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Evidence of adaptive tolerance to nickel in isolates of *Cenococcum geophilum* from serpentine soils

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Abstract Selection for metal-tolerant ecotypes of ectomycorrhizal (ECM) fungi has been reported in instances of metal contamination of soils as a result of human activities. However, no study has yet provided evidence that natural metalliferous soils, such as serpentine soils, can drive the evolution of metal tolerance in ECM fungi. We examined in vitro Ni tolerance in isolates of *Cenococcum geophilum* from serpentine and non-serpentine soils to assess whether isolates from serpentine soils exhibited patterns consistent with adaptation to elevated levels of Ni, a typical feature of serpentine. A second objective was to investigate the relationship between Ni tolerance and specific growth rates (μ) among isolates to increase our understanding of possible tolerance/growth trade-offs. Isolates from both soil types were screened for Ni tolerance by measuring biomass production in liquid media with increasing Ni concentrations, so that the effective concentration of Ni inhibiting fungal growth by 50% (EC_{50}) could be determined. Isolates of *C. geophilum* from serpentine soils exhibited significantly higher tolerance to Ni than non-serpentine isolates. The mean Ni EC_{50} value for serpentine isolates (23.4 μg ml^{-1}) was approximately seven times higher than the estimated value for non-serpentine isolates (3.38 μg ml^{-1}). Although there was still a considerable variation in Ni sensitivity among the isolates, none of the serpentine isolates had EC_{50} values for Ni within the range found for non-serpentine isolates. We found a negative correlation between EC_{50} and μ values among isolates (r=−0.555). This trend, albeit only marginally significant (P=0.06), indicates a potential trade-off between tolerance and growth, in agreement with selection against Ni tolerance in "normal" habitats. Overall, these results suggest that Ni tolerance arose among serpentine isolates of *C. geophilum* as an adaptive response to Ni exposure in serpentine soils.

Keywords Adaptive evolution · *Cenococcum geophilum* · Ectomycorrhizal fungi ecology · Nickel toxicity · Serpentine ecotypes · Ultramafic soils

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Introduction

Metal-contaminated soils, the result of either human activities or their natural geological origin, harbor a variety of organisms that have the ability to withstand metal toxicity. Serpentine soils are characterized by high levels of heavy metals (notably Ni), low levels of macronutrients, and an unbalanced (extremely low) Ca:Mg ratio (Proctor and Woodell 1975; Brooks 1987). This unique soil chemistry has triggered the adaptive evolution of higher plants, e.g., to Ni toxicity (Mengoni et al. 2001; Berglund et al. 2004; Bratteler et al. 2006).

In herbaceous plants, metal tolerance can evolve rapidly (Al-Hiyaly et al. 1993). However, in long-lived

organisms such as trees evolution of metal tolerance is most likely a slow process (Miller and Cumming 2000; Wright 2007). Trees are thought to cope with elevated metal soil concentrations by means of a large phenotypic plasticity and mycorrhizal symbiosis (Wilkinson and Dickinson 1995). Ectomycorrhizal (ECM) fungi can assist trees in handling soil metal toxicity (reviewed in Wilkins 1991; Wilkinson and Dickinson 1995; Godbold et al. 1998; Jentschke and Goldbold 2000). As Taylor (2000) emphasized, trees such as pine or oak are unlikely to colonize a site that is too toxic to support ECM fungi as they depend strongly on their root associates for mineral nutrition. Consequently, in low-fertility soils, such as serpentine, Ni tolerance in ECM fungi may be of great value to the plant even if metal uptake by the plant is not reduced.

Studies on ECM fungal communities in serpentine soils are surprisingly scarce (Maas and Stuntz 1969; Moser et al. 2005; Brearley 2006; Urban et al. 2008). Moser et al. (2005) compared the diversity of ECM fungi associated with *Quercus garryana* growing on and off serpentine soils in Oregon, USA. Although some ECM species were unique to serpentine soil, *Cenococcum geophilum* Fr. was found in both soil types (Moser et al. 2005). Similarly, Panaccione et al. (2001) isolated *C. geophilum* from both serpentine and non-serpentine soils in Maryland, USA.

The presumed occurrence of serpentine-tolerant ecotypes of wide-ranging ECM species like *C. geophilum* is of special ecological interest because the association with adapted root symbionts is thought to be a major component of the survival strategy of trees in metal-contaminated soils (Adriaensen et al. 2004, 2006). Metal-tolerant isolates of ECM fungi have frequently been isolated from polluted soils (e.g., Brown and Wilkins 1985; Colpaert and Van Assche 1987; Jones and Hutchinson 1988; Egerton-Warburton and Griffin 1995; Colpaert et al. 2000, 2004). Overall, however, it is still uncertain whether ECM fungi colonize metal-contaminated soils because they evolve metal tolerance (Colpaert and Van Assche 1992; Egerton-Warburton and Griffin 1995) or rather because of their constitutively widespread tolerance (Brown and Wilkins 1985; Denny and Wilkins 1987; Jones and Hutchinson 1988; Blaudez et al. 2000). Clearly, a large intraspecific variation in metal sensitivity exists within some species, including *C. geophilum* (McCreight and Schroeder 1982; Thompson and Medve 1984; Tam 1995; Fomina et al. 2005; Gonçalves et al. 2007).

