td>
<td>This work</td>
<td>OmMa15</td>
</tr>
<tr>
<td>II.O. maius A</td>
<td>Poland</td>
<td>Unpolluted</td>
<td><em>V. myrtillus</em></td>
<td>Martino <em>et al.</em> (2000)</td>
<td>OmA</td>
</tr>
<tr>
<td>II.O. maius 091</td>
<td>Canada</td>
<td>Unpolluted</td>
<td><em>V. angustifolium</em></td>
<td>Dalpè (1986)</td>
<td>Om91</td>
</tr>
</tbody>
</table>
Culture conditions

For DNA extraction, fungal cultures were grown in Czapek dox liquid medium (Oxoid) for 30 days under shaking conditions (120 r.p.m. in conical flasks) at 25 °C.

For growth assays in the presence of metal compounds, fungal isolates were grown on 3% Czapek–sucrose medium (sucrose 30 g L⁻¹, NaNO₃ 3 g L⁻¹, K₂HPO₄ 3H₂O 1.31 g L⁻¹, MgSO₄·7H₂O 0.5 g L⁻¹, FeSO₄·7H₂O 0.01 g L⁻¹, KCl 0.5 g L⁻¹) in 50-mL flasks containing 40 mL of liquid medium. When required, the medium was supplemented with either 0.19 mM (0.05 g L⁻¹) K₂Cr₂O₇ (C. Erba, 99% purity), 0.32 mM (90 mg L⁻¹) NiSO₄ (C. Erba, 99% purity), 10 mM ZnSO₄·7H₂O (Fluka, 99% purity) or 0.05 mM 3CdSO₄·8H₂O (Sigma, 98% purity). The culture medium was adjusted to pH 5 using 20 mM 2-(N-morpholino) ethane sulphonic acid before autoclaving. Metal solutions were filter-sterilized (0.2 μm pore size). At least three replicates were prepared for each experimental condition and each experiment was repeated at least two times. Fungal cultures were maintained at 25 °C on an orbital shaker at 120 r.p.m. After 1 month, the fungal biomass was separated by vacuum filtration, collected on filter paper and dried until reaching a constant weight. The growth results for each fungal isolate in the presence of each metal compound were tested for significance by means of one-way anova (with the Tukey post hoc test) using the software systat version 11. Principal component analysis (PCA), performed using the xlstat software, was used to compare the growth of the fungal isolates on all the metal tested as well as in the presence of a given metal compound.

Fungal isolation from blueberry roots and identification

Group I strains (Table 1) were isolated from roots of blueberry (V. myrtillus L.) plants harvested in a serpentine area of about 2500 m² located in the Mont Avic Natural Park (Col de la Croix, 2286 m.a.s.l., Aosta Valley, Italy). Roots were cleaned accurately from soil residues (12 h under running tap water) and their mycorrhizal fungal colonization was verified by light microscopy before fungal isolation. Roots were then sterilized for 1 min with 1% NaClO and finally gently homogenized through a glass potter to obtain a root cell suspension. After three washes with sterile water, followed by centrifugation at 1000 g the root suspension was plated on 2% malt agar containing 15 mg L⁻¹ streptomycin and 50 mg L⁻¹ chloramphenicol. The plates were kept at 25 °C until fungal colonies began to grow. Each fungal colony from the root suspension plates was transferred on 2% malt agar glass culture tubes, and microscope slides were prepared for morphological identification. Morphological fungal identification was performed using taxonomic keys (Barron, 1962; Domsch et al., 1980).

