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ORIGINAL PAPER

Genetic diversity and differential in vitro responses to Ni in *Cenococcum geophilum* isolates from serpentine soils in Portugal

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Abstract Amplified fragment length polymorphism (AFLP) analysis was used to investigate the genetic diversity in isolates of the ectomycorrhizal fungus *Cenococcum geophilum* from serpentine and non-serpentine soils in Portugal. A high degree of genetic diversity was found among *C. geophilum* isolates; AFLP fingerprints showed that all the isolates were genetically distinct. We also assessed the in vitro Ni sensitivity in three serpentine isolates and one non-serpentine isolate. Only the non-serpentine isolate was significantly affected by the addition of Ni to the growth medium. At 30 μg g^{-1} Ni, radial growth rate and biomass accumulation decreased to 73.3 and 71.6% of control, respectively, a highly significant inhibitory effect. Nickel at this concentration had no significant inhibitory effect on serpentine isolates, and so the fitness of serpentine isolates, as evaluated by radial growth rate and biomass yield, is likely unaffected by Ni in the field. In all isolates, the Ni concentration in the mycelia increased with increasing Ni concentration in the growth medium, but two profiles of Ni accumulation were identified. One serpentine isolate showed a linear trend of Ni accumulation. At the highest Ni exposure, the concentration of Ni in the mycelium of this isolate was in the hyperaccumulation range for Ni as defined for higher plants. In the remaining isolates, Ni accumulation was less pronounced and seems to approach a plateau at 30 μg g^{-1} Ni. Because two profiles of Ni accumulation emerged among our Ni-insensitive serpentine isolates, this result suggests that different Ni detoxification pathways may be operating. The non-serpentine isolate whose growth was significantly affected by Ni was separated from the other isolates in the genetic analysis, suggesting a genetic basis for the Ni-sensitivity trait. This hypothesis is further supported by the fact that all isolates were maintained on medium without added Ni to avoid carry-over effects. However, because AFLP analysis failed to distinguish between serpentine and non-serpentine isolates, we cannot conclude that Ni insensitivity among our serpentine isolates is due to evolutionary adaptation. Screening a larger number of isolates, from different geographical origins and environments, should clarify the relationships between genetic diversity, morphology, and physiology in this important species.

Keywords AFLP · *Cenococcum geophilum* · Ectomycorrhizal fungi · Nickel sensitivity and accumulation · Serpentine

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Introduction

Cenococcum geophilum Fr. (Class Ascomycetes) is an ectomycorrhizal (ECM) fungus with a worldwide distribution. It colonizes a broad range of host species and habitats and is one of the most frequent, often dominant, ECM types (Trappe 1964; Horton and Bruns 2001; Richard et al. 2005). This fungus lacks sexual and asexual spores, but it produces sclerotia that may be dispersed by water or animals (Massicotte et al. 1992; LoBuglio et al. 1996).

Serpentine soils are typically characterized by an unbalanced quotient Ca/Mg, low levels of N, P, K, and phytotoxic concentrations of heavy metals such as Ni, Cr, and Co. Serpentine soils are also shallow stony soils, with low water retention capacity (Menezes de Sequeira and Pinto da Silva 1992; Proctor 1999). These soils support a characteristic flora with many endemic species and probably also sustain a characteristic mycoflora. Thus, as pointed out by Panaccione et al. (2001), serpentine soils provide an opportunity to study the population biology and physiology of mycorrhizal fungi colonizing plants on natural metalliferous sites. In an early survey of mycorrhizas in Portuguese serpentine soils, Gonçalves et al. (1997) found abundant *C. geophilum* mycorrhizas in *Quercus ilex* subsp. *ballota*, the dominant tree in these areas (Menezes de Sequeira and Pinto da Silva 1992). It was then suggested that the fungal isolates involved in the symbiosis in serpentine soils could be ecotypes tolerant to Ni, accounting for the overall fitness of its plant host. In serpentine soils, establishing ECM with adapted fungal partners is likely to be important for tree adjustment to edaphic limitations (Adriaensen et al. 2004).

Significant inter- and intraspecific variation in sensitivity to heavy metals by ECM fungi has been extensively reported (Colpaert and Van Assche 1987, 1992; Denny and Wilkins 1987; Jones and Hutchinson 1988; Egerton-Warburton and Griffin 1995; Hartley et al. 1997; Blaudez et al. 2000; Colpaert et al. 2000). For example, the EC_{50} (i.e., the effective concentration of metal that inhibits growth by 50%) values for Cd differed by over three orders of magnitude among the four ECM species studied by Hartley et al. (1997). In turn, Blaudez et al. (2000) reported wide variation in Cd sensitivity among isolates of different ECM species, with EC_{50} values of *Suillus luteus* isolates ranging from 0.04 to > 1 ppm. Several studies have compared the response of ECM isolates of the same species from contaminated and uncontaminated sites in an attempt to relate the degree of sensitivity to specific metals with the concentration of those metals in the soil of origin (Brown and Wilkins 1985; Denny and Wilkins 1987; Jones and Hutchinson 1988; Colpaert and Van Assche 1987, 1992; Egerton-Warburton and Griffin 1995; Blaudez et al. 2000; Colpaert et al. 2000). Blaudez et al. (2000) investigated the response of isolates of *Paxillus involutus*, *Pisolithus tinctorius*, *Suillus bovinus*, *S. variegatus*, and *S. luteus* to Cd, Co, Ni, and Zn and found no significant differences between EC_{50} values of isolates from different soil categories (low, medium, or high metal concentration). By contrast, however, Colpaert et al. (2000) reported that isolates of *S. luteus* from a site polluted with Zn and Cd were more tolerant to these metals than isolates from an unpolluted site. Therefore, it seems that tolerance to heavy metals in ECM fungi can be either constitutive or adaptive. The in vitro response to heavy metals in *C. geophilum* has been shown to vary among isolates (McCreight and Schroeder 1982; Thompson and Medve 1984; Tam 1995; Fomina et al. 2005), although isolates from contaminated and uncontaminated areas were, to our knowledge, never compared.