Gonçalves et al. (1997) found abundant *C. geophilum* ECM in *Quercus ilex* subsp. *ballota* growing in Portuguese serpentine areas and suggested the presence of Ni-tolerant fungal ecotypes. Subsequent studies failed to detect genetic divergence between serpentine and non-serpentine isolates of this species collected from both a serpentine and a non-serpentine site in northeast Portugal (Portugal et al. 2001, 2004; Gonçalves et al. 2007). Nonetheless, we did observe differential in vitro responses to Ni in *C. geophilum* isolates originated in serpentine soil, in comparison to a non-serpentine isolate: the non-serpentine isolate was the only isolate whose growth was significantly inhibited by the addition of Ni to the culture medium (Gonçalves et al. 2007). Conversely, Pannacione et al. (2001) showed that isolates of *C. geophilum* growing in serpentine soils (dominated by *Pinus virginiana*) and non-serpentine soils in Maryland were genetically distinct. Based on this result, these authors advocated that serpentine factors had selected for serpentine-tolerant fungal ecotypes. Interestingly, in a previous study, Miller and Cumming (2000) had found no evidence of ecotypic differentiation in *P. virginiana* growing on serpentine soil and, thus, speculated that the association with ECM fungi in the field could reduce the selective pressure exerted by the chemical properties of serpentine soils on the trees. The results from Panaccione et al. (2001) provided support to this idea, but without physiological evidence this hypothesis cannot be confirmed. This is especially important given that the evolution of metal tolerance in ECM fungi does not necessarily imply a reduction of the genetic diversity of the tolerant populations or a genetic differentiation between tolerant and non-tolerant populations (Muller et al. 2004, 2007).

Whilst compelling evidence of selection for metal-tolerant ecotypes has recently been provided in instances of metal pollution of anthropogenic origin, both in ericoid and ECM fungi (Colpaert et al. 2000; Sharples et al. 2001; Colpaert et al. 2004; Adriaensen et al. 2005), no study has yet demonstrated a statistical difference in metal sensitivity within a single ECM fungal species in response to natural metal contamination in soils.

In this study, we compared Ni sensitivity in 12 isolates of *C. geophilum*, seven isolates from serpentine soils and five isolates from non-serpentine soils, and from distant geographical origins: Portugal and the USA. Isolates from Portugal included the ones previously surveyed (Gonçalves et al. 2007), while isolates from the USA were among those studied by Panaccione et al. (2001) and by Douhan and Rizzo (2005). Because it was suggested, based on the allocation principle (Levins 1968), that tolerance may have a metabolic "cost" to ECM fungi (Hartley et al. 1997b), we were also interested in determining the relationship, if any, between specific growth rates and Ni sensitivity, among isolates. The hypotheses being tested were (1) that isolates from serpentine sites would exhibit lower sensitivity to Ni than isolates from the non-serpentine sites, irrespectively of their geographical origin, and (2) that Ni-tolerant isolates would have lower specific growth rates, in control conditions.

Materials and methods

Sampling sites and isolates origin

Isolates of *C. geophilum* coded 119M, 217M, 715M, 743M, and 747M (former 1,19MT9, 2,17MT5, 7,15MT5, 7,43MT5, and 7,47MT5, respectively; codes were changed for ease of reading) were obtained from sclerotia collected from serpentine soil samples in a site near the village of Morais, northeast Portugal (39° 42′ N, 04° 34′ W). A non-serpentine isolate, 428R (former 4,28CT5), was obtained close to the village of Rabal, situated at about 40 km from the serpentine area (39° 44′ N, 04° 06′ W). In both sites, *Q. ilex* subsp. *ballota* is the dominant tree species. A detailed description of the methods of isolation and maintenance of these isolates can be found in Gonçalves et al. (2007). The Ni concentration (ammonium acetate extracts) in the soil samples from which these isolates were obtained was measured by Nabais (2000). In serpentine samples, values ranged between 4.80 and 14.6 μg Ni g^{-1}, whereas in the non-serpentine samples the concentration of Ni was significantly lower ($t_{0.05(2),4}$=2.785, P<0.05) and varied between 0.60 and 3.00 μg Ni g^{-1} (Gonçalves et al. 2007).

