

Subcellular distribution and chemical forms of cadmium in a dark septate endophyte (DSE), *Exophiala pisciphila*

Fangdong Zhan^{1,2} · Yongmei He¹ · Yuan Li¹ · Tao Li² · Yun-Ya Yang³ · Gural S. Toor³ · Zhiwei Zhao²

Received: 1 April 2015 / Accepted: 1 July 2015 / Published online: 14 July 2015
© Springer-Verlag Berlin Heidelberg 2015

Abstract Our objective was to understand the cadmium (Cd) tolerance mechanisms by investigating the subcellular distribution, chemical forms of Cd and adsorptive groups in the mycelia of *Exophiala pisciphila*. We grew *E. pisciphila* in the liquid media with increasing Cd concentrations (0, 25, 50, 100, 200, and 400 mg L⁻¹). Increased Cd in the media caused a proportional increase in the Cd uptake by *E. pisciphila*. Subcellular distribution indicated that 81 to 97 % of Cd was associated with the cell walls. The largest amount and proportion (45–86 %) of Cd was extracted with 2 % acetic acid, and a concentration-dependent extraction was observed, both of which suggest that Cd-phosphate complexes were the major chemical form in *E. pisciphila*. A large distribution of phosphate and Cd on the mycelia surface was observed by scanning electron microscopy-energy dispersive spectrometer (SEM-EDS). The precipitates associated with the mycelia were observed to contain Cd by transmission electron microscopy-energy dispersive X-ray spectroscopy (TEM-EDX). Fourier transform infrared (FTIR) identified that hydroxyl, amine, carboxyl, and phosphate groups were responsible for binding Cd. We conclude that Cd associated

with cell walls and integrated with phosphate might be responsible for the tolerance of *E. pisciphila* to Cd.

Keywords Cadmium · *Exophiala pisciphila* · Subcellular distribution · Adsorptive groups · Cd-phosphate

Introduction

Cadmium (Cd) contamination in soils is a serious environmental problem due to Cd release from the industrial processes and farming practices (Loganathan et al. 2012). Cd is a heavy metal and is toxic to soil fungi even at a low concentration (Moulis and Thevenod 2010). However, some fungi present cellular mechanisms to tolerate Cd stress and survive in heavy metals polluted soils (Bellion et al. 2006; Hafnerburg and Kothe 2007).

The cellular mechanisms of Cd tolerance in fungi have been widely demonstrated, and it was found that the subcellular distribution of Cd in the fungal mycelia is closely related to toxic metal tolerance (Vijver et al. 2004; Bellion et al. 2006). Accumulation of Cd was found mainly in the mycelium cell walls of *Phoma* sp. F2 (Yuan et al. 2007) and *Glomus intraradices* (Gonzalez-Guerrero et al. 2008) by transmission electron microscopy and energy-dispersive X-ray (TEM-EDX) analysis. In *Neurospora crassa*, approximately 62 % of total Cd content was found in the cell walls (Bhanoori and Venkateswerlu 2000). The binding of Cd to cell walls alleviates the toxic effects of Cd by effectively lowering the intracellular Cd concentration and is regarded as an essential metal-detoxification mechanism for fungi (Blaudez et al. 2000).

In addition, the biological toxicity of Cd in cells is also closely related to its chemical forms, which can be successively extracted by designated solutions (80 % ethanol, deionized

Responsible editor: Elena Maestri

✉ Zhiwei Zhao
zhaozhw@ynu.edu.cn

- ¹ College of Resources and Environment, Yunnan Agricultural University, Kunming 650201, People's Republic of China
- ² Laboratory for Conservation and Utilization of Bio-Resources, and Key Laboratory for Microbial Resources of the Ministry of Education, Yunnan University, Kunming 650091, People's Republic of China
- ³ Soil and Water Quality Laboratory, Gulf Coast Research and Education Center, University of Florida, Wimauma, FL 33598, USA

water, 1 M NaCl, 2 % acetic acid, and 0.6 M HCl) (Yang et al. 2000). For instance, water-soluble Cd in inorganic (extracted by 80 % ethanol) and organic forms (extracted by deionized water) had higher activity and mobility. They were more toxic to plant cells, while the undissolved Cd in pectates/protein (extracted by 1 M NaCl) and phosphate (extracted by 2 % acetic acid) forms were less toxic (Fu et al. 2011; Xu et al. 2011). A higher concentration of Cd in inorganic and water-soluble forms was found in a Cd-sensitive barley genotype, while there was more Cd in pectates/protein forms in three Cd-resistant genotypes (Wu et al. 2005). The larger percentages of Cd in the forms of pectates/protein and phosphate were responsible for the adaptation of ramie to Cd stress (Wang et al. 2008). Therefore, the chemical forms of Cd are closely associated with Cd tolerance in plants.

Among the chemical forms of Cd, cadmium-phosphate complexes are insoluble and have lower in vivo mobility and toxicity, which plays an important role in Cd tolerance (Qiu et al. 2011). Cd forms precipitates with phosphate and causes a significant increase in the concentration of phosphate in the roots of *Azolla* (Sela et al. 1988). An increase in phosphorus application in the media enhanced the percentage of Cd combined with phosphate and led to enhanced tolerance in corn and wheat (Yang et al. 2000). It has also been confirmed that Cd mainly exists as Cd-phosphate complexes in the roots and leaves of *Mirabilis jalapa* (Yu and Zhou 2009). Furthermore, Cd tolerance was correlated with an increase in undissolved Cd-phosphate complexes in the leaves of two grasses: *Poa pratensis* and *Festuca arundinacea* (Xu and Wang 2013). These studies were mostly conducted in plants, and a little research is conducted on the chemical forms of Cd in fungal mycelia.

Dark septate endophytes (DSE), a diverse group of ascomycetes, are dominant colonizers in plant roots (Jumpponen and Trappe 1998; Jumpponen 2001) and play an important role in enhancing the tolerance of plants to heavy metals (Li et al. 2011). They are found in various habitats but are especially common in stressful environments, particularly in heavy metal-polluted areas, such as in the vicinity of the nickel-copper smelters (Ruotsalainen et al. 2007), zinc slag heaps (Deram et al. 2008), lead-zinc slag heaps (Zhang et al. 2013), and lead-zinc mine wastelands (Zhang et al. 2008; Li et al. 2012). Previous research has shown that DSE isolated from heavy metal-polluted soils has a high tolerance to Cd (Zhang et al. 2008; Ban et al. 2012); however, the Cd tolerance mechanisms of DSE are not well elucidated.

