

ZINC RESISTANCE OF SIX DARK SEPTATE ENDOPHYTIC FUNGI AND THE GROWTH RESPONSE OF TOMATO

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Abstract. Dark septate endophytic fungi (DSEF) can promote the growth response of host plants and can increase their resistance to heavy metals. In this study, six isolated DSEFs were proven to be able to form microsclerotia with plants. The objective of this study was to investigate the promoting and inoculation effects of these six DSEFs. Molecular analysis showed that the DSEFs may be new additions to the fungal flora of Taiwan and may be new species in the world. Screening and selection of Zn-resistant DSEFs showed that only two strains, CkDB5 and RrtHH10, were Zn-resistant. Evaluation of the Zn resistance range of CkDB5 and RrtHH10 revealed that the former presented lower EM₅₀ values than the latter, and that both strains showed the same minimum inhibitory concentration values. As the Zn concentration increased, twisting and looping of the mycelial morphology of the strains was observed. Resynthesis experiments proved that CkDB5 and RrtHH10 could promote the growth response of tomato. The biomasses of CkDB5-inoculated plants was up to four times higher than that of the controls. This study showed that, of the six DSEFs, only CkDB5 and RrtHH10 showed Zn-resistance. CkDB5 is a fungus with particularly high Zn resistance, and it could facilitate tomato growth.

Keywords: *dark septate endophytic fungi, growth response, half maximal effective, resynthesis, Zn-resistant*

Introduction

Heavy metals are some of the most toxic inorganic substances known to mankind, and they can contaminate the environment (Upadhyaya et al., 2010). Phytoremediation removes heavy metals through synthesis with plants and microorganisms; to date, it remains the best strategy for heavy-metal recovery with the least harmful effects to the environment (Diagne et al., 2015; Helmisaari et al., 2007). Mycorrhizae promote the growth (Azcón-Aguilar and Barea, 1997; Schmid et al., 1995) and tolerance (Gibson and Mitchell, 2005; Lin et al., 2011) of host plants. Mycorrhizal associations have also been shown to enhance the tolerance of host plants to Zn and Cu (Bradley et al., 1981). Some fungi can alleviate toxic metal stress under certain circumstances (Smith and Read, 1997).

Dark septate endophytic fungi (DSEFs), which are commonly found in the roots of healthy plants, present dark pigmentation and septate hyphae (Mathew and Malathy, 2008) and are often sterile in culture (Newsham, 1999; Piercey et al., 2004). DSEFs refer to miscellaneous groups of anamorphic ascomycetous fungi that can colonize the intracellular or intercellular tissues of roots, and maintain an important functional relationship with host plants. DSEFs can enhance the growth and mineral nutrition of hosts (Fernando and Currah, 1996; Jumpponen et al., 1998; Lin, 2016; Shivanna et al., 1994). Several studies have indicated that some endophytes could promote the growth of host plants in soils that have been contaminated by heavy metals (Monnet et al., 2001; Sun et al., 2011; Xu et al., 2015; Zhang et al., 2010).

Li et al. (2012) suggested that fungal endophyte colonization in Pb- Zn contaminated plants is moderately abundant and that some isolates adapt remarkably well to Pb²⁺ and

Zn²⁺. These findings indicate a potential application in phytoremediation. Diao et al. (2013) indicated that Zn is an essential micronutrient required by a wide variety of cellular processes. However, excessive Zn can be toxic to organisms (Jaekel et al., 2005). Zn is also proposed in the 13-metal contamination list published by the US Environmental Protection Agency (Ramos et al., 2002). In the laboratory, many researchers have demonstrated that DSEF isolates could facilitate the growth of host plants. However, the Zn resistance of these DSEFs to various metal concentrations remains unknown. The purpose of this study is to examine the response of six DSEFs to Zn.