Isolates S1–8 and S3–9 (serpentine) and N2–6 (non-serpentine) came from Maryland, at the Soldiers Delight Natural Environment area in Owings Mills (39° 24′ N, 76° 50′ W). Isolates were originally trapped from soils on roots of *P. virginiana*, but *Quercus* species were also present on the sampling sites (Panaccione et al. 2001). In serpentine soils at Soldiers Delight, a grassland/savannah, Ni concentration extracted with Mehlich III was significantly higher than in the non-serpentine soils, with mean concentrations 59.3 and 13.5 μg Ni g^{-1}, respectively (Panaccione et al. 2001). Three additional non-serpentine isolates (1-1-3, 1-5-2 and 3-10-6) came from a savannah woodland in Sierra Nevada foothills (39° 15′ N, 121° 17′ W), California, dominated by *Quercus douglasii* (Douhan and Rizzo 2005). More information regarding the sampling sites as well as detailed information concerning isolation and culturing procedures of *C. geophilum* isolates from the USA can be found elsewhere (Dahlgren et al. 1997; Panaccione et al. 2001; Douhan and Rizzo 2005).

In total, we used 12 isolates of *C. geophilum*; seven from serpentine soils and five from non-serpentine soils. Table 1 presents the origin sites of the studied isolates as well as their probable hosts. All the isolates are kept in our collection on potato dextrose agar (PDA; Difco, USA) medium without added Ni.

Table 1 Origin sites and potential hosts of *Cenococcum geophilum* studied isolates

Isolates	Origin site	Potential host
119M 217M 715M 743M 747M	Morais, Trás-os-Montes, Portugal, serpentine site	*Quercus ilex* subsp. *ballota*
428R	Rabal, Trás-os-Montes, Portugal, non-serpentine site	*Quercus ilex* subsp. *ballota*
1-1-3 1-5-2 3-10-6	Sierra Nevada foothills, CA, USA, non-serpentine site	*Quercus douglasii*
S1–8 S3–9	Soldiers Delight, Owing Mills, MD, USA, serpentine site	*Pinus virginiana*, *Quercus* spp.
N2–5	Soldiers Delight, Owing Mills, MD, USA, non-serpentine site	*Pinus virginiana*, *Quercus* spp.

Growth curves

All glassware were washed in 10% HNO_3 and thoroughly rinsed in ultrapure water (18 MΩ) before use in the experiments. Stock solutions were also prepared with ultrapure water with reagent-grade chemicals (Sigma, USA).

Determination of growth curves was carried out in liquid modified Fries medium (Fries 1978) containing (mM): D-glucose, 28; ammonium tartrate, $(NH_4)_2C_4H_4$, 5.4; KH_2PO_4, 1.5; $MgSO_4{\cdot}7H_2O$, 0.4; $CaCl_2{\cdot}2H_2O$, 0.2; NaCl, 0.3. Microelements included (μM): $FeCl_2{\cdot}6H_2O$, 4.0; $ZnSO_4{\cdot}7H_2O$ 3.0; $MnSO_4{\cdot}H_2O$, 6.0; $CuSO_4{\cdot}5H_2O$, 0.8; $Na_2MoO_4{\cdot}2H_2O$, 0.8; *myo*-inositol, 56; biotin, 0.1; pyridoxine–HCl, 0.5; riboflavin, 0.3; nicotinamide, 0.8; *p*-amino-benzoic acid, 0.7; thiamine–HCl, 0.3; and Ca-pantothenate, 0.2. Media were adjusted to pH 5.5 and autoclaved for 15 min at 121°C. After cooling, vitamins and *myo*-inositol previously sterilized by filtration (0.2 μm) were added.

For each isolate, 30 Petri dishes were inoculated using the following method. Mycelial plugs, 5 mm in diameter, were taken with a sterilized borer from the edge of actively growing colonies. In order to obtain uniform inocula, a large number of plugs were placed on fresh PDA (Difco, USA) medium plates. As soon as hyphal growth was visible to the naked eye, plugs showing hyphae emerging in all directions were transferred to the experimental plates. Three discs of fungal mycelium were inoculated into 90-mm Petri dishes containing 25 ml of fresh Fries solution. Petri dishes were double wrapped in Parafilm® and incubated at 22°C in the dark. Nine plugs of each isolate were immediately harvested and the dry weight for three disks was determined. This established the mean dry weight of the mycelia at the start of the experiment. Inoculated Petri dishes were randomized within the incubator.

Three replicates of each isolate were harvested after incubation during 2, 4, 8, 12, 16, 20, 24, 28, and 34 days (in two cases also at day 40). Mycelial mats were removed from

the medium and dried to a constant weight at 50°C. The dry weight increase over time was plotted as a growth curve for each isolate. Data were analyzed and graphically displayed using SigmaPlot 8.02 (SPSS 2002). Several models of growth were tested and the best fitted curve selected.

Dose–response curves

In vitro Ni tolerance was tested using the same procedures as in the previous experiment, but with liquid medium amended with eight concentrations of Ni supplied as nickel sulfate ($NiSO_4 \cdot 7H_2O$). Aliquots of a stock solution containing 2,000 μg Ni ml^{-1} were added to the growth medium to give final concentrations of 0, 0.5, 2, 10, 20, 50, 100, and 200 μg Ni ml^{-1}. Three replicates were used for each concentration.