In the present study, the objective was to study the subcellular distribution, chemical forms, and adsorptive groups of Cd in the mycelia of a DSE fungus (*Exophiala pisciphila*) to determine the mechanisms responsible for the Cd tolerance in fungi. We used subcellular fractionation, sequential solvent extraction, scanning electron microscopy-energy dispersive spectrometry (SEM-EDS), TEM-EDX spectroscopy, and

Fourier transform infrared (FTIR) analyses to unravel Cd tolerance mechanisms. Our working hypothesis was that *E. pisciphila* developed strategies of metal sequestration in the cell walls to minimize Cd toxicity.

Materials and methods

DSE strain and cultivation

A DSE fungus, *Exophiala pisciphila* H93, was isolated from the roots of *Arundinella bengalensis* (Poaceae), naturally growing in an old mine smelting site in Huize County, Yunnan Province, Southwest China (103° 36' E, 26° 55' N), and the fungus was identified based both on its morphology and molecular method (Zhang et al. 2008, 2013). The fungus is now preserved in the Agricultural Culture Collection Center of China (accession number ACCC32496). Furthermore, we found that it could form typical DSE structures when H93 was inoculated in the roots of maize (*Zea mays* L.) and enhanced the tolerance of maize to heavy metals when it colonized in the roots of maize (Li et al. 2011).

The strain was maintained on potato glucose agar (PDA) slants by subculture every 2 months and stored at 4 °C in a refrigerator. MMN medium (CaCl₂·2H₂O 0.05 g L⁻¹, NaCl 0.025 g L⁻¹, glucose 10.0 g L⁻¹, maltose 3.0 g L⁻¹, NaCl 0.025 g L⁻¹, K₂HPO₄ 0.5 g L⁻¹, VB₁ 0.1 mg L⁻¹, MgSO₄·7H₂O 0.15 g L⁻¹, 1 % FeCl₃ solution 1.2 mL L⁻¹, NaNO₃ 3.0 g L⁻¹; pH 5.5) was used for inoculums (Zhan et al. 2011).

The treatments with Cd concentrations of 0, 25, 50, 100, 200, and 400 mg L⁻¹ in MMN medium were obtained by adding 0, 0.25, 0.5, 1, 2, and 4 mL of CdCl₂·2.5H₂O stock solution (10 g L⁻¹), respectively. A subsample of *E. pisciphila* mycelia was transferred from the slant to a PDA plate and cultured for 10 days to form a fungal colony. A fungal block of 6 mm diameter was taken from the plate and cultured in liquid MMN medium at 28±1 °C, 120 rpm for 7 days based on a growth experiment that found that the fungal biomass reached the maximum on day 7. The mycelia in liquid media were isolated by filtration, washed with distilled water three times, and suction filtrated to obtain fresh mycelia samples. Part of the fresh mycelia was dried at 70–75 °C for 48 h to obtain dry mycelia samples.

Total cadmium analysis

A subsample (0.20 g) of the dry mycelia was digested in 15 mL of an ultrapure mixture of concentrated HNO₃/HClO₄ (3:1) (v/v) on a thermo block at 200–250 °C to obtain a transparent solution, which was diluted to 50 mL with 0.2 % HNO₃. Total concentrations of Cd were determined using atomic absorption spectrometer (TAS-990, Beijing Puxi Instrument Factory, Beijing, People's Republic of China).

Appropriate quality controls included the use of CdCl_2 as a standard solution.

Cadmium subcellular distribution

Fresh mycelia (0.50 g) were homogenized in an extraction buffer (0.25 mol L^{-1} sucrose, Tris-HCl buffer solution (pH 7.5), and 1.0 mmol L^{-1} DL-dithioerythritol) according to the previous studies (Wang et al. 2008; Weigel and Jager 1980) with some modifications. In brief, the homogenate was centrifuged at 600 rpm for 15 min, and the precipitation was designated as “cell wall fraction,” consisting mainly of cell walls and cell wall debris. The resulting supernatant solution was further centrifuged at $28,620\times g$ for 45 min. The resultant precipitate was referred to as the “cell organelles fraction,” and the supernatant solution was referred to as the “soluble fraction.” All steps were performed at 4 °C. The three separated fractions were then dried and wet digested for Cd analysis.

Scanning electron microscopy-energy dispersive spectrometer analysis

After rinsing with 0.1 mM phosphate buffer (pH 7.2), a subsample of the fresh mycelia was fixed in 2.5 % glutaraldehyde solution at 4 °C for 3 h. The mycelia were washed with 0.1 mM phosphate buffer three times for 20 min each. Then, the mycelia were successively dehydrated with 30, 50, 70, 90, and 100 % ethanol for 15 min each to produce dehydrated mycelia, which were then stored in absolute ethanol. A subsample of the dehydrated mycelia was air-dried and coated with a thin layer of gold using a sputter coater (SCD005, BAL-TEC, Finland). The samples were observed under a thermal field-emission environmental scanning electron microscope (SEM, QUANTA200, FEI, USA) combined with an energy dispersive spectrometer (EDS, EDAX, APOLLO, USA).

Transmission electron microscopy-energy dispersive X-ray spectroscopy analysis

A subsample of the dehydrated mycelia was embedded in 618 epoxy resin, and ultrathin sections (70 nm) were obtained using an ultramicrotome (EM UC6, LEICA, Germany). The ultrathin sections were observed using a transmission electron microscope (H-7650, HITACHI, Japan) and analyzed for the metal components in the black precipitates surrounding the mycelia using a transmission electron microscope (TEM, JEM-2100, OXFORD, UK) combined with an energy dispersive X-ray spectroscopy (EDX, INCA, OXFORD, UK).

Fourier transform infrared analysis

A subsample of the dry mycelia was ground with infrared quality KBr (1:10) and pressed into disks in a vacuum using a Spectra Lab Pelletiser. The Fourier transform infrared (FTIR) spectra ($4000\text{--}400\text{ cm}^{-1}$) were recorded in a Thermo Nicolet Avatar 360 FTIR spectrophotometer.

