The Journal of Microbiology, December 2008, p. 624-632
DOI 10.1007/s12275-008-0163-6

Vol. 46, No. 6

Dark Septate Endophyte (DSE) Fungi Isolated from Metal Polluted Soils: Their Taxonomic Position, Tolerance, and Accumulation of Heavy Metals *In Vitro*

Yujie Zhang[1], Yan Zhang[1], Maojun Liu[1], Xiaodong Shi[2], and Zhiwei Zhao[1]*

[1]*Laboratory for Conservation and Utilization of Bio-Resources, and Key Laboratory for Microbial Resources of the Ministry of Education, Yunnan University, Kunming 650091, P. R. China*
[2]*Department of Biology, Qujing Normal University, Qunjing 655011, P. R. China*

(Received July 1, 2008 / Accepted September 18, 2008)

To understand the possible role of the plant root associated fungi on metal tolerance, their role in the uptake of heavy metals and the potential transfer of these metal ions to the plant, three strains of dark septate endophytic (DSE) fungi were isolated from a waste smelter site in southwest China, and one strain was isolated from a non-contaminated site. According to molecular phylogenetic analysis of the ITS 1-5.8S rDNA-ITS 2 gene regions and morphological characteristics, one is identified as *Exophiala pisciphila,* and the other three are non-sporulating fungi under the experiment condition with the nearest phylogenetic affinities to the *Thysanorea papuana* strain EU041814. Tolerance and accumulation abilities of the three DSE strains for metals were investigated in liquid culture. Minimum inhibitory concentrations (MIC) of Pb, Zn, and Cd were determined. It was demonstrated that the tolerance of the DSE strains varied between metal species and strains. The *E. pisciphila* strain is able to accumulate lead and cadmium over 20% and 5% of dry weight of biomass, respectively. Partial of the sequestrated metals can be washed with $CaCl_2$. Morphological and enzyme activity changes taking place in the presence of excessive Pb, Cd, and/or Zn also indicate that the mechanism of heavy metal tolerance and accumulation of the DSE strains would be a complex process. The findings indicated promising tolerance and accumulation of the DSE strains with potential values in metal cycling and restoration of soil and water system.

***Keywords*:** dark septate endophytes (DSE), *Exophiala pisciphila*, metal tolerance, accumulation

Excessive heavy metals in soil have detrimental effects on ecosystems and pose risks to human health as they enter the food chain via agricultural products or contaminated drinking water (Cerbasi and Yetis, 2001). Among soil organisms, fungi are highly resistant to heavy metal pollution (Jordan and Lechevalier, 1975), and play important roles in element cycling, mineral transformations, and fungal-plant interactions (Massaccesi *et al*., 2002; Gadd, 2007).

In nature, the majority of plants associate with soil fungi to form symbioses (Smith and Read, 1997). Dark septate endophytes (DSE) are conidial or sterile ascomycetes colonizing plant roots (Jumpponen and Trappe, 1998) and have been reported from more than 600 plant species worldwide (Barrow and Aaltonen, 2001; Rains *et al.*, 2003; Addy *et al.*, 2005). Mandyam and Jumpponen (2005) reviewed the DSE abundance data in different habitats and data about their possible roles in ecosystems, including the facilitation of mineral nutrient uptake of the host plant, utilization of wider organic nutrient pools through symbiotic DSE, alteration of host water uptake and environmental tolerance, and involvement in host drought and heat tolerance. In addition, DSE were regularly isolated from metal polluted soils. Cevnik *et al.* (2000) reported that DSE are the dominant fungi from healthy fine roots of *Erica herbacea* in lead, cadmium, and zinc-polluted soil. Ruotsalainen *et al.* (2007) reported that DSE hyphal colonisation in *Deschampsia flexuosa* in polluted site was not affected by the elevated heavy metals compared with that in the control site, and higher numbers of intracellular DSE sclerotia (13.3 vs 3.4% of the control site) indicate a survial strategy in the unfavourable environment. During the surveying of the arbuscular mycorrhizal fungi in a lead and zinc mine smelting site in Yunnan, southwest China, we also found the high hyphal colonization of DSE in healthy plant roots and a lot of DSE strains were isolated and identified in laboratory.

In order to understand the possible impact of the plant associated fungi on metal tolerance, their role in the uptake of heavy metals and the potential transfer of these metal ions to the plant, the survival and tolerance and the accumulation capabilities of these fungi to heavy metals require assessment *in vitro*. Several experiments have proved that ectomycorrhizal fungi can stand high lever metal concentrations and accumulate these metals in the mycelia, and protect their host from heavy metal poisoning (Leyval *et al.*, 1997; Colpaert *et al.*, 2000) while the behavior of the DSE is less certain. This article reports on the metal-tolerance and accumulative potentials of several DSE strains in liquid culture. In addition, morphological changes of the DSE strains under metal stresses were observed.

* To whom correspondence should be addressed.
(Tel) 86-871-5033-824; (Fax) 86-871-5034-799
(E-mail) zhaozhw@ynu.edu.cn

