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## RESEARCH ARTICLE

# Plant growth promotion, metabolite production and metal tolerance of dark septate endophytes isolated from metal-polluted poplar phytomanagement sites

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**One sentence summary:** Metal-tolerant dark septate endophytic fungi can use complex carbon sources and promote plant growth through the release of auxin and volatile organic compounds.

**Editor:** Angela Sessitsch

## ABSTRACT

Numerous studies address the distribution and the diversity of dark septate endophytes (DSEs) in the literature, but little is known about their ecological role and their effect on host plants, especially in metal-polluted soils. Seven DSE strains belonging to *Cadophora*, *Leptodontidium*, *Phialophora* and *Phialocephala* were isolated from roots of poplar trees from metal-polluted sites. All strains developed on a wide range of carbohydrates, including cell-wall-related compounds. The strains evenly colonized birch, eucalyptus and ryegrass roots in re-synthesis experiments. Root and shoot growth promotion was observed and was both plant and strain dependent. Two *Phialophora* and *Leptodontidium* strains particularly improved plant growth. However, there was no correlation between the level of root colonization by DSEs and the intensity of growth promotion. All strains produced auxin and six also stimulated plant growth through the release of volatile organic compounds (VOCs). SPME-GC/MS analyses revealed four major VOCs emitted by *Cadophora* and *Leptodontidium*. The strains exhibited growth at high concentrations of several metals. The ability of metal-resistant DSE strains to produce both soluble and volatile compounds for plant growth promotion indicates interesting microbial resources with high potential to support sustainable production of bioenergy crops within the context of the phytomanagement of metal-contaminated sites.

**Keywords:** dark septate endophytes; plant growth promotion; metal tolerance; auxin; volatile organic compounds

## INTRODUCTION

Dark septate endophytes (DSEs) are polyphyletic ascomycetes mainly characterized by brown to blackish mycelium. Due to their singular morphology, they were initially described as ‘mycelium radices atrovires’ or ‘pseudomycorrhizal’ fungi (Melin 1921), and later introduced as fungi with sterile and dark septate—DS hyphae—that occur in roots of various plants (Read and Haselwandter 1981).

DSEs are widespread: they have been observed in more than 600 plant species spanning 100 plant families. They colonize both herbaceous and woody plants, and more particularly roots of metal hyperaccumulators, halophytes, orchids, or marine macrophytes (Mandyam and Jumpponen 2005). They occur in diverse habitats such as arid, arctic, boreal, alpine, temperate forest or tropical ecosystems (Jumpponen and Trappe 1998a; Mandyam and Jumpponen 2005).

Pathogenic or beneficial interactions between DSEs and host plants have been discussed in a number of studies (Mandyam and Jumpponen 2005; Newsham 2011; Mayerhofer, Kernaghan and Harper 2013). These studies concluded that responses to DSEs were variable and depended on the host-symbiont combination. Several studies suggest that DSEs produce a variety of extracellular enzymes that break down complex forms of carbon, nitrogen and phosphorus commonly present in soils, and thus potentially provide originally unavailable nutrients for plants (Jumpponen and Trappe 1998a; Jumpponen, Trappe and Mattsson 1998; Caldwell, Jumpponen and Trappe 2000; Usuki and Narisawa 2007; Upson, Read and Newsham 2009; Mandyam, Loughin and Jumpponen 2010).

DSEs have received much attention in the recent years because they colonize plants growing in stressed environments such as elevated CO<sub>2</sub>, poor nutrient and high salinity (Alberton, Kuyper and Summerbell 2009; Sonjak et al. 2009). Moreover, abundant and diverse DSEs have been found associated to the roots of a large range of plant species growing on metal-contaminated soils (Vrálstad, Schumacher and Taylor 2002; Ruotsalainen, Markkola and Kozlov 2007; Regvar et al. 2010; Deram, Languereau and Haluwyn 2011; Zhang, Li and Zhao 2013). Interestingly, mycorrhizal colonization by *Salix caprea* decreased under metal pollution, whereas DSE colonization was not affected (Likar and Regvar 2009). Similarly, mycorrhizal colonization of herbaceous species drastically decreased in highly polluted soils, whereas infection by DSEs remained unchanged (Ruotsalainen, Markkola and Kozlov 2007; Deram, Languereau and Haluwyn 2011). Despite these numerous indications about the worldwide distribution and diversity of DSEs in the literature, little is known about their ecological role and their effect on host plants in polluted soils.