Chemical form extraction and analysis

Cadmium associated with different chemical forms was successively extracted by designated solutions in the following order (Yang et al. 2000): (i) 80 % ethanol extracting inorganic Cd, including nitrate, chloride, and aminophenol Cd; (ii) deionized water extracting soluble Cd-organic acid complexes and $\text{Cd}(\text{H}_2\text{PO}_4)_2$; (iii) 1 M NaCl extracting Cd integrated with pectates and protein; (iv) 2 % acetic acid extracting insoluble CdHPO_4 , $\text{Cd}_3(\text{PO}_4)_2$, and other Cd-phosphate complexes; (v) 0.6 M HCl extracting Cd oxalate; and (vi) Cd in residues.

A subsample (0.40 g) of the fresh mycelia was homogenized in extraction solution with a mortar and a pestle, diluted to 1:50 (w/v), and shaken for 22 h at 25 °C. Then, the homogenate was centrifuged at $1789\times g$ for 10 min, obtaining the first supernatant solution. The sedimentation was resuspended twice in extraction solution and shaken for 2 h at 25 °C, centrifuged at $2795\times g$ for 10 min, and then the supernatant of the three suspensions and centrifugation steps was boiled for each of the five extraction solutions. Each of the pooled supernatant solutions was then evaporated on an electric plate at 70 °C to a constant weight and digested at 145 °C with an acid oxidative mixture of $\text{HNO}_3/\text{HClO}_4$ (3:1, v/v). Concentrations of Cd associated with different chemical forms were determined.

Statistical analysis

All data are presented as means of three replicated measurements. The results are expressed as means \pm standard deviation (SD). Statistical analyses were performed using the Duncan's new multiple range method by SPSS 22.0. Least significant difference (LSD) at a 0.05 probability level was used to detect the differences between treatment means. The Pearson's correlation coefficient of the mean data was applied to determine the Cd relationship between subcellular distribution and chemical forms.

Results

Subcellular distribution of cadmium in the mycelia

Cadmium content in the mycelia of *E. pisciphila* increased with increase in the initial concentration of Cd in the media

Table 1 Cadmium contents (mg g⁻¹) in the subcellular fraction of *E. pisciphila* mycelia

Cd in the media (mg L ⁻¹)	Cell wall	Cell organelles	Soluble fraction	Recovery (%)	Total
25	3.0 ^d ±0.74 (81.0)	0.3 ^b ±0.05 (9.2)	0.4 ^b ±0.09 (9.8)	102.1	3.6 ^d ±0.58
50	5.5 ^d ±1.55 (87.3)	0.4 ^b ±0.07 (5.9)	0.4 ^b ±0.08 (6.8)	98.8	6.4 ^d ±0.57
100	18.4 ^c ±3.85 (95.1)	0.5 ^b ±0.08 (2.4)	0.5 ^b ±0.17 (2.5)	102.6	18.9 ^c ±4.26
200	34.7 ^b ±6.83 (97.0)	0.5 ^b ±0.12 (1.5)	0.5 ^b ±0.15 (1.5)	93.8	38.1 ^b ±5.00
400	51.8 ^a ±2.96 (97.0)	0.8 ^a ±0.22 (1.5)	0.8 ^a ±0.24 (1.5)	103.8	51.4 ^a ±8.04

The content of Cd in the table is based on dry weight of fungal mycelia; data are means±SD ($n=4$). Different letters in the same column indicate a significant difference at the 5 % level. Figures in parentheses are percent of total cadmium. Recovery (%)=(cell wall+cell organelles+soluble fraction)×100/total

(Table 1). The highest content of Cd (51.4 mg g⁻¹) was observed in the mycelia grown in the medium containing 400 mg L⁻¹ Cd.

The subcellular distribution (cell wall, cell organelles, and soluble fraction) of Cd in the mycelia of *E. pisciphila* is shown in Table 1. The majority of Cd was stored in the cell walls (81–97 %). A small amount of Cd was stored in cell organelles (1.5–9.2 %) and in the soluble fraction (1.5–9.8 %). Cd content in the cell walls increased significantly with an increase in Cd in the medium, while the Cd content in the cell organelles and soluble fraction increased very little (nonsignificant) and only significantly increased in the 400 mg L⁻¹ Cd treatment. In other words, the percentage of Cd stored in the cell walls increased, whereas the proportion stored in cell organelles and the soluble fraction decreased with an increase in Cd concentration in the media (Table 1).

Chemical forms of cadmium in the mycelia

The content of Cd in different chemical forms in the *E. pisciphila* mycelia is shown in Table 2. Most of the Cd (45–86 %) was bound with the phosphate (extracted by 2 % acetic acid), of which both the content and percent of total Cd increased in a concentration-dependent manner following the increase in Cd in the media. Approximately 11–40 % of the

Cd was in pectates/protein (extracted by 1 M NaCl), of which the content increased with the increase in Cd in the media, while the proportion decreased. The remainder of the Cd was in the organic form (1.3–7.4 %) extracted by deionized water and in the oxalate (0.9–5.7 %) extracted by 0.6 M HCl. The contents of both of these forms showed a small increase as the concentration of Cd increased in the media, while proportion of total Cd decreased. The lowest amounts of Cd were in inorganic form extracted by 80 % ethanol and Cd in residues.

