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Improved tolerance of maize (*Zea mays* L.) to heavy metals by colonization of a dark septate endophyte (DSE) *Exophiala pisciphila*

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ABSTRACT

Dark septate endophytes (DSE) are ubiquitous and abundant in stressful environments including heavy metal (HM) stress. However, our knowledge about the roles of DSE in improving HM tolerance of their host plants is poor. In this study, maize (*Zea mays* L.) was inoculated with a HM tolerant DSE strain *Exophiala pisciphila* H93 in lead (Pb), zinc (Zn), and cadmium (Cd) contaminated soils. *E. pisciphila* H93 successfully colonized and formed typical DSE structures in the inoculated maize roots. Colonization of *E. pisciphila* H93 alleviated the deleterious effects of excessive HM supplements and promoted the growth of maize (roots and shoots) under HM stress conditions, though it significantly decreased the biomass of inoculated maize under no HM stress. Further analysis showed that the colonization of *E. pisciphila* H93 improved the tolerance of maize to HM by restricting the translocation of HM ions from roots to shoots. This study demonstrated that under higher HM stress, such a mutual symbiosis between *E. pisciphila* and its host (maize) may be an efficient strategy to survive in the stressful environments.

1. Introduction

All plants in natural ecosystems appear to be symbiotic with fungal endophytes (Rodriguez et al., 2009). Among this highly diverse group of endophytic fungi, dark septate endophytes (DSE) have received much attention in the recent years (Peterson et al., 2004). DSE comprise a heterogeneous group of root-associated endophytic fungi which are characterized by melanized intercellular and intracellular running hyphae and so-called microsclerotium (aggregation of dark, thick-walled, closely packed inflated cells) within epidermis and cortex of plant roots (Jumpponen and Trappe, 1998; Mandyam and Jumpponen, 2005; Silvani et al., 2008). Increasing evidence suggests that DSE may benefit their host plants by facilitating the uptake of plant mineral nutrients including P, N and water (Haselwandter and Read, 1982; Mullen et al., 1998; Newsham, 1999; Upson et al., 2009), suppressing the infection of plant pathogens (Narisawa et al., 2000; 2004; Barrow, 2003) and alleviating the harmful effects of severely stressful environments (Treu et al., 1996). Subsequently, experiments conducted by Usuki and Narisawa (2007) further confirm that DSE (*Heteroconium chaetospira*) can form a mutualistic symbiosis with Chinese cabbage (*Brassica campestris*) by nutrient exchange (the associated fungi providing organic nitrogen in exchange for their host plant carbon), though some controversial and speculative aspects of deleterious, neutral DSE-plant associations are still debated (Mandyam and Jumpponen, 2005; Peterson et al., 2008; Smith and Read, 2008).

Increasing field studies reveal that DSE are ubiquitous root-associated fungi, and especially common in stressful environments such as cool, nutritionally poor, alpine or subalpine ecosystems, high saline environments and polar regions (Read and Haselwandter, 1981; Hambleton and Currah, 1997; Mandyam and Jumpponen, 2005; Newsham et al., 2009; Sonjak et al., 2009). Even in severely stressful heavy metal (HM) polluted soils (5705 mg kg^{-1} Pb, 58007 mg kg^{-1} Zn and 77 mg kg^{-1} Cd) of the Ancient Lead and Zinc Smelting Site of Huize, Yunnan Province, southwest China, where non-ferrous metals Pb and Zn have been smelted by ancient smelting methods for hundreds of years (Xia et al., 1980), most plants of natural revegetation are colonized by DSE (Liang et al., 2007). Recent studies show that DSE are one of the most common root-associated fungi in metal polluted soils (Likar and Regvar, 2009; Regvar et al., 2010), and these DSE have an inherent tolerance (Zhang et al., 2008) and a low sensitivity to HM (Gibson and Mitchell, 2006). While many strides have been made in understanding the ecological significance of mycorrhizal fungi, our knowledge about the functions of DSE in HM polluted environments is poor (Likar and Regvar, 2009).

