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SHORT NOTE

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Influence of pH on copper and zinc sensitivity of ericoid mycobionts in vitro

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Abstract The effect of pH on growth, metal uptake and toxicity in four isolates of ericoid mycobionts (two *Hymenoscyphus ericae* from unpolluted heathland sites and two *H. ericae*-type mycobionts from metal-contaminated mine spoil) was assessed in vitro. These isolates were incubated in liquid medium (10% Rorison's medium, glucose at 10 g l^{-1}) containing either 0.25 mM Cu or 2.0 mM Zn and adjusted to pH 2, 3, 4, 5 or 6. After 30 days incubation, dry mass and mycelial metal content were determined and growth was expressed as tolerance index, i.e. dry mass in the presence of metal as a percentage of dry mass in the absence of metal. Initial medium pH had a significant effect on both tolerance index and metal accumulation. Tolerance indices were highest at pH 2, with several isolates showing a stimulation of growth (i.e. tolerance index >100%) at this pH. Tolerance index decreased at higher initial pH values and growth of two mycobionts was completely inhibited (tolerance index=0) in the Cu-supplemented media at pH 6. Reduction in tolerance index coincided with an increase in mycelial accumulation of Cu and Zn. Practical and environmental implications of these results are discussed.

Keywords pH · Ericoid mycobionts · Mine spoil · Copper · Zinc · Metal sensitivity

Introduction

Ericoid mycobionts are considered to be adapted to acidic metal-contaminated sites such as disused mine sites by having a low heavy metal sensitivity (Bradley et al. 1982; Denny and Ridge 1995; Martino et al. 2000b; Cairney et al. 2001; Sharples et al. 2001) and possessing mechanisms that allow them to utilize nutrients under these conditions (Martino et al. 2000a, 2003; Gibson and Mitchell 2004, 2005). The bioavailability and consequent fungitoxicity of heavy metals are affected by the pH status of the growth media (Starkey 1973; Babich and Stotzky 1977; Gadd and Griffiths 1980; Gadd and White 1985; Jongbloed and Borst-Pauwels 1992). Despite this, the effect of pH on the interaction of heavy metals with ericoid mycorrhizal fungi has only rarely been considered (Martino et al. 2003) and assessment of metal sensitivity is routinely carried out at pH values greater than those encountered by these organisms in their typical habitats. In order to determine the effect of pH on metal uptake and toxicity to mycobionts in vitro, four ericoid mycobionts were grown in liquid media adjusted to a range of different pH values (2–6, encompassing those typical of acid heathland soils, i.e. pH <4 and acidic mine spoils, i.e. pH <3) and supplemented with either Cu or Zn.

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Materials and methods

Ericoid mycobionts

The fungi included two isolates of *Hymenoscyphus ericae* (Read) Korf and Kernan (*H. ericae* A and *H. ericae* 101) and two sterile, slow-growing, dark septate ericoid mycobionts (Avoca and Parys mycobionts) isolated from root systems of seedlings of *Calluna vulgaris* (L.) Hull growing on mine spoil at two abandoned mine sites, Avoca Copper Mines (County Wicklow, Ireland; Nat. Grid Ref. T190812) and Parys Mountain Copper Mines (Anglesey, UK; Nat. Grid Ref. SH440903), respectively. The culture of *H. ericae* A is a single ascospore isolate. The two mine isolates (Avoca and Parys mycobionts) were observed to have similar cultural characteristics on 5% malt extract agar (MEA) as the two *H. ericae* isolates, including a tendency of hyphae to segment under low nutrient conditions to form chains of arthroconidia. All isolates had the ability to form typical ericoid mycorrhizas with rooted cuttings of *C. vulgaris*. Preliminary PCR of the ITS region using ITS1 and ITS4 primers have revealed the presence of bands of identical size in *H. ericae* 101, Parys mycobiont and Avoca mycobiont. The cultures have been deposited in the genetic resource collection of CABI Bioscience (IMI, Egham, UK). The accession codes for *H. ericae* A, *H. ericae* 101, Avoca mycobiont and Parys mycobiont are IMI392237, IMI392238, IMI392239 and IMI392236, respectively.

