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Diversity and Metal Tolerance of Nematode-Trapping Fungi in Pb-Polluted Soils

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The diversity of nematode-trapping fungi (NTF) in two lead (Pb) mines in Yunnan Province, China was investigated in 2004. In total, 20 species belonging to five genera were identified from 500 samples collected at the Lanping and the Huize mines. Pb concentrations ranged from 216~7,150 mg/kg for the former and 132~13,380 mg/kg for the latter, respectively. The fungi were divided into five groups based on different trapping mechanisms. The trapping-net producer group contained the largest number of species, with nine. Two predators, *Dactylellina ellipsosporum* and *Arthrobotrys oligospora,* were found at frequencies of 32.85% and 15.41%, respectively. The diversity indexes of NTF were positively correlated with Pb pollution levels in both the Lanping Mine (r=0.66) and the Huize Mine (r=0.72), suggesting that the distribution of NTF was not negatively affected by Pb contamination. For most strains of a given species, there was no significant difference (P>0.01) in the Pb tolerance between the strains isolated from habitats with low or high Pb concentrations. However, Pb toxicity exerted adverse effects on trap formation and predacious capability of fungi. We discuss the possible metal tolerance mechanisms and their relationships to the survival strategy of NTF in Pb-polluted environments.

***Keywords*:** Nematode-trapping fungi, Pb toxicity, heavy metal tolerance

With ever increasing industrial and agricultural activities worldwide, more wastes are released into the environment. As one of the more serious pollution sources, metalliferous minesgenerate a large quantity of heavy metals and byproducts such as mine tailings and dusts which can find its way into soil from waste water and airborne dust particles. Heavy metals have been reported to exert an adverse effect on the qualitative and quantitative structures of microbial communities, including the diversity, biomass and activity of soil microorganisms (Brookes and McGrath, 1984; Aoyama and Nagumo, 1997; Smit *et al*., 1997; Wuertz and Mergeay, 1997; Val *et al*., 1999; Kozdroj and Elsas, 2001). The heavy metal lead, which has the symbol *Pb*, has historically been mined in many countries for its various uses, including in tools, construction and architectural purposes, weapons, paints and a range of other materials. However, the potential public health impact of Pb mining and smelting operations have been highlighted given cases of air, soil and aquatic ecosystem contaminations.

Inorganic Pb can inhibit the growth and photosynthesis of marine algae and cyanobacteria (Malanchuk and Gruendling, 1973), nitrogen fixation of cyanobacteria (Henriksson and DaSilva, 1978), germination of spores and mycelial growth of fungi (Somers, 1961; Smith, 1977), and the growth, trap formation and collagenase activity of predacious fungi (Rosenzweig and Pramer, 1980).

Nematode-trapping fungi (NTF) are a group of hyphomycetes capable of capturing, killing and digesting nematodes by special modified hyphal structure names trapping devices. The trapping devices include sticky nets, sticky knobs, sticky branches, constricting rings, and non-constricting rings (Barron, 1992). These fungi are abundant in many habitats examined so far, with only a few species showing restricted geographical distribution (Duddington, 1951a, 1951b; Gray, 1987). Though several previous studies have reported the general ecological characteristics of NTF, relatively little attention has been given to the distribution of these fungi in heavy-metal polluted environments. In a previous study, we showed that NTF were widely distributed in substrates with high concentrations of Pb, including sites with mine tailings (Mo *et al*., 2006). The activity and evolutionary or ecological adaptation of NTF in Pb-polluted areas, however, are not well understood. The objective of this study is to characterize the diversity of NTF from two Pb mines in order to obtain a greater understanding of the possible mechanisms involved in fungal metal tolerance.

Materials and Methods

Sampling procedure

Samples were collected from two separate Pb mines in September 2004. The Lanping Pb mine (26°42′N, 99°42′E, altitude 2410 m) and the Huize Pb mine (26°65′N, 103°72′E,

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altitude 2246 m) are both situation in Yunnan Province, China. The Lanping mine has the largest Pb reserve in China and the second largest in Asia (Zu *et al.*, 2004). Around each mine, 5 sampling sites located about 1 km from each other were selected. At each site, 50 soil samples (each about 200 g from a depth of 5~10 cm) were collected in an area of about 100 m^2. All samples were kept in sterile plastic boxes at 4°C before use.

