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RESEARCH ARTICLE



## Dark septate endophytes colonizing the roots of 'non-mycorrhizal' plants in a mine tailing pond and in a relatively undisturbed environment, Southwest China

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### ABSTRACT

Dark septate endophytes (DSEs), one of the most common fungal colonizers of roots, are considered to overlap in function with mycorrhizal fungi. However, there is little knowledge on the distribution and identity of DSEs in 'non-mycorrhizal' plants. In the current study, colonization and diversity of DSEs colonizing the roots of eight typically 'non-mycorrhizal' families were assessed. In total, 120 root samples of 31 plant species were all colonized by DSEs. Intensity of DSE colonization varied greatly among different plant species, with a range of 0.56–47.56%, 8.13% on average. *Cladosporium*, *Cyphellophora* and *Phialophora* were the dominant genera, with a relative abundance of more than 60% over a total of 90 isolates. Our results showed that diverse DSE species colonized the roots of 'non-mycorrhizal' plants, especially they were more common in degraded mine tailings than in the undisturbed site, but their integral roles to the functional roots are in need of further experimental demonstration.

### ARTICLE HISTORY

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'Non-mycorrhizal' plants; dark septate endophyte (DSE); colonization characteristics; *Cladosporium*; *Cyphellophora*; *Phialophora*

### Introduction

In natural ecosystems, the plant roots are regarded as a large reservoir of many types of mutualistic microorganisms, including bacteria, archaea, fungi, collectively designated as the plant–microbiome or phytomicrobiome (Quiza et al. 2015), the most common of which are the arbuscular mycorrhizal (AM) fungi, formed symbiotic association intimately with the roots of more than 80% terrestrial plants, growing inter- and intracellularly in the root cortex (Smith & Read 2008). Both fossil evidence and molecular data indicate that this widespread AM association existed in the earliest land plants, and may have enabled plants to colonize land (Bravo et al. 2016; Remy et al. 1994; Wang et al. 2010). However, it is found interestingly that some plants, for example, most of the plants of Caryophyllaceae, Brassicaceae, Chenopodiaceae, Juncaceae and Polygonaceae are insusceptible to infection with mycorrhizal fungi (Newman & Reddell 1987), and defined as the 'possibly non-mycorrhizal or rarely mycorrhizal' plants, which do not usually form mycorrhizas of any of the well-recognized kinds (Smith & Read 2008). But, one must keep in mind that some families have both mycorrhizal and non-mycorrhizal members and, even in predominantly non-mycorrhizal groups, some mycorrhizal species do occur. For example, Miller et al. (1999) survey the mycorrhizal status of the genus *Carex* (belonging to the generally considered 'non-mycorrhizal' Cyperaceae), and find both the obligately non-mycorrhizal species and AM members of *Carex*, and find that mycorrhizal colonization appears to occur in response to many factors, both environmental and phylogenetic.

Increasing evidence reveals that a miscellaneous group of ascomycetous anamorphic fungi called dark septate endophytes (DSEs), a term broadly classified as conidial and sterile septate fungal endophytes with diverse morphological features

such as dark septate hyphae and microsclerotia, dominantly colonize root tissues intracellularly and intercellularly from the tropics to arctic and alpine habitats (Jumpponen & Trappe 1998; Vohník et al. 2015; Casazza et al. 2017; Hewitt et al. 2017). Even under some extreme environments, for example, old slag heaps resulting from the processing of zinc ores, DSE gradually becomes the most prevailing root colonizers with the decreasing AM colonization at increasing environmental stresses (Deram et al. 2008, 2011). Recently, Huusko et al. (2016) also report a shift of fungal community from an AM-dominated to a DSE-dominated colonization in *Deschampsia flexuosa* roots along a postglacial primary successional land uplift gradient. More importantly, the increasing evidence supports the conclusion that the prevalence of DSEs in extreme habitats functionally and ecologically overlaps with mycorrhizal fungi. For example, with regard to the alleged roles in protecting plants from abiotic and biotic stresses via habitat adapted symbiosis, the DSE can enhance nutrient availability to plants, and contribute to plant stress resistance to pathogens, metal pollution, etc. (Jumpponen 2001; Mandyam & Jumpponen 2014; Newsham 2011; Wang et al. 2016).

Although there has been a continual increase of interest in DSE fungi (Card et al. 2016; Monica et al. 2015; Jumpponen & Trappe 1998; Newsham 2011), there is a need for studies on the characterization of DSE in the different 'non-mycorrhizal' species or families, which may have evolved compensatory mechanisms of DSE for the effective nutrient uptake. In the present study, 120 root samples of 31 plant species belonging to 8 'non-mycorrhizal' families were studied in Kunming Changchong Mountain (CM) and in Niubahuang Sn/Pb/Zn/ mine tailings pond in Gejiu, in Yunnan Province, Southwest (SW) China. The colonization characteristics and diversity of DSE colonizing the roots of these typically 'usually

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non-mycorrhizal or rarely mycorrhizal plant families' were assessed and the ecological roles of DSE were also discussed.

