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Effects of Zn and Ni metals on the growth of different isolates of *Pythium* species isolated from metal-contaminated and non-contaminated soils

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Abstract

Some species of fungi growing on serpentine and calamine soils accumulate heavy metals in their mycelia and show poor growth in non-contaminated soil. This study tests the hypothesis that these fungi growing in heavy metal contaminated soil are tolerant and decrease the pollution from the soil. In present study, *Pythium mamillatum* Meurs and *P. splendens* Braun were isolated from Ni and *Pythium ultimum* Trow from Zn polluted soils whereas only *P. ultimum* was isolated from non metal-contaminated soils. The effect of different concentrations of Zn and Ni on mycelial growth and biomass *P. mamillatum*, *P. splendens* and *P. ultimum* were studied. *P. ultimum* isolated from zinc contaminated soil was more tolerant than species isolated from non-contaminated or nickel-contaminated soil. *P. mamillatum* and *P. splendens* isolated from high nickel soil were more resistant to nickel than *Pythium* species isolated from non-contaminated or Zn-contaminated soil. The production of oospores of *P. mamillatum* was greater in Zn concentrations as compared to the *P. ultimum* isolated from Zn-contaminated soil. However, the comparative study of *P. ultimum* and *P. mamillatum* shows that the production of oospores increases with increasing Zn and Ni concentrations up to a certain level and then decreases.

Introduction

Metals are involved in all aspects of fungal growth, metabolism and differentiation. Many metals such as K, Mg, Ca, Mn, Fe, Cu and Zn are essential. Nickel may be beneficial for some plants (Dalton *et al*., 1988). Fungal cells can accumulate all these elements by transport systems of varying specificity. Most essential and non-essential metals exhibit toxicity above a certain concentration, which will vary depending on the organism, the physico-chemical properties of the metal and environmental factors (Gadd, 1988).

A range of fungi from all major taxonomic groups may be found in metal-polluted habitats and an ability to survive and grow in the presence of potentially toxic concentrations is frequently encountered (Turnau, 1991). In general terms, toxic metals are believed to affect fungal communities by reducing abundance and species diversity and selecting for resistant/tolerant populations (Duxbury, 1985).

In Zn- polluted soils, species of *Geomyces* and *Paecilomyces* and some sterile forms have been shown to increase with increasing pollution level, whereas *Penicillium* and *Oidiodendron* spp. declined at polluted sites (Nardgren *et al*., 1983). It seems, therefore, that elevated concentrations of heavy metals can affect the qualitative and quantitative composition of fungal populations though it must be stressed that it may be difficult to separate metal effects from those of other environmental components. Polluted soils may be nutrient poor, of variable pH and may also contain additional toxicants (Gadd and Griffiths, 1978). These factors may affect fungal populations.

Higher concentrations of Pb, Cd, Zn and Hg are found in macro fungi from urban and industrial areas although there are differences in uptake abilities between different species and for different metals (Brown and Hall, 1990). Most of the studies discussed above relied upon the dilution plate technique for isolating fungi from soil. *Pythium* species are rarely isolated by this technique, but may be encouraged to grow on selective media (Robertson, 1973). This study was designed with two aims in mind, do pathogenic fungi grow in metal contaminated soil and heavy metals in metalliferous soil affect pathogenic fungi in the same manner? It concentrated on effects of heavy metals on root-infecting fungi and interaction between fungi and metal accumulating plants.

Materials and Methods

Pythium mamillatum, P. splendens, and *P. ultimum* isolated from Nickel and Zinc contaminated soils obtained from Clough Wood, Derbyshire and Grey Hill, Scotland respectively as

well as *P. ultimum* isolated from non-contaminated soil obtained from the University of Sheffield (U.K) culture collection. These species of *Pythium* were isolated by using special media and baiting technique (Hendrix and Campbell, 1970).