Material and methods

Strains

Six DSEFs were used in this study. Strains CkDB2 and CkDB5 were previously isolated from the roots of *Cinnamomum kanehirae* (120°47'37.33" E, 23°28'16.12" N) from a plantation located in Dabang Township, Chiayi County, Taiwan (Hong et al., 2014). Strain CoLG3 was previously isolated from the roots of *C. osmophloeum* (120°47'15" E, 23°43'47" N) in a plantation located in Lugu Township, Nantou County, Taiwan. Strain MoAL5 was previously isolated from the roots of *Mahonia oiwakensis* (120°48'45" E, 23°30'46" N) in a plantation located in Alishan Township, Chiayi County, Taiwan (Tan et al., 2016). Strain RhYM6 was previously isolated from the roots of *Rhododendron hyperythrum* (121°33'40" E, 25°09'20" N) in a plantation located in Beitou District, Taipei City, Taiwan. Finally, strain RrtHH10 was previously isolated from the roots of *R. rubropilosum* var. *taiwanalpinum* (121°17'21" E, 24°09'58" N) in a plantation located in Renai Township, Nantou County, Taiwan. These six strains were deposited at the Tree Mycorrhiza Laboratory of National Chiayi University.

Seeds

The seeds of ARIA tomatoes (*Solanum lycopersicum* L.) used in this experiment, were kindly provided by the Known-You Seed Co., Ltd.

DNA extraction, sequencing and phylogenetic analysis

The methods described by Phosri et al. (2007) were followed. Mycelia for deoxyribonucleic acid (DNA) extraction, amplification, and sequencing were scraped from the surfaces of PDA cultures. Genomic DNA was extracted using Puregene Proteinase K. Total fungal DNA was used as the template for amplification with primers ITS1-F and ITS4 (Gardes and Bruns, 1993). Polymerase chain reaction (PCR) products were sequenced by Genomics BioSci and Tech Company. Sequences were assembled and related sequences were analyzed using the basic local alignment search tool (BLAST) searches. The phylogenetic relationships were analyzed by Molecular Evolutionary Genetics Analysis software (Tamura et al., 2011). Bootstrapping was performed using neighbor-joining (Saitou and Nei, 1987).

Screening and selection of Zn-resistant DSEFs

Screening and selection of Zn-resistant DSEFs was performed using the methods of Ban et al. (2012) and Zeng et al. (2008) with modifications. The Zn-resistance of the six DSEFs was determined by placing two 0.5 mm³ plugs of the mycelium from each DSEF

into an Erlenmeyer flask (125 mL) containing 25 mL of modified Melin-Norkrans liquid medium (MMN) (Marx, 1969). Each DSEF was placed in 6 flasks, 3 with Zn (ZnCl_2 , 1 mg/mL) and another 3 without. After 14 days of cultivation, the flasks were autoclaved (liquid cycle at 121 °C for 20 min), harvested by filtration (filter paper, AdvanTec No. 1), dried to a constant mass at 70 °C, and weighed.

Evaluation of metal tolerance range

Strains that survived cultivation in Zn medium were evaluated for their Zn tolerance range using the method of Ban et al. (2012) with modifications. The sensitivity of select DSEFs to Zn was evaluated by measuring their minimum inhibitory concentration (MIC) and the effective concentration inhibiting 50% of mycelial growth (EC_{50}). Petri dishes with MMN medium (Marx, 1969) were used to cultivate the selected DSEFs for 14 days. Each DSEF was exposed to six Zn concentrations (0, 0.4, 0.8, 1.2, 1.6, and 2.0 mg/mL) in three replicates. Two 0.5-cm diameter plugs were removed from the edge of 14-day-old colonies and placed in flasks. All treated samples were harvested after incubation in the dark for 14 days, after which the flasks with medium were autoclaved (liquid cycle at 121 °C for 20 min), filtered (filter paper, AdvanTec No. 1), dried to a constant mass at 70 °C, and weighed.

Observation of mycelial morphology under different concentrations of Zn

All mycelium treatments were observed. After 14 days of cultivation in different concentrations of Zn, several mycelia were obtained and carefully placed onto slides. Lactophenol cotton blue (BD BBL™) was applied to stain hyphae, and morphological observations were made using a light microscope; photos were taken for recording purposes (Hutton et al., 1994; Piercey et al., 2002; Sigler and Flis, 1998).