## Materials and methods

### Sample sites and sample collection

In total, two sample sites including a natural ecosystem of CM in Kunming city, and an old abandoned Niubahuang Sn/Pb/Zn mine tailings pond in Gejiu city, were selected in Yunnan province, SW China (Figure 1). CM is located at 102°42'31" E, 25°13'11" N, in Kunming city, where has low-latitude monsoon climate with sunlight of 2198 h, frost-free period of 230 days, annual mean temperature of 14.9°C, and mean annual precipitation of about 1190.3 mm, nearly 88% occurring from May to October. The forest is co-dominated by warm temperate coniferous, semi-humid evergreen broadleaf and mixed conifer–broadleaf trees (Zhang 2011). With a distance of approximately 220 km, Niubahuang Sn/Pb/Zn mine tailings pond (NTP, 103°10'42.2" E, 23°17'41.7" N) is an old abandoned polymetallic tin ore tailings pond, near to the world-class tin production region, known as the super large tin copper polymetallic mining area (~300 Mt of Sn, and ~700 Mt of Cu, Pb, Zn, Sb, Ag, Mo, Au and Bi) (Cheng et al. 2010). The climate of the area belongs to tropical and subtropical monsoon climate with mean annual temperature of 15.9°C and average annual precipitation of 1069.7 mm. The native vegetation is rare for the massive deforestation and rapid pollution until now in NTP, scattered with sparse *Pinus yunnanensis*, shrubs and herbs.

In total, 120 root samples of 31 plant species belonging to 8 'non-mycorrhizal' families were collected from the two samples sites. Roots and rhizosphere soils (5–25 cm depth) of each plant species with at least 3 replicates were collected randomly, including 20 plants species of 8 families from CM and 15 plants species of 7 families from NTP, respectively. The sampled roots were immediately brought back to



**Figure 1.** Geographic localization of the two collecting sites, Kunming CM and Gejiu Niubahuang Sn/Pb/Zn mine tailings pond (NTP), in SW China.

the laboratory and subjected to anatomical observation of DSE colonization and the isolation of fungal endophytes described as follows.

### DSE colonization

For anatomical observation of DSE colonization, the roots (with tips) were firstly washed thoroughly under tap water, and then cleared in 10% (w/v) KOH at 90°C in a water bath for 1 hour, and then stained with acid fuchsin (Berch & Kendrick 1982). Compared with the red-stained AM fungal structures (i.e. vesicles, arbuscules, hyphae, etc.), the dark and septate hyphae and intracellular microsclerotia of DSE were observed without visually stained color. For each sample, at least eight 2-cm-long root fragments were chosen for examination under a compound-light microscope (OLYMPUS-BX31) at 400× magnification. The colonization status, including the DSE hyphae or microsclerotia, was observed. The total root colonization intensity of DSE (DSE%), including the intensity by intraradical DSE hyphae (H%) and intracellular microsclerotia (M%), were estimated using the magnified intersection method by checking over 200 intersections for each sample (Massicotte et al. 2005; McGonigle et al. 1990).

### DSE isolation

Fresh root samples were carefully cleaned under the running tap water to remove soil particles and sterilized with 75% ethanol for 5 min and 10% NaClO for 5 min, respectively. Then, the sterilized roots were rinsed three times in sterile distilled water, finally air-dried under sterile conditions, cut into 3–5 mm pieces and cultured on the both malt extract agar (MEA) and potato dextrose agar (PDA) isolation media described as follows (Arnold et al. 2000). Over 80 root pieces of each plant species were deposited on 1% MEA (malt extract 20 g, peptone 5 g, agar 18 g, and water 1000 mL, pH 6.4) and PDA (potato extract 200 g, dextrose 20 g, agar 18 g and water 1000 mL) at 28°C in the dark for 30 days, respectively. Streptomycin (100 mg L<sup>-1</sup>) and ampicillin (100 mg L<sup>-1</sup>) were also added to the above two isolation media to avoid bacterial contamination (Silvani et al. 2008). During cultivation, root samples were checked every other day, and only new melanized fungal colonies growing out of the plant root tissues were transferred to PDA slants. The isolation ratio was calculated as the total number of endophytic fungal isolates divided by the total number of DSE-colonized plant species. Also, the relative abundance (RA%) was calculated as the number of fungal isolates in one clade divided by the total number of all fungal isolates (Li et al. 2012).