Effect of initial pH on growth of ericoid mycobionts exposed to copper or zinc

Rorison's solution (10%) contained the following components (in mg l^{-1}): $CaSO_4 \cdot 2H_2O$, 34.4; $MgSO_4 \cdot 7H_2O$, 24.6; K_2HPO_4, 17.4; Fe-EDTA, 2.5; $MnSO_4 \cdot 4H_2O$, 0.2; H_3BO_3, 0.3; $ZnSO_4 \cdot 7H_2O$, 0.04; $CuSO_4 \cdot 5H_2O$, 0.04 (Hewitt 1966); and glucose (10 g l^{-1}). The media were supplemented with either 0.25 mM Cu or 2 mM Zn. Previous studies have shown that growth of the four ericoid mycobionts was reduced at these concentrations of Cu and Zn at pH 4.5 (Gibson 2004). Unamended controls were also prepared without elevated Cu and Zn. Alanine (sole N source) was sterilized by passing through a sterile 0.2-μm filter (Pall, USA) and added to the autoclaved media at ca. 50°C at a nitrogen concentration equivalent to that of 10% Rorison's medium. Alanine, rather than ammonium, was chosen as a suitable N source for this experiment because ammonium assimilation can have a marked effect on medium pH (Roos and Luchner 1984). pH was adjusted to 2, 3, 4, 5 or 6 with sterile 1 M NaOH or 1 M HCl. Medium (20 ml) was transferred to 9.0-cm-diameter Petri dishes and each dish was inoculated with a 6-mm mycelial plug removed from the edge of an actively growing colony of each isolate on 5% MEA. Each culture was harvested after 30 days incubation stationary in the dark at 20°C and dry mass determined after oven drying at 80°C for 24 h. The results were expressed as tolerance indices, i.e. dry mass of each ericoid mycobiont in metal-supplemented media expressed as a percentage of dry mass in a control medium (standard 10% Rorison's medium containing alanine as N source and glucose at 10 g l^{-1} as C source). pH of media was determined at time zero and after 30 days incubation using a calibrated Orion 420A pH meter.

Determination of Cu and Zn contents of mycelia

Fungal mycelia were digested in Teflon bombs (15 ml volume) containing 2 ml concentrated acid mixture (HNO_3: HCl; 4:1). The Teflon bombs were incubated at 140°C for 7 h (Otte et al. 1995). The bombs were then allowed to cool and the digested samples were diluted to 10 ml with double deionised water and filtered through 0.45-μm filter papers (Gelman, Michigan). Samples were analysed for Cu and Zn in a flame atomic absorption spectrophotometer (Solar Unicam 929, UK) after the appropriate dilution.

Statistical analysis

Analysis of variance (ANOVA) was carried out using StatView statistics package for windows (SAS Institute Inc., Cary, NC). Comparisons between means of samples (n=3) were undertaken using one-way ANOVA. Data showing significant differences ($P \leq 0.05$) were subjected to Fisher's protected least significant difference (PLSD) multivariate test.

Results

Changes in pH of the media after 30 days incubation

The final pH values of the media, adjusted initially to pH 2, were unchanged, whereas a negligible reduction in final pH of the media, initially adjusted to pH 3, was observed (Table 1). The final pH values of the remaining media varied, ranging from 3.3 to 3.7 except in the Cu-supplemented medium at the initial pH of 6, in which the reduction in pH was less pronounced (pH 4.8–5.0) due to the poor growth of the ericoid mycobionts.

Table 1 Final pH of the medium after growth of ericoid mycobionts for 30 days in 10% Rorison's liquid medium adjusted to different initial pH levels and supplemented with 0.25 mM Cu or 2.0 mM Zn

Mycobionts	Initial pH	Final pH of medium after 30 days		
		Control	+ Cu	+ Zn
H. ericae A	2.0	2.0±0.01 b	2.1±0.01 a	2.0±0.00 b
	3.0	2.9±0.02 a	2.9±0.00 a	2.9±0.01 a
	4.0	3.6±0.04 a	3.5±0.04 ab	3.4±0.01 b
	5.0	3.7±0.02 b	3.9±0.01 a	3.6±0.06 c
	6.0	3.7±0.02 b	4.9±0.13 a	3.6±0.02 c
H. ericae 101	2.0	1.9±0.00 b	2.0±0.00 a	2.0±0.00 a
	3.0	2.9±0.00 a	2.9±0.01 a	2.9±0.01 a
	4.0	3.5±0.01 a	3.5±0.02 a	3.5±0.01 a
	5.0	3.6±0.02 b	3.7±0.04 a	3.7±0.03 a
	6.0	3.6±0.02 c	5.0±0.07 a	3.7±0.02 b
Parys mycobiont	2.0	1.9±0.01 b	2.0±0.00 a	2.0±0.00 a
	3.0	2.9±0.01 a	2.9±0.00 a	2.9±0.01 a
	4.0	3.5±0.01 a	3.5±0.03 a	3.4±0.03 b
	5.0	3.7±0.05 a	3.7±0.08 a	3.4±0.14 a
	6.0	3.7±0.05 b	4.8±0.18 a	3.6±0.02 b
Avoca mycobiont	2.0	2.0±0.00 a	2.0±0.00 a	2.0±0.01 a
	3.0	2.9±0.00 a	2.9±0.01 a	2.9±0.03 b
	4.0	3.5±0.02 a	3.4±0.03 b	3.3±0.03 c
	5.0	3.5±0.05 a	3.5±0.03 a	3.4±0.07 a
	6.0	3.6±0.04 b	5.0±0.14 a	3.4±0.02 c