DSEs could be considered for the phytomanagement of metal-contaminated soils by trees and their associated microorganisms. For that purpose, there is a need to take into account (i) the wide range of plants potentially colonized by DSEs, especially trees with high biomass productivity, (ii) their growth promotion capabilities and (iii) their tolerance to metal pollution. As compared to mycorrhizal fungi or clavicipitaceous endophytes, only a limited number of DSEs have been characterized. Among the few studies about the mechanisms involved in plant growth promotion and tolerance to stress, very few address DSEs. Thus, there is a need to better characterize the diversity, metabolism and interactions of DSEs with host plants to investigate their potential use in phytomanagement studies.

In the present work, we first isolated fungal endophytic strains including non-DSEs (Lacercat-Didier et al. 2016), and DSEs from poplar trees grown on different phytomanagement plots

of metal-contaminated sites. Here, based on their taxonomy, we focused our work on DSE *sensu stricto* (Jumpponen and Trappe 1998a) belonging to four genera (*Phialophora*, *Cadophora*, *Leptodontidium* and *Phialocephala*) of the Helotiales. The main objectives of this study were (i) to study their ability to metabolize various carbon sources, and to grow in the presence of different metals, (ii) to investigate their ability to colonize different plant species, (iii) to demonstrate the production of plant-growth-promoting (PGP) metabolites such as auxin and volatile organic compounds (VOCs) and (iv) to determine their overall impact on plant growth in mesocosm experiments.

## MATERIALS AND METHODS

### Isolation and culture of root-colonizing DSE fungi

Root samples were collected from poplar trees growing in three metal-polluted sites in France: Fresnes-sur-Escaut (Nord; N 50°25'46" E 3°35'4"), MetalEurop (Pas-de-Calais; N 50°26'17" E 3°03'54") and Pierrelaye (Val d'Oise; N 49°1'21" E 2°9'1"). Sediments from the dredging of the Escaut River have been dumped at the Fresnes-sur-Escaut site for several decades. The major pollutants of this site are Cd, Pb and Zn (1.0, 57 and 191 mg of total element per kilogram of sediment, respectively, pH 7.8). The MetalEurop site is a former agricultural area that was strongly contaminated by Cd, Pb and Zn (3.1, 176 and 294 mg of total element per kilogram of soil, respectively, pH 6.5) from atmospheric particles generated by the activity of the MetalEurop smelter (Sterckeman et al. 2002). The experimental site of Pierrelaye is an agricultural plain of ca 1200 ha that was irrigated for almost one century by the non-treated waste waters from Paris, hence contamination of top soils by Cd, Pb and Zn (1.5, 139 and 318 mg of total element per kilogram of soil, respectively, pH 7.4) (Lamy et al. 2006).

Roots were first washed with tap water, surface-sterilized by immersion in hydrogen peroxide (35% v/v) for 30 s and finally rinsed three times with sterile distilled water. Root segments (5 mm) were placed on malt extract agar (MEA, malt extract: 12 g l<sup>-1</sup>; agar: 15 g l<sup>-1</sup>, pH 5.5) at 24°C in the dark. Ampicillin (100 µg ml<sup>-1</sup>) and chloramphenicol (100 µg ml<sup>-1</sup>) were also added to the medium to avoid bacterial contamination. After 2 weeks of growth, mycelium growing out of the roots was isolated and sub-cultured in new MEA plates.

Two other endophytic strains from unpolluted soils (*Piriformospora indica* DSM 11827 and *Leptodontidium* sp. PMI.412) were also included in our study. They were supplied by P. Franken (Humboldt University of Berlin, Germany) and by G. Bonito (Michigan State University, USA), respectively.

### Molecular identification of DSEs

Fungal DNA was extracted using a REDextract-N-Amp™ Plant PCR kit (Sigma-Aldrich, Saint-Quentin Fallavier, France) according to the manufacturer's protocol. The internal transcribed spacer (ITS) region was amplified using the primers ITS1 and ITS4 (White et al. 1990). Twenty microliters of a mixture containing 0.2 µM of each primer, 4 µl of genomic DNA, 5.2 µl of water and 10 µl of REDextract-N-Amp PCR ready mix were used for PCR amplification. The following PCR program was used: 3 min denaturation at 94°C, followed by 40 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, and 10 min final extension at 72°C. PCR products were cloned into the pGEM-T® vector system I (Promega, Charbonnières-les-Bains, France), and the corresponding plasmids were sequenced. The ITS sequences were used to retrieve similar sequences from the GenBank

using the NCBI BLAST program. Sequence alignment was carried out using Clustal X. Bootstrap analysis was performed with 1000 replications. The maximum-likelihood (ML) tree was drawn with the kimura 2-parameter model, and the neighbor-joining (NJ) tree was drawn with the p differences model and gamma for rate heterogeneity using MEGA 6 (Tamura et al. 2013).