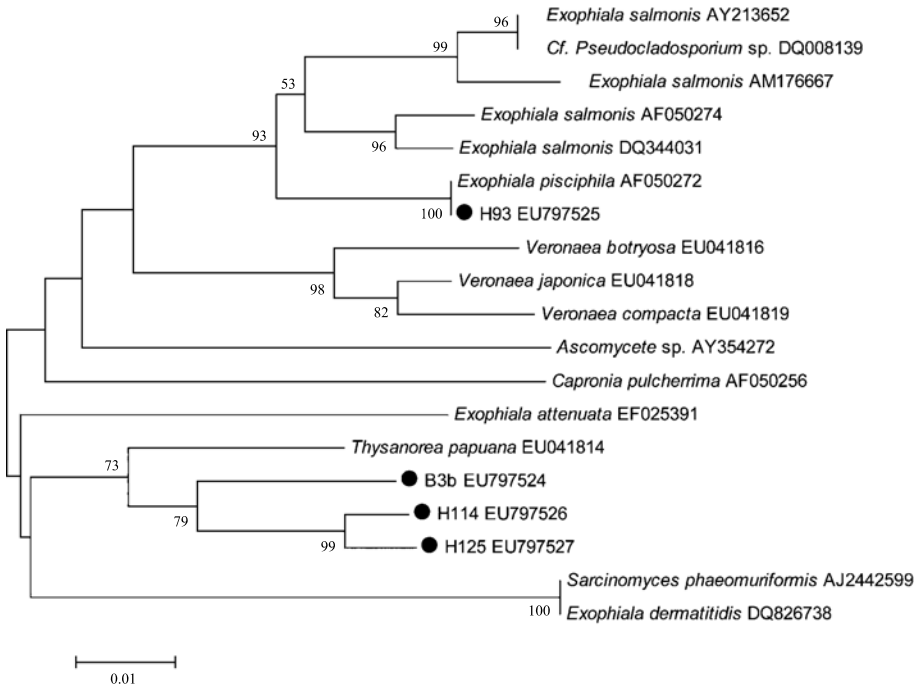

Fig. 1. NJ tree of the tested DSE strains (The tree is unrooted. Based on the analysis of 1,000 bootstrap replicates, nodes with support above 50% are indicated at the branches).

Materials and Methods

Isolation of the DSE strains

Three DSE strains were isolated from an old Pb-Zn mine smelting site, in Huize, Yunnan province, southwest China (103°03′ E- 103°55′, 25°48′ N- 27°04′). The heavy metal levels (HCl extractable metal concentrations) in the soil can be classified as moderate concentrations for Cu (25~70 μg/g dry soil) and high concentrations for Pb (260~430 μg/g), Zn (5,540~7,460 μg/g) and Cd (15~23 μg/g) according to SEPA (1997). *Arundinella bengalensis* and *Artemisia carvifolia* are two of the main plant species naturally regenerating in the Pb-Zn mine tailings, and the DSE hyphal colonization is high in the root of the two plant species [44%, for *Arundinella bengalensis*, 41% for *Artemisia carvifolia*, detected by magnified intersection method according to McGonigle *et al*. (1990)]. One DSE strain, isolated from *Curculigo capitulate* in a non-contaminated environment, was used for comparison. Healthy plant roots were collected and immediately processed. DSE strains were isolated according to the method of Silvani *et al.* (2008).

DNA extraction and PCR amplification

Fresh mycelia of the isolated DSE strains were obtained from cultures grown on Potato Dextrose Agar (PDA) plates for 7 days at 25°C in the dark. Fungal DNA was extracted with a DNeasy Plant Mini Kit (QIAGEN, USA) according to the manufacturer's protocol. Extracted DNA was used as the template for the Polymerase Chain Reaction (PCR). Primers ITS5 and ITS4 were used to amplify the ITS 1-5.8S rDNA-ITS 2 region (White *et al.*, 1990). The PCR product was analyzed by electrophoresis. EaZy Nucleic Acid Gel Extraction Kit (Omega bio-tek, USA) was used for purification of PCR products.

DNA sequencing and phylogenetic analysis

Amplified DNA products were sequenced with an ABIPRISM 3700 DNA sequencer using standard protocols provided by the manufacturer (PE Biosystems, USA). Sequence data obtained in this study were deposited in GenBank. GenBank sequences with the nearest phylogenetic affinities were selected for constructing a phylogenetic tree. Reference fungal species and GenBank accession no. are shown in Fig. 1. Multiple alignment searches were performed using the program CLUSTAL X (Thompson *et al.*, 1994). Phylogenetic analyses were performed by the neighbor-joining (NJ) method using MEGA version 4 (Kumar *et al.*, 2004). The NJ tree was constructed using Kimura's two-parameter method (Kimura, 1980). Bootstrap support was determined from 1,000 replications (Felsenstein, 1985).

Heavy-metal tolerance of the DSE strains

DSE strains were activated on PDA plates for at least one week before inoculation. Tolerance to heavy metals was determined as the minimum inhibitory concentration (MIC) against the tested fungi (Zafar *et al.*, 2007). Briefly, basal liquid medium (containing 0.4 g KH_2PO_4, 0.2 g $MgCL_2 \cdot 6H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 2 mg $ZnCl_2 \cdot H_2O$, 5 mg $MnCl_2 \cdot 4H_2O$, 3 mg $FeCl_2 \cdot 4H_2O$, 0.05 mg $CuCl_2 \cdot 2H_2O$, 150 mg L-lysine·HCl, 150 mg L-cystine, 150 mg DL-methionine, 100 mg L-phenylalanine, and 15 g sucrose per liter medium), modified referring to Caldwell *et al.* (1991) and Mandyam and Jumpponen (2005), were prepared and amended with various amounts of mono heavy metals to achieve the de-

Table 1. Minimum inhibitory concentration (MIC) of the DSE fungi (data are recorded as the lowest metal concentration that no biomass was obtained within two weeks of cultivation), and the EC50 of some DSE strains

DSE strains	Pb^{2+}		Cd^{2+}		Zn^{2+}	
	MIC (mg/ml)	EC50 (mg/ml)	MIC (mg/ml)	EC50 (mg/ml)	MIC (mg/ml)	EC50 (mg/ml)
H93	2.3	0.8	0.51	0.27	3.1	1.5
H125	1.2	0.6	0.015	-	0.1	-
H114	1.0	-	0.01	-	0.08	-
B3b	0.9	-	0.025	-	0.1	-

-, showed that the parallel EC50 was not determined

sired concentrations ranging from 0 to 10 mg/ml for Pb^{2+}, Zn^{2+}, and 0 to 1 mg/ml for Cd^{2+} with lead chloride, $ZnCl_2 \cdot 2H_2O$ and $CdCl_2 \cdot 2.5H_2O$ (analytical grade) respectively. Metal concentrations were adjusted to meet the requirements for the determination of EC50 (the effective concentration inhibiting growth by 50%). The pH of different liquid media was adjusted to 5.5 and autoclaved. Activated DSE strains were inoculated in triplicates in different flaks. For the sporulating isolates, spores were suspended in a small quantity of liquid medium (10^6 CFU/ml) first. Equal volume of suspended spores (100 μl) was seeded in triplicates in 100 ml basal liquid medium with different concentrations of heavy metals. For the non-sporulating fungi, hyphal segments were regulated according to colony form unit (CFU). Then the inoculated flasks were incubated at 28±1°C with shaking (120 rpm) for two weeks. The lowest concentrations of the metals, in which the tested fungi can not grow within two weeks of cultivation, were recorded as MICs. And the EC50s of the representative strains were also recorded in Table 1.