The genetic structure of *C. geophilum* might reflect its physiological diversity (Panaccione et al. 2001; Jany et al. 2002; Douhan and Rizzo 2005), with natural selection of genotypes adapted to particular soil conditions (Ennos and McConnell 1995). Previous studies using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis of rDNA loci and inter-simple sequence repeats (ISSR) revealed high genetic variability in *C. geophilum* isolates from serpentine and non-serpentine sites in Portugal, especially among isolates of different morphological types (Portugal et al. 2001; Portugal et al. 2004). However, these analyses failed to detect genetic divergence between serpentine and non-serpentine isolates. In this study, genetic diversity among these isolates was further investigated by amplified fragment length polymorphism (AFLP) analysis. The AFLP method is useful to search for genetic markers for adaptive traits because variability is assessed at a large number of independent loci and variations are revealed in any part of the genome (Vos et al. 1995; Majer et al. 1996; Bensch and Åkesson 2005). In a previous AFLP analysis of *C. geophilum*, isolates from serpentine and non-serpentine soils clustered separately (Panaccione et al. 2001). These authors suggested that the serpentine soil factors were responsible for the observed genetic divergence.

In this work, our objectives were to characterize *C. geophilum* serpentine and non-serpentine isolates using AFLP genetic markers and to investigate whether genetic diversity relates to in vitro Ni tolerance.

Materials and methods

Study sites

Isolates of *C. geophilum* were obtained in 1996 and 1997 from two sites approximately 40 km apart, one near to the village of Morais (39°42′N, 04°34′W) and a second site close to the village of Rabal (39°44′N, 04°06′W), both within a 20-km radius of the city of Bragança, northeast

Portugal (Table 1). In Morais, the soil is an orthi-eutric leptosol without a B horizon. It is derived from ultramafic rocks and has an intermediate texture between loam and silt loam (serpentine soil). The non-serpentine soil of Rabal is classified as an umbric leptosol, with an A horizon of 10 to 30 cm. It develops from schist and has a sandy loam texture (Agroconsultores and Coba 1991). Both sites are under Mediterranean-type climate, with a marked dry season in summer and precipitation occurring mainly from early autumn to mid spring. The vegetation encompasses sclerophyllous communities dominated by the evergreen oak species *Q. ilex* subsp. *ballota*.

Fungal material

At both sites, three samples of soil were collected at 1-m distant points from the main stem of five randomly selected *Q. ilex* trees. Samples were taken from the soil surface to a depth of 10–15 cm. On average, the distance between sampled trees was 10 m. The three samples from each tree were pooled together, placed in air-tight plastic bags, and kept at 4°C until processed.

Isolates of *C. geophilum* were obtained from sclerotia using a procedure adapted from Trappe (1969). Viable sclerotia (non-floating in water) were surface-sterilized in 3% (*w*/*v*) calcium hypochlorite for 15 min, rinsed in sterilized water, and individually transferred to fresh Potato Dextrose Agar (PDA; Difco, USA) medium. Mycelium arising from the sclerotia with the typical morphological characteristics of *C. geophilum* was sub-cultured in PDA. Many isolations were attempted but not many were successful: sclerotia isolations from the non-serpentine site yielded only two isolates in pure culture. Isolates were separated in different morphological types (Table 1), according to their macroscopical appearance. Characteristics such as the color of the colony, mycelium surface texture, margin appearance, and pattern of ramification were used.

Cultures were kept in PDA slants covered with the cryoprotector, 10% glycerol (*v*/*v*), in a deep freezer (−80°C), at the Department of Botany, University of Coimbra.

Table 1 Code and site of origin of *Cenococcum geophilum* isolates used in this study and Ni concentration in the soil samples from which sclerotia were collected

Code	Site of origin	Ni concentration (μg g^{-1})[a]
4,74CT1	Rabal (non-serpentine soil)	0.60
4,28CT5	Rabal	3.00
7,43MT5	Morais (serpentine soil)	4.80
2,17MT5	Morais	11.9
7,47MT5	Morais	11.9
5,37MT5	Morais	11.9
1,19MT9	Morais	14.6
7,15MT5	Morais	13.1
6,19MT5	Morais	14.6

The code of each isolate was assigned according to the following nomenclature: sclerotium reference number, Petri dish reference number, and tree reference number, from serpentine (*M*) or from non-serpentine or control areas (*C*), belonging to morphological type X (*T1* morphological type 1; *T5* morphological type 5; *T9* morphological type 9).