Table 1. List of ericoid mycorrhizal fungal isolates and their origin

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin</th>
<th>Site</th>
<th>Host plant</th>
<th>Reference</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>II.O. maius I.Ib/11</td>
<td>Italy</td>
<td>Unpolluted</td>
<td>Calluna vulgaris</td>
<td>Perotto et al. (1996)</td>
<td>OmI</td>
</tr>
<tr>
<td>II.O. maius I.Ib/5</td>
<td>Italy</td>
<td>Unpolluted</td>
<td>C. vulgaris</td>
<td>Perotto et al. (1996)</td>
<td>OmII</td>
</tr>
<tr>
<td>II.O. maius I.Ib/1</td>
<td>Italy</td>
<td>Unpolluted</td>
<td>C. vulgaris</td>
<td>Perotto et al. (1996)</td>
<td>OmIII</td>
</tr>
<tr>
<td>III.O. maius Zn</td>
<td>Poland</td>
<td>Industrial pollution</td>
<td>V. myrtillus</td>
<td>Martino et al. (2000)</td>
<td>OmZn</td>
</tr>
<tr>
<td>III.O. maius Cd</td>
<td>Poland</td>
<td>Industrial pollution</td>
<td>V. myrtillus</td>
<td>Martino et al. (2000)</td>
<td>OmCd</td>
</tr>
<tr>
<td>III.O. maius Al-1</td>
<td>Poland</td>
<td>Industrial pollution</td>
<td>V. myrtillus</td>
<td>Lacourt et al. (2000)</td>
<td>OmAl1</td>
</tr>
<tr>
<td>III.O. maius Cd-2</td>
<td>Poland</td>
<td>Industrial pollution</td>
<td>V. myrtillus</td>
<td>Lacourt et al. (2000)</td>
<td>OmCd2</td>
</tr>
<tr>
<td>III.O. maius Cd-3</td>
<td>Poland</td>
<td>Industrial pollution</td>
<td>V. myrtillus</td>
<td>Lacourt et al. (2000)</td>
<td>OmCd3</td>
</tr>
<tr>
<td>III.O. maius Cd-4</td>
<td>Poland</td>
<td>Industrial pollution</td>
<td>V. myrtillus</td>
<td>Lacourt et al. (2000)</td>
<td>OmCd4</td>
</tr>
</tbody>
</table>
Measurement of the soil metal content in the sampling sites

The metal content of four soil samples from the Mont Avic site was measured after acid digestion using the emission spectroscopic technique inductivity-coupled plasma–optical method system, with a technical sensitivity comprised between 0.01 and 200–300 ng L⁻¹ (analyses were performed by the Biochemical Laboratory of the Torino Chamber of Commerce). The results are shown in Table 2. The Cr, Ni and Co contents were above the Italian law limit (d.lg. 471/1999), while the other metals analysed were below the legal limit.

Table 2. Serpentine soil metal content (values are expressed as mg/kg and they are the average of four replicates ± SD)

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Mean values Mont Avic area</th>
<th>Italian law threshold (d. lg. 471/1999)</th>
<th>Italian soils' values (Leita et al., 1998)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromium</td>
<td>504 ± 252</td>
<td>150</td>
<td>70</td>
</tr>
<tr>
<td>Manganese</td>
<td>390 ± 147</td>
<td>–</td>
<td>NA</td>
</tr>
<tr>
<td>Nickel</td>
<td>312 ± 119</td>
<td>120</td>
<td>50</td>
</tr>
<tr>
<td>Cobalt</td>
<td>35 ± 13</td>
<td>20</td>
<td>NA</td>
</tr>
<tr>
<td>Copper</td>
<td>12 ± 4</td>
<td>120</td>
<td>50</td>
</tr>
<tr>
<td>Zinc</td>
<td>61 ± 18</td>
<td>150</td>
<td>110</td>
</tr>
<tr>
<td>Lead</td>
<td>42 ± 28</td>
<td>100</td>
<td>18</td>
</tr>
<tr>
<td>Cadmium</td>
<td>&lt;1</td>
<td>2</td>
<td>0.6</td>
</tr>
</tbody>
</table>