Metal analysis of the mycelia

Mycelia for heavy metal analysis were filtrated and rinsed extensively with distilled water, dried to stable weight at 70°C. Part of the water-washed mycelia of representative treatments were selected to be further washed with 1 N $CaCl_2$ (shaking at 28°C, 120 rpm for 30 min) and filtrated and rinsed again with distilled water, dried to stable weight at 70°C. According to the method of Lu (2000), 0.1~0.2 g of dried mycelia was treated with 10 ml of HNO_3+3 ml of $HClO_4$. Digestion was carried out on a hot plate until dense fume evolved and a clear solution was obtained. The filtrate was used for determination of various metals in the dried mycelia by atomic absorption spectrophotometer (AAS, model TA990) according to the standard protocols provided by the manufacturer (Beijing Purkinje General Instrument Co., Ltd.).

Observation of fungal morphology under metal stress

The growth and morphological characteristics of hyphae and spores of the tested fungi in basal liquid medium with different Pb^{2+}, Zn^{2+}, and Cd^{2+} concentrations were observed under microscope in every growth phase.

Enzyme activity changes of H93 under metal stress

The isolate of H93 was selected as representative to be cultured further for the detection of enzyme activities of superoxide dismutase (SOD) and catalase (CAT), the two main enzymes that were usually involved in the responses of organisms to metal stresses (Choudhary *et al.*, 2007). Triplicates were inoculated for every concentration of Cd^{2+} and Pb^{2+}. After a week of incubation, mycelia were filtrated, rinsed extensively and frozen at -70°C. The frozen mycelia were grinded on ice and dissolved in cold Tris-buffer (30 mM, pH 7.6). Cell debris and precipitated proteins were removed by centrifugation (10,000×g, 15 min, 4°C), and the supernatant was used for the detection of enzyme activities of SOD and ACT. Relative enzyme activities of SOD and CAT were determined with SOD Assay Kit and CAT Assay Kit as recommended by the manufacturer (Biotechnology institute, China).

Statistical analysis

Data on mycelium biomass, heavy metal content in the mycelia and enzyme activities in hyphae were analyzed by the computer software package, SPSS 11.5 (SPSS Inc., USA), and the results were expressed as Mean±SEM.

Results

Taxonomic position of the DSE strains

The DSE strain H93 was isolated from *Arundinella bengalensis*. H114 and H125 were from *Artemisia carvifolia*. B3b was isolated from *Curculigo capitulate* in a non-contaminated site. The ITS 1-5.8S rDNA-ITS 2 gene region sequence data of these DSE stains were deposited to GenBank with accession numbers of EU797524 (B3b), EU797525 (H93), EU797526 (H114), and EU797526 (H125). The data matrix

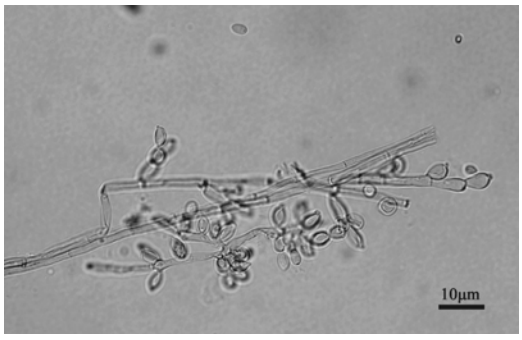

Fig. 2. Morphology of H93 cultured on PDA medium for two weeks.

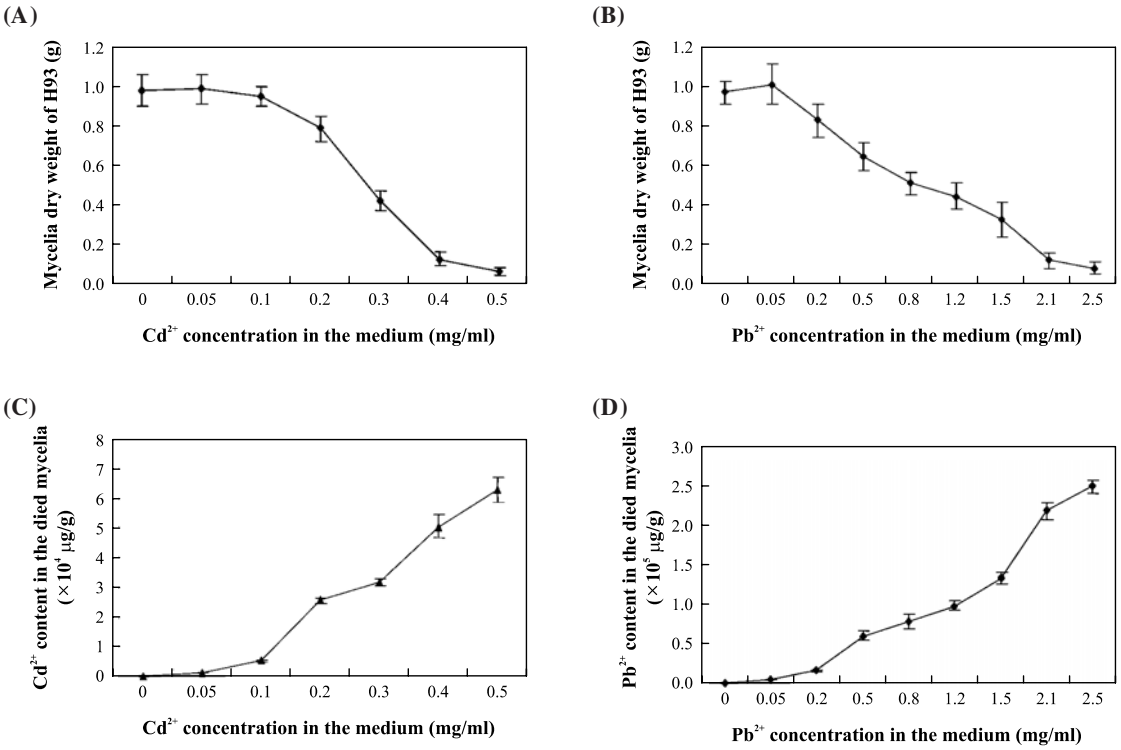

Fig. 3. Growth curves of H93 cultivated for two weeks in liquid basal medium supplemented with different concentrations of Pb^{2+} or Cd^{2+}, and increasing metal content in the mycelia; Data are the Mean±SEM of triplicates of one experiment.