[a] Ammonium acetate extract

AFLP analysis

For each isolate, total DNA was extracted from fresh mycelium. This was performed in two separate occasions according to either Möller et al. (1992) or to the fungal and plant DNA extraction kit *nucleon® phytopure* (Amersham Pharmacia Biotech, UK). Extracted DNA was solubilized in ultrapure water and stored at −20°C until use. The AFLP analysis was performed using the procedure described by Gräser et al. (2000) with some modifications. Restriction fragments for amplification were generated in 40 μl reaction volumes. Genomic DNA (500 ng) was digested with 5 U each of *Eco*RI (Pharmacia Biotech, Sweden) and *Mse*I (New England Biolabs, Canada) at 37°C, during 3–5 h. Then, the ligation mixtures (10 μl) were prepared by adding 5 pM *Eco*RI and 50 pM *Mse*I adapters (Applied Biosystems, USA), 1 U of T4 DNA ligase, and 1× ligase buffer (Gibco, Germany). The ligation reactions were incubated at room temperature for 3 h. After ligation, the reaction mixtures were diluted 1:10. For the selective amplification of the *Eco*RI–*Mse*I fragments, we used the pair of primers *Eco*RI–TGC and *Mse*I–CTA (5′–GACTGCGTACCAATTCTGC–3′ and 5′–GATGAGTCCTGAGTAACTA–3′) (MWG Biotech AG, Ebersberg, Germany). The primer *Eco*RI–TGC had been labeled with 6-FAM (6-carboxifluorescein) on the 5′ end. The amplification reactions were performed in 25 μl reaction volumes, containing 8 μl of the 1:10 diluted ligation mixture as the template, 1× *Taq* buffer, 200 μM (each) dNTPs, 1.25 U of *Taq* polimerase (Pharmacia Biotech, USA), and 25 pmol of AFLP primers. Reactions were run in an Applied Biosystems thermocycler 9600 (Norwalk, USA) through 36 cycles as follows: denaturing at 94°C for 30 s, annealing for 30 s, and extension at 72°C for 1 min. The annealing temperature of 65°C in the first cycle was subsequently reduced by 0.7°C for each of the next 12 cycles and was kept at 56°C for the remaining 23 cycles. Fragment detection was done by capillary electrophoresis in an automated sequencer ABI Prism™ 310 (Applied Biosystems, USA), using the internal molecular weight marker ROX-500 (Corradini et al. 2002).

The presence/absence of polymorphic fragments with 30–500 base pairs (bp) was determined, and a genetic distance matrix was constructed using Nei's distance coefficient (Nei and Li 1979). A phenogram was constructed with the