DNA extraction and PCR amplification

Genomic DNA was extracted using the DNeasy Plant mini kit (Qiagen) from about 100 mg (fresh weight) of fungal mycelium, following the manufacturer's instructions. The integrity of isolated DNA was assessed through electrophoresis in a 0.8% (w/v) agarose gel. PCR amplifications were carried out in a final volume of 50 μL in a thermal cycler GeneAmp System 9700 (Applied Biosystem) using the following mix: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.1 mM MgCl₂, 0.01% (w/v) gelatin, 0.1 mM dNTPs, 0.6 μM of each primer and 2 U of RedTaq DNA polymerase (Sigma). To amplify the ITS region, the primers ITS1f (Gardes & Bruns, 1993) and ITS4 (White et al., 1990) were used. To amplify the Cu,ZnSOD region, the primer pair czsodf1 (GTCGAAGGTCGCTTGAGG) and SODr (CCCCATCATGCAAGAATGCC, modified from Vallino et al., 2009) were used, with an annealing temperature of 55 °C. The amplified products were purified (Qiaquick PCR purification kit, Qiagen) and sequenced (Genelab, Rome and DiNAMYCODE, Turin, Italy). Sequences were deposited in GenBank under the accession numbers: FN661500–FN661518, FN812707, FN812708, FN812710–FN812713 for the ITS sequences, FN662649–FN662667, FN823031–FN823037 for the SOD gene sequences.

Amplified fragment length polymorphism (AFLP) analysis

The AFLP protocol was adapted from Vos et al. (1995), as described by Lanteri et al. (2004). Briefly, 5 μL (200–250 ng) DNA was double-digested with EcoRI and MseI and ligated to adapters. Digested and ligated DNA fragments were preamplified with primers carrying one selective base (EcoRI+A=E+A and MseI+C=M+C), and selectively amplified using primers carrying two selective bases. Initially, 16 primer pairs (four EcoRI combined with four MseI primers) were tested against six templates, and the outcome of this pilot experiment resulted in the choice of the four
primer combinations E+AA/M+CG, E+AA/M+CT, E+AC/M+CA and E+AT/M+CA. AFLP amplicons were resolved through 5% denaturing polyacrylamide gels, and visualized by silver staining as described by Bassam et al. (1991). These four primers' combinations were used for all the subsequent AFLP experiments on the 15 ericoid isolates of Mont Avic. One isolate (OmMa2) was used twice as an internal control of the repeatability of the technique. The presence/absence of bands was evaluated for each primer combination by considering only the upper part of the gel (above 60–70 bp). A matrix based on the presence (1) or the absence (0) of each band was generated.

Molecular data analysis

Sequences were edited using the program sequencher version 4.1.4 (Gene Codes Corporation). Nucleotide sequences were analysed through the blast (Altschul et al., 1997) searching program on the NCBI site http://www.ncbi.nlm.nih.gov. Phylogenetic analysis was performed for the single-locus with mega 3.1, using the neighbour-joining method, by means of the Kimura 2 parameter. Bootstrap tests were performed with 500 replicates (nodes with bootstrap values inferior to 50% were eliminated). The binary matrix obtained from AFLP data was imported into the nexus format and imported in mrbayes (Ronquist & Huelsenbeck, 2003). The Bayesian inference was performed with 1 000 000 generations with default parameters. The consensus tree was visualized and edited using figtree v1.1.2 (Rambaut, 2008).

The proportion of distinguishable genets (PD –Ellstrand & Roose, 1987) was calculated as the ratio between the number of genets and the total number of isolates.

Sequence polymorphism was calculated as the proportion of polymorphic nucleotide sites (p-distance) by dividing the number of polymorphic nucleotides in the sequence alignment by the total number of nucleotides.

Results

Genetic diversity of *O. maius* isolates from the serpentine site

A total of 138 fungal isolates from the roots of *V. myrtillus* plants collected in the Mont Avic Park were transferred in pure culture. According to the taxonomic key of Barron (1962), 69 of these colonies could be attributed to the species *O. maius*. The remaining isolates did not sporulate in culture. Out of the 69 *O. maius* isolates, 15 were chosen for further analyses. PCR amplification with the ITS1f and ITS4 primers (White et al., 1990) yielded a band around 600 bp, as expected, for all samples. The comparison of the ITS sequences with the GenBank database confirmed that all the 15 isolates belonged to *O. maius* species.