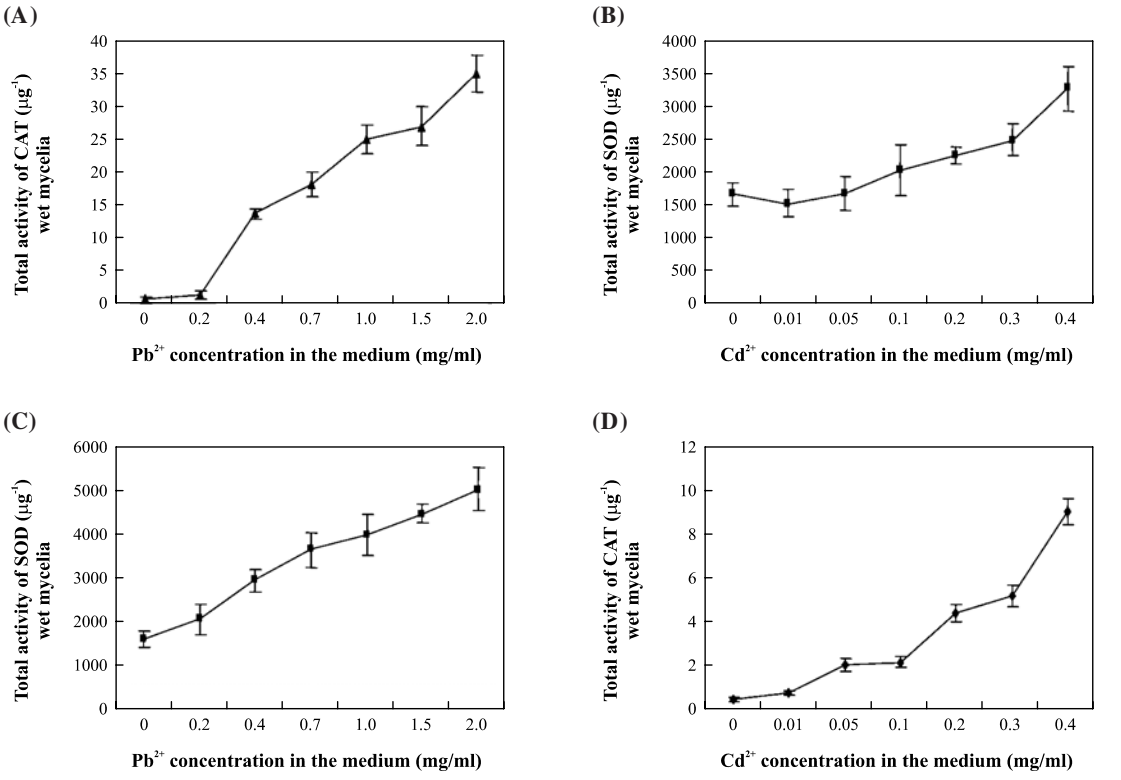

Fig. 4. Enzyme activities of SOD and CAT in the hyphae of H93 cultivated for a week in liquid basal medium supplemented with a series of increasing metals. Data are the Mean±SEM of triplicates of two experiments.

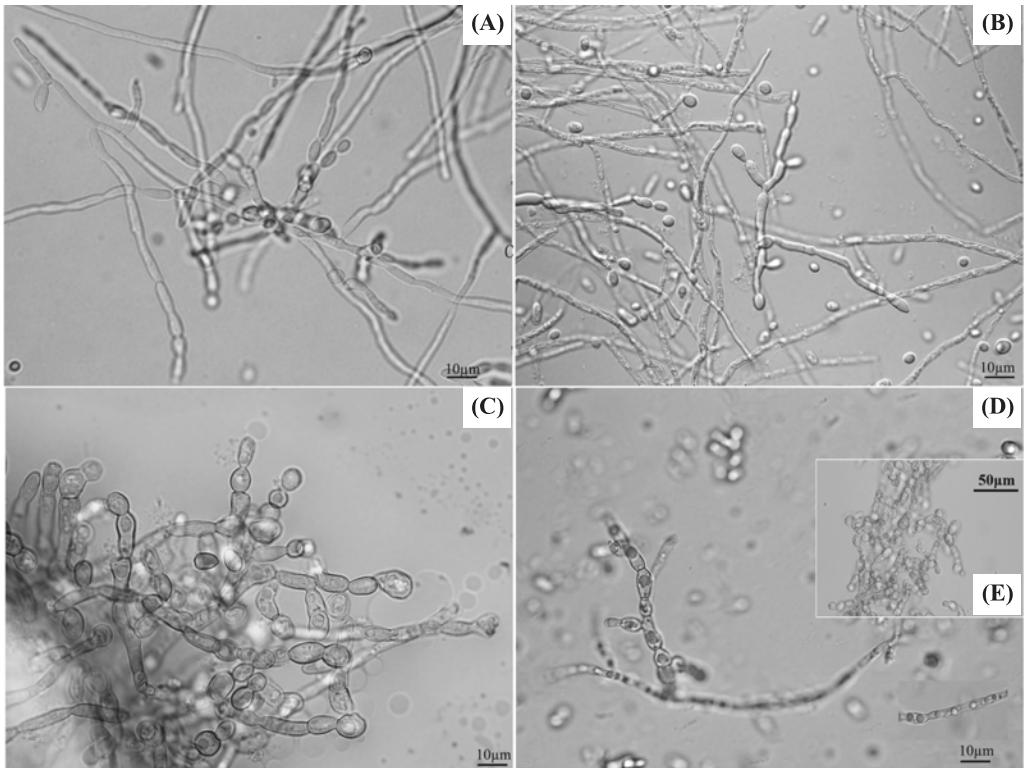

Fig. 5. Morphological changes of H93 cultivated under different conditions and different culture period (A) hyphae and spores of H93 cultured for two weeks in liquid basal medium; (B) hyphae and spores cultured for 30 days in basal liquid medium; (C) the swollen mycelia of H93 cultured for two weeks in liquid basal medium supplemented with 0.35 mg/ml of Cd^{2+}; (D) growth-restoring hyphae and spores of H93 cultivated for 30 days in the medium supplemented with 0.35 mg/ml of Cd^{2+}; (C) the saccharomyces-like structures of H93 cultivated for two weeks in the medium supplemented with 0.5 mg/ml of Cd^{2+}.