Isolation of NTF

For each of the 500 soil samples, 1 g of soil was spread on a Corn Meal Agar (extract of 20 g cornmeal, agar 18 g, adding water to the final volume of 1,000 ml, CMA) plate and approximately 200 nematodes (*Panagrellus redivivus*) were added as the bait for NTF. Each sample was plated on 3 replica petri dishes, which resulted in 150 plates for 50 samples from each collection site. After incubation at room temperature (about 20~28°C) for 20 days, the presence of NTF was recorded and identified according to the taxonomic system of Scholler *et al.* (1999).

Analysis of lead concentration

The mixed soil sample of each site was air-dried and passed through a 2 mm mesh sieve, and the fine soil particles were then pulverized and passed through a 180 μm mesh sieve. After drying for 2 h at 105°C, the samples were digested in aqua regia following the method of McGrath and Cunliffe (1985), and the total concentrations of Pb analyzed with an atomic absorption spectrophotometer (Model Z-8000, Hitachi, Japan). For controls, we used reagent blanks, duplicates, and the standard reference material (NIST 2709).

Effect of lead on mycelial growth of NTF

Nine isolated species, each including 1~2 isolates from Pb-polluted soils and one from the Pb-free soils, were tested for their susceptibility to 0.35 mM $PbCl_2$ following the protocol described in Mo *et al.* (2006).

The effect of lead on trap formation and predacious activity of NTF

The effect of Pb on trap formation and nematode trapping activity of NTF was performed according to the methods of Rosenzweig and Pramer (1980), with slight modifications. Briefly, strains were grown at 28°C on 2% CMA plates with 0.15 mM $PbCl_2$ or without any Pb. After incubation for 7 days, a suspension of nematodes (containing about 500 *Panagrellus redivivus*) was added to the fungal colony. Each treatment was conducted in triplicate. The plates were incubated for an additional 24~72 h and examined microscopically for the presence of traps. Trap density was estimated visually and scored, from low to high, as follows: 0, +1, +2, +3, +4, +5, and +6. Additionally, the predacious capability of NTF was estimated by their rate of capture (CR). The CR was calculated by the following formula when more than 100 nematodes were counted under a microscope:

$$CR=\frac{\text{Number of nematodes captured by traps}}{\text{Total number of nematodes counted}}\times 100\%$$

Relationship between predacious behavior and metal tolerance of NTF

To test whether NTF can obtain nutrition while avoiding metal toxicity by preying nematodes, the metal tolerance of two mutants, Tms2316 and Tms3583, and their wild type parental strain of *Monacrosporium sphaeroides* were examined. Tms2316 and Tms3583 were unable to form trapping device under the condition of nematode inducement (Xu *et al.*, 2005). The performance of toxic effect on mycelial growth followed the protocol described above with additional 200 nematodes added into each plate.

Statistical analysis

Because NTF could not form discernable colonies on CMA medium when the soil was directly spread onto the plate, the occurrence of individuals in each species was estimated as the number of soil samples in which the species occurred. For example, as mentioned above, a soil sample was plated on 3 replicated dishes. When a species was found in one or more of the 3 replicated plates, we recorded it as 1. Fungal taxa with an occurrence frequency higher than 10% were classified as frequent species. Shannon index (H′) was used to estimate the diversities of NTF. The above data were calculated using the following formulas:

$$OF=\frac{\text{Individual number of a species}}{\text{Individual number of all species}}\times 100\%$$

$$H'=\sum_{i=1}^{s} Pi\ \log_e Pi,\ \text{where}\ Pi=\frac{Ni}{N}$$

Ni = the individual number of *i*th species,
N = the individual number of all species,
Pi = the proportion of *i*th species,
Log_ePi = the natural logarithm of P.

The data were comparatively analyzed with analysis of variance (ANOVA) using the SPSS software package (SPSS 11.01 Inc., USA). Least significant differences (LSD) test were performed with a P value of <0.01 used to indicate difference between treatments.