AFLP analysis of the 15 isolates resulted in 170 amplified fragments, of which 128 were polymorphic. On the basis of the binary matrix, a tree was generated (Fig. 1). As expected, the two repetitions of OmMa2 were identical. Ten genets out of the 15 isolates were distinguished, grouped into three clusters. The sample OmMa3 remained separated from the other serpentine soil samples. The proportion of distinguishable genets (PD) was 0.73.
Figure 1. AFLP phylogeny on Mont Avic isolates. The phylogeny was inferred by Bayesian analysis corresponding to the consensus of 7293 trees obtained with mrbayes.

**Growth assays indicate a different response of O. maius isolates to metal compounds**

Figure 2 shows the results of the growth assay in the presence of metal compounds performed on the 15 isolates from the serpentine area and on two isolates from Niepolomice plots polluted with industrial wastes mainly containing Cd and Zn (Table 1). Four isolates (OmMa 1, 3, 4 and 11) from the serpentine area showed a better growth on Cr- and Ni-containing medium than on the unamended medium. The most tolerant isolate to Cr was OmMa3, whose growth was significantly higher than all the other isolates. The two isolates from the Niepolomice site (OmZn and OmCd) showed the highest growth on Zn and Cd, respectively, whereas they were not particularly tolerant to Cr or Ni (Fig. 2). There was no correlation between fungal genotype and growth on the metal-amended media. For example, isolates OmMa12, 14 and 15, which were identical in the AFLP profile (Fig. 1), showed different growth abilities (Fig. 2).
Figure 2. Growth rate of the ericoid mycorrhizal isolates on Cr-, Ni-, Zn- and Cd-amended media. The growth rate is indicated as the ratio between the fungal biomass formed on metal-amended and unamended medium, respectively, and a higher value indicates a lower growth inhibition.

*Significant difference ($P<0.05$) among each isolate and all the other 16 isolates for the growth on a specific metal compound.

PCA showed that the O. maius isolates from both polluted sites responded differently to the metals most abundant/toxic in the sites of origin (Fig. 3a). The most significant separation was between OmZn and OmMa3 for growth on Zn and on Cr, respectively (Fig. 3b).

The functional Sod1 gene shows a higher sequence polymorphism than the neutral ITS region
To investigate within-species gene divergence in stressful environments, we evaluated the sequence polymorphism of the ITS of the nuclear ribosomal gene and of the \textit{Sod1} gene, coding for the \textit{O. maius} Cu,ZnSOD. To reveal within-species gene diversity possibly related to pollution, we compared the sequences obtained from \textit{O. maius} isolates (Table 1) representative of the different \textit{O. maius} genets identified for the serpentine site (by AFLP, this work) and for the Niepolomice site (by random amplified polymorphic DNA (RAPD), Lacourt \textit{et al.}, 2000), as well as from unpolluted sites (by RAPD, Perotto \textit{et al.}, 1996). The results (Fig. 4a) showed that all isolates, irrespective of the site of origin, displayed a higher frequency of polymorphisms in the \textit{Sod1} sequence than in the ITS region. However, the frequency of polymorphisms in the \textit{Sod1} sequence of the Niepolomice isolates was much higher when compared with isolates from the other two sites. Similarly, the Niepolomice isolates also showed a higher genetic variation in the ITS region.

![Sequence polymorphism](image)

**Figure 4.** Sequence polymorphism of the ITS and the SOD loci. (a) p-Distance of the ITS region and the SOD in the three groups of fungal isolates listed in Table 1. (b) p-Distance of different regions of SOD locus in three groups of fungal isolates. (c) Details of the nucleotide polymorphism of different regions of SOD locus in three groups of fungal isolates.