Evaluation of the growth responses of tomato inoculated with DSEFs

After the desired DSEFs were screened, they were applied to the roots of ARIA tomatoes under different environments with or without Zn. Pure resynthesis was done through the method proposed by Dalpé (1986). After surface cleaning, tomato seeds were sterilized with 10% sodium hypochlorite solution for 15 min, rinsed 3 times with sterilized distilled water, and then incubated in glass jars containing 1% agar for germination. Germinated seedlings were transplanted to 100 g of peat substrate (containing 100 mL MMN medium with or without Zn (ZnCl_2 , 1 or 0 mg/mL)) in a glass jar ($95 \times 73 \text{ mm}^2$). Five aseptic tomato seedlings were inoculated with each DSEF. Growth was then allowed to proceed for 42 days in a growth chamber (22 °C, 65% RH, and 16 h of light with a maximum illumination of 5,000 lx). To determine the growth responses of the plants, the seedlings were carefully removed from the jar after incubation.

Evaluation of Zn concentrations in tomato

Evaluation of Zn concentrations in the seedlings was performed following the method described by Yang et al. (2015). Each plant sample was washed thoroughly with tap water and rinsed five times with deionized water to remove surface dust and soil. The cleaned samples were then dried at 80 °C in an oven for 48 h until a constant

weight was achieved. Afterward, the samples were crushed using a microphyte disintegrator.

The concentrations of Zn in the plant samples were determined using the method described by Allen (1989). Samples were completely digested in concentrated HNO₃ (16 mol/L) and HClO₄ (12 mol/L) at a ratio of 4:1 (v/v). The digestion tubes were removed from the digestion block to cool. After the solution had cleared, it was filtered through AdvanTec No. 1 filter paper and transferred quantitatively into a 10-mL volumetric flask by addition of distilled water. Metal concentrations were determined by flame atomic absorption spectrometry (FAAS).

Statistical analyses

Statistical Package for the Social Sciences (version 12.0) (Illinois, USA) for Windows was used for statistical analysis. Means of 3 or 5 separate experiments \pm standard deviations ($n = 3$ or 5) were calculated from the collected data. Tukey's multiple range test at the $p \leq 0.05$ significance level was used to analyze differences between treatments.

Results

Molecular identification of six DSEFs

The data matrix used to construct the neighbor-joining tree was comprised of 18 taxa and 704 aligned characters (including gaps/missing data) (*Fig. 1*). Based on the results of the phylogenetic analysis, the internal transcribed spacer (ITS) sequences of RrtHH10 clustered with those of *Phialocephala*, forming a cluster with 100% bootstrap support. RrtHH10 shared the closest sequence identity with *Phialocephala fortinii* (GQ302683). The ITS sequences of CoLG3 were similar to those of CkDB2 and clustered with those of *Wiesneriomyces laurinus* (KR822217; KP057801), forming a cluster with 100% bootstrap support. The ITS sequences of RhYM6 were clustered with those of Herpotrichiellaceae sp. (KJ471529) and Eurotiomycetes sp. (KM520020), forming a cluster with 98% bootstrap support. The ITS sequences of MoAL5 were clustered with those of *Cladophialophora*, forming a cluster with 95% bootstrap support. MoAL5 shared the closest sequence identity with *Cladophialophora chaetospira* (EU035406). The ITS sequences of CkDB5 were clustered with those of *Scolecobasidium*, forming a cluster with 99% bootstrap support. CkDB5 shared the closest sequence identity with *Scolecobasidium humicola* (AY265334).

Screening of Zn-resistant DSEFs

After 14 days of incubation, the samples were harvested and dried at 70 °C until a constant weight was achieved (*Table 1*). In treatments without Zn, all six DSEFs survived; in contrast, only strains CkDB5 and RrtHH10 survived in treatments with Zn.

In treatments without Zn (*Table 1*), strain CkDB2 showed the highest dry weight of 74.0 ± 6.4 mg. By comparison, the dry weights of MoAL5, RrtHH10, RhYM6, CoLG3, and CkDB5 were 65.4 ± 5.7 , 59.8 ± 6.3 , 57.7 ± 4.9 , 44.7 ± 1.9 , and 39.2 ± 3.2 mg, respectively.

In treatments with Zn (*Table 1*), only strains CkDB5 and RrtHH10 survived; the dry weights of these strains were 28.8 ± 2.8 and 29.5 ± 1.2 mg, respectively.