For each isolate, we established a standard point 50% along the growth curve as the time to harvest the colonies. The purpose was to ensure that all isolates were harvested at the same stage of growth. Based on this procedure, the estimated times of harvest were: 119M, 17 days; 217M, 18 days; 715M, 17 days; 743M, 23 days; 747M, 22 days; 428R, 19 days; 1-1-3, 21 days; 1-5-2, 17 days; 3-10-6, 23 days; S1–8, 15 days; S3–9, 17 days; N2–6, 13 days. At their pre-determined harvest date, mycelium was collected by filtration through a Buchner funnel onto filter paper pads, washed a few times with ultrapure water, and dried to a constant weight at 50°C. The dry weight increase during the test period was calculated. For ease of comparison, dry weight increases were expressed as a percentage of the control (also referred as tolerance indices, TI). The effective concentration of Ni inhibiting fungal growth by 50% (EC_{50}) was calculated by fitting the best curves to the data with SigmaPlot (SPSS 2002).

Biomass increase data were analyzed by two-way analysis of variance (ANOVA) followed by the Holm–Sidak multiple comparison test. Variable factors were habitat (two levels=serpentine and non-serpentine) and Ni concentration (eight levels=0, 0.5, 2, 10, 20, 50, 100, and 200 μg ml^{-1}). We used a *t* test to compare mean EC_{50} values between serpentine and non-serpentine isolates. A paired *t* test was used to test the hypothesis that there was not a significant difference between biomass increase at 50% in basal medium (first assay) and biomass increase at harvest date in control medium (no added Ni) in the dose–response assay. Each variable was checked for normality and homogeneity of variance before tests were performed. All analyses were carried out with the computer package SigmaStat 3.0 (SPSS 2003).

Growth/tolerance trade-offs

For each isolate growing in basal medium, a linear curve was fitted to the logarithmic values of the dry weight data along the exponential phase of growth and the specific growth rates (μ, day^{-1}) were calculated as the slopes of the lines (Griffin 1994). The relationship between the μ values and the Ni EC_{50} values among the fungal isolates was tested with the Pearson product-moment correlation coefficient, after checking for parametric analysis assumptions, using SigmaStat 3.0 (SPSS 2003).

Results

Growth curves

The growth of the isolates was best described by a sigmoid shaped curve, which is typical of micro-organisms grown in batch culture. The growth curves of six isolates of both soil types are shown as example (Fig. 1a–f). The biomass increase in basal medium was variable. For instance, isolate 217M produced a biomass of 82.10 mg after growth for 34 days, more than twice the biomass yield in isolate 715M (40.37 mg) during the same period (Fig. 1a,b). Isolates achieved approximately 50% of their final biomass, as calculated by the model fitted curve, after 13 to 23 days.

Dose–response curves

Mycelia were harvested before nutrient exhaustion, and lag phases were avoided. At least at the control treatment (no added Ni), the time of harvest at 50% growth was similar in both assays ($t_{0.05(2),11}=1.569$, $P=0.145$).

Increasing Ni concentrations resulted in an inhibition of biomass yield for all isolates, with TI values <5% at the highest Ni exposure (200 μg ml^{-1}). However, the significant interaction effect between habitat (serpentine vs. non-serpentine) and Ni concentration in the two-way ANOVA analysis ($P<0.05$) showed that serpentine and non-serpentine isolates were inhibited to a different degree in the presence of Ni (Fig. 2). The mean EC_{50} for Ni was significantly higher ($t_{0.05(2),10}=3.966$, $P<0.01$) in serpentine isolates than in non-serpentine isolates: 23.38 vs. 3.376 μg Ni ml^{-1}, respectively (Fig. 3). In fact, all serpentine isolates had EC_{50} values higher than those recorded in the non-serpentine isolates. The highest Ni EC_{50} among the isolates was observed in serpentine isolate 747M (39.90 μg ml^{-1}) and the lowest in the non-serpentine isolate 1-5-2 (1.353 μg ml^{-1}). The serpentine isolate 715M was capable of growth at extreme Ni concentrations; at 100 μg Ni ml^{-1}, the TI value of this isolate was >25%.

The average TI values and respective range for each group of isolates, serpentine and non-serpentine, are presented in Table 2. The difference between the two groups was significant at 2, 10, and 20 μg Ni ml^{-1}. Moreover, at 10 and 20 μg Ni ml^{-1}, there was no overlap in