used in constructing of the NJ tree comprised 19 taxa and 588 aligned characters (including gaps/missing data). Based on the result of the phylogenetic analysis, H93 (a sporulating strain) grouped with *E. pisciphila* strains (AF050272) in a single clade with 100% bootstrap support and 99% sequence identities (Fig. 1) and sister to *Exophiala salmonis* stain (DQ344031) with 98% identities. H93 was also supported as an *E. pisciphila* strain by morphological characteristics (Fig. 2). It produces dark grey colonies under cultivation conditions on potato agar with dextrose. The anellidoconidia are round in shape with a rounded distal end and cut outgrowth. The septated conidia are 2×5 μm in size. Sporulation is good on potato agar with dextrose and bad on the Sabouraud's agar. H114 and H125 grouped together with 99% support in a clade (with sequence identities of 97%) sister to B3b (identities of 93%). The three non-sporulating strains grouped together again sister to the *Thysanorea papuana* strain (EU041814), but with only 91% sequence identities.

Metal tolerance of the DSE isolates

Metal tolerance of the DSE isolates varied between strains and metal species (Table 1). H93 can survive metals of Pb^{2+} (over 2 mg/ml), Zn^{2+} (over 3 mg/ml), and Cd^{2+} (0.5 mg/ml), respectively, the concentration of metals that was lethal to many of other species. The non-sporulating strains (H114, H125, and B3b) tolerate 1 mg/ml of Pb^{2+} and 0.1 mg/ml of Zn^{2+}, respectively, but they can not survive when Cd^{2+} concentration in the medium is above 0.025 mg/ml (Table 1). Compared with H114 and H125, mycelia growth of B3b, the non-polluted-site-derived strains, was very slow during the first 3 weeks when Pb^{2+} concentration in the medium was over 0.3 mg/ml.

EC50s to specific metal of the tolerant strains were also recorded in Table 1.

Metal accumulation in mycelia

Element analysis was carried out on mycelia cultivated in liquid media without additional metals and in media supplemented with elevated concentrations of Pb^{2+}, Zn^{2+}, and Cd^{2+}. No metals of Pb^{2+} and Cd^{2+} and a small quantity of Zn^{2+} were detected in mycelia from control cultures. Pb content in the mycelia of H93 (the most metal-tolerant strain) grown in the Pb^{2+} supplemented media can be over 25% dry mass of the mycelia (100∼300 times of its concentration in the medium). Metal content of Cd^{2+} and Zn^{2+} in mycelia of H93 can be 4.9% and 16%, respectively (10∼100 times of its concentration in the medium).

With increasing metal concentrations in the medium, fungal growth was inhibited, but metal content in the mycelia increased. We obtained a series of decreasing values of fungal biomasses (0∼1 g dry weight) and increasing values of metal content of Pb^{2+} (0∼25%) in the mycelia when H93 was cultured for two weeks in liquid medium supplemented