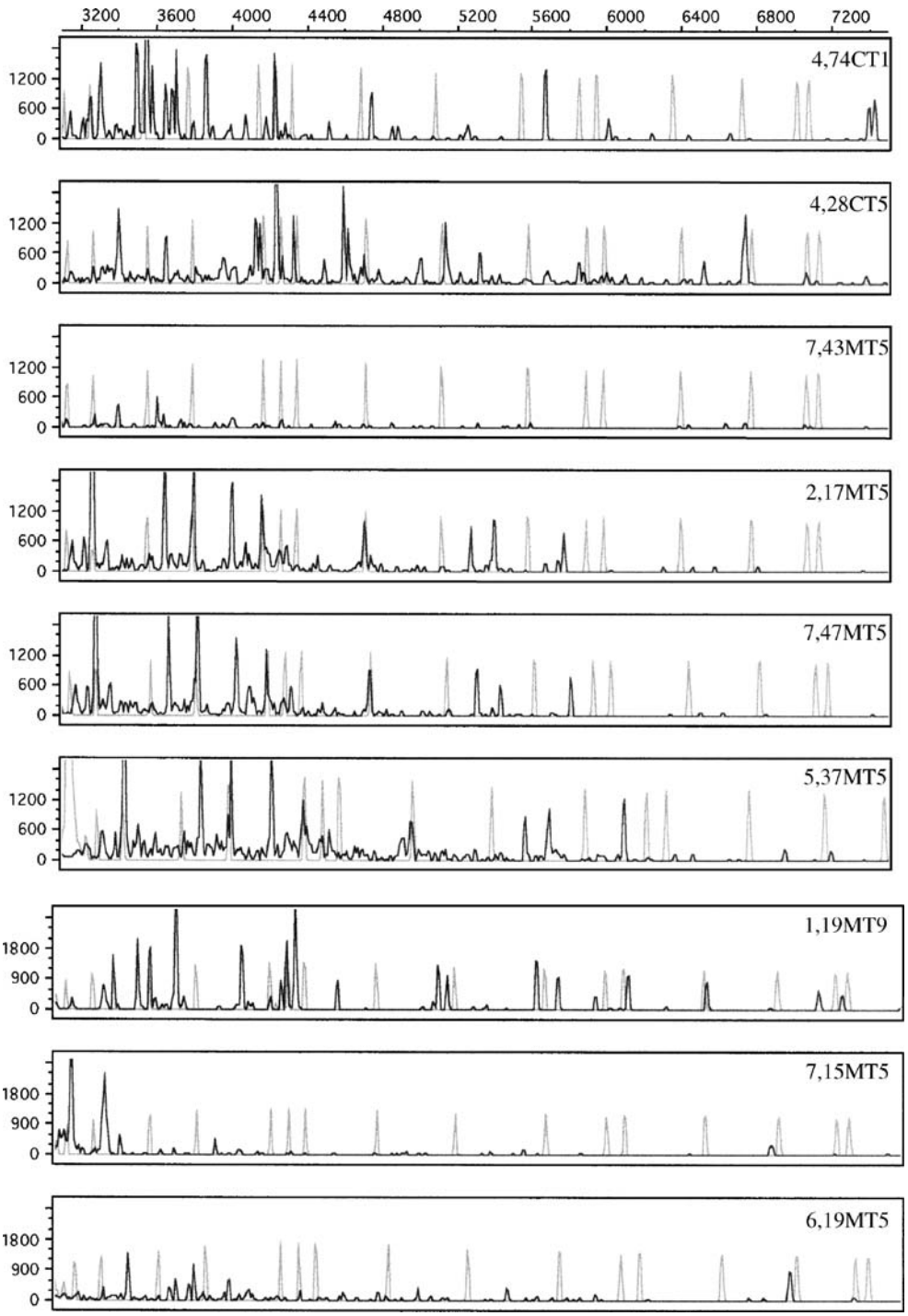

Fig. 1 AFLP electropherograms obtained with selective primers pair *Eco*RI–TGC and *Mse*I–CTA for *Cenococcum geophilum* isolates. The *X*-axis represents the time line, while the *Y*-axis represents the peak height. In all electropherograms, light gray color is attributed to internal molecular weight marker ROX–500 and dark gray color to the peaks that correspond to AFLP detected fragments

unweighted pair group method with arithmetic mean (UPGMA) algorithm in the PHYLIP software package, and the robustness of the phenogram topology was assessed by bootstrap analysis (Felsenstein 1993).

Ni sensitivity analysis

In vitro Ni sensitivity was assessed in five isolates of *C. geophilum* from the two studied sites, serpentine (isolates 2,17MT5, 1,19MT9, and 7,43MT5) and non-serpentine (isolates 4,74CT1and 4,28CT5) sites (Table 1). These were chosen as representative isolates of both sites and of the three recognized morphological types (T1, T5, and T9). Isolates were kept in culture medium without Ni for more than 3 years before the following assay.

Plugs (∅ 5 mm) were cut from the edges of actively-growing fungal colonies and placed on PDA test plates prepared with native soil water filtrate and amended with

Ni as $NiSO_4 \cdot 6H_2O$ at 0, 5, 10, 15, and 30 μg g^{-1} Ni (final pH 5.5). Plates were incubated in the dark, at room temperature (approximately 21°C) for 8 weeks. At the end of weeks 4 and 6, colony diameters (means of two perpendicular measurements) were recorded, and the radial growth rate for each colony during this 2-week period was determined. After incubation, mycelia were harvested to determine their biomass. Agar was removed according to Colpaert et al. (2000), and the colonies were dried to a constant mass at 60°C and weighed. A tolerance index (TI) for both radial growth rate (mm/week) and final biomass (mg) was calculated as the percentage of the radial growth rate (or biomass) retained on the Ni-amended media compared with performance on the control medium (Colpaert and Van Assche 1987).

Eight replicates were started for each isolate-treatment combination. However, some were lost due to contamination, and this resulted in unbalanced sample size. Therefore, a conservative statistical approach was adopted. For each isolate, differences in radial growth rate and biomass yield between Ni treatments and control treatment (no Ni added) were analyzed by non-parametric analysis of variance (Kruskal–Wallis test) followed by a Dunn's post-hoc test at $P<0.05$. The analyses were performed using SigmaStat statistical package 3.0.1 (SPSS 2003).

Ni quantification

Nabais (2000) quantified the Ni concentration (ammonium acetate extracts) in the soil samples from which *C. geophilum* isolates were obtained. Values in the serpentine samples ranged between 4.80 and 14.6 μg g^{-1} Ni, whereas in the non-serpentine soil samples, the concentration was significantly lower ($t_{0.05\ (2),\ 4}=2.785$, $P=0.05$) and varied between 0.60 and 3.00 μg g^{-1} Ni (Table 1).

At the end of the Ni-sensitivity assay, we quantified the total Ni concentration in the mycelium of each isolate–Ni combination at 5, 10, and 30 μg g^{-1} Ni levels. Because of the low amount of mycelium per replicate, a composite sample was used for each isolate–Ni combination. Dried mycelia (approximately 500 mg) were ground in liquid nitrogen. The resulting powder was combusted for 6 h in a muffle furnace at 600°C, and the ash was dissolved in 2 ml 0.5 M HCl (Colpaert et al. 2000). Samples and blanks (with acid only) were further diluted with ultrapure water up to 10 ml and analyzed by atomic spectroscopy (Perkin Elmer Aanalyst 100, USA).