Results

Lead concentration in soil samples

The soils from around Lanping and Huize mines were heavily polluted by Pb with a concentration ranging from 216 to 7,150 mg/kg and from 132 to 13,380 mg/kg, respectively (Table 1). These Pb levels were significantly higher than the average background value (23.6 mg/kg) of Chinese soils.

Distribution and diversity of NTF in lead-polluted soil

In total, 20 species of NTF belonging to 5 genera (*Arthrobotrys* Corda, *Dactylellina* Morelet, *Datylella* Grove, *Drechslerella* Subram., *Gamsylella* Scholler, Hagedorn and Rubner) were isolated from the 500 samples (Table 2 and 3). These fungi were partitioned into five groups according to the different trapping devices. The most abundant type of trap recorded was the adhesive net (OF=41.43%, S=9), followed by ad-

hesive knob (OF=33.9%, S=2), constricting rings (OF=7.95%, S=1), sticky branches (OF=7.78%, S=3), and non-constricting rings combined with adhesive knobs (OF=1.87%, S=1). In the two Pb-polluted regions, the NTF predators *Da. ellipsospora* (OF=32.85%) and *A. oligospora* (OF=15.41%), were the most frequent species found in all sites.

In the Lanping mine, the highest diversity of NTF was found in site 1 (H′=1.89; S=10; N=149), followed by site 2 (H′=1.76; S=10; N=143), site 3 (H′=1.70; S=8; N=122), site 5 (H′=1.37; S=7; N=96) and site 4 (H′=1.31; S=6; N=79). In the Huize mine, NTF diversity in descending order were: site 8 (H′=1.92; S=10; N=176), site 7 (H′=1.86; S=11; N=84), site 6 (H′=1.82; S=9; N=171), site 10 (H′=1.38; S=8; N=114) and site 9 (H′=1.26; S=7; N=100). The diversity of NTF in Pb-polluted soils was found to correlate positively with the Pb concentration in the Lanping (r=0.67) and Huize mines (r=0.72) (Table 5).

Effect of lead on mycelial growth of NTF

We tested for effects of Pb on mycelial growth of 24 strains, in 9 NTF species (Table 4). Our preliminary results showed that none of the fungi tested was significantly affected by $PbCl_2$ at concentrations lower than 0.05 mM. Therefore, the susceptibility of NTF to Pb was determined using a series of $PbCl_2$ concentrations (0.05, 0.1, 0.25, 0.35, 0.70, 1.0, 1.2, 1.8 mM). In Pb concentration of 0.1~1.2 mM, the NTF exhibited significant differences in mycelial growth. All tested

Table 1. Characteristics of sampling sites from the Lanping lead mine (Site 1~Site 5) and the Huize lead mine (Site 6~Site 10)

Sampling site	GPS value	Substrate	Vegetation	pH value	Pb concentration (mg/kg)
Site 1	26° 41′ N, 99° 42′ E	soil	bush	6.14	3,490
Site 2	26° 41′ N, 99° 42′ E	soil	pine	6.03	3,120
Site 3	26° 41′ N, 99° 41′ E	soil	herbage	6.00	7,150
Site 4	26° 42′ N, 99° 40′ E	soil	pine	6.20	747
Site 5	26° 43′ N, 99° 40′ E	soil	herbage	6.07	216
Site 6	26° 649′ N, 103° 725′ E	mine tailing	moss	6.20	5,670
Site 7	26° 65′ N, 103° 71′ E	mine tailing	none	5.62	8,590
Site 8	26° 64′ N, 103° 72′ E	soil	pine	5.76	13,380
Site 9	26° 47′ N, 103° 62′ E	mine tailing	none	6.24	5,460
Site 10	26° 48′ N, 103° 62′ E	soil	pine	6.30	132