**Different sequence polymorphism frequency is found in the coding and noncoding regions of the Sod1 locus**

The sequence polymorphism of coding and noncoding regions of the \textit{Sod1} locus was compared in the \textit{O. maius} isolates from polluted and unpolluted sites. Within the \textit{Sod1} locus (Fig. 4b), when compared with isolates from the unpolluted or from the serpentine soils, isolates from the industrially polluted site showed a high frequency of polymorphisms in the \textit{Sod1} promoter region (Fig. 4b). Out of the 63 polymorphisms found in the promoter region of the Niepolomice isolates, 28 were single-nucleotide polymorphism (SNPs) and 35 were insertions/deletions (InDels) (Fig. 4c). Of the 35 InDels, three corresponded to single InDels and 32 formed a nucleotide string. It
cannot be excluded that the last occurred as a single event, but the number of SNPs alone would still be about threefold higher in the Niepolomice isolates than in fungi from the serpentine site, and sixfold higher than fungi from unpolluted sites. *Oidiodendron maius* isolates from the serpentine and the unpolluted sites showed a similar genetic variation in the intron region, but a higher (about twofold) number of polymorphisms was found in the isolates from the Niepolomice site (Fig. 4b).

By contrast, the region coding for the functional protein (exon region) was highly conserved in isolates derived from all three sites. Among isolates from unpolluted sites, all mutations were synonymous. In the Mont Avic isolates, one mutation out of eight SNPs was nonsynonymous: OmMa3 isolate has a glutamic acid instead of glutamine in position 111 of the aminoacid sequence. In the Niepolomice isolates, one mutation out of nine SNPs was nonsynonymous: isolates OmZn and OmCd3 have an aspartic acid instead of glutamic acid in position 133.

The complete sequences of the *Sod1* locus were also used to build a phylogenetic tree in which six different haplotypes were clearly distinguished (Fig. 5). The *Sod1* sequences of isolates from serpentine soils formed a single cluster, with the exception of OmMa3, which formed a separate cluster together with two isolates (OmII and OmIII) from an unpolluted site. The *Sod1* sequences of the three other isolates from unpolluted sites (Om91, OmA and OmI) formed a separate cluster (Fig. 5). The *Sod1* sequences of isolates from the Niepolomice site formed two separate clusters, with OmZn and OmCd2 forming the most distant group (Fig. 5). These two fungal isolates also formed a distinct cluster based on their ITS region (not shown).
Figure 5. Phylogenetic tree of the SOD gene. The analysis was performed on the complete sequences of the Sod1 locus using mega 3.1 software with the neighbour joining method, by means of the Kimura 2 Parameter. Bootstrap tests were performed with 500 replicates (nodes with bootstrap values superior to 50 are shown).

Discussion

Environmental stress is a force shaping the adaptation and evolution of organisms living in changing environments, and the impact of human activities on natural environments has caused rapid and often stressful and deteriorating changes. The occurrence of adaptive processes is clearly shown by the fact that many organisms have adapted quite rapidly to manmade changes in the environment (e.g. Bradshaw, 1952; Woods, 1981; Macnair, 1997). In particular, adaptation of fungi to heavy metal soil pollution of anthropic origin is supported by several studies (Leyval et al., 1997; Martino et al., 2000; Meharg & Cairney, 2000; Colpaert et al., 2004; Adriaensen et al., 2005, 2006; Zarei et al., 2008; Gamalero et al., 2009; Krznaric et al., 2009), although the molecular mechanisms of adaptation are unclear. Also, it is unclear whether the mechanisms of adaptation to soil conditions that have rapidly changed because of human activities may be different from those driving evolution in more stable soils, such as the naturally metalliferous soils. We have addressed this question in ericoid mycorrhizal fungi, for which several isolates of the common symbiont O. maius were already available from industrially polluted sites, by isolating conspecific strains from a naturally contaminated serpentine soil.