To understand the possible functions of this poorly understood group of root-associated fungi in HM polluted soils, on the basis of known information we hypothesized that, DSE as well as mycorrhizal fungi, could improve tolerance of host plants to heavy metals beyond nutrient acquisition and resultant positive host growth responses. To test this hypothesis, one of the most dominant and highly metal resistant DSE strains, *Exophiala pisciphila* H93, isolated from the roots of *Arundinella bengalensis* naturally growing in Ancient Lead and Zinc

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Smelting Site of Huize, China, was used for inoculum. Greenhouse pot experiments were conducted under different concentrations of HM stress (Pb, Zn, Cd) to assess the effects of inoculation with *E. pisciphila* H93 on (1) maize biomass, (2) the absorption, translocation and accumulation of heavy metals in shoots and roots of maize and (3) the tolerance of host plants to HM. Possible mechanisms of DSE enhancing the tolerance of their host plants to HM are discussed.

2. Materials and methods

2.1. Preparation of fungal colonized and non-colonized maize seedlings

This experiment was performed to get uniform fungal (*E. pisciphila* H93) colonized and non-colonized maize (*Zea mays* L.) seedlings to be used for the subsequent pot experiments (2.2).

Maize seeds were surface-sterilized by dipping in 75% ethanol for 5 min and then in 10% sodium hypochlorite for 10 min under agitation. Sterilized seeds were thoroughly rinsed with sterile water and then aseptically planted onto the water agar medium (agar 8 g L^{-1}) contained in Petri dishes (90 mm) for germinating at 25 °C, and 6 seeds were used per Petri dish.

After 3 days of incubation, the germinating seeds were transplanted into sterile glass bottles (Φ60 × 200 mm) containing culture substrata for fungal inoculation. Each glass bottle was filled with about 130 g culture substrata that 100 g river sand mixed with 30 mL improved MS liquid medium (containing in mg L^{-1}: NH_4NO_3 1650, KNO_3 1900, $CaCl_2 \cdot 2H_2O$ 440, $MgSO_4 \cdot 7H_2O$ 370, KH_2PO_4 170, KI 0.83, H_3BO_3 6.2, $MnSO_4 \cdot 4H_2O$ 22.3, $ZnSO_4 \cdot 7H_2O$ 8.6, $Na_2MoO_4 \cdot 2H_2O$ 0.25, $CuSO_4 \cdot 5H_2O$ 0.025, $CoCl_2 \cdot 6H_2O$ 0.025, $FeSO_4 \cdot 7H_2O$ 27.8, Na_2-EDTA $\cdot 2H_2O$ 37.3, linositol 100, V_{B3} 0.5, V_{B6} 0.5, V_{B1} 0.5, Glycine 2, supplemented with 20 g L^{-1} sucrose). Before using, river sand was sieved through a 0.2 cm sieve and washed by tap water to remove the soil silt and nutrients, and placed in the 80 °C oven and dried to constant weight (this usually required 5–12 h) and then autoclaved for 2 h at 121 °C (three times with 2-day intervals). The germinating seeds were planted into the culture substrata contained in the bottles (2 seeds for each bottle) and each seed was inoculated with a fungal disk (Φ 0.5 cm) cut from a 14-day-old PDA culture (potato 200 g, dextrose 20 g, agar 18 g, and water 1000 mL), by attaching the fungal disk to the root of maize. For a control treatment, other germinating seeds were also inoculated with a disk (Φ 0.5 cm) cut from PDA plate without fungus and planted into the bottle as above. All the inoculation processes were operated in an SW-CJ-1DF clean bench (Airtech, China). The glass bottles were covered with sterile AeraSeal films (150 × 150 mm) (Mycomebio (Beijing) Bio-medical Science Technology Center, China) and cultivated under a day temperature of 25 °C, a night temperature of 18 °C with a photoperiod of 12 h for 4 weeks, and watered with 5 ml deionized–distilled water every 5 days per bottle. After 4 weeks' cultivation, the maize seedlings were ready for the subsequent pot experiments (2.2).