### DSE identification

For the identification of fungi, phylogenetic analyses of fungal internal transcribed spacer (ITS) rDNA sequences (ITS1-5.8S-ITS2) from all fungal isolates were used. Initially, the total genomic DNA of each DSE strain was extracted and purified using the cetyltrimethyl ammonium bromide (CTAB) method (Sharrock et al. 2004). The primer set ITS1-F/ITS4 was used to amplify the ITS1-5.8S-ITS2 regions (White et al. 1990). The PCR reactions were carried out in a total volume of 50 µL containing 39 µL sterile distilled water, 5 µL 10× PCR buffer (containing Mg<sup>2+</sup>), 2 µL dNTP, 1 µL of

each 10  $\mu\text{M}$  primers, 0.25  $\mu\text{L}$  Taq DNA polymerase (5U  $\mu\text{L}^{-1}$ , Takara Biotechnology Co. LTD., Japan), and 1  $\mu\text{L}$  DNA template. The amplifications were performed on a Mastercycler (Eppendorf, Germany) under the following conditions: an initial pre-denaturing at 95°C for 4 min, 32 cycles at 94°C for 45 s, 55°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 5 min. The amplification products were electrophoresed in 1% agarose gel and purified using Gel Extraction Kit (UNIQ-10, Sangong, Shanghai, China), then sequenced on an ABI 3730 DNA Analyzer (Applied Biosystems, Inc.). Subsequently, a dataset including the ITS sequences and the

similar sequences retrieved from GenBank using the NCBI BLAST program (Altschul et al. 1997) was aligned by the MegAlign program of Lasergene Package (DNASTar Inc., Madison, WI, USA) and subjected to a neighbor-joining (NJ) analysis using PAUP\*4.0b10 (Swofford 2002) with support values estimated using 1000 bootstrap. Modeltest 3.06 was used to determine the evolutionary model that best fit the dataset (Posada & Crandall 1998). The ITS gene sequences reported in this study have been deposited in GenBank under accession numbers KY987519–KY987540. Only representative sequences were deposited.

**Table 1.** Colonization intensity of DSE (mean  $\pm$  SE,  $n \geq 3$ ) in plant roots in Kunming CM and Gejiu Niubahuang Sn/Pb/Zn mine tailings pond (NTP), SW China.