Table 2. NTF from lead contaminated soil around the Lanping mine

Taxa and diversity analysis	Ni (Individual number of *i*th species)					OF
	Site 1	Site 2	Site 3	Site 4	Site 5	
A. conoides	2	4	6		4	2.72
A. gampsospora	2	1				0.51
A. musiformis				20	17	6.28
A. oligospora	26	32	43	6	40	24.96
A. reticulata	2	4	1			1.19
A. superba	12	5	8			4.25
Da. drechsleri	3				1	0.68
Da. ellipsospora	43	49	32	41	28	32.77
Da. haptotyla		2		1	5	1.36
Da. leptospora					1	0.17
Dr. dactyloides	20	27	16	4		11.38
D. clavata		3				0.51
D. sp_1	10					1.70
D. sp_2			5			0.85
G. gephyropaga	29	16	11	7		10.7
N	149	143	122	79	96	
S	10	10	8	6	7	
H′	1.89	1.76	1.70	1.31	1.37	
Pb concentration	3,490	3,120	7,150	747	216	

A, *Arthrobotrys*; Da, *Dactylellina*; Dr, *Drechslerella*; D, *Datylella*; G, *Gamsylella*

Table 3. NTF from lead contaminated soil around the Huize mine

Taxa and diversity analysis	Ni (Individual number of *i*th species)					OF
	Site 6	Site 7	Site 8	Site 9	Site 10	
A. conoides	1	2				0.67
A. microscaphoides				1		0.17
A. oligospora	25	8	5	3	2	7.19
A. superba	24	6	4	1	1	6.02
A. thaumasia	4	13	16	28	26	14.55
A. vermicola	37	20	15	13	20	17.56
Da. haptotyla	11	2	2			2.51
Da. drechsleri	1	1	7			1.51
Da. ellipsospora	48	24	41	49	50	35.45
Da. leptospora		2				0.34
Dr. dactyloides	20	27	30	4		5.18
D. panlongna	20		1			3.51
D. sp_3			2			0.34
G. gephyropaga		1	21	2	6	5.02
N	171	84	176	100	144	
S	9	11	10	7	8	
H′	1.82	1.86	1.92	1.26	1.38	
Pb Concentration	5,670	8,590	13,380	5,460	132	

Table 4. Effect of lead on mycelial growth of nematode-trapping fungi

Taxa	Strains	Pb concentration of soil From where the strain come from (mg/kg)	IGR (Mean IGR±SE, n=3)
A. conoides	2-20-2	3,120 (site 2)	14.35±1.19
	2-18-1	3,120 (site 2)	13.73±0.09
	1.9	11.23	14.61±0.17
A. musiformis	4-16-2	747 (site 4)	10.99±0.04
	1.122	11.23	11.25±0.01
A. oligospora	1-23-1	3,490 (site 1)	11.61±0.11[a]
	5-45-3	216 (site 5)	14.61±0.17
	7-35-2	8,590 (site 7)	14.78±0.18
	1.12	11.23	14.25±0.12
A. superba	7-29-2	8,590 (site 7)	7.03±0.60[a]
	2-42-3	3,120 (site 2)	13.89±0.74[a]
	1.16	11.23	16.68±0.40
A. thaumasia	8-10-3	13,380 (site 8)	14.74±0.37
	9-47-3	5,460 (site 9)	14.35±0.78
	1.547	11.23	13.33±0.49
A. vermicola	9-19-1	5,460 (site 9)	17.80±0.30
	1.533	11.23	18.27±0.09
Da. haptotyla	7-32-1	8,590 (site 7)	12.00±0.50
	5-6-3	216 (site 5)	11.50±0.50
	1.543	11.23	11.95±0.28
Da. leptospora	5-21-3	216 (site 5)	13.46±0.22
	1.114	11.23	12.14±0.60
G. gephyropaga	3-10-3	7,150 (site 3)	13.39±0.89
	1.569	11.23	14.18±0.12

[a] Significant difference in mean is indicated by $P<0.01$. The presence of '[a]' on IGR line indicates the multiple comparisons among strains of the same species.