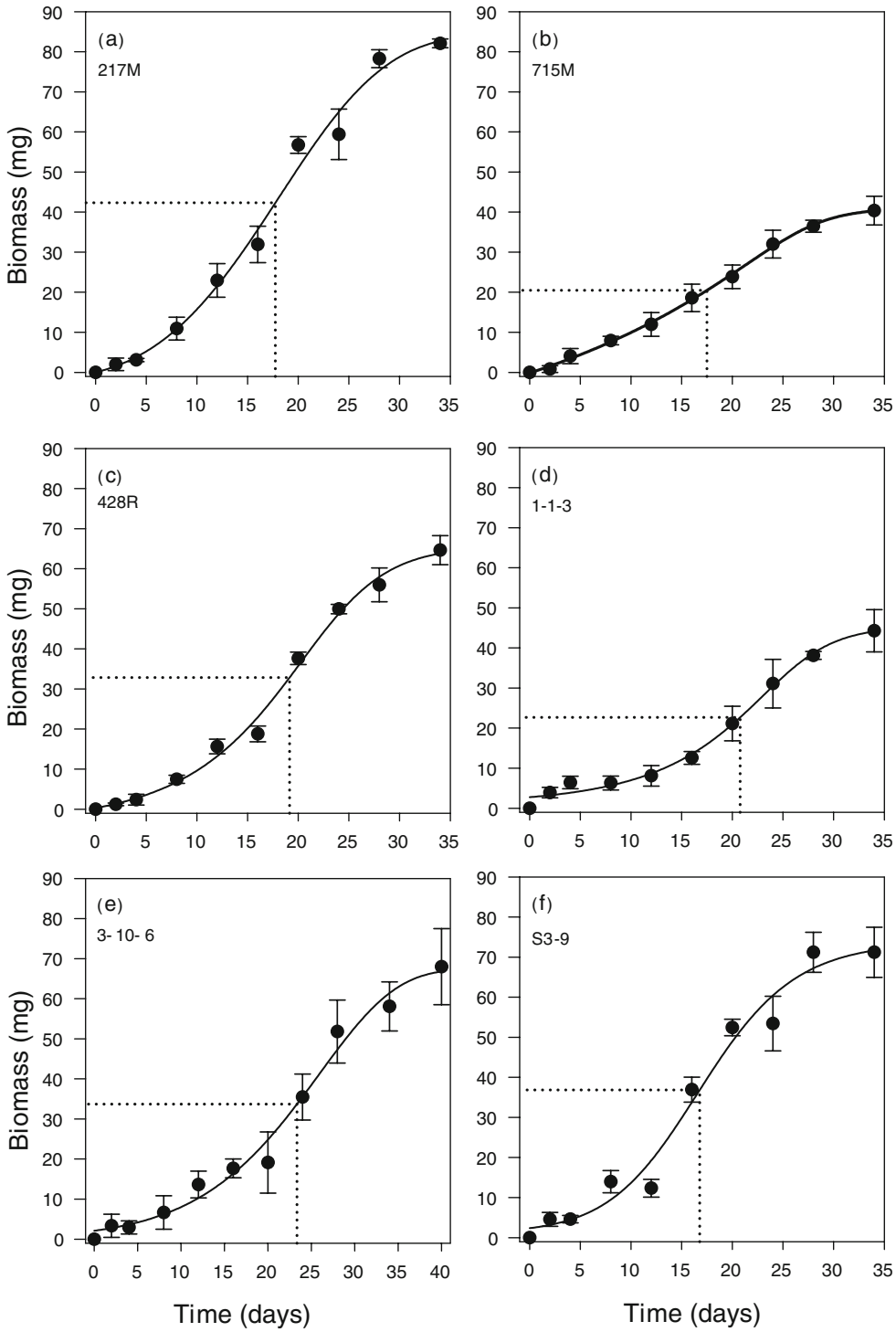

Fig. 1 Growth curves of *Cenococcum geophilum* isolates **a** 217M, **b** 715M, **c** 428R, **d** 1-1-3, **e** 3-10-6, and **f** S3–9. Each *point* represents the mean of three replicates, *bars* represent the SE of the mean; curves were fitted using a sigmoid equation. *Line drops* indicate the time of growth (days) at a standard point 50% along the growth curve of each isolate

the TI values of the two groups of isolates (Table 2, Fig. 4a, b).The presence of 10 μg Ni ml^{-1} significantly inhibited the biomass yield of non-serpentine isolates. At this concentration, there were two non-serpentine isolates (1-1-3 and N2–6) whose growth has already ceased (TI=0, Fig. 4a). In contrast, serpentine isolates were unaffected by the presence of Ni up to 20 μg ml^{-1} and total inhibition of growth was first recorded at 100 μg Ni ml^{-1}, in isolates 217M and S3–9 (Table 2).