Plants	Sites	DSE colonization intensity			Fungal isolates
		H%	M%	DSE%	
<b>Amaranthaceae</b>					
<i>Achyranthes bidentata</i> <sup>a</sup>	CM	0.33 $\pm$ 0.19	0.44 $\pm$ 0.44	0.56 $\pm$ 0.56	
<i>Achyranthes aspera</i>	NTP	3.89 $\pm$ 0.59	0	3.89 $\pm$ 0.59	G59109, G59214, G59211, G59207, G59291, G59213, G59297
<i>Amaranthus retroflexus</i>	NTP	1.78 $\pm$ 0.49	0	1.78 $\pm$ 0.49	
Average		2.00 $\pm$ 1.69	0.26 $\pm$ 0.52	2.19 $\pm$ 1.91	
<b>Caryophyllaceae</b>					
<i>Cucubalus baccifer</i>	NTP	9.89 $\pm$ 2.15	1.67 $\pm$ 1.20	10.11 $\pm$ 2.23	G5805
<i>Dianthus</i> sp.	NTP	3.11 $\pm$ 0.73	0	3.11 $\pm$ 0.73	G63305, G63306, G63313, G63395, G63396
<i>Silene gallica</i> <sup>a</sup>	CM	13.44 $\pm$ 1.64	4.22 $\pm$ 3.42	16.56 $\pm$ 1.44	
<i>Stellaria media</i> <sup>a</sup>	CM	17.89 $\pm$ 2.06	0.67 $\pm$ 0.67	18.00 $\pm$ 2.14	P1401, P1402, P1405, P1406, P1410, M1403, M1405, M1406, M1407, M1409, M1410, M1414
Average		11.08 $\pm$ 6.20	1.64 $\pm$ 3.20	11.95 $\pm$ 6.68	
<b>Chenopodiaceae</b>					
<i>Chenopodium album</i>	NTP	3.55 $\pm$ 0.22	0	3.55 $\pm$ 0.22	G48215, G480201, G48101, G48216
	CM	2.45 $\pm$ 1.31	0.33 $\pm$ 0.33	2.45 $\pm$ 1.31	
<i>Ch. ambrosioides</i>	CM	2.44 $\pm$ 0.95	0	2.44 $\pm$ 0.95	
	NTP	3.89 $\pm$ 0.49	0	3.89 $\pm$ 0.49	
Average		2.81 $\pm$ 1.52	0.11 $\pm$ 0.33	2.81 $\pm$ 1.52	
<b>Commelinaceae</b>					
<i>Commelina communis</i> <sup>a</sup>	NTP	35.56 $\pm$ 1.46	2.00 $\pm$ 0.33	35.56 $\pm$ 1.46	
<b>Cruciferae</b>					
<i>Brassia campestris</i>	CM	2.56 $\pm$ 1.16	0	2.56 $\pm$ 1.16	
<i>Brassica juncea</i>	NTP	1.39 $\pm$ 0.42	0	1.39 $\pm$ 0.42	
<i>Lepidium virginicum</i> <sup>a</sup>	CM	4.44 $\pm$ 1.06	0.89 $\pm$ 0.89	4.55 $\pm$ 1.13	
Average		2.45 $\pm$ 1.87	0.22 $\pm$ 0.77	2.47 $\pm$ 1.93	
<b>Cyperaceae</b>					
<i>Carex baccans</i> <sup>a</sup>	NTP	5.34 $\pm$ 0.88	0	5.34 $\pm$ 0.88	G703, G9113, G6104, G6105, G802, G8103, G8107, G8193
<i>Cyperus rotundus</i>	CM	4.67 $\pm$ 0.51	0	4.67 $\pm$ 0.51	
Average		5.05 $\pm$ 1.39	0	5.05 $\pm$ 1.39	
<b>Juncaceae</b>					
<i>Juncus effusus</i>	NTP	6.46 $\pm$ 1.78	0	6.46 $\pm$ 1.78	
	CM	2.22 $\pm$ 0.29	0	2.22 $\pm$ 0.29	P1013, M1001, P1009, P1007, P1012, P1010, P1008, P1005, P1003
<i>Juncus inflexus</i> <sup>a</sup>	CM	4.34 $\pm$ 0.88	0	4.34 $\pm$ 0.88	P1104, P1101, M1104, P1103, M1102, M1101, P1102
Average		4.34 $\pm$ 2.53	0	4.34 $\pm$ 2.53	
<b>Polygonaceae</b>					
<i>Fagopyrum dibotrys</i> <sup>a</sup>	CM	1.89 $\pm$ 0.49	0.22 $\pm$ 0.22	1.89 $\pm$ 0.49	P61
	NTP	3.45 $\pm$ 1.26	0	3.45 $\pm$ 1.26	G55308, G55105, G55413, G55315, G55313, G45201, G45207, G55415, G55416, G45205, G45291, G45297
<i>Fallopia multiflora</i> <sup>a</sup>	CM	1.33 $\pm$ 0.77	0.22 $\pm$ 0.22	1.55 $\pm$ 0.97	M71
<i>Polygonum campanulatum</i> <sup>a</sup>	NTP	40.78 $\pm$ 2.95	2.11 $\pm$ 0.59	40.78 $\pm$ 2.95	
<i>Polygonum capitatum</i> <sup>a</sup>	NTP	47.56 $\pm$ 2.13	3.78 $\pm$ 0.59	47.56 $\pm$ 2.13	
<i>Polygonum chinense</i> <sup>a</sup>	CM	4.89 $\pm$ 0.40	0	4.89 $\pm$ 0.40	P1501, P1502, P1503, P1504, M1501
<i>Polygonum cuspidatum</i> <sup>a</sup>	NTP	4.00 $\pm$ 0.58	0	4.00 $\pm$ 0.58	
<i>Polygonum lapathifolium</i> var. <i>lanatum</i> <sup>a</sup>	NTP	9.60 $\pm$ 2.65	0	9.60 $\pm$ 2.65	G308
<i>Polygonum molle</i> <sup>a</sup>	NTP	8.56 $\pm$ 0.43	1.01 $\pm$ 0.81	9.33 $\pm$ 0.72	G18101, G16212, G18205, G16210, G18204, G17216, G18295
<i>Polygonum nepalense</i>	NTP	5.94 $\pm$ 1.98	0	5.94 $\pm$ 1.98	
<i>Rumex nepalensis</i>	NTP	7.78 $\pm$ 1.24	0	7.78 $\pm$ 1.24	G34305, G34304, G34395, G34394
<i>Polygonum</i> sp1.	NTP	17.00 $\pm$ 0.39	0.22 $\pm$ 0.22	17.00 $\pm$ 0.39	G195, G15207, G25208, G15201
<i>Polygonum</i> sp2.	NTP	4.55 $\pm$ 0.40	0	4.55 $\pm$ 0.40	G33301
<i>Rumex dentatus</i>	CM	3.22 $\pm$ 1.06	0	3.22 $\pm$ 1.06	
<i>Rumex hastatus</i>	CM	1.33 $\pm$ 0.69	0.56 $\pm$ 0.56	1.45 $\pm$ 0.78	M41
Average		10.98 $\pm$ 12.85	0.49 $\pm$ 1.19	10.08 $\pm$ 12.84	
The total average		7.98 $\pm$ 10.63	0.49 $\pm$ 1.39	8.13 $\pm$ 10.68	

Note: H%, M% and DSE% represent the colonization intensity of DSE hyphae, microsclerotia and the total values, respectively.