Linkage between subcellular distribution and chemical forms of cadmium

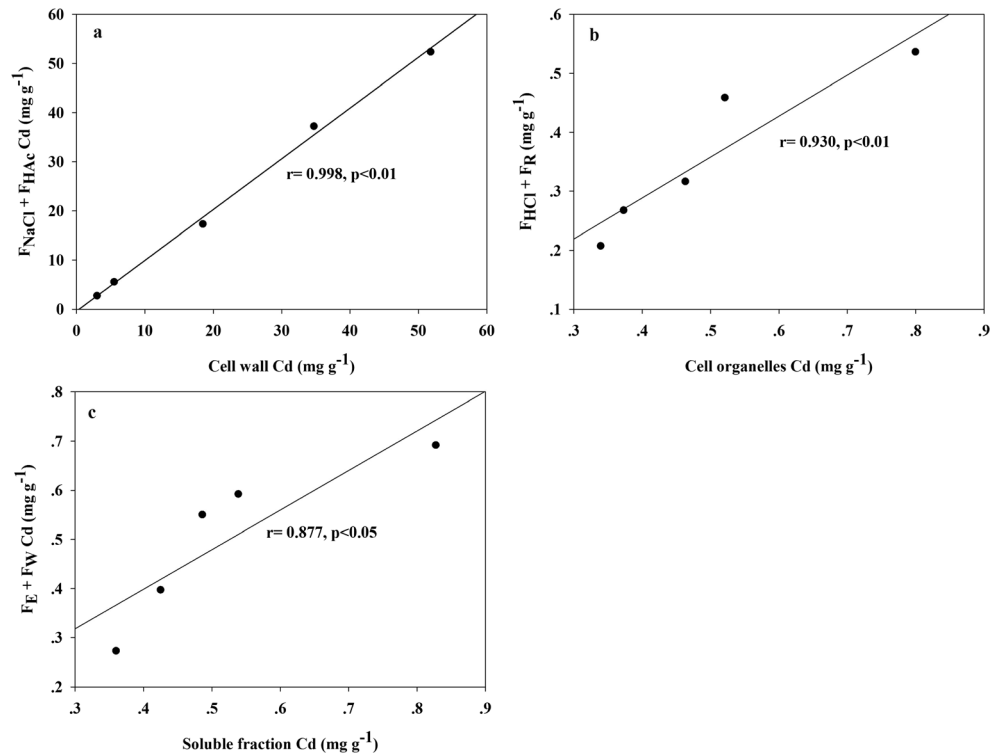
The correlations of Cd concentrations between different subcellular fractions and chemical forms in the mycelia were determined (Fig. 1). Very significant correlation of Cd concentrations was found between cell walls and the chemical forms extracted by NaCl and acetic acid ($r=0.998$, $p<0.01$), and cell organelles and the chemical forms extracted by HCl and residues ($r=0.930$, $p<0.01$). Significant correlation of Cd concentrations was found between the soluble fraction and the chemical forms extracted by ethanol and water ($r=0.887$, $p<0.05$). These results indicated that the Cd in the cell walls was mainly in the form of pectates/protein and phosphate and that the Cd in the cell organelles was in the form of oxalate.

Table 2 Cadmium contents (mg g⁻¹) in different chemical forms of *E. pisciphila* mycelia

Cd in the media (mg L ⁻¹)	Ethanol extractable	Water extractable	NaCl extractable	Acetic acid extractable	HCl extractable	Residue	Total recovery (%)
25	0.03 ^c ±0.010 (1.04)	0.2 ^c ±0.02 (7.4)	1.3 ^b ±0.32 (39.7)	1.5 ^d ±0.52 (45.4)	0.2 ^c ±0.08 (5.7)	0.02 ^c ±0.004 (0.75)	89.8
50	0.04 ^c ±0.007 (0.69)	0.4 ^{bc} ±0.02 (5.7)	1.9 ^b ±0.79 (29.7)	3.7 ^d ±0.66 (59.6)	0.2 ^c ±0.04 (3.7)	0.04 ^b ±0.005 (0.57)	98.3
100	0.07 ^b ±0.007 (0.40)	0.5 ^{ab} ±0.11 (2.6)	2.7 ^b ±0.72 (14.6)	14.7 ^c ±3.49 (80.6)	0.3 ^{bc} ±0.02 (1.5)	0.04 ^{ab} ±0.006 (0.21)	96.3
200	0.010 ^a ±0.005 (0.25)	0.5 ^{ab} ±0.13 (1.3)	5.2 ^a ±0.95 (13.5)	32.1 ^b ±1.81 (83.7)	0.4 ^{ab} ±0.13 (1.1)	0.04 ^{ab} ±0.003 (0.10)	100.5
400	0.11 ^a ±0.006 (0.21)	0.6 ^a ±0.15 (1.1)	6.1 ^a ±1.11 (11.3)	46.3 ^a ±3.44 (86.4)	0.5 ^a ±0.15 (0.9)	0.05 ^a ±0.003 (0.09)	104.2

The content of Cd in the table is based on dry weight of fungal mycelia. Values are means±SD ($n=4$). Different letters in the same row denote significant difference ($P<0.05$). Figures in parentheses are percent of total cadmium. Recovery (%)=(all extracted forms)×100/total

Fig. 1 Correlation of Cd between subcellular distribution and chemical forms. F_E -ethanol extractable form, F_W -water extractable form, F_{NaCl} -NaCl extractable form, F_{HAc} -acetic acid extractable form, F_{HCl} -HCl extractable form, and F_R -residual form



Scanning electron microscopy-energy dispersive spectrometer analysis

The point scan of SEM-EDS was carried out on the mycelia of *E. pisciphila* grown at two Cd concentrations of 50 mg L⁻¹ (Fig. 2a) and 100 mg L⁻¹ (Fig. 2b) Cd. According to the point scan, the relative content of carbon (C) was greatest at 66 and 70 %, followed by oxygen (O) at 28 and 23 % at 50 and 100 mg L⁻¹ Cd, respectively. Among mineral elements, phosphorus (P) was the highest in the mycelia, followed by Cd, Ca, S, Na, and K. For example, when the Cd concentration in the

media was 50 mg L⁻¹, the relative contents of P, Cd, Ca, S, Mg, Na, and K were 2.76, 1.97, 1.38, 0.82, 0.58, 0.35, and 0.25 %, respectively. At 100 mg L⁻¹ Cd treatment, the relative contents of P, Cd, Ca, S, Mg, and Na were 2.42, 2.31, 0.63, 0.73, 0.48, and 0.29 %, respectively.

The line scans of SEM-EDS were also conducted on the mycelia of *E. pisciphila* grown at two Cd concentrations of 50 mg L⁻¹ (Fig. 2c) and 100 mg L⁻¹ (Fig. 2d) Cd. Cd signal was detected over almost the entire mycelia surface. There were 295 point analyses in the line scan on the mycelia grown at 50 mg L⁻¹ Cd. In these point analyses, 23 points showed no