Growth/tolerance trade-offs

Specific growth rates (μ, day^{-1}) varied among isolates, from 0.05 to 0.13 day^{-1}. In general, fungal isolates with

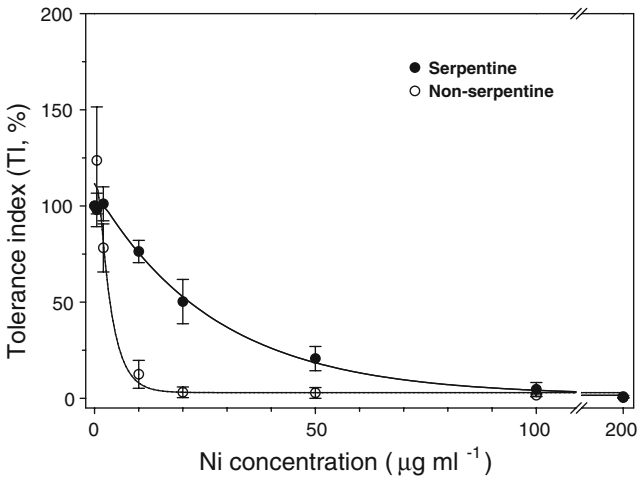

Fig. 2 Tolerance indices (TI, %) of serpentine (*solid circles*, $n=7$) and non-serpentine (*open circles*, $n=5$) isolates of *Cenococcum geophilum* over a range of Ni concentrations (0.5, 2, 10, 20, 50, 100, and 200 µg ml^{-1}). Values are means ± SE; sigmoid curves were fitted to the data

higher Ni EC_{50} values had lower characteristic μ values although this relationship was only marginally significant ($r=-0.555$, $P=0.06$).

Discussion

In vitro Ni tolerance of serpentine and non-serpentine isolates of *C. geophilum* varied significantly, suggesting that metal tolerance is an adaptive response to Ni exposure in serpentine soils (Table 2, Fig. 2). The mean EC_{50} value for Ni in non-serpentine isolates was ca. seven times lower than the EC_{50} mean value for serpentine isolates (Fig. 3). These results corroborate our previous analysis (Gonçalves et al. 2007) that suggested that Ni-tolerant ecotypes of *C. geophilum* might have evolved in serpentine soils. At the time, only one non-serpentine isolate of *C. geophilum* was compared with three serpentine isolates. In this study, we screened in vitro Ni tolerance in 12 isolates; seven from serpentine soils and five from non-serpentine soils. At 50 µg Ni ml^{-1}, three out of five non-serpentine isolates had TI=0, whereas the seven serpentine isolates were all able to grow at this level of Ni exposure, further emphasizing the different performance between the two groups. We included isolates previously studied by Panaccione et al. (2001): S1–8, S3–9, and N2–6. The results provide physiological support to their hypothesis of ecotypic differentiation between serpentine and non-serpentine populations of *C. geophilum* at Soldiers Delight. The biomass yield was inhibited by 50% at 16.16, 9.292, and 1.916 µg Ni ml^{-1} in isolates S1–8, S3–9 (both serpentine), and isolate N2–6 (non-serpentine), respectively. Isolates 1-1-3, 1-5-2, and 3-10-6 collected from non-serpentine habitats in Sierra Nevada foothills were originally isolated and characterized by Douhan and Rizzo (2005). These isolates were included in our study with the objective of balancing the number of serpentine vs. non-serpentine isolates in the experiment. As expected, these were Ni sensitive.

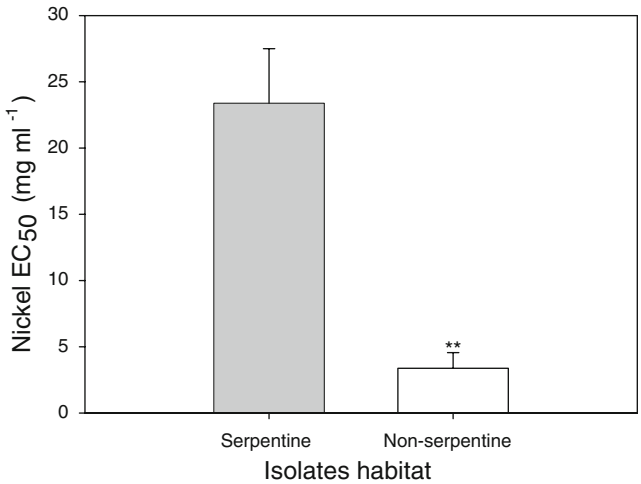

Fig. 3 Effective concentrations of Ni inhibiting biomass yield by 50% (EC_{50}) in serpentine ($n=7$) and non-serpentine ($n=5$) isolates of *Cenococcum geophilum*. Values are means±SE; $^{**}P<0.01$, as analyzed by *t* test

Although there was still a considerable variation in Ni sensitivity among the isolates, the highest EC_{50} value determined for a non-serpentine isolate (7.868 µg Ni ml^{-1}) was nonetheless lower than the lowest EC_{50} value registered among serpentine isolates, 9.292 µg Ni g^{-1}. This suggests that the serpentine isolates do not simply represent a subset of a "normal" *C. geophilum* population. Moreover, because isolates from distant geographical origins were screened (including two serpentine soils located in different continents), it is unlikely that results are merely an artifact of local history. Therefore, the results enable us to suggest a causal relationship between Ni tolerance and fungal habitat (serpentine vs. non-serpentine). Furthermore, all isolates in this study were kept on culture medium without Ni, in some cases for more than 5 years before the experiments were performed, so that Ni tolerance could not have arisen from physiological adaptation. Because *C. geophilum sensu lato* is such a diverse species at the population level (Panaccione et al. 2001; Jany et al. 2002; LoBuglio and Taylor 2002; Portugal et al. 2004; Douhan and Rizzo 2005; Gonçalves et al. 2007), however, more isolates should be screened from the same and other replicate locations (serpentine, non-serpentine) before we can conclusively demonstrate adaptive tolerance to Ni in populations of *C. geophilum* in serpentine habitats.