<sup>a</sup>The species with the first report of DSE status.

## Data analysis

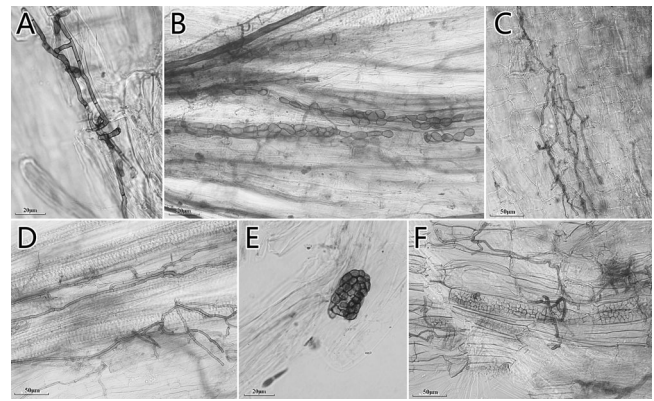
The statistical analysis was performed using SPSS software (version 22.0). The assays were carried out in at least three replicates and the results for colonization intensity of DSE hyphae (H%), microsclerotia (M%) and the total colonization intensity of DSE (DSE%) are expressed as mean values  $\pm$  standard deviations ( $n \geq 3$ ). Then, the data on the DSE colonization intensities including H%, M% and DSE% were subjected to independent *T*-test to evaluate the differences between all the samples from two sample sites. The differences of the totally average DSE colonization intensity were considered statistically significance if the *p* value was less than .05.

## Results

### DSE colonization

Of the 31 species analyzed, only 16.1% were colonized by AM fungi (5 plant species belonging to Polygonaceae and Commelinaceae families). On the contrary, all root samples were found to be colonized by typical 'dark and septate' endophytic fungi (Table 1), and various morphological structures of DSEs were observed (Figure 2). Under compound-light microscope, sparse melanized or slightly dark running hyphae and a variety of morphological microsclerotia of DSE, such as band-shaped, peloton-like, brain-like, colonized the epidermis and cortex cells of most roots, including the most commonly encountered plant species in Caryophyllaceae and Polygonaceae surveyed (Figure 2). The total colonization intensity of DSE (DSE%) showed the minimum value (0.56%) in the roots of *Achyranthes bidentata* from CM, and peaked in *Polygonum capitatum* from Niubahuang Sn/Pb/Zn mine tailings pond with the maximum colonization intensity of 47.56% (Table 1), and there was an average colonization of  $8.13\% \pm 10.68\%$  in total. Interestingly, we noted that 53.33% of the 'non-mycorrhizal' families (8/15 families) were colonized by DSE microsclerotia in Gejiu NTP site, but this value was reduced to 30% (6/20 families) in Kunming CM site.

In Kunming CM site, the majority of plants had a low colonization intensity of DSE, ranging from 0.56% to 18%, with an average of  $4.76\% \pm 0.80\%$ . While, in Gejiu NTP site, the average DSE colonization intensity was  $10.15 \pm 1.44\%$ , which was significantly higher than that in Kunming CM site (Table 2,  $p < .01$ ). Interestingly, we found that there were approximately 55% plant species (11/20) with above 5% colonization intensity in Gejiu NTP site, and there were 5 plant species (25%) with above 10% DSE colonization intensity (Table 1). Meantime, we also noted that DSE colonization intensity varied greatly across plant species and even across different ecotypes of the same plant species from different ecological habitats. For example, the mean DSE colonization of *Juncus effusus* from Kunming CM site ( $2.22\% \pm 0.29\%$ ) was lower than that from Gejiu NTP site ( $6.46\% \pm 1.78\%$ ). Similar trends of DSE colonization were also observed in the roots of *Chenopodium album*, *Fagopyrum dibotrys* and *Chenopodium ambrosioides* from Gejiu NTP site (with a 3.55%, 3.45% and 3.89% DSE colonization intensity, respectively), significantly higher compared with the average DSE colonization of 2.45%, 1.89% and 1.44% in Kunming CM site, respectively. We also noted a distinct pattern of DSE colonization intensity in the 8 plant families surveyed, and the maximum value of  $11.95 \pm 6.68\%$  occurred in