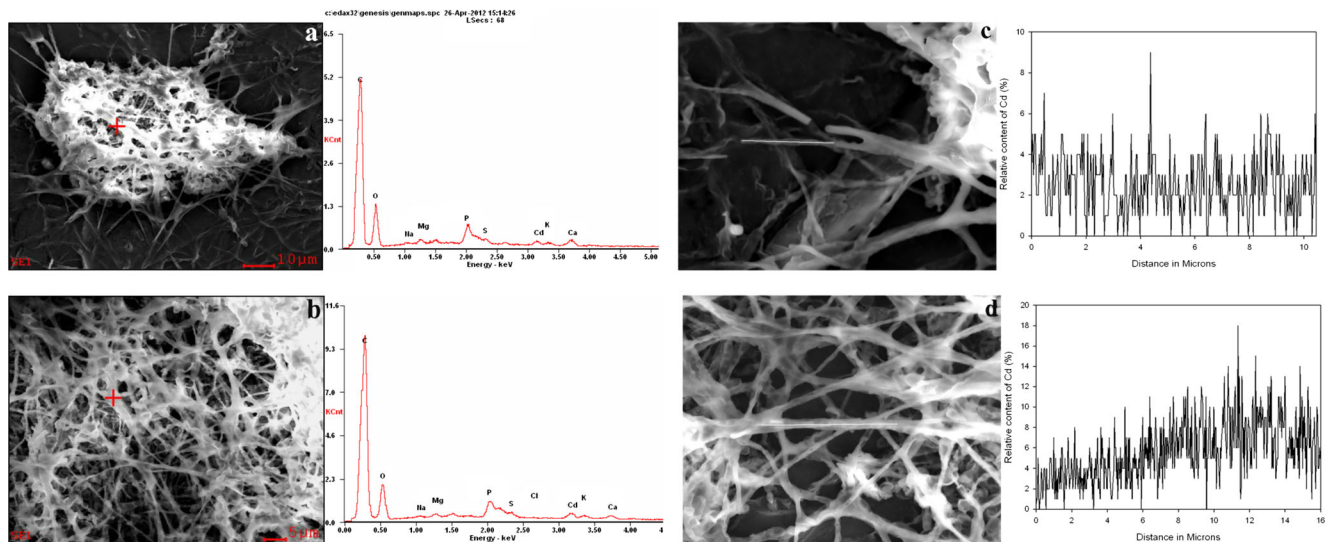


Fig. 2 Point (a 50 mg L⁻¹ Cd, b 100 mg L⁻¹ Cd) and line (c 50 mg L⁻¹ Cd, d 100 mg L⁻¹ Cd) scan of SEM-EDS on the mycelia of *E. pisciphila*

Cd, while 244 and 23 points showed relative Cd contents of 1–4 and >4 %, respectively. At 100 mg L⁻¹ Cd treatment, the line scans included 436 points. Of these, only 6 points did not detect Cd; 101, 169, 107, and 53 points had relative Cd contents of 1–3, 4–6, 7–9, and >10 %, respectively. These results showed that the content of Cd and its distribution on the mycelia surface of *E. pisciphila* increased with an increase in Cd in the media.

Transmission electron microscopy-energy dispersive X-ray spectroscopy analysis

The ultramicrotomes of *E. pisciphila* mycelia grown at 50 and 200 mg L⁻¹ Cd were observed by TEM (Fig. 3). There were many black precipitates (designated by arrows in the figure) with a high electron density around the cell walls. At 50 mg L⁻¹ Cd treatment, the black precipitates were evenly distributed around the cell walls. At 200 mg L⁻¹ Cd treatment, the black precipitates gathered as a group (see arrows) and were located near the cell walls. In addition, there were no visible black precipitates in the cells. The TEM indicated that Cd was mainly deposited in the cell walls.

The black precipitates deposited in the cell walls of *E. pisciphila* mycelia grown at 200 mg L⁻¹ Cd was further analyzed by TEM-EDX (Fig. 4). The signals of Cd (see arrow) were strong in the spectrum, which showed that the black precipitates deposited in the cell walls contained a large amount of Cd.

Fourier transform infrared analysis

Fourier transform infrared analysis (FTIR) spectra of the *E. pisciphila* mycelia grown at 0, 100, and 400 mg L⁻¹ Cd were obtained (Fig. 5). The broad band at 3400–3200 cm⁻¹ was due to the overlapping of hydroxyl (–OH) and amine (–NH) stretching vibrations. The peak at 2924 cm⁻¹ is indicative of –CH stretch. The band peaks at 1743 and 1641 cm⁻¹ may be attributed to asymmetric and symmetric stretching vibrations of carboxyl (–C=O) groups. The characteristic peak at 1078–1026 cm⁻¹ represents the presence of phosphate groups (–P=O). The shifting of IR peaks (0 Cd treatment) from 1036 to 1026 cm⁻¹ (100 and 400 mg L⁻¹ Cd treatment) suggests the involvement of phosphate bonds. Thus, hydroxyl, amine, carboxyl, and phosphate groups were involved in the binding of Cd to the cell walls of *E. pisciphila*.

Discussion

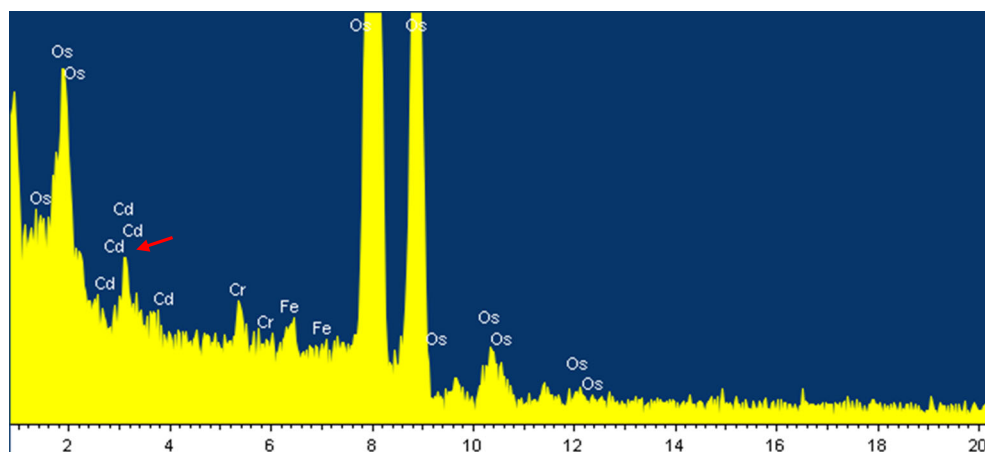
Subcellular distribution analysis showed that 81–97 % of total Cd was stored in the cell walls of *E. pisciphila* mycelia. SEM-EDS analysis confirmed the Cd distribution on the mycelia surface. TEM-EDX analysis found that precipitates deposited



Fig. 3 TEM analysis of the mycelia of *E. pisciphila* (**a** 50 mg L⁻¹ Cd treatment, **b** 200 mg L⁻¹ Cd treatment). The red arrows pointed black precipitates with a high electron density around the cell walls

on the cell walls contained Cd. These results indicated that most of Cd was binding to the cell walls. Binding of heavy metal ions onto cell walls is a common and effective way to prevent heavy metal ions from entering the cell (Bellion et al. 2006; Douchiche et al. 2010). The presence of Cd mainly in the cell walls was a general subcellular distribution pattern in different fungi, such as *Pisolithus arrhizus* (Turnau et al. 1994), *Oidiodendron maius* (Bardi et al. 1999), *Neurospora crassa* (Bhanoori and Venkateswerlu 2000), *Paxillus involutus* (Blaudez et al. 2000), *Glomus intraradices*