In the present study, unlike others (e.g., Egerton-Warburton and Griffin 1995; Blaudez et al. 2000; Colpaert et al. 2000, 2004), liquid media were preferred over solid agar medium for assessing Ni tolerance. Reviewing the

Table 2 Mean and range of tolerance indices (TI, %) for Ni calculated for the biomass increase (mg) of *Cenococcum geophilum* isolates, from serpentine and non-serpentine soils

	Ni concentration (μg ml^{-1})[a]						
	0.5	2	10	20	50	100	200
Serpentine (n=7)	97.94 a	101.11 a	76.33 a	50.28 a*	20.67 a*	4.677 a*	0.327 a*
	(64.15–131.80)	(68.81–125.02)	(52.16–99.91)	(18.53–103.28)	(0.791–44.89)	(0–25.81)	(0–1.458)
Non-serpentine (n=5)	123.67 a	78.17 b	12.49 b*	3.129 b*	2.816 a*	1.569 a*	0.881 a*
	(55.97–203.26)	(45.00–106.79)	(0–39.70)	(0–14.20)	(0–14.20)	(0–14.08)	(0–3.373)

For each column, different letters indicate significant differences at $P<0.05$ according to the Holm–Sidak post hoc test. For each line, * denotes significant differences in relation to control (no added Ni) at $P<0.05$ according to the Holm–Sidak post hoc test

[a] Mole equivalent, 1 μg Ni ml^{-1} =17.0 μM

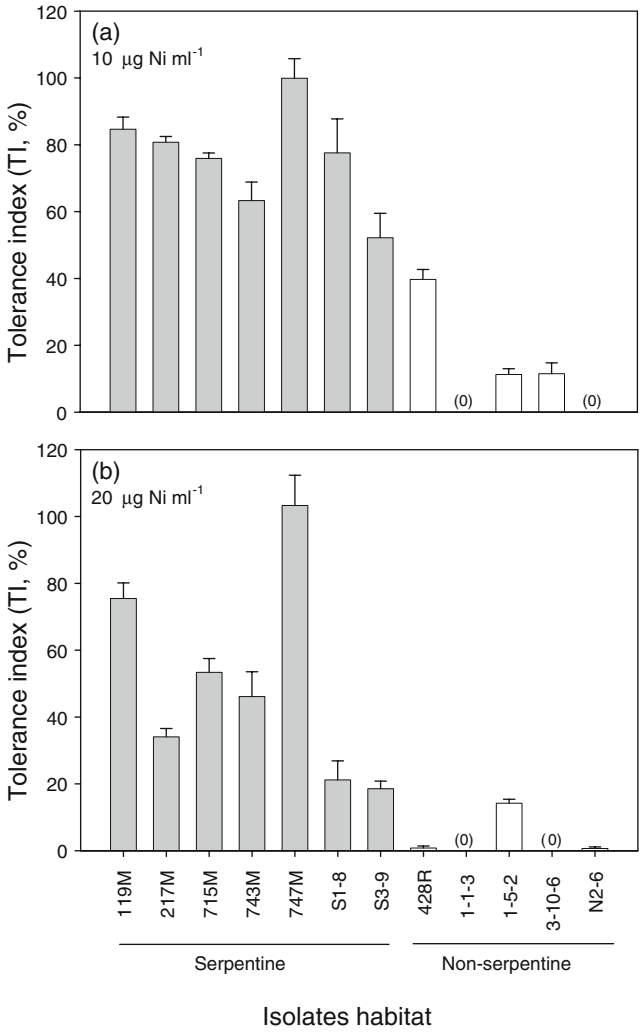

Fig. 4 Tolerance indices (TI, %) of each isolate of *Cenococcum geophilum* surveyed for biomass increase in the presence of **a** 10 and **b** 20 μg Ni ml^{-1}. Values are means of three replicates ± SE

methodology commonly used for assessing metal sensitivity in ECM fungi, Hartley et al. (1997b) pointed out the problems associated with the use of agar such as complexation of metals within the substrate that can alter the concentration of metal available to the fungi. Nevertheless, because metal interaction with medium components is still a concern in liquid culture, we took special care to reduce the inorganic phosphate concentration in the medium in order to minimize Ni precipitation. The phosphate concentration in our liquid modified Fries media was 1.5 mM while the modified Melin–Norkrans (MMN) medium, frequently used in studies of ECM fungi response to metals (e.g., Egerton-Warburton and Griffin 1995; Hartley et al. 1997a), contains up to 6 mM phosphate. In our previous work (Gonçalves et al. 2007), Ni sensitivity of isolates 428R, 217M, and 743M was assessed in PDA medium, which is a well-known rich organic solid medium. Therefore, it is not surprising that the TI value reported