**Figure 2.** Morphological characteristics of the DSE colonizing the roots of the studied 'non-mycorrhizal' plants in Kunming CM and Gejiu Niubahuang Sn/Pb/Zn mine tailings pond (NTP), SW China. (A) dark and septate hyphae in *Stelaria media* in CM; (B) microsclerotia in *Chenopodium album* in NTP; (C) mycelial labyrinth in *Silene gallica* in CM; (D) dark and septate hyphae in *Polygonum* sp.1 in NTP; (E) brain-like microsclerotium in *Polygonum capitatum* in NTP; (F) dark and septate hyphae and microsclerotia in *Dianthus* sp. in NTP.

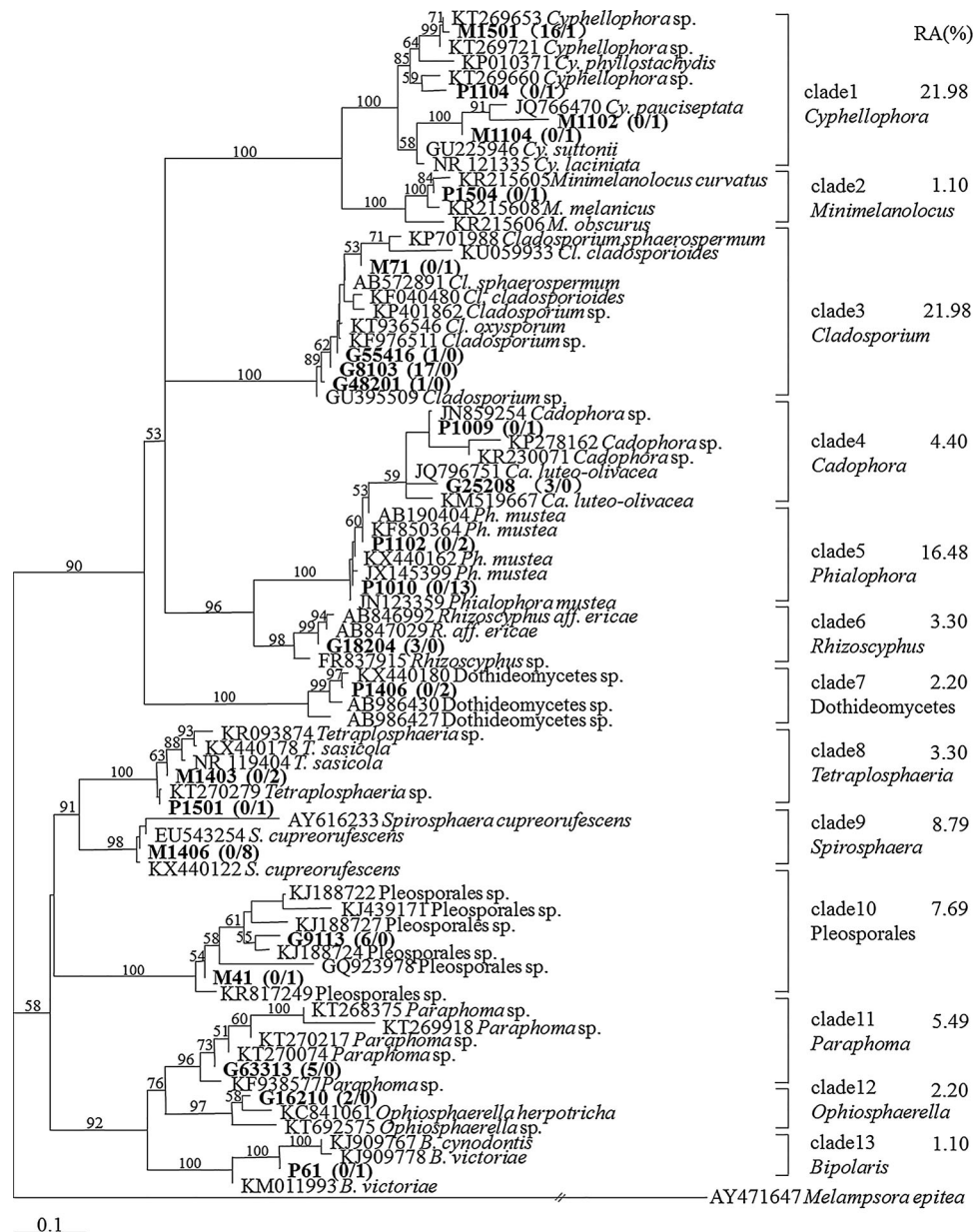
**Table 2.** Results of independent *T*-test to compare the total average DSE colonization intensity (mean  $\pm$  SE) in the plant roots from Kunming CM ( $n = 45$ ) and GejiuNiubahuang Sn/Pb/Zn mine tailings pond (NTP,  $n = 75$ ), SW China, including hyphae (H%), microsclerotia (M%) and the total DSE structures (DSE%).