Each value represents the mean of three replicates ±SE. One-way ANOVA was carried out and values (along each row) sharing the same letter are not significantly different at the 5% level as determined by Fisher's PLSD test

Effect of initial pH on tolerance indices of ericoid mycobionts to elevated copper or zinc

A comparison of the growth of the ericoid mycobionts on the control media (without elevated Cu or Zn), showed that *H. ericae* A and *H. ericae* 101 had optimum growth at pH 4, whereas growth of the Avoca and Parys Mountain mycobionts was unaffected by the initial pH of the medium (data not shown). Response of the ericoid mycobionts to the presence of elevated Cu and Zn in the media was dependent on the initial pH of the medium. Stimulation of growth, i.e. with a tolerance index >100%, of all ericoid mycobionts was observed at pH 2 in the Cu-supplemented medium and at pH 3 for *H. ericae* A (Fig. 1). Only the Parys Mountain and Avoca mycobionts had a tolerance index greater than 100% on Zn-amended media, and only at pH 2 (Fig. 1). Tolerance index decreased with increasing pH of the Cu- and Zn-supplemented media and all ericoid mycobionts showed a low or negligible tolerance index at pH 6 (Fig. 1).

Effect of initial pH on metal accumulation in mycelia of ericoid mycobionts at elevated copper or zinc

Copper accumulation in mycelium of *H. ericae* A and the Avoca mycobiont could not be determined at pH 6 due to the low mycelial yield at this pH. In both Cu- and Zn-supplemented media, an increase in metal accumulation was observed as initial pH in the medium was raised, although accumulation of Zn by *H. ericae* 101 appeared to be unaffected by changes in initial pH (Fig. 1). Cu accumulation at pH 2 was negligible. At pH 6, either Cu or Zn increased to a concentration of 6–8 mg g^{-1} (Fig. 1).

Discussion

The ericoid mycobionts' responses to Cu and Zn were significantly affected by the initial pH of the medium. Tolerance indices of all mycobionts decreased significantly as initial pH of the medium increased from 2 to 6. At an initial pH of 2, a relative stimulation of growth was observed in all mycobionts growing in the Cu-supplemented medium, but only the two mine site mycobionts showed a high tolerance on Zn-amended media at pH 2. The decline in growth was associated with an increase in Cu or Zn accumulation in the mycelium, and the ericoid mycobionts that accumulated the most Cu and Zn were those in which growth was most reduced. These results are in contrast to those of another study in which isolates of *H. ericae* that accumulated the most Zn were found to be the most tolerant to this metal (Denny and Ridge 1995).

Previous studies have reported an increased sensitivity of fungi to heavy metals at higher pH. A relative increase in Cu sensitivity of *Scytalidium* sp. was demonstrated as initial pH was raised from 2 to 7 and this was particularly evident in media containing ammonium as the sole N source (Starkey 1973). Sensitivity of *Aureobasidium pullulans* to 4 mM Cu was also found to be greater at pH 4.5 than at 3.2 (Gadd and Griffiths 1980). Likewise, a decrease in dry mass of *Aspergillus niger*, *Rhizopus stolonifer* and *Trichoderma viride* occurred in the presence of Cd (10 μg ml^{-1}) in media