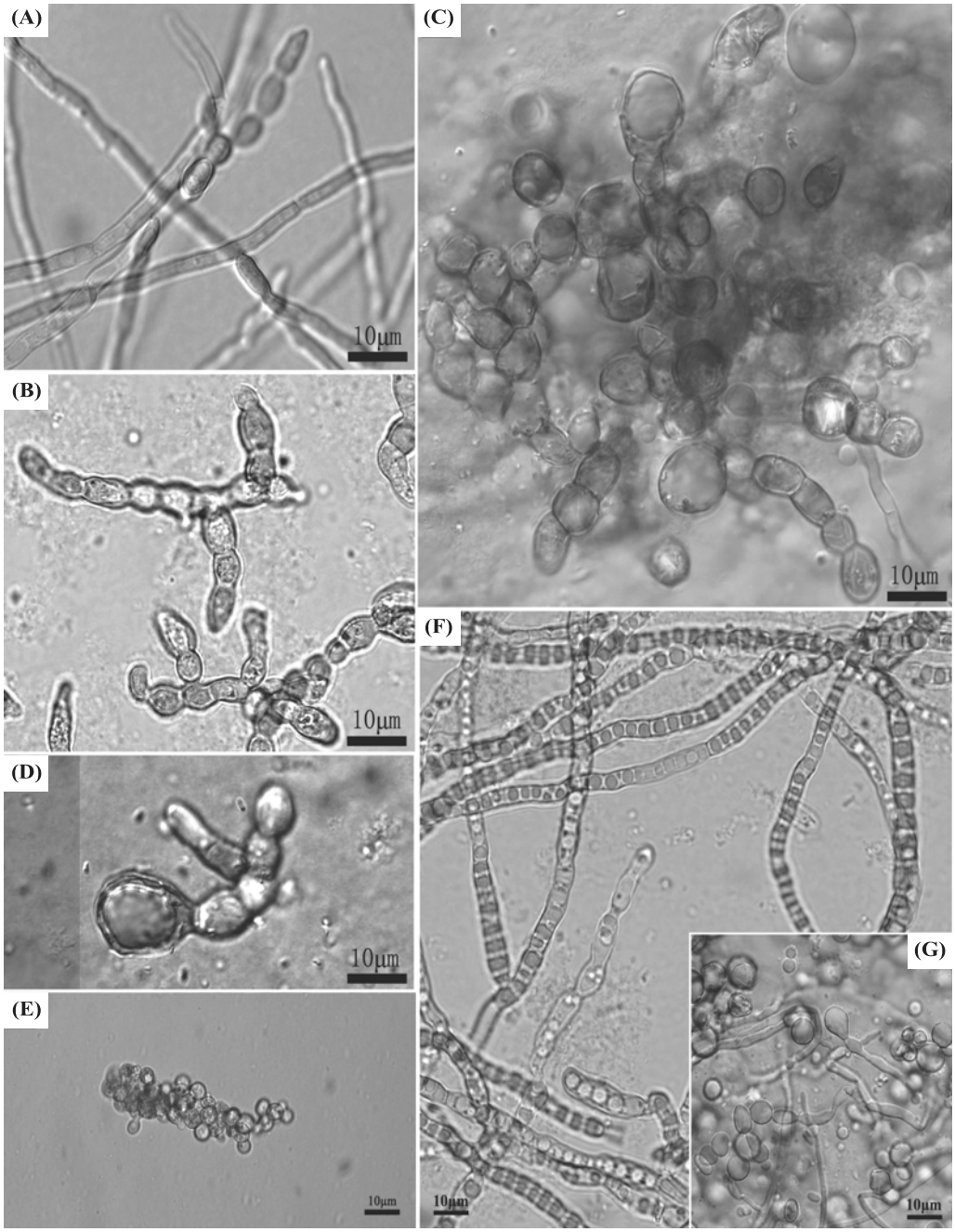

Fig. 6. Morphological changes of H93 and H125 at different culture conditions: (A) hyphae and spores of H93 cultured for one week in liquid basal medium with no metal stresses; (B) hyphae cultured for one week in the medium supplemented with 1 mg/ml of Pb^{2+}; (C and D) hyphae cultured for 30 days after inoculation of spores of H93 in the liquid medium supplemented with 1 mg/ml of Pb^{2+}; (E) saccharomyces-like structures of H93 cultivated for two weeks in the medium supplemented with 3 mg/ml of Zn^{2+}; (F) hyphae of H125 cultivated for 30 days in the medium supplemented with 1 mg/ml of Pb^{2+}. (G) A great deal of secreted granulas deposited among the hyphae when H93 was cultured for 30 days in the liquid medium supplemented with 1 mg/ml of Pb^{2+}.

with increasing Pb^{2+} concentration (Fig. 3). Contents of Cd^{2+} in the mycelia cultivated for two weeks in liquid medium supplemented with Cd^{2+} from 0 to 0.4 mg/ml were from 0 to 49,330 mg/kg (0~4.93%) of its biomass. The Zn^{2+} content in mycelia of H93 was 155,660 mg/kg (15.56% dry weight) when the fungus was inoculated in basal medium supplemented with 2 mg/ml Zn^{2+} and cultured for 10 days.

With time, metal content in the mycelia increased. Pb^{2+} content in the mycelia of H93 cultured for 30 days in basal medium supplemented with 1 mg/ml Pb^{2+} was 31.8% (dry weight). Cd^{2+} content cultured for a month in the medium supplement with 0.4 mg/ml of Cd^{2+} was 6.3%. While Pb^{2+} content in mycelia of H125 was 125,140 mg/kg (12.51%) when it was cultured for 30 days in the medium supple-

mented with 1 mg/ml Pb and cultured for the same time. When the mycelia were then washed with $CaCl_2$ solution, Pb content in the mycelia of H93 decreased from 25% to 17.9%, Cd from 4.93% to 3.57, Zn from 15.56% to 11%. Pb content in the mycelia of H125 decreased from 12.5% to 2.4%.

Morphological changes observed

During the process of cultivation with each treatment, morphological changes were observed under compound microscope at different growth phases. It was found that the three metal species induced similar morphological changes for the sporulating strain including a decrease in overall mycelial length and the size of fungal colony, and an increase in number of hyphae branches in response to the increase of metal concentrations in the media. When the metal concentrations are low, hyphal wall was smooth and mycelia growth is relatively normal without apparent morphological changes. When Zn^{2+} or Pb^{2+} concentration in the medium was above 0.7 mg/ml, and Cd^{2+} concentration was above 0.3 mg/ml, the cell and hyphae became swollen, and conidia formation was inhibited. When metal concentration in the medium was extreme (0.5 mg/ml Cd^{2+}, 3 mg/ml Pb^{2+} or 3 mg/ml Zn^{2+}), the whole cultures of H93 grew into a kind of chains of spherical spore-like (Fig. 5C) and/or saccharomyces-like structures with irregular deposits in the cells (Fig. 5E and 6E). We followed the whole processes of the cultivation of H93 in liquid basal medium supplemented with 0.35 mg/ml of Cd^{2+} and in the medium supplemented with 1 mg/ml of Pb^{2+}.

When H93 was cultured under Cd^{2+} concentration of 0.35 mg/ml, it grew very slowly (about 0.07 g biomass dry weight was obtained) and did not produce spores in the first three weeks. Hyphae of this period became swollen, and it is likely that the cell wall thickness increased and most of the hyphae changed into chains of spherical spore-like structures (Fig. 5C). Three weeks later haphal growth speeded up and began to produce spores normally. Hyphal wall again became smooth, with no phenomenon of cell swelling, but many hard granules in the mycelia can be seen (Fig. 5D).

When Pb^{2+} concentration in the medium was 1 mg/ml, mycelia of H93 grew slowly from the second day after the spores were inoculated in the medium. The inoculated spores germinated and branched at several directions (Fig. 6B). Mycelium growth was inhibited by excess Pb^{2+} and all the hypae were short segments and swelled to the utmost (Fig. 6B). With time, hyphae deformed further and many big granules formed in the cells and secreted from the broken tip of the hyphae at last (Fig. 6C and D). A great deal of secreted granules deposited among the deformed hyphae (Fig. 6C and G). No similar morphological changes were observed in the control culture of H93 (with no supplement Pb in the medium) (Fig. 5A, 5C, and 6A)

The morphological changes under metal stresses of the non-sporulating fungi were not like those of the sporulating fungi. The hyphae also swelled and changed into a kind of shape of extreme abnormality as shown in Fig. 6F. But we did not find the chains of spore-like structures in the course of cultivation.

Enzyme activity changes in the mycelia of H93 cultured for one week under different metal stress conditions

Enzyme activities of SOD and CAT in H93 were measured to detect the cellular oxidative stress grew under metal stressed conditions. The results of two independent experiments are summarized in Fig. 3. Repeated experiments showed that SOD and CAT activities in the hyphae of H93 have positive correlations with Pb^{2+} (r_{SOD}=0.994, P<0.01; r_{SOD}=0.933, P<0.01) and Cd^{2+} (r_{CAT}=0.982, P<0.01; r_{CAT}= 0.986, P<0.01) concentrations in the medium.