	Total average colonization intensity		<i>F</i>	df	<i>p</i>	Sig.
	CM	NTP				
H%	$4.50 \pm 0.73$	$10.06 \pm 1.44$	13.41	105.39	.001	**
M%	$0.50 \pm 0.26$	$0.49 \pm 0.13$	0.01	118.00	.954	NS
DSE%	$4.76 \pm 0.80$	$10.15 \pm 1.44$	12.15	109.07	.001	**

the plants of Caryophyllaceae, then followed by Polygonaceae, Cyperaceae, Juncaceae, Chenopodiaceae, Cruciferae, and there was the lowest colonization intensity of  $2.19\% \pm 1.91\%$  in Amaranthaceae averagely. While, we also noted that the total DSE colonization intensity reached  $35.56 \pm 1.46\%$ , but only one species of *Commelina communis* in Commelinaceae with three replicates from Gejiu NTP site.

### Isolation and identification of DSE strains

In total, 90 root-associated melanized fungi were isolated, including 54 strains isolated from Gejiu NTP site and 36 strains from Kunming CM site (Table 1). Moreover, the isolation ratios did not differ significantly in Gejiu NTP site (the total isolation ratio was 2.7:1) from the Kunming CM site (the total isolation ratio was 2.4:1). According to the phylogenetic analysis, 90 fungal isolates were identified. Interestingly, the most common fungi colonizing the roots of these 'non-mycorrhizal' plants were related to the well-known DSE fungi, for example, *Phialophora mustea*, *Cadophora luteo-olivacea*. We found that the fungal species of *Cladosporium*, *Cyphellophora* and *Phialophora* were the most abundant with a total RA over 60% (Figure 3). The RA of fungi from *Spirosphaera*, Pleosporales, *Paraphoma* and *Cadophora* was above 4%, while the remaining species were closely related to the fungi of *Minimelanolocus*, *Rhizocyphus*, Dothideomycetes, *Tetraplophaeria*, *Ophiosphaerella* and *Bipolaris* and were present at very low percentages (Figure 3).



**Figure 3.** NJ phylogram of ITS sequences of cultured melanized fungal isolates colonizing the roots of 'non-mycorrhizal' plants in Kunming CM and Geju Niubahuang Sn/Pb/Zn mine tailings pond (NTP), SW China, based on the TrN + G substitution model determined by Modeltest 3.06 (Posada and Crandall 1998), rooted tree by *Melampsora epitea* as outgroup. Numbers above the nodes indicate the bootstrap support in NJ analysis, 1000 times. RA here represents the ratio of the numbers of fungal isolates in a given clade relative to the total number of fungal isolates. Arabic numbers in parentheses indicate the number of fungal isolates originating from NTP (above slash) and from CM (below slash), respectively.

## Discussion

In the present study, plant species belonging to Caryophyllaceae, Chenopodiaceae, Cyperaceae, Polygonaceae, Juncaceae, Cruciferae, Amaranthaceae and Commelinaceae were studied, which are conventionally accepted as non-AM families (Smith & Read 2008). In contrast to the almost absence of a mycorrhizal colonization, we found that all the root samples surveyed in this study were frequently colonized by DSEs, which suggested that these fungi were one of the most common fungal colonizers of the roots of the 'non-mycorrhizal' plant families. Increasing evidence showed that DSEs are ubiquitous, occurring in a variety of habitats ranging from South African coastal plains and lowlands to tropical, temperate, subalpine, alpine, maritime Antarctic and arctic zones (Jumpponen & Trappe 1998; Peterson et al. 2004; Vohník et al. 2015; Casazza et al. 2017; Hewitt et al. 2017). In fact, the co-existence between DSE and AMF symbionts occupying terrestrial environments have

been extensively documented, but more field surveys also supported that a given plant species in specific microhabitats tends to favor the colonization by DSE over AM fungi (Weishampel & Bedford 2006). For example, Angel et al. (2016) found that *Poa attenuata*, a plant growing at an elevation of 6150 m a.s.l., was intensively colonized by DSE, instead of AM fungi. Similarly, Vohník et al. (2016) also reported the DSE association with the roots of *Posidonia oceanica*, a submerged marine macrophyte without mycorrhizal colonization in the northwest Mediterranean Sea.