Results

AFLP analysis

Electropherograms of all isolates exhibited clear and easily identifiable peaks, corresponding to different DNA fragments (Fig. 1). Duplicate DNA extractions of the same isolate yielded consistent results. The size of the 122 identified fragments ranged from 30 to 500 bp. Only isolates 4,74CT1 and 1,19MT9 had peaks that corresponded to fragments slightly greater than 500 bp (two in isolate 4,74CT1 and one in isolate 1,19MT9). Each isolate displayed a distinct AFLP pattern, resulting in a proportion of distinguishable genets (PD)=1.00. Thirteen fragments (approximately 10.7%) were exclusive of control (non-serpentine) isolates. On the other hand, some fragments were only present in serpentine isolates, e.g., one fragment of 84 bp. Approximately 14.9% of all fragments were common to the three 7M serpentine isolates (2,17MT5, 7,47MT5, and 5,37MT5).

Genetic distance between every pair of isolates was calculated using the Nei's index (Table 2). Distance coefficients varied between 0.13 and 0.85. The phenogram separated the non-serpentine isolate 4,28CT5 from all the others. The remaining isolates clustered in two groups: one that included isolates 2,17MT5, 7,47MT5, and 5,37MT5 (7M isolates), and a second group that included isolates 4,74CT1, 1,19MT9, and the remaining isolates with type 5 morphology: 6,19MT5, 7,43MT5, and 7,15MT5 (Fig. 2).

Table 2 Distance matrix calculated with Nei's coefficient based on AFLP fragments obtained with selective primers pair *Eco*RI–TGC and *Mse*I–CTA

	4,74CT1	4,28CT5	7,43MT5	2,17MT5	7,47MT5	5,37MT5	1,19MT9	7,15MT5
4,28CT5	0.71							
7,43MT5	0.44	0.8						
2,17MT5	0.68	0.85	0.69					
7,47MT5	0.63	0.8	0.56	0.13				
5,37MT5	0.87	0.73	0.98	0.34	0.4			
1,19MT9	0.49	0.73	0.35	0.69	0.58	0.85		
7,15MT5	0.41	0.8	0.14	0.66	0.53	0.85	0.4	
6,19MT5	0.61	0.68	0.39	0.74	0.63	0.73	0.54	0.34

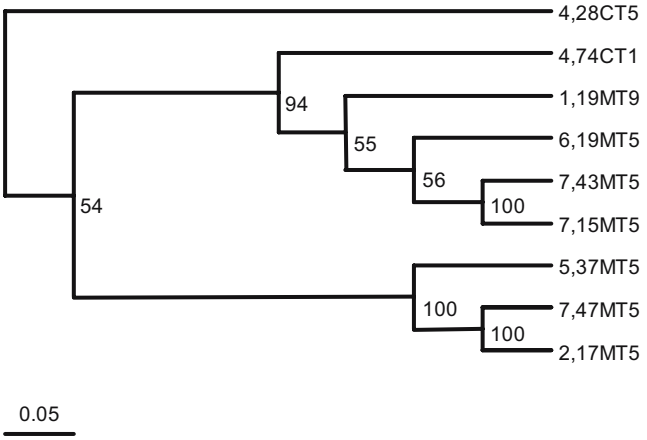

Fig. 2 Bootstrap consensus UPGMA tree obtained for AFLP detected fragments with selective primers pair *Eco*RI–TGC and *Mse*I–CTA of the *Cenococcum geophilum* isolates (100 replicates)

Within this group, the three morphological types (T1, T5, and T9) were separated, and moreover, the isolate 4,74CT1 (non-serpentine) diverged from the others (Fig. 2). The phenogram showed a robust topology as demonstrated by bootstrap values, always greater than 50% and in three of seven cases equal to 100% (Fig. 2).

Ni sensitivity and accumulation

Isolate 4,74CT1 failed to grow under the experimental conditions of the Ni sensitivity assay, and therefore, no results on this isolate are reported. A large heterogeneity was observed in radial growth rate and biomass yield among isolates (Table 3). In control medium (no Ni added), radial growth rate ranged between 0.20 mm/week in isolate 1,19MT5 and 0.60 mm/week in isolate 7,43MT5; values of biomass after 8 weeks varied between 164.3 and 299.4 mg in isolates 2,17MT5 and 7,43MT5, respectively.

Sensitivity to Ni varied among isolates: the non-serpentine isolate 4,28CT5 was the only isolate whose growth was significantly affected by the addition of Ni to the culture medium at the concentrations tested (Table 3, Fig. 3a,b). Radial growth rate decreased significantly at 20 and 30 μg g^{-1} Ni ($H=31.62$, $P<0.01$), while final biomass was significantly reduced at 30 μg g^{-1} Ni ($H=18.42$, $P<0.01$; Table 3). The addition of 20 μg g^{-1} Ni resulted in a 21% inhibition of radial growth rate in this isolate, as compared to the control treatment, whereas at 30 μg g^{-1} Ni, the inhibitory effect was even stronger, with growth approximately 26% lower than control (Table 3, Fig. 3a). Biomass was reduced from 260.6 mg in the control treatment to 186.5 mg at 30 μg g^{-1} Ni, a 28% inhibition (Table 3, Fig. 3b). In serpentine isolates, neither radial growth rate nor biomass yield was significantly affected by Ni addition ($P>0.05$; Table 3). Results were variable, with slight inhibition or stimulus of growth occurring over the whole range of concentrations tested (Table 3, Fig. 3a,b). The isolate that performed best was 7,43MT5. At the highest Ni amendment (30 μg g^{-1}), TI values of this isolate were 105.5 and 117.3% for radial growth rate and biomass yield, respectively (Fig. 3a,b).