Discussion

Although heavy metals of relatively high concentration in a bio-available form are toxic for most fungal isolates, some exceptional fungi possess the ability to survive and accumulate these metals under conditions that are lethal for other biota (Zapotoczny *et al.*, 2007). According to the point of view of Wainwright and Gadd (1997), H93 seems to belong to the group of highly tolerant fungi, being also much more tolerant to Pb, Zn, and Cd than the other non-sporulating fungi isolated from the same habitats and from non-polluted site. In the mean time, all of the sterile fungi including the non-contaminated site derived strain also belong to the highly tolerant fungi to lead but low tolerant fungi to cadmium and zinc. H114, H125, and B3b are different DSE strains with near phylogenetic affinities. They carried similar tolerance to Pb, Zn, and Cd, though the growth of B3b was delayed for a more period under the stresses of these heavy metals. The EC50 values also indicate a strong growth rate of the DSE fungi cultured at the condition of relatively high metal concentrations.

All metals exert toxicity when present above certain threshold concentrations in bio-available forms (Gadd, 1993). Mechanisms of metal tolerance in fungi include reduction of metal uptake and/or increased efflux, metal immobilization, e.g. cell-wall adsorption, extracellular binding by polysaccharides, and intracellular sequestration by metallothioneins and phytochelatins, vacuolar localization, and etc (Collin-Hansen *et al.*, 2003, 2005; Gadd, 2000, 2007). A particular organism may directly and/or indirectly rely on several survival strategies. For example synthesis of metallothioneins or c-glutamyl peptides is a mechanism of Cu^{2+} resistance in *Saccharomyces cerevisiae*, and Cu binding or precipitation around the cell wall and intracellular transport are also mechanisms of the total cellular response (Gadd and White, 1989). A 25% increase in cell wall yield was reported for *Neurospora crassa* grown in the presence of cadmium, compared to a controlled cadmium-free growth (Bhanoori and Venkateswerlu, 2000). Martino *et al.* (2000) reported that one of the mechanisms of pigmented ericoid mycorrhizal fungi to tolerate heavy metals is deposition of melanin on the cell wall and secretion to the media.

In the present study, mechanisms of survival of the strains under metal stress conditions would be a complex strategy. Surface adsorption contributes to part of the metals sequestrated by the mycelia. The proportion of the sequestrated Pb^{2+} that can be exchanged by Ca^{2+} was more in the mycelia of H125 than that in the mycelia of H93, while, tightly

combined and/or absorbed metals account for the most of the metals sequestrated by the mycelia of H93. It is likely that the mycelia cell walls of H93 thickened in response to cadmium and lead (Fig. 5C, 6B, and 6D). The cell wall components should play important roles in the tolerance and sequestration of the metals. In the meantime, metal accumulation of the DSE mycelia should be a process from outside the cells to the cell wall and even inside the cells. Intracellular transportation, precipitation, and localization (Fig. 5C, D, and E; Fig. 6B, C, and D) would also occurred under Cd^{2+} or Pb^{2+} stresses. When H93 was cultivated in the medium supplemented with 1 mg/ml of Pb^{2+}, some of the metal ions may be transported into the cells (Fig. 6B) and then be accumulated and a great deal of big granules formed and secreted from the tip of the hyphae at last (Fig. 6C and D). This may be a kind of toxic metal effusing. Morphological changes of hyphae of H93 under higher metal concentrations were more serious. All the hyphae changed into chains of spherical spore-like and saccharomyces-like structures (Fig. 5C and E; Fig. 6B, C, and D) in response to the increase of metals in the medium. But this kind of transfiguration was not found in the cultivation of the sterile strains (Fig. 6F).

Positive correlations between enzyme activities of SOD and CAT with the metal concentrations in the medium indicate that increased SOD and CAT were synthesized to remove the increasing reactive oxygen species (ROS) generated in the mycelia of H93 with the increase of the metals (Cd^{2+} or Pb^{2+}) in the medium. Increased cellular oxidative stresses can destroy many of the functional proteins and other important molecules and display toxicity in the cells. The most important enzymes for the removal of ROS are superoxide dismutase (SOD) and catalase (CAT) (Collin-Hansen *et al.*, 2005). Many studies have reported the involvement of SOD and CAT in response to metal stresses by organisms including microbes (Schützendübel and Polle, 2002; Trotter *et al.*, 2006).

Apparently, whatever mechanism these DSE strains employed, they can tolerant and sequestrate high concentration of the heavy metals in the cells or/and through the cell-wall combination. These dark septate endophytic fungi can undoubtedly exert impact to the host plants growing in the metal polluted soil. Whether they can absorb and sequestrate the metals in the mycelia lest that the toxic ions inter the plant organism, or it can assimilate the metals and transmit them to host plants and contribute to phytoremediation, further investigation should be conducted to study the effects of the inoculated DSE strains on their host plants. And more indexes should be detected to estimate their value in environmental biotechnology.

In conclusion, sequence data of ITS ITS 1-5.8S rDNA-ITS 2 gene regions and morphological characteristics of four DSE isolates showed that they are different DSE strains, belonging to different taxa. All of the tested DSE strains were lead tolerant. Only H93 (the sporulting strain) isolated from the smelter area had significant tolerance to Cd^{2+} and zinc. Tolerant DSE stains can accumulate high levels of the corresponding metals. Mechanisms of metal survival of these DSE strains would be a complex strategy. Increased enzyme activities are also one of the means of tolerance of the DSE fungi. Further studies are necessary to understand the mechanisms of the metal tolerance and accumulation of these DSE strains. In addition, the effects of the inoculated DSE stains to their hosts should be investigated.

Acknowledgements

This work was financially supported by The National Natural Science Foundation of China (NSFC3770052, NSFC40763003) and National Program on Key Basic Research Projects (Special Item 2005CCA05700).

References

Addy, H.D., M.M. Piercey, and R.S. Currah. 2005. Macrofungal endophytes in roots. *Canadian J. Botany* 83, 1-13.

Barrow, J.R. and R.E. Aaltonen. 2001. Evaluation of the internal colonization of *Atriplexcanescens* (Pursh) Nutt. roots by dark septate fungi and the influence of host physiological activity. *Mycorrhiza* 11, 199-205.

Bhanoori, M. and M. Venkateswerlu. 2000. *In vivo* chiti-cadmium complexation in cell wall of *Neurospora crassa*. *Biochim. Biophys. Acta.* 152, 21-28.