Table 5. Correlation between lead concentration of soil and Shannon index (H′) of nematode-trapping fungi

Sampling area	x	y	Equation	r
Lanping mine	Pb concentration	Nematode	y=−21.24x+276.35	−0.89
		H′	y=6E-0.5x+1.4205	0.67
Huize mine	Pb concentration	Nematode	y=−2.07x+169.93	−0.087
		H′	y=0.045x+1.35	0.72

Table 6. Effect of lead on trap formation and predatory capability of nematode-trapping fungi

Taxa	Strain	Trap density		Predacious capability (100%) (Mean±SE, n=3)	
		Without Pb^{2+}	With Pb^{2+}	Without Pb^{2+}	Addition of Pb^{2+}
A. conoides	2-20-2 (site 2)	+1	+2	57±4	76±4[a]
	5-11-3 (site 5)	+1	+2	56±1	61±4
	1.9	+1	+2	59±2	57±1
A. oligospora	7-35-2 (site 7)	+4	+3	97±3	81±3[a]
	1-23-1 (site 1)	+5	+3	96±3	87±1[a]
	1.12	+5	+3	91±1[a]	98±2
A. superba	7-29-2 (site 7)	+5	+4	100±0	100±0
	2-42-3 (site 2)	+5	+4	100±0	94±0
	1.16	+5	+5	94±1	100±0
A. vermicola	9-19-1 (site 9)	+2	+1	78±2	51±1[a]
	1.533	+2	+1	85±2	67±4[a]
A. musiformis	4-16-2 (site 4)	+2	+1	86±3	54±3[a]
	1.122	+2	+1	64±1[a]	59±4[a]
A. thaumasia	8-10-3 (site 8)	+4	+1	100±0	55±1[a]
	9-47-3 (site 9)	+4	+1	100±0	46±3[a]
	1.547	+3	+1	100±0	58±3[a]

[a] Significant difference in mean is indicated by $P<0.01$. The presence of 'a' in the 'without Pb^{2+}' column indicates the multiple comparisons of strains belonging to same species, while in the 'with Pb^{2+}' coloumn indicates the multiple comparisons of same strain under the condition with or without Pb^{2+}.

strains stopped their growth at concentrations over 1.8 mM (data not shown). For the NTF that grew slowly at concentrations above 0.5 mM of $PbCl_2$, another concentration, 0.35 mM, was selected to compare the effect of metal toxicity on mycelial growth. On CMA medium containing 0.35 mM of $PbCl_2$, the values of IGR varied between 7.03∼18.27%. NTF usually showed different tolerance to Pb toxicity at the species level. Among strains of the same species, there was no significant difference ($P>0.01$) in metal tolerance, regardless of where these strains were derived from, including habitats without Pb pollution. Except for three strains (1-23-1 of *A. oligospora*, 7-29-2 and 2-42-3 of *A. superba*), which showed more tolerance than the Pb-free soil strain ($P<0.01$), NTF from Pb-polluted soils exhibited similar tolerance to Pb toxicity as those of Pb-free soils.

Effect of lead on trap formation and predacious activity of NTF

Six species (each having 1∼2 strains from Pb-polluted soil and one strain from soil without Pb contamination) were used in this experiment (Table 6). At concentration of 0.15 mM $PbCl_2$, the trap densities of all species and strains tested decreased significantly compared to that without Pb. The exception was 3 strains of *A. conoides* and one strain (strain 1.16) of *A. superba.* Distinct differences in trap formation ability were observed among species, but not among strains of the same species. This observation was consistent regardless of differences in the origins of strains and Pb concentrations in their original niches.

In most cases, the strains of NTF significantly decreased their capacity to capture nematodes under Pb stress ($P<0.01$). However, in some cases, Pb toxicity did not significantly affect the capability of NTF, including the tested strains of *A. superba*, *A. conoides*, and strain 1.12 of *A. oligospora*. In one instance, the presence of Pb significantly increased the trap formation and predacious capability of the strain 2-20-2 of *A. conoides* ($P<0.01$). Within a given species, we observed little significant differences among strains in their predacious ability, the exception being two strains (1.12 of *A. oligospora* and 1.122 of *A. musiformis*, both were derived from the habitats without Pb-pollution) which showed a lower capability in capture nematodes than the other strains of the same species.