The Ni concentration in the mycelia increased with increasing Ni concentration in the growth medium, but Ni accumulation was more pronounced in serpentine isolate 1,19MT9 (Fig. 4). From the 5 to the 30 μg g^{-1} Ni treatment, the concentration of Ni in the mycelium of this isolate showed an 11-fold increase, from 0.21 to 2.32 mg g^{-1} Ni DW (Fig. 4). Isolates 2,17MT5, 7,43MT5 (serpentine), and isolate 4,28CT5 (non-serpentine) accumulated less Ni. At 30 μg g^{-1} Ni, the concentration of Ni in the mycelium of these isolates ranged between 0.58 and 1.21 mg g^{-1} DW, about twice the concentration values at 5 μg g^{-1} Ni (Fig. 4).

Table 3 Radial growth rate (mm/week) and biomass yield (mg) of *Cenococcum geophilum* isolates

	Isolates	Ni amendment (μg g^{-1})[a]					
		0	5	10	15	20	30
Radial growth rate[b]	4,28CT5	0.38±0.02(6)[c]	0.38±0.01(8)	0.38±0.01(6)	0.34±0.01(8)	0.30±0.01*(8)	0.28±0.01**(8)
	2,17MT5	0.40±0.06(5)	0.33±0.02(8)	0.38±0.06(5)	0.31±0.03(5)	0.35±0.02(4)	0.29±0.04(5)
	1,19MT9	0.20±0.00(8)	0.18±0.00(7)	0.20±0.00(8)	0.20±0.00(8)	0.19±0.01(7)	0.19±0.00(8)
	7,43MT5	0.60±0.01(8)	0.59±0.02(8)	0.60±0.01(8)	0.62±0.01(8)	0.61±0.01(8)	0.63±0.01(8)
Biomass	4,28CT5	260.6±4.94(4)	248.5±11.4(8)	257.0±8.86(6)	245.5±10.4(8)	226.7±8.12(6)	186.5±7.80**(6)
	2,17MT5	164.3±47.8(2)	209.7±10.7(8)	231.0±7.32(4)	199.4±23.3(4)	164.8±30.0(4)	151.1±66.9(3)
	1,19MT9	167.5±1.95(6)	198.8±16.7(7)	175.7±3.40(8)	172.4±6.53(8)	171.5±13.7(7)	182.9±12.1(6)
	7,43MT5	299.4±12.7(6)	314.6±12.4(8)	312.1±13.4(8)	354.0±29.0(8)	332.9±6.69(8)	351.1±7.59(6)

Values are means±SE of 2 - 8 replicates.
[a] Mole equivalent, 1 μg g^{-1} Ni=17.0 μM
[b] Calculated between weeks 4 and 6
[c] Number of replicates
Significance levels between Ni treatment and control: *$P<0.05$; **$P<0.01$

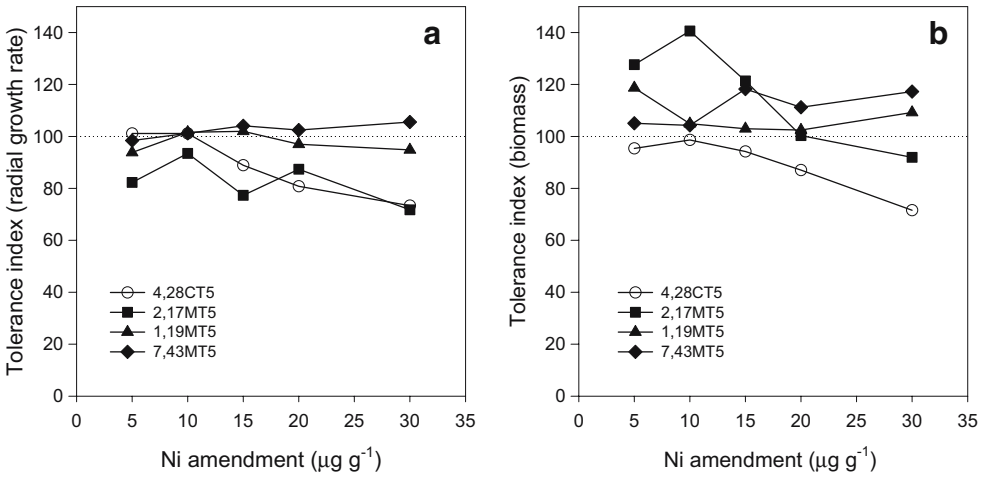

Fig. 3 Nickel tolerance indices (TI, %) of *Cenococcum geophilum* isolates exposed to increasing Ni concentrations (5, 10, and 30 µg g^{-1}) in the growth medium; **a** TI based on radial growth rate (measured between weeks 4 and 6) and **b** TI based on biomass yield after 8 weeks of growth

Discussion

Using AFLP analysis, we observed a high genetic diversity in *C. geophilum* isolates. Furthermore, the AFLP profiles made possible to discriminate 100% of the isolates (Fig. 1). Other authors using the same selective primer pair (*Eco*RI-TGC and *Mse*I–CTA) in *Trichophyton* spp., also in the Ascomycetes, obtained a PD value of only 0.13 (Gräser et al. 2000), indicating that this primer combination was appropriate for detecting polymorphism in *C. geophilum*. Although unexpected, as this species has no known sexual stage (LoBuglio 1999), this result is consistent with recent analysis of *C. geophilum* populations. In the study by Panaccione et al. (2001), AFLP profiling of *C. geophilum* detected 12 genotypes out of 13 isolates. Other authors, using other molecular markers, also obtained a high genetic diversity within and between populations of *C. geophilum* (Jany et al. 2002; LoBuglio and Taylor 2002; Douhan and Rizzo 2005).