here for isolate 428R was at 20 μg Ni ml^{-1} of only 0.08%, whereas in our previous work it was still of 28% at 30 μg Ni g^{-1}. In the present work, Ni EC_{50} value of this isolate was established as 7.868 μg Ni ml^{-1}. Similarly, the biomass yield of isolates 217M and 743M was not significantly affected at the range of Ni concentrations tested then, but in this study the mean biomass yield of these isolates was reduced by 50% at 16.97 and 18.99 μg Ni g^{-1}, respectively. While illustrating the effect of the culture medium in studies of ECM fungi response to metals, these findings also confirm our previous results in terms of relative performance of the isolates.

Given our interest in determining the existence of possible trade-offs between Ni tolerance and growth, it was important to determine the growth rates of isolates in relative terms. The specific growth rate (μ) is a basic parameter in the analysis of growth for micro-organisms grown in batch culture and, if all conditions are optimal, then the maximum specific growth rate, μ_{max}, is obtained. This is characteristic of a particular isolate (Deacon 2006). In filamentous fungi, this parameter can be used if the mycelium remains loose and fluffy, and nutrients flow freely around the hyphae (Griffin 1994; Deacon 2006). Since the formation of spherical pellets was not observed in our experiment, it seems the former conditions were satisfied. Even if determination of μ values can be questionable under the plug inoculation method of our experiments (certainly we cannot assure that optimal growth conditions were met), we used it on a relational basis to make first estimate comparisons between "fast" and slow-growing isolates. Our results are in agreement to predictions for a cost of tolerance to ECM fungi (Hartley et al. 1997b), giving further support to our hypothesis of adaptive tolerance to Ni in serpentine isolates of *C. geophilum*. In fact, if we assumed that the Ni tolerance trait was constitutive, no trade-off was to be expected and, in the absence of Ni, serpentine isolates would be predicted to grow as much as non-serpentine isolates. Our results, though, are contrary to this suggestion and sustain our second hypothesis. Although only marginally significant (P=0.06), the negative correlation between Ni EC_{50} values and μ values among isolates (r=−0.555) indicates a potential trade-off between tolerance and growth. Clearly, further research is required before generalizations can be made about the relationship between growth and tolerance in *C. geophilum*. Nevertheless, it is noteworthy that not one single Ni-tolerant isolate was found among our non-serpentine isolates, which would not be entirely unexpected (if Ni tolerance was indeed a constitutive trait) given the high genetic diversity of this species, possibly a species complex (Douhan and Rizzo 2005; Douhan et al. 2007). This result suggests selection against metal tolerance in "normal" habitats: the expression of Ni tolerant gene(s) in individuals from uncontaminated sites seems to be costly in terms of energy expenditure.

Serpentine edaphic limitations are not restricted to soil chemistry. The typical coarse texture and shallowness also decreases soil water-holding capacity (Proctor and Woodell 1975; Brooks 1987). Because of this, it has long been recognized that adjustment to water stress drought is pervasive in serpentine taxa and serpentine ecotypes of species of wider distribution (e.g., Hughes et al. 2001; Rajakaruna et al. 2003). Greater tolerance to drought of *C. geophilum* relative to other ECM fungi has been demonstrated experimentally (Mexal and Reid 1973; di Pietro et al. 2007). Could *C. geophilum* adaptation to drought represent a pre-requisite for successful colonization of serpentine soils? We suggest that, in *C. geophilum*, pre-adaptation to drought-prone soils and the evolution of Ni tolerance combine, enabling this fungus to successfully colonize serpentine soils. In an analogous situation, populations of the ericoid mycorrhizal fungus *Hymenoscyphus ericae* from As/Cu-contaminated soils have been shown to evolve tolerance to As, while expressing constitutive tolerance to Cu (Bradley et al. 1982; Sharples et al. 2001).

Conclusions

Our study provides evidence of adaptive tolerance to Ni in isolates of *C. geophilum* from serpentine soils. Adaptive tolerance to metals has been previously reported for ericoid and ECM fungi in response to metal pollution of human origin, such as in old mining sites (Egerton-Warburton and Griffin 1995; Colpaert et al. 2000; Sharples et al. 2001; Colpaert et al. 2004). However, this is the first study showing that natural metalliferous soils, such as serpentine soils, can drive the evolution of metal tolerance in ECM fungi.

Although Ni EC_{50} values from this study should not be directly extrapolated to a field situation, previous studies suggest that in vitro screenings can predict growth differences of fungal isolates in symbiosis (cf. Colpaert et al. 2000; Adriaensen et al. 2004). Therefore, and because the concentrations of Ni tested in our study are environmentally realistic, we hypothesize that symbiosis with Ni-tolerant isolates of *C. geophilum* confer an adaptive advantage to the host trees growing in serpentine soils.

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