Relationship between predacious mechanism and metal tolerance of NTF

On CMA plates containing 0.35 mM of $PbCl_2$, the wild type strain of *M. sphaeroides* easily formed sticky nets to trap

nematodes, while mutants Tms2316 and Tms3583 did not. The IGR values of Tms2316 and Tms3583 were 24.93± 1.93% and 22.58±1.58%, respectively, which were significantly higher that of the wild-type strain (16.77±0.06%) (P<0.01). This result suggested that the two mutants had lower levels of Pb resistance than the wild-type strain.

Discussion

NTF are common in soils with cultivated and non-cultivated crops (Gray, 1987; Barron, 1992). In this study, fungi were found to be distributed widely in Pb-polluted soils, and even in mine tailings (e.g., site 6~7). In contrast to common findings that heavy metals exerted adverse influence on microbial population and activities, we found that the species diversities of NTF in Pb-polluted soils were positively correlated with Pb concentration at the Lanping Pb mine (r=0.67) and the Huize Pb mine (r=0.72). Although the scope of this study meant that we could not ascertain if Pb-pollution was beneficial for species diversity of studied fungi, the results suggest that NTF were abundant in Pb-polluted soils and that Pb pollution appeared not to be the primary factor restricting the distribution of NTF. These results supported our previous findings which concluded that the distribution of NTF could not be restricted by Pb pollution (Mo *et al*., 2006). The non-linear effect of heavy metals on microbial properties was also reported by Rost *et al*. (2001) and Chander *et al*. (2001).

Natural populations thriving in heavy metal contaminated ecosystems are often subjected to selective pressures for an increased resistance to toxic metals. Evolutionary adaptation to heavy metals is a well-documented process in several different groups of organisms including bacteria (Diels and Mergeay, 1990), animals (Levinton *et al*., 2003), marine algae (Nielsen *et al*., 2003), mosses (Shaw, 1988) and fungi (Colpaert *et al*., 2004). Fungi inhabiting environments containing high concentrations of heavy metals for extended periods can be selected for investigating metal tolerance.

A few suilloid fungi (e.g., *Sillus luteus)* isolated from Zinc-polluted regions were found tolerant to Zn (Colpaert *et al*., 2004). The authors found that the frequency of Zinc-tolerant genotypes decreased with the increasing distance from the smelters. Similarly, Colpaert and Assche (1992) found that *S. bovines* and *S. luteus,* from metal-contaminated soils, were more tolerant to heavy metals than isolates from unpolluted soils. Willenborg *et al*. (1990) observed that *Amanita muscaria* and *Hebeloma crustuliniforme,* from polluted areas, were more tolerant to Cadmium and Zinc. However, in a previous report (Mo *et al*., 2006), as well as in this study, we found no significant difference in Pb tolerance of NTF between strains of same species isolated from soils with different level of Pb concentrations (P>0.01). Our results indicate that natural thriving populations of NTF in Pb contaminated ecosystems did not increase their metal resistance.

The NTF are generally poor competitors in the soil and that a simple labile equilibrium between nematode density and trapping activity do not exist (Cooke, 1968). Though NTF can prey on nematodes as a food source, under conditions with sufficient nutrition, they have similar nutritional requirements as other saprophytic fungi in media (Satchuthananthavale and Cooke, 1967a, 1967b) and in soil environment (Cooke, 1963; Gray, 1987). Researchers have proposed that NTF trap soil nematode to obtain nitrogen and thereby compete saprophytically for carbon and energy in nitrogen-poor environments (Jaffee, 2004). In this study, in high Pb concentration environments, the wild type strain of *M. sphaeroides* easily formed devices to trap nematodes and was more tolerant to Pb than its derived mutants, which showed no capability in develop trapping devices to trap nematodes. Considering the difference in nutrition uptake between NTF and most common fungi, NTF can obtain nutrition not only saprophytically but also by preying on nematodes. It's likely that in environments containing abundant heavy metals, NTF can keep their populations thriving by preying on nematodes, thus avoiding other poisonous food. Of course, this hypothesis needs further experimental support, especially from data on the local food web.

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