2.2. Greenhouse pot cultivation

Pot culture experiments in a greenhouse were established to determine the effects of *E. pisciphila* H93 on maize (*Zea mays* L.) growing under different kinds and levels of HM (Pb, Zn, Cd) stress. River sand containing different kinds and concentrations of HM (Pb, Zn, Cd) were applied as pot culture substrata. According to the environmental quality standard for soils of China (GB15618-1995) and the contents of HCl-extractable Pb, Zn and Cd in the field soils where the test fungus was isolated (Liang et al., 2009), three HM polluted levels of each HM species (Pb, Zn and Cd) and one non-polluted level (control) were conducted and thus formed four test treatments in total (Table 1). River sand was cleaned and dried as above before using. To make the four groups of pot culture substrata, 1 kg of the dried river sand was mixed with 150 ml different concentrations of HM salt solutions to achieve the concentrations shown in Table 1, respectively. For example, the 1000 mg kg^{-1} of Pb (II) substrata means 1 kg dried sand contained 1000 mg Pb (II). Therefore, to make the 1000 mg kg^{-1} of Pb (II) substrata, 1 kg of the dried sand was mixed with 150 ml solution that contained 1000 mg Pb (II). Similarly, to make the 500 mg kg^{-1} of Zn (II) substrata, 1 kg of the dried sand was mixed with 150 ml solution that contained 500 mg Zn (II). To make the non-polluted substrata (control), 1 kg of the dried sand was mixed with 150 ml of deionized-distilled water. The concentrations of HCl-extractable Pb (II), Zn (II), Cd (II) from the sand were trivial, being 4.3, 4.5 and 0.006 mg kg^{-1}respectively, and thus were omitted in the subsequent analysis. Plastic pots (20 cm diameter × 16 cm height) each filled with 2 kg different pot culture substrata were used for plant growing.

Table 1
Heavy metal (HM) concentrations of cultural substrata in the four test groups.

Groups	HM concentrations (mg kg^{-1} DW)		
	$Pb(NO_3)_2$	$ZnSO_4 \cdot 7H_2O$	$CdSO_4 \cdot 8H_2O$
I	0	0	0
II	1000	500	50
III	2000	1000	100
IV	3000	1500	150

Before the transplantation of the above 4-week-old maize seedlings from the bottles to pots, the colonization intensity of *E. pisciphila* H93 in maize roots was evaluated. Five 0.5 cm root fragments were randomly collected from each maize seedling and cleaned in 5 mL 10% (w/v) KOH contained in a test tube for 2 h at 90 °C in a water bath and then stained with 0.5% acid fuchsin (Berch and Kendrick, 1982). Colonization intensity of *E. pisciphila* H93 was determined by using the magnified intersection method under a compound-light microscope (Olympus-BX51) at 200× magnification (McGonigle et al., 1990). Fungal colonization intensity, i.e. the percentage of the presence of *E. pisciphila* H93 in all intersections observed (more than 150 intersections were used for each sample) was determined for each sample.

The fungal colonized (only those seedlings each had 93–95% root colonization intensity from inoculation of *E. pisciphila* H93 were used) and non-fungal colonized (without inoculation) maize seedlings were transplanted into pots filled with different pot culture substrata as described in Table 1. Three plants (one plant per pot) were used for each treatment (each group × each metal species), and thus a total of 60 pots were used (3 HM × 3 groups × 2 treatments × 3 replicates + 1 non-HM × 1 group × 2 treatments × 3 replicates). Then maize seedlings were grown in a glasshouse with a day temperature varying between 16 and 28 °C, a night temperature varying between 8 and 16 °C. Seedlings were regularly watered with deionized–distilled water and each pot received 100 mL sterile Hoagland's nutrient weekly.

2.3. Harvest and analysis of plant HM

After 3 months of growth, the leaves of maize at the time of male flowering were turning brownish yellow and all maize was harvested. Fungal colonization intensity was evaluated at this stage for each plant as described above. Shoots (leaves + stems) and roots were collected, respectively, and roots were thoroughly washed by tap water to remove the soil silt. The shoots and roots then were placed in the 80 °C oven and dried to constant weight and weighed. All dried root and shoot samples were ground separately by a mini-vegetation disintegrator (FZ102, Tianjing City Test Instrument Co. Ltd, China), and 0.5 g shoot and 0.5 g root powder of each sample were then subsampled by the usual method of coning and quartering. These representative subsamples were digested by $HNO_3 + HClO_4$ respectively and the concentrations of HM ions were determined by the flame atomic absorption spectrometry (FAAS) using a Z2000 polarized Zeeman atomic absorption spectrophotometer (Hitachi, Japan). Quantification was carried out with a