Fig. 4 TEM-EDX analysis of the black precipitates attached to the mycelia of *E. pisciphila* grown at 200 mg L⁻¹ Cd



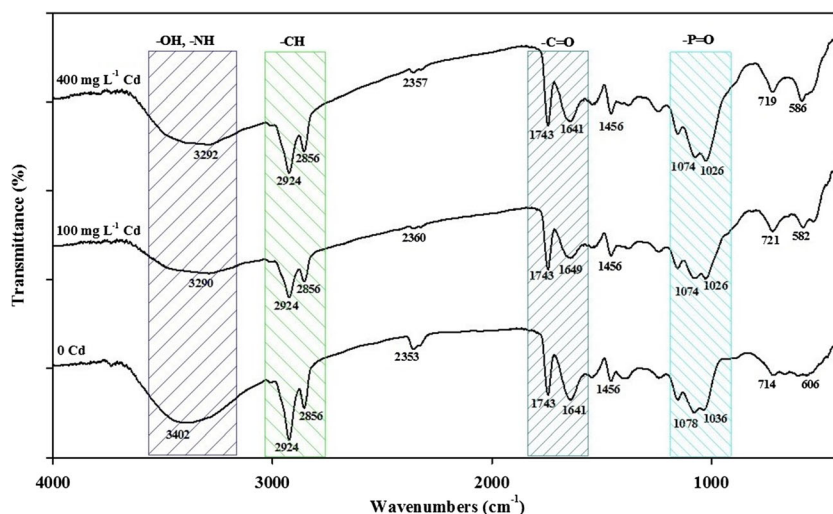
(Gonzalez-Guerrero et al. 2008), and *Phoma* sp. F2 (Yuan et al. 2007). Furthermore, the proportion of Cd stored in the cell walls increased, whereas the percent stored in the cell decreased with an increase in Cd in the media. This result indicated that the cell walls prevented Cd entry into the cells. The cell walls can work as a barrier and effectively sequester heavy metal ions (Macfie and Welbourn 2000; Latha et al. 2005; Meychik et al. 2011). Therefore, the cell walls could be a key factor in heavy metal exclusion mechanisms for fungi.

The strong affinity of heavy metals for fungal cell walls is closely related to the cell wall constituents. Fungal cell walls are mainly comprised of glycoproteins and polysaccharides, mainly glucan and chitin (Bowman and Free 2006; Latge 2007), which have a significant potential metal-binding ability. Metal ions can be bound to cell wall polymers, such as chitin and glucan (Baik et al. 2002; de Franco et al. 2004), and form the chitin-Cd complex in vivo (Bhanoori and Venkateswerlu 2000). In addition, some specific compounds such as melanins in the fungal cell walls also take part in the metal binding. The dark mycelia of DSE mycelia contain lots

of melanins in the cell walls (Zhan et al. 2011), which have a strong biosorptive capacity for metal ions (Fogarty and Tobin 1996; Buszman et al. 2006). Therefore, adsorption of Cd by melanized fungal cell walls of DSE could protect DSE from Cd toxicity.

The tight binding of Cd with fungal mycelia was closely related with functional groups present in fungal cell walls, as revealed by FTIR spectroscopy. In general, the broad peak at 3400–3200 cm⁻¹ was considered to represent overlapping hydroxyl (–OH) and amine (–NH) stretching vibrations (Akar and Tunali 2005; Bayramoglu et al. 2006; Das et al. 2008; Xiao et al. 2010). The peak at 2924 cm⁻¹ represented the –CH₂ stretching vibration of alkyl chains (Majumdar et al. 2008). The peaks at 1743 and 1641 cm⁻¹ were asymmetric and symmetric carboxyl (–C=O) stretching vibrations of carboxyl groups (Das et al. 2008; Xiao et al. 2010). The phosphate groups showed the characteristic peak at 1078–1026 cm⁻¹ (Das et al. 2008; Majumdar et al. 2008). In particular, the FTIR peaks shifting from 1036 to 1026 cm⁻¹ indicated the involvement of phosphate bonds (Duran et al. 2011). Therefore, the FTIR spectra indicated the presence of

Fig. 5 FTIR spectra of the *E. pisciphila* mycelia



hydroxyl, amine, carboxyl, and phosphate groups in the cell walls of *E. pisciphila*.

The chemical forms of Cd are closely related to their biological toxicity. For instance, water-soluble Cd in inorganic form (extracted by 80 % ethanol) and organic form (extracted by deionized water) have greater harmful effects than the undissolved Cd in pectates/protein (extracted by 1 M NaCl) and phosphate (extracted by 2 % acetic acid) (Wang et al. 2008; Fu et al. 2011). For *E. pisciphila*, the major chemical form of Cd was the undissolved Cd-phosphate complexes, followed by Cd in pectates/protein. The proportion of total Cd of the Cd-phosphate form increased markedly with the increase in Cd in the media. The Cd forms of pectates/protein and phosphate were mainly associated with the cell walls of the mycelia. Forming Cd-phosphate complexes could be an important detoxification mechanism for fungi. It has been reported that *Rhodotorula* sp. Y11 shows an increase in P content in the cells under Cd toxicity (Li et al. 2008). Polyphosphate accumulation in extraradical hyphae could enhance the Cd resistance of *Rhizophagus irregularis* (Yao et al. 2013). *Cunninghamella elegans* may accumulate polyphosphate as a detoxification mechanism to precipitate and tolerate Cd (de Lima et al. 2013). *Penicillium chrysogenum* deposited Cd inside or outside of the cells by forming $Cd_3(PO_4)_2$ with phosphoryl (Sarret et al. 1998). The conspicuous phosphorus peak in the EDAX spectra for *Rhizopus oligosporus* indicated that Cd was deposited as cadmium-phosphate (Aloysius et al. 1999).

We suggest that *E. pisciphila* mainly form undissolved Cd in phosphate on the cell walls to decrease Cd toxicity by forming Cd-phosphate. Forming Cd-phosphate complexes would allow *E. pisciphila* to tolerate Cd stress by accumulating Cd on the cell walls of the mycelia. To understand that Cd-phosphate complexes are involved in Cd tolerance is helpful in elucidating the survival strategy of *E. pisciphila* in heavy metal-polluted soils. However, the linkage between phosphate or polyphosphate and Cd-detoxifying mechanisms in *E. pisciphila* is currently not known and needs further study.