Effect of zinc and nickel on radial growth

Two percent water agar was autoclaved at 120 °C for 20 minutes for maintaining cultures of *Pythium mamillatum, P. splendens,* and *P. ultimum* isolated from nickel and zinc contaminated soils. Water agar was used to avoid complexion of metal ions. Zn and Ni solutions were prepared from the salts $ZnSO_4$ $7H_2O$ and $NiSO_4$ $4H_2O$ respectively, and sterilized by Millipore filtration. The metal solution was added to 20 ml aliquots of molten water agar at 45°C in 9 cm diameter petri dishes for the determination of radial growth rate of the test fungi. The final metal concentrations were 0, 2, 4, 6, 8 and 10 μg Zn ml-1 and 0, 0.5, 1, 2, 3, 4, and 5 μg Ni ml-1. These concentrations were chosen to reflect the range of concentrations likely to be found under field conditions. The petri dishes were rotated to mix the solution with the water agar. Three replicates were prepared for each metal concentration. After solidifying the water agar, each petri dish was inoculated centrally with an 8 mm disc cut from the margin of a 3 day old culture of each of the species of *Pythium* on cornmeal agar (CMA). These cultures were maintained at 25 °C in an incubator. The diameter of each colony was determined at 24h intervals and radial growth rate was calculated.

Effect of zinc and nickel on biomass

Nutrient media containing different concentrations of zinc and nickel were prepared by adding filter-sterilized metal solution to 20 ml aliquots of autoclaved Rorison's nutrient solution containing 0.1% D-glucose in 50 ml conical flasks for the determination of biomass. The final metal concentrations were 0, 2, 4, 6, 8 and 10 μg Zn ml^{-1} and 0, 0.5, 1, 2, 3, 4, and 5 μg Ni ml^{-1}. Three replicates were prepared for each metal concentration. Each conical flask was inoculated with an 8 mm disc cut from the margin of a 3 day old culture of each of the species of *Pythium*. Flasks were incubated at 25 °C. Mycelial dry weight was determined at 8-day intervals. Mycelial mats together with any oospore and/or sporangia were harvested by vacuum filtration through a filter paper, which had been previously oven-dried and weighed. Each mycelial mat with its filter paper was dried at 70 °C for 48 hrs, and re-weighed.

Effect of zinc and nickel on oospore production

In order to determine the oospore production, the potato-carrot broth was autoclaved at 120 °C for 20 minutes. Twenty ml aliquots of sterilized potato-carrot broth with Zn and Ni solutions were added to 9 cm standard sterilized petri dishes. The final concentrations of Zn and Ni was similar as used for determination of biomass. The dishes were swirled gently to mix the metal solution with the potato-carrot broth properly. Two 8 mm discs cut from the margin of a 3-day old culture of *P. ultimum* were placed in the centre of each petri dish. Cultures were maintained at 25 °C. After 15 days the mycelial mats with any oospores were harvested, transferred to a sterilized vial containing 5 ml sterile water and blended in a homogenizer for two minutes. The resultant suspension was passed through three layers of muslin cloth to remove the mycelial fragments. The liquid containing the oospores was centrifuged for 15 minutes at 2000 rpm. The supernatant liquid was poured off and the residue was again mixed with 10 ml sterilized water. Centrifugation was repeated until the oospore suspension was free from hyphal fragments. The final residue was mixed with 5 ml of sterilized water and the number of oospores in each treatment determined using a Haemacytometer slide (Rod Fuchs-Rosenthal). The number of oospores from the *P. mamillatum* isolated from serpentine soil was also determined to compare the production of oospores in Ni- and Zn-contaminated soil.

Statistical analysis was made by using Duncan's Multiple Range test (Steel and Torrie, 1980).

Results

Effect of zinc and nickel on radial growth

Pythium ultimum from Zn-contaminated soil and *P. mamillatum* from Ni-contaminated soil grew well over the range of 0-6 μg Zn ml^{-1} and *P. splendens* over the range of 0-4 μg Ni ml^{-1}. Above these limits growth rate of all species decreased with increasing concentration of Zn, although the growth rate of *P. ultimum* at 10 μg ml^{-1} was not significantly different from that at 8 μg Zn ml^{-1}. *P. ultimum* from non-contaminated soil was more sensitive to Zn concentrations compared to all isolates of *Pythium* from contaminated soils. This *Pythium* species could only grow in 0-2 μg ml^{-1} Zn. Above this level growth rate decreased with increasing concentrations of Zn (Table 1).

Table 1: Influence of zinc on radial growth rate of different species of *Pythium* (mm/day).