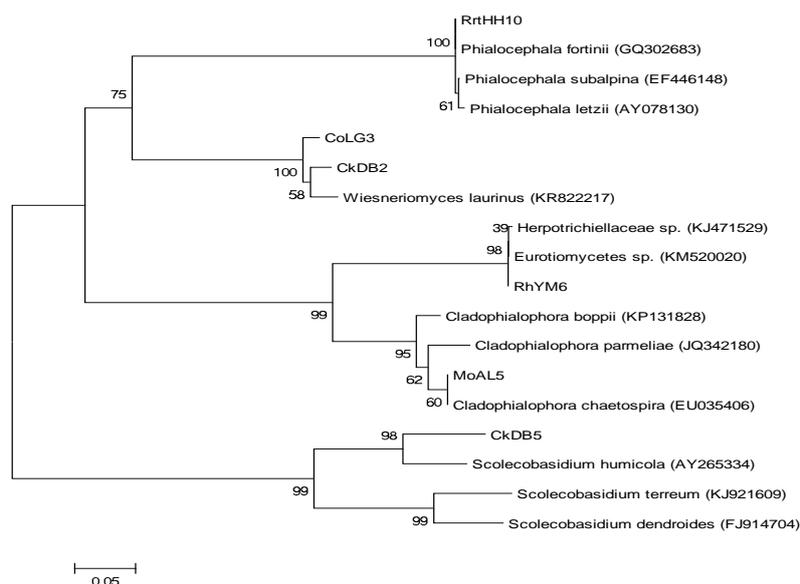


Figure 1. The neighbor-joining phylogenetic tree of the six strains of interest. The tree was obtained using the maximum composite likelihood model for pairwise distance measurements based on ITS sequences. Numbers on branches indicate values generated from 1,000 bootstrap replica

Table 1. Dry weights of six DSEFs in MMN medium with and without Zn

Treatment (strain)	Dry weight (mg)	
	Zn-free	Zn
CoLG3	44.7 ± 1.9 ^b	-
CkDB2	74.0 ± 6.4 ^a	-
CkDB5	39.2 ± 3.2 ^b	28.8 ± 2.8 ^a
MoAL5	65.4 ± 5.7 ^{ab}	-
RhYM6	57.7 ± 4.9 ^{ab}	-
RrtHH10	59.8 ± 6.3 ^{ab}	29.5 ± 1.2 ^a

All values represent the means ± standard deviations of three replicate cultures. Values in the same column with different letters indicate a difference at the 5% significance level

Evaluation of Zn tolerance range

Strains CkDB5 and RrtHH10 produced varying dry weights in media with different Zn concentrations after 14 days of incubation (Table 2).

Strain CkDB5 showed significantly different dry weights under different Zn concentrations (Table 2). The highest dry weight value in 0 mg/mL is 28.4 ± 1.8 mg, compared to 0.4 mg/mL (22.1 ± 4.2 mg), 0.8 mg/mL (19.1 ± 3.9 mg), 1.2 mg/mL (16.9 ± 1.9 mg), 1.6 mg/mL (16.0 ± 2.5 mg), 2.0 mg/mL (4.9 ± 0.6 mg) and 2.4 mg/mL (0 mg).

Strain RrtHH10 also showed significantly different dry weights under different Zn concentrations (Table 2). The highest dry weight value in 0 mg/mL is 53.4 ± 8.1 mg, compared to 0.4 mg/mL (42.4 ± 16.0 mg), 0.8 mg/mL (34.9 ± 6.2 mg), 1.2 mg/mL (11.5 ± 1.7 mg), 1.6 mg/mL (7.7 ± 1.9 mg), 2.0 mg/mL (5.9 ± 2.3 mg) and 2.4 mg/mL (0 mg).