Caldwell, B.A., M.A. Mastellano, and R.P. Griffiths. 1991. Fatty acid esterase production by ectomycorrhizal fungi. *Mycorrhiza* 33, 233-236.

Cerbasi, I.H. and U. Yetis. 2001. Biosorption of Ni (ii) and Pb (ii) by *Phanerochaete chrysosporium* from binary metal system - Kinetics. *Water Res.* 27, 15-20.

Cevnik, M., M. Jurc, and D. Vodnik. 2000. Filamentous fungi associated with the fine roots of *Erica herbacea* L. from the area influenced by the Zerjav lead smelter (Slovenia). *Phyton. Ann. Rei. Bot.* 40, 61-64.

Choudhary, M., U.K. Jetley, M.A. Khan, S. Zutshi, and T. Fatma. 2007. Effect of heavy metal stress on proline, malondialdehyde, and superoxide dismutase activity in the cyanobacterium *Spirulina platensis*-S5. *Ecotoxicol. Environ. Saf.* 66, 204-209.

Collin-Hansen, C., R.A. Andersen, and E. Steinnes. 2003. Isolation and N-terminal sequencing of a novel cadmium-binding protein from *Boletus edulis*. *J. Phys. IV* 107, 311-314.

Collin-Hansen, C., R.A. Adersen, and E. Steinnes. 2005. Molecular defense systems are expressed in the king bolete (*Boletus edulis*) growing near metal smelters. *Mycologia* 97, 973-983.

Colpaert, J.V., P. Vandenkoornbuyse, K. Adriaensen, and J. Vangeonsveld. 2000. Genetic variation and heavy metal tolerance in the ectomycorrhizal basidiomycete *Suillusluteus*. *New Phytol.* 147, 367-379.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783-791.

Gadd, G.M. 1993. Interactions of fungi with toxic metals. *New Phytologist* 124, 25-60.

Gadd, G.M. 2000. Bioremedial potential of microbial mechanisms of metal mobilization and immobilization. *Curr. Opin. Biotechnol.* 11, 271-279.

Gadd, G.M. 2007. Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. *Mycol Res.* 111(Pt 1), 3-49.

Gadd, G.M. and C. White. 1989. The removal of thorium from simulated acid process streams by fungal biomass. *Biotechnol. Bioeng.* 33, 592-597.

Jordan, M.J. and M.P. Lechevalier. 1975. Effects of zinc-smelter emissions on forest soil microflora. *Can. J. Microbiol.* 21, 1855-1865.

Jumpponen, A. and J.M. Trappe. 1998. Dark septate endophytes: a review of facultative biotrophic root colonizing fungi. *New*

Phytologist 140, 295-310.
Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16, 111-120.
Kumar, S., K. Tamura, and M. Nei. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform.* 5, 150-163.
Leyval, C., K. Turnau, and K. Haselwandter. 1997. Effect of heavy metal pollution on mycorrhizal colonization and function : physiological, ecological, and applied aspects. *Mycorrhiza* 7, 139-153.
Lu, R.K. 2000. Methods of Soil and Agricultural Chemical Analysis. China Agricultural Scientech Press, Beijing, China.
Mandyam, K. and A. Jumpponen. 2005. Seeking the elusive function of the root-colonising dark septate endophytic fungi. *Studies Mycol.* 53, 173-189.
Martino, E., K. Turnau, M. Girlanda, P. Bonfante, and S. Perotto. 2000. Ericoid mycorrhizal fungi from heavy metal polluted soils: their identification and growth in the presence of zinc ions. *Mycol. Res.* 104, 338-344.
Massaccesi, G., M.C. Romero, M.C. Cazau, and A.M. Bucsinszky. 2002. Cadmium removal capacities of filamentous soil fungi isolated from industrially polluted sediments, in La Plata (Argentina). *World J. Microbiol. Biotechnol.* 18, 817-820.
McGonigle, T.P., M.H. Miller, D.G. Evans, G.L. Fairchild, and J.A. Swan. 1990. A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. *New Phytol*. 115, 495-501.
Rains, K.C., N.M. Nadkarni, and C.S. Bledsoe. 2003. Epiphytic and terrestrial mycorrhizas in a lower montane Costa Rican cloud forest. *Mycorrhiza* 13, 257-264.
Ruotsalainen, A.L., A. Markkola, and M.V. Kozlov. 2007. Root fungal colonisation in *Deschampsia flexuosa*: Effects of pollution and neighbouring trees. *Environ. Pollut.* 47, 723-728.
Schützendübel, A. and A. Polle. 2002. Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. *J. Exp. Bot.* 53, 1351-1365.
SEPA (State environmental protection administration of China). 1997. Soil quality-Ddtermination of copper, zinc-Flame atomic absorption spectrophotometry. GB/T 17138.
Silvani, V.A., S. Fracchia, L. Fernández, M. Pérgola, and A. Godeas. 2008. A simple method to obtain endophytic microorganisms from field-collected roots. *Soil Biol. Biochem.* 40, 1259-1263.
Smith, S.E. and D.J. Read. 1997. Mycorrhizal symbiosis, 2nd ed. Academic Press, London, UK.
Thompson, J.D., D.G. Higgins, and T.J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673-4680.
Trotter, E.W., E.J. Collinson, I.W. Dawes, and C.M. Grant. 2006. Old yellow enzymes protect against acrolein toxicity in the yeast *Saccharomyces cerevisiae. Appl. Environ. Microbiol.* 72, 4885-4892.
Wainwright, M. and G.M. Gadd. 1997. Fungi and industrial pollutants, p. 85-97. *In* D.T. Wicklow and B. Söderström (eds.), The Mycota IV. Environmental and Microbial Relationships. Springer, Berlin, Heidelberg, Germany.
White, T.J., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315-322. *In* M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White (eds.), PCR protocols: a guide to methods and applications, Academic Press, San Diego, USA.
Zafar, S., F. Aqil, and I. Ahmad. 2007. Metal tolerance and biosorption potential of filamentous fungi isolated from metal contaminated agricultural soil. *Biores. Technol.* 98, 2557-2561.
Zapotoczny, S., A. Jurkiewicz, G. Tylko, T. Anielska, and K. Turnaud. 2007. Accumulation of copper by *Acremonium pinkertoniae*, a fungus isolated from industrial wastes. *Microbiol. Res.* 162, 219-228.