Concentration. of Zn (μg ml^{-1})	*P. ultimum* *	*P. ultimum* **	*P. mamillatum*	*P. splendens*
0	11.25 ± 0.14 a	10.75 ± 0.14 b	10.58 ± 0.08 a	12.33 ± 0.08 a
2	10.92 ± 0.17 a	12.16 ± 0.30 a	10.00 ± 0.14 a	12.41 ± 0.17 a
4	9.5 ± 0.29 b	10.58 ± 0.17 b	10.08 ± 0.30 a	12.41 ± 0.08 a
6	9.16 ± 0 b	10.50 ± 0.14 b	9.92 ± 0.08 a	11.33 ± 0.22 b
8	6.75 ± 0.14 c	8.33 ± 0.08 c	7.08 ± 0.42 b	6.08.08 ± 0.08 c
10	4.1 ± 0.44 d	8.17 ± 0.08 c	4.73 ± 0.17 c	4.25 ± 0.25 d

Values in the same column followed by the same letter are not significantly different.
**P. ultimum* isolated from non contaminated soil.
** *P. ultimum* isolated from Zn-contaminated soil.
P. mamillatum and *P. splendens* isolated from Ni-contaminated soils.

Table 2: Influence of nickel on radial growth rate of different species of *Pythium* (mm d/day).

Concentration of Ni (μg ml^{-1})	*P. ultimum* *	*P. ultimum* **	*P. mamillatum*	*P. splendens*
0	11.25 ± 0.14 a	10.75 ± 0.14 a	10.58 ± 0.08 a	12.33 ± 0.08 a
0.5	10.75 ± 0.14 a	10.33 ± 0.22 ab	10.75 ± 0.25 a	12.08 ± 0.42 a
1	9.83 ± 0.08 b	10.08 ± 0.08 b	9.58 ± 0.08 ab	11.67 ± 0.08 ab
2	6.3 ± 0.41 c	8.33 ± 0.08 c	10.22 ± 0.11 a	10.83 ± 0.17 b
3	5.58 ± 0.08 c	7.33 ± 0.08 d	9.88 ± 0.05 a	7.83 ± 0.08 c
4	3.5 ± 0 d	5.08 ± 0.17 e	10.27 ± 0.56 a	6.33 ± 0.30 d
5	2.25 ± 0.14 e	3.25 ± 0.14 f	8.66 ± 0 b	4.67 ± 0.08 e

Values in the same column followed by the same letter are not significantly different.

Nickel had little effect on the linear growth rate of *P. mamillatum* until the concentration reached 4 μg ml^{-1}. Above this value extension rate significantly decreased. By contrast, the growth rate of *P. ultimum* from high-Zn and *P. splendens* from high-Ni soil showed a significant decline with increasing Ni concentrations above 1 μg ml^{-1}. *P. ultimum* from non metal-contaminated soil had shown resistance only up to 0.5 μg Ni ml^{-1}. Increasing concentrations of Ni brought about significant reduction in radial extension of *P. ultimum* from non-contaminated soil (Table 2).

Effect of zinc and nickel on biomass

Dry weight yield of *P. ultimum* was not significantly affected by Zn concentrations up to 8 μg ml^{-1}. Dry weight of *P. mamillatum* was significantly reduced by Zn concentrations above 2 μg ml^{-1}, with the exception of 8 μg ml^{-1}. Dry weight of *P. splendens* was very low in all treatments as compare to *P. ultimum* and *P. mamillatum* whereas zinc appeared to have little effect. Dry weight of *P. ultimum* from non metal-contaminated soil was reduced by zinc. The biomass at 2 μg Zn ml^{-1} was significantly lower than the control but further increase in Zn to 10 μg ml^{-1} brought about no further reduction (Table 3).

Dry weight yield of *P. ultimum* isolated from Zn-contaminated soil was very sensitive to Ni concentration. At 0.5 μg Ni ml^{-1} the biomass was much lower than the control and the dry weight is not significantly different up to 4 μg ml^{-1} however this value is significantly different from Ni 5 μg ml^{-1}. Nickel did not affect the biomass of *P. mamillatum* until the concentration exceeded 4 μg ml^{-1}. Biomass of *P. splendens* was very much lower than the other species; Ni concentrations above 1 μg ml^{-1} significantly reduced the dry weight of this species. *P. ultimum* from non-metal-contaminated soil was very sensitive to Ni concentration. Biomass fell dramatically with increasing Ni concentrations to 1 μg ml^{-1}; thereafter there was a more gradual reduction in dry weight (Table 4).