Table 2. Dry weights of strains *CkDB5* and *RrtHH10* in MMN medium with different Zn concentrations

Zn concentrations (mg/mL)	Dry weight (mg)	
	<i>CkDB5</i>	<i>RrtHH10</i>
0	28.4 ± 1.8 ^a	53.4 ± 8.1 ^a
0.4	22.1 ± 4.2 ^{ab}	42.4 ± 16.0 ^a
0.8	19.1 ± 3.9 ^b	34.9 ± 6.2 ^a
1.2	16.9 ± 1.9 ^b	11.5 ± 1.7 ^b
1.6	16.0 ± 2.5 ^b	7.7 ± 1.9 ^b
2.0	4.9 ± 0.6 ^c	5.9 ± 2.3 ^b
2.4	0 ^d	0 ^c

All values represent the means ± standard deviations of three replicate cultures. Values in the same column with different letters indicate a difference at the 5% significance level

Morphological changes of the DSEFs under different Zn concentrations

The mycelial morphology of strains *CkDB5* and *RrtHH10* were observed under a light microscope after cultivation in MMN medium with different Zn concentrations for 14 days. Strains *CkDB5* (Fig. 2A–C) and *RrtHH10* (Fig. 2D–F) showed notable morphological changes in the presence of different Zn concentrations.

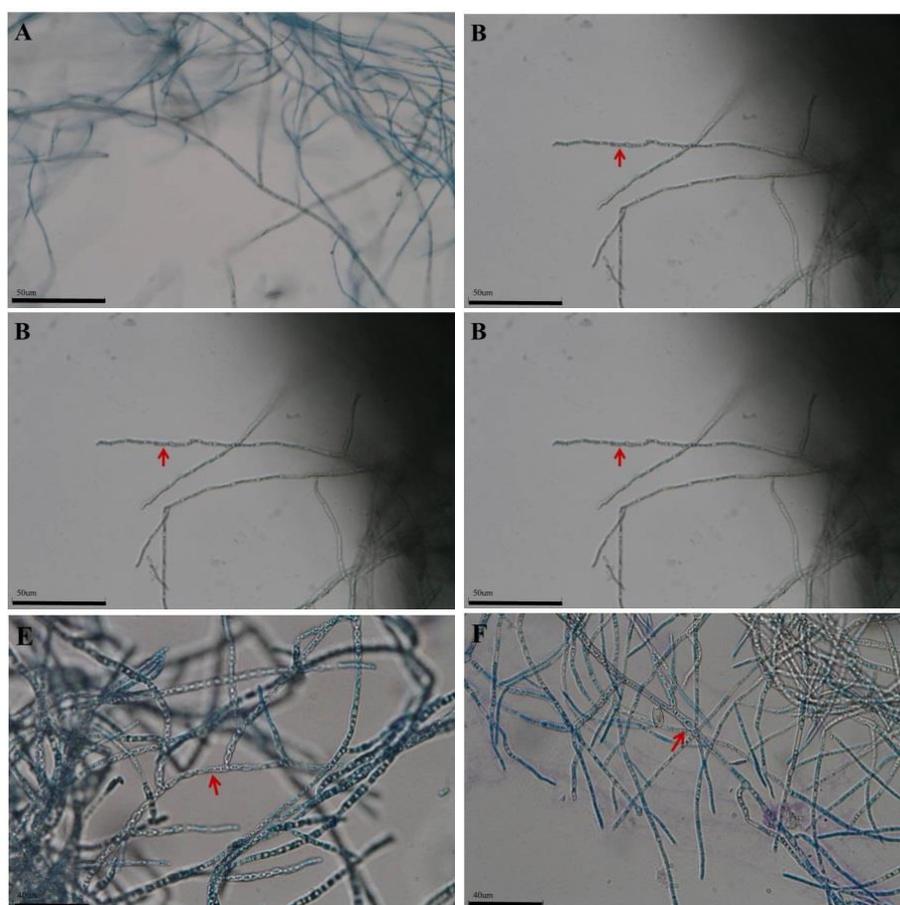


Figure 2. Mycelial morphology of strains *CkDB5* and *RrtHH10* in different Zn concentrations. A–C. Strain *CkDB5*. D–F. *RrtHH10*. A and D, 0 mg/mL. B and E, 0.8 mg/mL. C and F, 1.6 mg/mL

Growth response

After 42 days of incubation, all treated tomato seedlings (with/without Zn) had survived (*Fig. 3*), and the fresh weights of all treatments showed significant differences ($p < 0.05$) (*Table 3*).