A previous ISSR analysis discriminated the *C. geophilum* isolates used in this work according to their morphological type (Portugal et al. 2004). The AFLP profiling, however, did not confirm that result because isolates from the three recognized morphological types (T1, T5, and T9) grouped in the same cluster. According to our AFLP data, only the non-serpentine isolate 4,28CT5 was separated from the remaining isolates. The other non-serpentine isolate, 4,74CT1, grouped together with serpentine isolates in a sub-cluster, although it diverged from them in that particular group (Fig. 2). In the study by Panaccione et al. (2001), *C. geophilum* isolates from serpentine and non-serpentine soils grouped in distinct clusters, but in this study, a genetic separation between serpentine and non-serpentine isolates was not observed. However, the low number of non-serpentine isolates in this study hindered the development of a robust analysis of the genetic structure of *C. geophilum* in the area.

The four isolates of *C. geophilum* in the in vitro Ni tolerance screening were all able to grow at the highest amendment of Ni, although serpentine isolates performed better than the non-serpentine isolate. Moreover, TI values were higher than 70% even at the maximum Ni level, which means that EC_{50} values of Ni among these isolates were beyond the range of concentrations tested (Fig. 3a,b). Comparisons of metal sensitivity of ECM reported by different studies can be misleading because of specific experimental conditions. Nevertheless, at the concentration range we used, Ni has been reported to significantly inhibit the growth of several ECM fungal species. For example, *Lactarius hibbardae* and *Scleroderma flavidum* were among the ECM species reported by Jones and Hutchinson (1988) whose growth was significantly inhibited at 25 µg g^{-1} Ni. In the work by Blaudez et al. (2000), isolates of *Suillus bovinus*, *S. luteus*, and *S. variegatus* were strongly inhibited by Ni; most of the isolates did not grow at all at

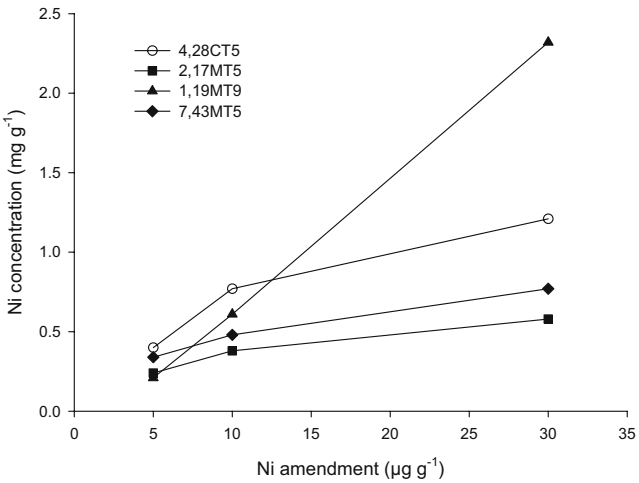

Fig. 4 Nickel concentrations in mycelia of *Cenococcum geophilum* isolates exposed to increasing Ni concentrations (5, 10, and 30 µg g^{-1}) in the growth medium for 8 weeks

50 μg g^{-1} Ni. In addition, a highly significant inhibition of growth (measured as biomass inhibition) was observed in many isolates of *Paxillus involutus* and *Pisolithus tinctorius* at this Ni concentration (Blaudez et al. 2000). Furthermore, *C. geophilum* isolates from our study seem to be less sensitive to Ni than previously reported for this species. In fact, an EC_{50} value of 1 μg g^{-1} Ni was established for a *C. geophilum* isolate by Tam (1995), while McCreight and Schroeder (1982) reported arrest of growth at 12–14 μg g^{-1} Ni for a *C. geophilum* isolate included in their study. Our results, thus, further illustrate the enormous variation in sensitivity to heavy metals in ECM fungi.

In our study, in vitro inhibitory effects of Ni were only observed in the non-serpentine isolate 4,28CT5 (Table 3, Fig. 3a,b). Both radial growth rate and biomass yield of this isolate were significantly inhibited. Radial growth was more sensitive to Ni than biomass yield, in agreement with previous results (Darlington and Rauser 1988; Colpaert et al. 2000). At 30 μg g^{-1} Ni, values for radial growth rate and biomass decreased to 73.3 and 71.6% of control, respectively, a highly significant inhibitory effect ($P<0.01$). Nickel, at this concentration, had no significant inhibitory effect on serpentine isolates (Table 3, Fig. 3a,b). As maximum values of available Ni in serpentine soils found by Nabais (2000) were approximately 29 μg g^{-1}, the fitness of serpentine isolates, as evaluated by radial growth rate and biomass yield, is likely unaffected by Ni in the field. Quite the contrary, performance of one serpentine isolate (7,43MT5) was stimulated by Ni at this concentration. Radial growth rate was slightly enhanced (TI=105.5%), and a similar trend was observed for biomass yield, which reached 117.3% of control at 30 μg g^{-1} Ni.