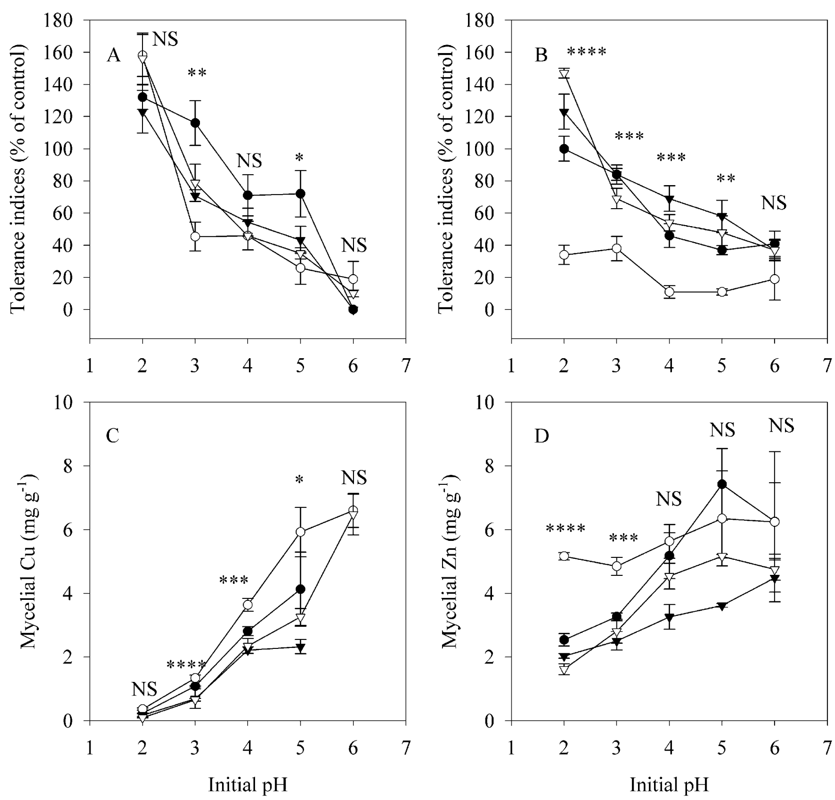

Fig. 1 Tolerance indices (based on biomass yields) and mycelial copper and zinc accumulation of *H. ericae* A, *H. ericae* 101, Avoca mycobiont and Parys mycobiont grown for 30 days in 10% Rorison's liquid medium supplemented with 0.25 mM Cu (**a** and **c**) or 2.0 mM Zn (**b** and **d**) and adjusted to pH 2, 3, 4, 5 or 6 before inoculation. One-way ANOVA (n=3) was carried out at each pH and *, **, *** and **** denote significant differences at the 5, 1, 0.1 and 0.01% level, respectively. NS denotes not significant. –•– *H.ericae* A, –○– *H. ericae* 101, –▾– Avoca mycobiont, –▿– Parys mycobiont

adjusted to higher pH values (Babich and Stotzky 1977). Also, *Penicillium ochro-chloron* had a greater intracellular Cu accumulation at higher pH levels (Gadd and White 1985). A rise in pH from 4 to 6.5 was shown to increase the Cd, Cu and Zn uptake by *Rhizopus arrhizus* and *Trichoderma viride* (Morley et al. 1996).

In vitro assessment of metal sensitivity is complicated by the numerous factors that are known to influence the bioavailability of metals in growth media. Such factors include the presence of inorganic anions such as phosphates and organic ligands such as peptone and yeast extract, carbon content, Eh and pH (Gadd 1993; Fomina et al. 2003). Any drop in pH within the medium may have been due to the release of organic acids, as opposed to proton extrusion, which is associated with ammonium uptake as demonstrated by Roos and Luchner (1984). pH may affect bioavailability of a metal by altering its speciation, i.e. change from the free cation form (M^{2+}) to an hydroxylated cation (MOH^+) or anion $[M(OH_4)^{2-}]$ form at higher pH values, and such hydroxylated species may be more likely to bind to fungal cell walls (Collins and Stotzky 1992). Under more acidic conditions, metal cations will be in competition with protons for adsorption and transport sites on the fungal cell wall and may reduce the negative surface potential of the wall. This has been suggested by Jongbloed and Borst-Pauwels (1992) to explain a reduced uptake of K^+ by the ectomycorrhizal fungi *Laccaria bicolor*, *Lactarius rufus* and *Lactarius hepaticus* as medium pH was reduced from 5 to 3.

Other investigations on metal tolerance of ericoid mycorrhizal fungi have invariably involved liquid media adjusted to pH values of 5 or greater. However, ericoid mycobionts are rarely found in habitats with such high pH and those mycobionts that occur on acidic mine spoils may experience pH values of 2.5 or lower (Gibson 2004). Thus, previous studies may have underestimated the insensitivity of these fungi to metals such as Cu and Zn in their typical habitats. It is concluded that adjustment of the initial pH in the growth medium is essential to provide a more accurate assessment of metal sensitivity of ericoid mycobionts.