Figure 3. Morphology of tomato seedlings after incubation for 42 days. A. Plants inoculated with CkDB5 without Zn. B. Plants inoculated with CkDB5 with Zn. C. Plants inoculated with RrtHH10 without Zn. D. Plants inoculated with RrtHH10 with Zn. E. Controls without Zn. F. Controls with Zn

In treatments without Zn (*Table 3*), plants inoculated with CkDB5 generated the highest fresh weights of 229.2 ± 5.1 mg compared to plants inoculated with RrtHH10 (81.5 ± 3.3 mg) and the control (54.7 ± 3.4 mg).

In treatments with Zn (*Table 3*), plants inoculated with CkDB5 also showed the highest fresh weights, 174.4 ± 11.4 mg, compared to plants inoculated with RrtHH10 (104.3 ± 11.2 mg) and the control (49.7 ± 4.0 mg).

Table 3. Biomasses of tomato seedlings grown for 42 days in different treatments

Treatment (mg mL ⁻¹)	Fresh weight (mg)		
	CkDB5-inoculation	RrtHH10-inoculation	Control
0	229.2 ± 5.1 ^{aA}	81.5 ± 3.3 ^{bB}	54.7 ± 3.4 ^{aC}
1	174.4 ± 11.4 ^{bA}	104.3 ± 11.2 ^{aB}	49.7 ± 4.0 ^{aC}

All values represent the means ± standard deviations of five replicate cultures. Values in the same row with different lowercase letters are significantly different at 5% significant level. Values in the same column with different capital letters are significantly different at 5% significant level

Evaluation of Zn concentrations in tomato

At harvest, the Zn concentrations of all treatments were determined by FAAS. The Zn concentrations of all treatments with Zn showed significant differences compared to all treatments without Zn ($p < 0.05$) (Table 4).

In treatments without Zn (Table 4), plants inoculated with CkDB5 showed the highest Zn concentration ($92.99 \pm 0.5 \mu\text{g/g}$) compared to the control ($56.4 \pm 4.5 \mu\text{g/g}$) and plants inoculated with RrtHH10 ($0 \mu\text{g/g}$).

In treatments with Zn (Table 4), plants inoculated with RrtHH10 demonstrated the highest Zn concentration ($1,083.03 \pm 13.0 \mu\text{g/g}$) compared to plants inoculated with CkDB5 ($912.83 \pm 6.2 \mu\text{g/g}$) and the control ($279.09 \pm 6.3 \mu\text{g/g}$).

Table 4. Zn concentrations of tomato seedlings grown for 42 days in different treatments

Treatment (mg mL ⁻¹)	Zn concentration ($\mu\text{g/g}$)		
	CkDB5-inoculation	RrtHH10-inoculation	Control
0	$92.99 \pm 0.5^{\text{bA}}$	0^{bC}	$56.4 \pm 4.5^{\text{bB}}$
1	$912.83 \pm 6.2^{\text{aB}}$	$1083.03 \pm 13.0^{\text{aA}}$	$279.09 \pm 6.3^{\text{aC}}$

All values represent the means ± standard deviations of five replicate cultures. Values in the same row with different lowercase letters are significantly different at 5% significant level. Values in the same column with different capital letters are significantly different at 5% significant level

Discussion

In our laboratory, six isolates were shown to be DSEFs, which are known to facilitate the growth of host plants. The six isolates belonged to five taxa (Fig. 1); CoLG3 and CkDB2 belonged to *W. laurinus*, RrtHH10 belonged to *P. fortinii*, CkDB5 belonged to *S. humicola*, RhYM6 belonged to Herpotrichiellaceae, and MoAL5 belonged to *C. chaetospora*.

All six DSEFs that were cultivated in treatments without Zn survived after incubation for 14 days, but only strains CkDB5 and RrtHH10 survived in treatments with Zn (Table 1). Although strains CkDB5 and RrtHH10 did not show significant growth in MMN medium without Zn, they unexpectedly survived in MMN medium with Zn. Therefore, strains CkDB5 and RrtHH10 were selected for further study (Table 1).