Distinct Ni accumulation patterns could be identified towards Ni exposure (Fig. 4). However, in contrast to previous reports (Colpaert et al. 2005), these patterns did not reflect the results on Ni sensitivity, i.e., the non-serpentine and Ni-susceptible isolate 4,28CT5 cannot be distinguished from the Ni-insensitive serpentine isolates on the basis of the Ni accumulation profiles. There are various mechanisms involved in metal tolerance in ECM fungi (Bellion et al. 2006) and that have been described in Ni-resistant mutants of yeasts and filamentous fungi (Joho et al. 1995). In this work, two profiles of Ni accumulation emerged among our Ni-insensitive serpentine isolates, suggesting that different mechanisms may be operating. At the range of concentrations tested (5–30 μg g^{-1} Ni), Ni concentration in the mycelium of the serpentine isolate 1,19MT9 increased proportionally with the increase of Ni in the growth medium (Fig. 4). This linear trend of Ni accumulation precludes Ni chelation by substances excreted by the fungus, e.g., organic acids, as one of the mechanisms involved in the maintenance of Ni homeostasis in this isolate (Ahonen-Jonnarth et al. 2000; Fomina et al. 2005). In this isolate, the Ni accumulation level (2.32 mg g^{-1} DW) at the highest Ni exposure far exceeds the threshold of hyperaccumulation of Ni as defined for higher plants (1,000 μg g^{-1} DW; Reeves and Baker 2000). The amino acid histidine has been identified as a strong ligand of Ni in hyperaccumulating plants (Krämer et al. 1996) and has also been implicated in the accumulation of Ni^{2+} ions in the vacuole of a Ni-resistant strain of *Saccharomyces cerevisae* (Joho et al. 1990). However, histidine was not detected in the mycelium of isolate 1,19MT9 after exposure to 50 μg ml^{-1} Ni in liquid culture (S.C. Gonçalves and M. Chalot, unpublished results). Other organic compounds could account for metal chelation inside the hyphae, e.g., metallothioneins and the non-protein thiol glutathione (Courbot et al. 2004; Bellion et al. 2007). In this study, the method used to determine the Ni concentration in the mycelia does not discriminate between Ni in cell walls and Ni inside the hyphae. Therefore, it is also possible that a large fraction of the Ni is binding to the cell wall, a mechanism that could contribute to metal tolerance (Meharg 2003). Although remarkably high, the levels of Ni accumulation observed in isolate 1,19MT9 must be interpreted with caution because this result derives from the analysis of only one composite sample. Nickel accumulation in the remaining isolates, including the non-serpentine and Ni-susceptible isolate 4,28CT5, was less pronounced and seems to approach a plateau at 30 μg g^{-1} Ni (Fig. 4). This result suggests the involvement of a metabolic-dependent detoxification mechanism although in isolate 4,28CT5 toxic effects of Ni were observed. Successful Ni detoxification pathways in isolates 2,17MT5 and 7,34MT5 may include decreased Ni influx, probably by modification of a Mg transport system (Joho et al. 1991), enhanced Ni efflux as found in the bacterium *Alcaligenes eutrophus* (Siddiqui and Schlegel 1987) or overexcretion of organic acids for extracellular Ni chelation as previously observed in higher plants (Yang et al. 1997).

The only Ni-susceptible isolate in our study was the non-serpentine isolate 4,28CT5 (Table 3, Fig. 3a,b). This isolate also clustered separately from the other isolates in the AFLP profiling (Fig. 2), suggesting a genetic basis for this physiological trait. This is further supported by the fact that all isolates were sub-cultured in growth medium without Ni for more than 3 years before the assay, and therefore, physiological adaptation is unlikely. All serpentine isolates screened for in vitro Ni sensitivity revealed to be Ni-insensitive at the range of concentrations tested (Table 3, Fig. 3a,b). Certainly, these isolates must be subjected to strong selective pressures in serpentine soils for increased fitness in this multi-stressed environment (Proctor 1999). However, we cannot conclude that Ni insensitivity among our serpentine isolates is due to adaptive evolution because results from AFLP profiling did not distinguish between serpentine and non-serpentine isolates of *C. geophilum*

(Fig. 2). Although low sensitivity to Ni among serpentine isolates is an indication of decreased Ni sensitivity in serpentine populations of *C. geophilum*, it may simply reflect constitutive variation in the response to Ni within this fungal species (Denny and Wilkins 1987; Blaudez et al. 2000). If we accept that sensitivity to Ni varies widely in *C. geophilum*, the differences we observed may only be due to the low number of isolates tested. Further studies with a larger number of isolates are needed to verify possible relationships between phenotypic diversity (morphological and physiological) and genetic variability within this species. Understanding the relationship between genetic diversity and functional traits in *C. geophilum* could provide important insights into the significance of intraspecific diversity in ECM species.

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