Oidiodendron maius represented about 50% of the strains isolated from mycorrhizal V. myrtillus plants growing in the Mont Avic serpentine site, thus suggesting that it is a common mycorrhizal endosymbiont in this environment. Despite the high metal content measured in the naturally polluted Mont Avic Park site, AFLP analysis revealed a rather high genetic diversity of O. maius, with 10 different genets out of 15 fungal isolates and a PD value of 0.73. These results are comparable to those obtained by Gonçalves et al. (2007), who found a high degree of genetic diversity in isolates of the ectomycorrhizal ascomycete Cenococcum geophilum from serpentine soils in Portugal. Concerning industrially polluted sites, both a lower (Colpaert et al., 2000; Lacourt et al., 2000) and a higher (Müller et al., 2004, 2007) genetic diversity have been reported for ectomycorrhizal and ericoid mycorrhizal fungal populations. The PD values calculated for O. maius in the serpentine site (0.73) are in the range of PD values calculated for ectomycorrhizal fungi characterized by a predominant sexual reproduction (i.e. PD=0.61 for Laccaria amethystina; Gherbi et al., 1999). By contrast, ectomycorrhizal fungi characterized by a predominant vegetative reproduction, such as Xerocomus chrysenteron (Fiore-Donno & Martin, 2001), show a lower PD value (0.007). Our data suggest therefore that O. maius mainly develops through sexual reproduction in this serpentine site.

Several studies on ectomycorrhizal fungi have investigated the possible correlations between the fungal sensitivity to specific metals and the amount of those same metals in the soil of origin, by comparing the fungal response of conspecific isolates from polluted and unpolluted sites (Colpaert & Van Assche, 1992; Egerton-Warburton & Griffin, 1995; Blaudez et al., 2000; Colpaert et al., 2000). For example, the experiments by Blaudez et al. (2000) revealed no significant differences in the level of tolerance to Cd, Cu, Ni and Zn among isolates of Paxillus involutus, Pisolithus tinctorius, Suillus bovinus, Suillus variegatus and Suillus luteus from soils with various (low to high) degrees of metal contamination. By contrast, the experiments by Colpaert et al. (2000) revealed higher tolerance to Zn and Cd for S. luteus isolates from a site polluted with these metals, when compared with isolates from a nonpolluted site. In the same way, Gonçalves et al. (2009) found that isolates of the ectomycorrhizal fungus Cenococcum geophyllum from serpentine soils exhibited significantly higher tolerance to Ni than nonserpentine isolates. Therefore, it seems that
tolerance to heavy metals in mycorrhizal fungi can be either constitutive or adaptive, and that pollution and/or metalliferous soils can drive the evolution of metal tolerance in some ectomycorrhizal fungi.

The ability of *O. maius* isolates to grow in the presence of the metal contaminants typical of the serpentine site (Cr and Ni) and of the industrially polluted site (Zn and Cd) suggests a different response of the isolates to the metals typical of the two sites. The isolates more tolerant to Cr and Ni were those that originated from the serpentine site, while the more tolerant isolates to Zn and Cd were those from the industrially polluted site characterized by these contaminants. PCA confirmed a different behaviour of the *O. maius* isolates on media containing these metals (Cd/Zn vs. Cr/Ni). In spite of the relatively small sample size, we can hypothesize a metal-specific adaptation of the *O. maius* isolates that reflects the specific contamination in the soil of origin.

AFLP analysis and growth assay indicate that organisms closely related in genetic terms do not necessarily display similar levels of stress resistance. These results confirm previous work by Lacourt et al. (2000), who identified genets in *O. maius* isolates from industrially polluted sites and found no direct correlations between genetic and functional diversity.