The sensitivity of strains CkDB5 and RrtHH10 to Zn was determined based on MIC and EC₅₀. Following the different Zn concentrations in MMN medium for evaluation of Zn tolerance range (Table 2), strain CkDB5 had shown significant differences at a lower Zn concentration (0.8 mg/mL), while strain RrtHH10 showed significant differences at a higher Zn concentration (1.2 mg/mL). The MIC of CkDB5 was over 2.0 mg/mL, and its ME₅₀ was over 1.6 mg/ml. The MIC of RrtHH10 was over 2.0 mg/mL, and its ME₅₀

was over 0.8 mg/mL. Strain CkDB5 showed the same Zn resistance as the fungal endophyte H93 (EU797525) ($ME_{50} = 1.5$ mg/mL) (Zhang et al., 2008). Therefore, strain CkDB5 could belong to a group of highly tolerant fungi based on the studies by Wainwright and Gadd (1997) and Zhang et al. (2008). The mycelial morphology of the strains was twisted and looped with increasing Zn concentrations (Fig. 2). Ban et al. (2012) and Zhang et al. (2008) reported similar results.

Although all treated tomato seedlings (with/without Zn) survived 42 days of incubation (Fig. 3; Table 3), the fresh weights of plants inoculated with CkDB5 and RrtHH10 were significantly higher than those of the control. The fresh weight of plants inoculated with CkDB5 without Zn (229.2 ± 5.1 mg) was significantly higher than that of plants inoculated with CkDB5 with Zn (174.4 ± 11.4 mg). Compared to the fresh weight of the control (54.7 ± 3.4 mg), the fresh weight of plants inoculated with CkDB5 without Zn was 4 times higher (229.2 ± 5.1 mg). Compared to the fresh weight of the control (54.7 ± 3.4 mg), the fresh weight of plants inoculated with RrtHH10 without Zn was only 1.6 times higher (81.5 ± 3.3 mg). DSEFs can enhance the growth of their host plant (Fernando and Currah, 1996; Jumpponen et al., 1998; Shivanna et al., 1994). While strain CkDB5 could increase the biomass of *C. kanehirae* cuttings by 200% (Lin, 2016), the same strain increased the biomass of inoculated tomatoes by up to 400% in this study (Table 3).

Of the six treatments (Table 4) studied, the Zn concentration of plants inoculated with RrtHH10 with Zn was 1,000 times higher than that of plants without Zn. The Zn concentration of plants inoculated with CkDB5 with Zn was 10 times higher than that of plants without Zn. The Zn concentration of the control with Zn was 5 times higher than that of plants without Zn.

Based on our results, only strains CkDB5 and RrtHH10 were Zn-resistant. While CkDB5 and RrtHH10 presented identical MIC values (Table 2), the former indicated higher ME_{50} values than the latter (Table 2). With or without Zn, CkDB5 could promote the growth response of tomato (Table 3). Ban et al. (2012) indicated that DSEFs are typical root endophytes, and they believed that potential effects of DSEFs on host plants should be considered. DSEFs can affect the heavy-metal uptake of their host plants and enhance the tolerance of these plants to heavy metal stress. DSEFs have also been shown to promote the growth response of their host plants (Gadd, 2007; Lin, 2016; Selosse et al., 2004). These fungi have been observed to transport absorbed nutrients to host plants to support their growth in harsh habitats (Deram et al., 2008; Likar and Regvar, 2009; Zhang et al., 2013).

Conclusion

Molecular analysis revealed that the six DSEFs studied in this work could be divided into five clusters; strain CoLG3 and CkDB2 were classified as *W. laurinus*, while strain RrtHH10 was classified as *P. fortinii*. Strain MoAL5 was classified as *C. chaetospora*, while strain RhYM6 was classified as Herpotrichiellaceae or Eurotiomycetes. Finally, strain CkDB5 was classified as *S. humicola*. These strains may be new species in the world or new species in the fungal flora of Taiwan. Of these six DSEFs, only strains CkDB5 and RrtHH10 were Zn-resistant and could promote the growth response of *S. lycopersicum*. Of the two endophytes, strain CkDB5 was more tolerant to Zn and showed better growth responses. CkDB5 is not only Zn-resistant, but it is also a promoter of tomato growth.

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