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References

Babich H, Stotzky G (1977) Sensitivity of various bacteria, including actinomycetes and fungi to cadmium and the influence of pH on sensitivity. Appl Environ Microbiol 33:681–695

Bradley R, Burt AJ, Read DJ (1982) The biology of mycorrhiza in the Ericaceae. VIII. The role of mycorrhizal infection in heavy metal resistance. New Phytol 91:197–209

Cairney JWG, van Leerdam DM, Chen DM (2001) Metal insensitivity in ericoid mycorrhizal endophytes from *Woolsia pungens* (Epacridaceae). Aust J Bot 49:571–577

Collins YE, Stotzky G (1992) Heavy metals alter the electrokinetic properties of bacteria, yeasts and clay minerals. Appl Environ Microbiol 58:1592–1600

Denny HJ, Ridge I (1995) Fungal slime and its role in the mycorrhizal amelioration of zinc toxicity to higher plants. New Phytol 130:251–257

Fomina M, Ritz K, Gadd GM (2003) Nutritional influence on the ability of fungal mycelia to penetrate toxic metal-containing domains. Mycol Res 107:861–871

Gadd GM (1993) Tansley review no. 47. Interactions of fungi with toxic metals. New Phytol 124:25–60

Gadd GM, Griffiths AJ (1980) Influence of pH on toxicity and uptake of copper in *Aureobasidium pullulans*. Trans Br Mycol Soc 75:91–96

Gadd GM, White C (1985) Copper uptake by *Penicillium ochrochloron*: influence of pH on toxicity and demonstration of energy-dependent copper influx using protoplasts. J Gen Microbiol 131:1875–1879

Gibson BR (2004) Heavy-metal sensitivity of mycorrhizal *Calluna vulgaris* and its mycobionts. PhD thesis, The National University of Ireland, Dublin

Gibson BR, Mitchell DT (2004) Nutritional influences on the solubilization of metal phosphate by ericoid mycorrhizal fungi. Mycol Res 108:947–954

Gibson BR, Mitchell DT (2005) Phosphatases of ericoid mycorrhizal fungi: kinetic properties and the effect of copper on activity. Mycol Res 109:478–486

Hewitt EJ (1966) Sand and water culture methods used in the study of plant nutrition. Commonwealth Agricultural Bureau, Farnham Royal, Slough

Jongbloed RH, Borst-Pauwels GWFH (1992) Effects of aluminium and pH on growth and potassium uptake by three ectomycorrhizal fungi in liquid culture. Plant Soil 140:157–165

Martino E, Coisson JD, Lacourt I, Favaron F, Bonfante P, Perotto S (2000a) Influence of heavy metals on production and activity of pectinolytic enzymes in ericoid mycorrhizal fungi. Mycol Res 104:825–833

Martino E, Turnau K, Girlanda M, Bonfante P, Perotto S (2000b) Ericoid mycorrhizal fungi from heavy metal polluted soils: their identification and growth in the presence of zinc ions. Mycol Res 104:338–344

Martino E, Perotto S, Parsons R, Gadd GM (2003) Solubilization of insoluble inorganic zinc compounds by ericoid mycorrhizal fungi derived from heavy metal polluted sites. Soil Biol Biochem 35:133–141

Morley GF, Sayer JA, Wilkinson SC, Gharieb MM, Gadd GM (1996) Fungal sequestration, mobilization and transformation of metals and metalloids. In: Frankland JC, Magan N, Gadd GM (eds) Fungi and environmental change. Cambridge University Press, Cambridge, pp 235–256

Otte ML, Kearns CC, Doyle MO (1995) Accumulation of arsenic and zinc in the rhizosphere of wetland plants. Bull Environ Contam Toxicol 55:154–161

Roos W, Luchner M (1984) Relationships between proton extrusion and fluxes of ammonium ions and organic acids in *Penicillium cyclopium*. J Gen Microbiol 130:1007–1014

Sharples JM, Meharg AA, Chambers SM, Cairney JWG (2001) Arsenate resistance in the ericoid mycorrhizal fungus *Hymenoscyphus ericae*. New Phytol 151:265–270

Starkey RL (1973) Effect of pH on toxicity of copper to *Scytalidium* sp. a copper tolerant fungus, and some other fungi. J Gen Microbiol 78:217–225