For understanding adaptation, evolution and biodiversity in a given species, one must explore the extent and causes of genetic variation. Adaptive evolution has long been regarded as the result of postmutational sorting by natural selection. We have therefore investigated the genetic variation in *O. maius* isolates from sites subjected to different environmental stress by focusing on a neutral gene (the ITS region) and on a gene encoding the Cu,ZnSOD, an enzyme that plays a known functional role in metal tolerance (Schützendübel & Polle, 2002; Sunkar et al., 2006; Lee et al., 2007). The higher molecular variation found in the functional Sod1 locus of isolates from all sites (as compared with the neutral ITS) indicates that mutations do not occur at random in the fungal genome. This is in accord with the observation made by Fox et al. (2008), who reported that genes whose product interacts with the extracellular environment tend to have high substitution rates in many mammalian lineages.

The discovery of stress-induced mutagenesis has revealed the existence of mutagenic mechanisms that differ from standard spontaneous mutagenesis and promote random mutations specifically when cells are poorly adapted to their environments (Metzgar & Wills, 2000; Tenaillon et al., 2004; Galhardo et al., 2007). A variety of environmental stresses can potentially induce genomic instability and accelerate adaptive evolution in bacteria, yeast and human cancer cells, generating fitter mutants (Galhardo et al., 2007). Reports of stress-induced mutagenesis in filamentous fungi are scanty, but differences in the mutation frequencies as a function of environmental stress have been observed for three different species of filamentous fungi from the ‘Evolution Canyon’ in Israel (Lamb et al., 1998, 2008), thus indicating that the phenomenon also applies to these organisms.

Among the *O. maius* isolates, those derived from the serpentine site showed a genetic variation, both in the ITS and in the Sod1 locus, fairly similar to isolates from unpolluted sites. By contrast, isolates from the Niepolomice site showed a higher number of polymorphisms in both genes. Previous reports indicate that isolates from harsher environments show a higher inherited frequency of spontaneous mutation (Lamb et al., 2008), thus suggesting a more stressful environment in the Niepolomice site. In contrast to the Mont Avic, a metalliferous site that featured a long and stable natural contamination, the Niepolomice site was subjected to a rapid and stressful change in the amount of industrial pollutants in the 1980s (Turnau, 1988).

Further analysis of genetic variation within the *Sod1* locus also suggests that the accumulation of mutations was not the result of a random process. This feature was particularly evident in the *O.
maius isolates from the Niepolomice site, which featured very high sequence polymorphism in the Cu,ZnSOD promoter region as compared with the coding region. The nucleotide polymorphism in the Sod1 promoter of the Niepolomice isolates was also very high when compared with isolates from the other two sites. A similar situation was found, as compared with a reference site (Janssens et al., 2007), in the promoter region of metallothionein genes in a cadmium-tolerant population of Orchesella cincta (Collembola).

Our observations in O. maius thus seem to indicate that, in addition to an increase in the number of random mutations, mutagenesis induced by environmental stress may target specific gene regions. Further work in a wider set of fungal isolates is needed to confirm these observations, and to verify a possible functional role of the different degree of polymorphism in the O. maius Sod1 promoter (e.g. a different transcriptional regulation).

In conclusion, our results would suggest that genes with a functional role in fungal growth and survival display a higher nucleotide polymorphism than neutral genes in the ericoid mycorrhizal fungus O. maius, and future work should investigate additional genetic loci to corroborate this finding. In addition, our data suggest that, in the Niepolomice site, environmental stress induced general mutagenesis and even preferentially directed mutations to specific sites within individual loci. It remains to be established whether these mutations improve fungal growth and survival (adaptive mutations).

Acknowledgements

The authors thank Luca Miserere for the valuable help with sample collection. M.V. and E.Z. acknowledge financial support by the University of Turin. M.P. received financial support from Regione Piemonte (SINAPSI fellowship). The results reported are part of the CEBIOVEM (D.M. 193/2003) programme. Research was also partly funded by Regione Piemonte (CIPE, 2004) and University grant (60%).

Authors' contribution

M.V. and E.Z. equally contributed to the paper.

References


**Mycol Res** **104**: 338-344.