Conclusions

Exophiala pisciphila was grown for 7 days in liquid media with increasing Cd concentrations. Cd content in the mycelia increased proportionally with an increase in the concentration of Cd in the media. Subcellular fractionation of Cd-containing tissues indicated that most (81–97 %) of the Cd was associated with cell walls. FTIR analysis indicated that hydroxyl, amine, carboxyl, and phosphate groups were responsible for the binding of Cd in the cell walls. The SEM-EDS analysis found that there was more phosphate and Cd on the mycelia surface, and the content of phosphate was higher than Cd. Around the cell walls, some precipitates associated with the mycelia were

observed by TEM and were found to contain Cd by TEM-EDX, which also confirmed Cd deposition in the cell walls. Furthermore, the largest amount and proportion (45–86 %) of Cd was extracted with 2 % acetic acid, and a concentration-dependent extraction was observed with increasing Cd in the media; both of which suggest that Cd-phosphate complexes were the major chemical form in *E. pisciphila*. Therefore, we conclude that binding of Cd to cell walls in Cd-phosphate complexes contributes to Cd tolerance in *E. pisciphila*.

Acknowledgments This work was financially supported by the National Natural Science Foundation of China (Nos. 41461093, 41101486, U1202236, 31160009, and 31460114).

References

- Akar T, Tunali S (2005) Biosorption performance of *Botrytis cinerea* fungal by-products for removal of Cd (II) and Cu (II) ions from aqueous solutions. *Miner Eng* 18:1099–1109
- Aloysius R, Karim M, Ariff AB (1999) The mechanism of cadmium removal from aqueous solution by nonmetabolizing free and immobilized live biomass of *Rhizopus oligosporus*. *World J Microbiol Biotechnol* 15:571–578
- Baik WY, Bae JH, Cho KM, Hartmeier W (2002) Biosorption of heavy metals using whole mold mycelia and parts thereof. *Bioresour Technol* 81:167–170
- Ban Y, Tang M, Chen H, Xu Z, Zhang H, Yang Y (2012) The response of dark septate endophytes (DSE) to heavy metals in pure culture. *PLoS One* 7, e47968
- Bardi L, Perotto S, Bonfante P (1999) Isolation and regeneration of protoplasts from two strains of the ericoid mycorrhizal fungus *Oidiodendron maius*: sensitivity to chemicals and heavy metals. *Microbiol Res* 154:105–111
- Bayramoglu G, Celik G, Arica MY (2006) Studies on accumulation of uranium by fungus *Lentinus sajor-caju*. *J Hazard Mater* 136:345–353
- Bellion M, Courbot M, Jacob C, Blaudez D, Chalot M (2006) Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi. *FEMS Microbiol Lett* 254:173–181
- Bhanoori M, Venkateswerlu G (2000) In vivo chitin–cadmium complexation in cell wall of *Neurospora crassa*. *Biochim Biophys Acta Gen Subj* 1523:21–28
- Blaudez D, Botton B, Chalot M (2000) Cadmium uptake and subcellular compartmentation in the ectomycorrhizal fungus *Paxillus involutus*. *Microbiology* 146:1109–1117
- Bowman SM, Free SJ (2006) The structure and synthesis of the fungal cell wall. *Bioessays* 28:799–808
- Buszman E, Pilawa B, Zdybel M, Wilczynski S, Gondzik A, Witoszynska T, Wilczok T (2006) EPR examination of Zn^{2+} and Cu^{2+} binding by pigmented soil fungi *Cladosporium cladosporioides*. *Sci Total Environ* 363:195–205
- Das SK, Ghosh P, Ghosh I, Guha AK (2008) Adsorption of rhodamine B on *Rhizopus oryzae*: role of functional groups and cell wall components. *Colloids Surf B* 65:30–34
- de Franco LO, de Maia RCC, Porto ALF, Messias AS, Fukushima K, de Campos-Takaki GM (2004) Heavy metal biosorption by chitin and chitosan isolated from *Cunninghamella elegans* (IFM 46109). *Braz J Microbiol* 35:243–247
- de Lima MA, de Franco LO, de Souza PM, do Nascimento AE, da Silva CA, de Maia RC, Rolim HM, Takaki G (2013) Cadmium tolerance