Effect of zinc and nickel on oospore production

The production of oospores was optimal in the range of 2-4 μg Zn ml^{-1} or 0.5 μg Ni ml^{-1}. oospore production at higher Zn concentrations was similar to that in control medium, but fell steeply when the concentration of Ni was increased. Oospore production by *P. mamillatum* was also stimulated by low concentrations of Zn and Ni, with optimal production at 4 μg Zn ml^{-1} and 3 μg Ni ml^{-1}. Increasing the concentration of either Zn or Ni above these values led to a marked reduction in the number of oospores (Table 5).

Table 3: Influence of zinc on biomass of different species of *Pythium* (mg/8day).

Concentration of Zn (µg ml^{-1})	*P. ultimum* *	*P. ultimum* **	*P. mamillatum*	*P. splendens*
0	13.83 ± 0.44 a	19.67 ± 1.76 ab	16.00 ± 1.15 a	4.67 ± 0.88 ab
2	7.83 ± 0.44 b	19.00 ± 0.57 ab	15.00 ± 1.15 ab	5.00 ± 0 a
4	8.00 ± 0 b	21.00 ± 2.5 a	11.00 ± 0 c	2.00 ± 0 c
6	7.83 ± 0.44 b	18.00 ± 0 ab	12.00 ± 0.58 bc	3.00 ± 0 bc
8	9.00 ± 0 b	15.67 ± 0.33 b	13.33 ± 0.33 abc	3.00 ± 0 bc
10	8.00 ± 0.57 b	10.33 ± 1.29 c	12.00 ± 0 bc	4.5 ± 0 ab

Values in the same column followed by same letter are not significantly different.

Table 4: Influence of nickel on biomass of different species of *Pythium* (mg/8day).

Concentration of Ni (µg ml^{-1})	*P. ultimum* *	*P. ultimum* **	*P. mamillatum*	*P. splendens*
0	13.83 ± 0.44 a	19.67 ± 1.76 a	16.00 ± 1.15 a	4.67 ± 0.88 a
0.5	5.4 ± 0.70 b	11.33 ± 0.33 b	16.2 ± 0.61a	4.5 ± 1.2 ab
1	2.5 ± 0.5 c	10.67 ± 0.88 b	16.3 ± 0.85 a	3.3 ± 0.87abc
2	2.5 ± 0.5 c	9.33 ± 0.88 b	16.00 ±0 a	2.3 ± 0.5 c
3	1.9 ± 0.49 c	7.00 ± 1.15 b	15.67 ± 0.33 a	3.00 ± 00 bc
4	1.63 ± 0.63 c	5.00 ± 0.57 bc	15.33 ± 1.4 a	3.00 ± 00 bc
5	0.9 ± 0 c	2.23 ± 0.57 c	11.33 ± 0.88 b	2.00 ± 00 c

Values in the same column followed by the same letter are not significantly different.

Table 5: Influence of Zn and Ni on production of oospores (No. of oospores/ml)

Concentration (µg ml^{-1})		*Pythium ultimum*		*P. mamillatum*	
Zinc	Nickel	Zinc	Nickel	Zinc	Nickel
0	0	1000	1000	1100	1100
2	0.5	1492	2218	1921	1151
4	1	1495	1189	2583	1180
6	2	986	431	2121	1200
8	3	931	151	1250	1239
10	4	864	43	867	985

Discussion

In order to compare the effects of toxic metals on fungal growth three types of measurements were made: radial extension growth rate, dry matter production and oospore production. Zinc was toxic to all isolates of *Pythium*, but these varied in their susceptibility to the metal. The most susceptible was *P. ultimum* isolated from non-contaminated soil. The radial growth rate and biomass production of this isolate were much reduced by increasing concentrations of zinc. The most tolerant species was *P. ultimum* isolated from a zinc-contaminated soil. The two species isolated from nickel-contaminated soil were intermediate in their tolerance of increasing zinc concentration in the medium. It appears that there is a correlation between zinc concentration in soil from which these fungi originally isolated and their tolerance to this metal.