Additionally, we also noted the difference in DSE colonization intensity among specimens of *Juncus effusus* collected in the two sample sites ( $6.46 \pm 1.78\%$  in NTP vs.  $2.22 \pm 0.29\%$  in CM). Evidently, differences in, for example, edaphic and climatic factors between the two different sample sites might also have influenced the process of DSE colonization, although the phylogenetic features of the host plants may play a decisive role in colonization (Miller et al. 1999;

Sivakumar 2013). For example, Binet et al. (2017) observed that by increasing the ambient temperature in Hummocks significantly enhanced the DSE root colonization of *Andromeda polifolia* but there was no effect on DSE colonization in warmed lawns, and they argued that the response of fungal root colonization to climate warming depends on the microhabitats. However, the abundant DSE colonization in 'non-mycorrhizal' plant roots observed in this study seems to suggest that root colonization by DSE and AMF and particularly the signaling, recognition and transduction process involved in these events relay on different molecular mechanisms and we think that it need further attention (Genre & Russo 2016).

In total, 90 endophytic fungi were isolated, and most fungi were closely related to the well-known DSE fungi, such as *Cadophora* spp. (anamorph, *Phialophora* spp.), *Leptodontidium* spp., *Pleosporales* spp. and *Cyphellophora* spp. (Newsham 2011). We found that *Cyphellophora*, *Cladosporium* and *Phialophora* were the most dominant genera colonizing the roots of all 'non-mycorrhizal' plant families surveyed in SW China. Pure-culture and culture-independent molecular analyses indicated that fungi from *Cyphellophora*, *Cladosporium* and *Phialophora* have been repeatedly isolated from various plant species (Bonfim et al. 2016; Coleman-Derr et al. 2016; Zhang et al. 2013). Accumulating evidence showed that under land cover changes or abrupt environmental shift, some habitats contained a rapid succession of fungi. For example, marine sediments in polluted conditions following the Deepwater Horizon Oil Spill, these fungi (e.g. *Cladosporium* spp.), who can extensively utilize hydrocarbon compounds, can thrive in hostile conditions, which appear to be intolerable for the other marine fungi (Bik et al. 2012). We argued that these common root-associated fungal assemblages, which presumably contain the inherit merits of rapid succession to abrupt environmental change, give way to dominant fungal communities in Niubahuang Sn/Pb/Zn mine tailing pond.

Interestingly, certain members from *Cladosporium* and *Cyphellophora* which were commonly characterized as fungal pathogens to animals and plants (Feng et al. 2013; Gao et al. 2015). However, our endophytic fungal isolates from *Cladosporium* and *Cyphellophora* were found to live inside healthy tissues as quiescent microthalli. It was consistent with our previous findings that *Exophiala pisciphila*, previously reported as a fish-pathogenic fungus, dominantly colonized the roots of numerous plants without any disease symptoms in an ancient Pb-/Zn- slag heap in China (Zhang et al. 2013), instead showed putatively mutualistic behavior by conferring a marked cadmium tolerance to its host plant (Wang et al. 2016). Recently, similar phenomena were reported by Hiruma et al. (2016) for the root endophytic *Colletotrichum tofieldiae*, a common member of destructive pathogens, which, under phosphorus-deficient conditions, confers plant fitness benefits by transferring the macronutrient phosphorus to shoots, promoting plant growth and increasing soil fertility.

Increasing reports of positive impacts of DSE colonization on their plant hosts support the view that DSEs indeed have a beneficial role in plant growth and development, plant nutrition and plant tolerance and resistance to abiotic and biotic stresses (Newsham 2011; Yuan et al. 2010). Direct evidence using isotope labeling revealed that a dematiaceous hyphomycete DSE, *Heteroconium chaetospira* formed a mutualistic

symbiosis with *Brassica campestris*, a non-mycorrhizal plant of the Cruciferae family, in which the fungus supplied host plants with essential mineral nutrients (mainly N), in exchange for photosynthetically fixed carbohydrates (Usuki & Narisawa 2007). Subsequently, more experimental data have provided new insights to understand further aspects of DSE functioning, for example, releasing of phosphorus (P) from soil insoluble phosphates, for example, calcium phosphate to crops (Spagnoletti et al. 2017), producing auxin volatile organic compounds to stimulate plant growth (Berthelot et al. 2016), increasing the plant tolerance against water deficit (Santos et al. 2016), and against fungal disease (Yuan et al. 2016). Moreover, differently from AM fungi, having a broad host spectrum and the axenic cultivability on economically viable synthetic media, make DSEs suitable for mass scale inoculum production for application in reforestation of mine spoils. However, more detailed laboratory experiments on the interactions among 'non-mycorrhizal' host plants and their root-associated DSE fungi in providing mutual nutritional benefits and protecting hosts against biotic and abiotic stresses are needed.

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