- and removal from *Cunninghamella elegans* related to the polyphosphate metabolism. *Int J Mol Sci* 14:7180–7192
- Deram A, Langueureau-Leman F, Howsam M, Petit D, Haluwyn CV (2008) Seasonal patterns of cadmium accumulation in *Arrhenatherum elatius* (Poaceae): influence of mycorrhizal and endophytic fungal colonisation. *Soil Biol Biochem* 40:845–848
- Douchiche O, Soret-Morvan O, Chaibi W, Morvan C, Paynel F (2010) Characteristics of cadmium tolerance in ‘Hermes’ flax seedlings: contribution of cell walls. *Chemosphere* 81:1430–1436
- Duran N, Marcato PD, Duran M, Yadav A, Gade A, Rai M (2011) Mechanistic aspects in the biogenic synthesis of extracellular metal nanoparticles by peptides, bacteria, fungi, and plants. *Appl Microbiol Biotechnol* 90:1609–1624
- Fogarty RV, Tobin JM (1996) Fungal melanins and their interactions with metals. *Enzym Microb Technol* 19:311–317
- Fu X, Dou C, Chen Y, Chen X, Shi J, Yu M, Xu J (2011) Subcellular distribution and chemical forms of cadmium in *Phytolacca americana* L. *J Hazard Mater* 186:103–107
- Gonzalez-Guerrero M, Melville LH, Ferrol N, Lott JN, Azcon-Aguilar C, Peterson RL (2008) Ultrastructural localization of heavy metals in the extraradical mycelium and spores of the arbuscular mycorrhizal fungus *Glomus intraradices*. *Can J Microbiol* 54:103–110
- Häferburg G, Kothe E (2007) Microbes and metals: interactions in the environment. *J Basic Microbiol* 47:453–467
- Jumpponen A (2001) Dark septate endophytes—are they mycorrhizal? *Mycorrhiza* 11:207–211
- Jumpponen A, Trappe JM (1998) Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. *New Phytol* 140:295–310
- Latge JP (2007) The cell wall: a carbohydrate armour for the fungal cell. *Mol Microbiol* 66:279–290
- Latha JNL, Rashmi K, Mohan PM (2005) Cell-wall-bound metal ions are not taken up in *Neurospora crassa*. *Can J Microbiol* 51:1021–1026
- Li Z, Yuan H, Hu X (2008) Cadmium-resistance in growing *Rhodotorula* sp. Y11. *Bioresour Technol* 99:1339–1344
- Li T, Liu MJ, Zhang XT, Zhang HB, Sha T, Zhao ZW (2011) Improved tolerance of maize (*Zea mays* L.) to heavy metals by colonization of a dark septate endophyte (DSE) *Exophiala pisciphila*. *Sci Total Environ* 409:1069–1074
- Li H, Li D, He C, Zhou Z, Mei T, Xu H (2012) Diversity and heavy metal tolerance of endophytic fungi from six dominant plant species in a Pb–Zn mine wasteland in China. *Fungal Ecol* 5:309–315
- Loganathan P, Vigneswaran S, Kandasamy J, Naidu R (2012) Cadmium sorption and desorption in soils: a review. *Crit Rev Environ Sci Technol* 42:489–533
- Macfie SM, Welbourn PM (2000) The cell wall as a barrier to uptake of metal ions in the unicellular green alga *Chlamydomonas reinhardtii* (Chlorophyceae). *Arch Environ Contam Toxicol* 39:413–419
- Majumdar SS, Das SK, Saha T, Panda GC, Bandyopadhyay T, Guha AK (2008) Adsorption behavior of copper ions on *Mucor rouxii* biomass through microscopic and FTIR analysis. *Colloids Surf B* 63:138–145
- Meychik NR, Nikolaeva YI, Komarynets OV, Ermakov IP (2011) Barrier function of the cell wall during uptake of nickel ions. *Russ J Plant Physiol* 58:409–414
- Moullis J, Thevenod F (2010) New perspectives in cadmium toxicity: an introduction. *Biometals* 23:763–768
- Qiu Q, Wang Y, Yang Z, Yuan J (2011) Effects of phosphorus supplied in soil on subcellular distribution and chemical forms of cadmium in two Chinese flowering cabbage (*Brassica parachinensis* L.) cultivars differing in cadmium accumulation. *Food Chem Toxicol* 49:2260–2267
- Ruotsalainen AL, Markkola A, Kozlov MV (2007) Root fungal colonisation in *Deschampsia flexuosa*: effects of pollution and neighbouring trees. *Environ Pollut* 147:723–728
- Sarret G, Manceau A, Spadini L, Roux J, Hazemann J, Soldo Y, Eybert-Berard L, Menthonnex J (1998) Structural determination of Zn and Pb binding sites in *Penicillium chrysogenum* cell walls by EXAFS spectroscopy. *Environ Sci Technol* 32:1648–1655
- Sela M, Tel-Or E, Fritz E, Huttermann A (1988) Localization and toxic effects of cadmium, copper, and uranium in *Azolla*. *Plant Physiol* 88:30–36
- Turnau K, Kottke I, Dexheimer J, Botton B (1994) Element distribution in mycelium of *Pisolithus arrhizus* treated with cadmium dust. *Ann Bot* 74:137–142
- Vijver MG, van Gestel CA, Lanno RP, van Straalen NM, Peijnenburg WJ (2004) Internal metal sequestration and its ecotoxicological relevance: a review. *Environ Sci Technol* 38:4705–4712
- Wang X, Liu Y, Zeng G, Chai L, Song X, Min Z, Xiao X (2008) Subcellular distribution and chemical forms of cadmium in *Bechmeria nivea* (L.) Gaud. *Environ Exp Bot* 62:389–395
- Weigel HJ, Jäger HJ (1980) Subcellular distribution and chemical form of cadmium in bean plants. *Plant Physiol* 65:480–482
- Wu F, Dong J, Qian QQ, Zhang G (2005) Subcellular distribution and chemical form of Cd and Cd–Zn interaction in different barley genotypes. *Chemosphere* 60:1437–1446
- Xiao X, Luo S, Zeng G, Wei W, Wan Y, Chen L, Guo H, Cao Z, Yang L, Chen J (2010) Biosorption of cadmium by endophytic fungus (EF) *Microsphaeropsis* sp. LSE10 isolated from cadmium hyperaccumulator *Solanum nigrum* L. *Bioresour Technol* 101:1668–1674
- Xu P, Wang Z (2013) Physiological mechanism of hypertolerance of cadmium in Kentucky bluegrass and tall fescue: chemical forms and tissue distribution. *Environ Exp Bot* 96:35–42
- Xu W, Shi W, Yan F, Zhang B, Liang J (2011) Mechanisms of cadmium detoxification in cattail (*Typha angustifolia* L.). *Aquat Bot* 94:37–43
- Yang ZY, Zheng S, Hu A (2000) Accumulation, chemical forms and physiological characterization of cadmium in plants affected by phosphorus. *Chin J Appl Environ Biol* 6:121–126 (In Chinese)
- Yao Q, Yang RH, Long LK, Zhu HH (2013) Phosphate application enhances the resistance of arbuscular mycorrhizae in clover plants to cadmium via polyphosphate accumulation in fungal hyphae. *Environ Exp Bot* 108:63–70
- Yu Z, Zhou Q (2009) Growth responses and cadmium accumulation of *Mirabilis jalapa* L. under interaction between cadmium and phosphorus. *J Hazard Mater* 167:38–43
- Yuan H, Li Z, Ying J, Wang E (2007) Cadmium (II) removal by a hyperaccumulator fungus *Phoma* sp. F2 isolated from blend soil. *Curr Microbiol* 55:223–227
- Zhan FD, He YM, Zu YQ, Li T, Zhao ZW (2011) Characterization of melanin isolated from a dark septate endophyte (DSE), *Exophiala pisciphila*. *World J Microbiol Biotechnol* 27:2483–2489
- Zhang YJ, Zhang Y, Liu MJ, Shi XD, Zhao ZW (2008) Dark septate endophyte (DSE) fungi isolated from metal polluted soils: their taxonomic position, tolerance, and accumulation of heavy metals *In Vitro*. *J Microbiol* 46:624–632
- Zhang Y, Li T, Zhao ZW (2013) Colonization characteristics and composition of dark septate endophytes (DSE) in a lead and zinc slag heap in Southwest China. *Soil Sediment Contam* 22:532–545