Nickel was more toxic with respect to Zn in all four isolates, but the pattern of susceptibility was different. The most susceptible was again *P. ultimum* isolated from non-contaminated soil. The most tolerant was *P. mamillatumm*, isolated from nickel-contaminated soil. *P. splendens* from nickel-contaminated soil and *P. ultimum* from zinc-contaminated soil were intermediate in their tolerance to nickel. *P. splendens* was less tolerant to nickel and zinc than *P. mamillatum*. Decrvalho and Milanez (1988) indicated that *P. splendens* was sensitive in vitro in CMA-10 medium, but not

in vivo in the soil, at 100 μg g-1 of Cu. These results collectively suggest that tolerance to nickel has been selected in those fungi isolated from a site rich in nickel. It is also interesting to note that isolates with resistance to one of the two metals also show greater tolerance to the other metal than does the isolate of *P. ultimum* from the uncontaminated site. Fungal density was reduced and there were alterations in species composition in polluted soil near a Zn smelter (Jordan and Lechevalier, 1975). Although we have no evidence of the mechanism of tolerance of *Pythium* species to zinc and nickel, it does appear that tolerance to one metal is linked in some way to tolerance of the other.

The effects of zinc and nickel on oospore production were more complex than on vegetative growth. *P. ultimum* isolated from zinc-contaminated soil and *P. mamillatum* isolated from nickel-contaminated soil produced more oospores when grown in low concentrations of either zinc or nickel than when grown in control medium. However, at high metal concentrations, oospore production declined. In control medium there was little difference in oospore production by the two species but *P. mamillatum* produced significantly more oospores than *P. ultimum* when either zinc or nickel concentration was increased above 1 μg ml^{-1}. At first sight this may seem somewhat surprising, since vegetative growth of *P. mamillatum* in media containing zinc was inhibited more strongly than that of *P. ultimum*. However, oospore production is stimulated by conditions of stress. In all these experiments nickel appears to be approximately twice as toxic as zinc when concentrations are expressed as μg ml^{-1}.

It is very much clear from the present study that fungi growing in metal polluted soils tolerate high concentrations of metal and reduce the metal pollution. However, the fungi isolated from non-contaminated soil was most susceptible with respect to metal polluted soil. It would be very interesting to investigate the mechanism of tolerance in these fungi.

References

Brown MT, Hall IR, 1990. Metal tolerance in fungi. In: *Heavy Metal Tolerance in Plants*: *Evolutionary Aspects* (AJ Shaw, ed.), pp. 95-104. CRC Press, New York.

Dalton DA, Russell SA, Evans, HJ, 1988. Nickel as a micronutrient element for plants. *Biofactors*, **1**: 11-16.

Decarvalho Y, Milanez A, 1988. Sensitivity of *Pythium* splendens Braun to heavy metals. *Revesta de Microbiologia*, **19**: 306-310.

Duxbury T, 1985. Ecological aspects of heavy metal responses of microorganisms In: *Advances in Microbial Ecology* (Marshal KC, ed.), pp.185-235. New York: Plenum Press.

Gadd GM, 1988. Accumulation of metals by microorganisms and algae. In: *Biotechnology: a Comprehensive Treatise, Special Microbial Processes* (Rehm HJ, ed.), Vol. 6b, pp.401-433. Weinheim: VCH Verlagsgesellschaft.

Gadd GM,Griffiths AJ, 1978. Microorganisms and heavy metal toxicity. *Microbial Ecology*, **4**: 303-317.

Hendrix FF, Campbell WA, 1970. Distribution of Phytophthora and Pythium spp. In soils the continental United States. *Canadian Journal of Botany*, **48**: 377-84.

Jordan MJ, Lechevalier MP, 1975. Effects of Zn smelter emissions on forest soil microflora. *Canadian Journal of Microbiology*, **21**: 1855-1865.

Nardgren A, Baath E, Soderstom B, 1983. Micro fungi and microbial activity along a heavy metal gradient. *Applied and Environmental Microbiology*, **45**: 1829-1837.

Robertson JI, 1973. Occurrence of *Pythium* spp. in New Zealand soils, pumices and peat, and on roots of container grown plants. *New Zealand Journal of Agricultural Research*, **16**: 357-365.

Steel RGD, Torrie JS, 1980. Principles and procedures of statistics. A biological approach. Second Edition. McGraw Hill Book Company Inc., New York. Toronto.

Turnaui K, 1991. The influence of Cd dust on fungi in a Pino-Quercetum forest. *Ekologia Polka*, **39**: 39-57.