### Metal tolerance tests

The sensitivity of the strains to different metals was evaluated by investigating the minimum inhibitory concentration (MIC) of As, Cd, Mn and Zn on fungal growth. Pachlewski medium (Pachlewski 1974) was selected as the growth medium to have maximal metal availability and avoid metal precipitation. Plugs (4 mm) were cut from the edge of actively growing 2-week-old colonies and placed on solid Pachlewski medium (pH 5.5) amended with metals at the following concentrations: 0–12 mM ZnCl<sub>2</sub> and MnCl<sub>2</sub>, 0–30 mM Na<sub>2</sub>HAsO<sub>4</sub> and 0–1.5 mM CdCl<sub>2</sub>. Growth was observed after 2 weeks of incubation at 24°C.

### Growth of DSE isolates on different carbon sources

The hydrolytic capacities of the fungi were determined in modified Pachlewski medium (NH<sub>4</sub>Cl 1.3 g l<sup>-1</sup>; KH<sub>2</sub>PO<sub>4</sub> 1 g l<sup>-1</sup>; MgSO<sub>4</sub>, 7H<sub>2</sub>O 0.5 g l<sup>-1</sup>; thiamine hydrochloride 10 μg l<sup>-1</sup>; pH 5.5) supplemented with the appropriate target substrates as the sole carbon source (8 g l<sup>-1</sup> C). The monosaccharides glucose, fructose, xylose and mannitol, the disaccharides sucrose, maltose and cellobiose, and the polymers lignin, cellulose, starch and xylan were tested. Triplicate flasks were shaken at 140 rpm and incubated for 2 weeks at 24°C. Mycelia were harvested and their dry biomass was determined. Data were expressed relatively to growth values on glucose. To determine cellulase activity, fungal plugs (4 mm) were placed on minimal medium containing 5 g l<sup>-1</sup> of carboxymethyl-cellulose (CMC) (Sigma-Aldrich) as the sole carbon source. After 2 weeks, the area of CMC hydrolysis was evaluated by flooding the plate with 3 ml of Congo red solution (0.1% w/v) for 5 min. Plates were subsequently washed three times with 15 ml of phosphate buffer (50 mM phosphate, 1 M NaCl; pH 7.0). Cellulase activity was estimated by calculating the 'diameter of the hydrolytic zone' over 'diameter of the colony' ratio.

### Determination of auxin secretion by DSEs

To estimate the ability of the strains to produce auxin (IAA), seven mycelial plugs (4 mm) were first grown in 50 ml of complete minimal medium (CM) (Hilbert et al. 2012). After 1 week of growth, the medium was supplemented with 2.5-mM tryptophan or not for 3 extra days, as previously described (Waqas et al. 2012). IAA concentrations were determined following the method of Salkowski (1885). Briefly, 3 ml of the culture were centrifuged, and 1-ml aliquots were mixed with 3 ml of Salkowski reagent (12 g FeCl<sub>3</sub> in 7.9 M H<sub>2</sub>SO<sub>4</sub>). The mixture was incubated for 20 min in the dark, and then a colorimetric assay was performed at 540 nm. A standard curve was generated with commercial IAA (Sigma-Aldrich) in CM minimal medium with or without tryptophan. The experiment was repeated with three independent cultures.

### Emission of VOCs by DSEs and SPME-GC/MS analyses

To study the emission of VOCs by DSEs, a *Nicotiana tabacum* assay was used according to a previously described test (Paul and

Park 2013). *Nicotiana tabacum* seeds were surface-sterilized for 4 h under a gaseous atmosphere produced by mixing NaClO 9% (w/v) and HCl 12% (v/v). After 1 week of growth on water agar (1%), tobacco seedlings were placed on MS agar (Murashige and Skoog 1962) amended with 3% sucrose in 24-well plates (one seedling per well). Twelve wells on the longer side of the plate were dedicated to the culture of the DSE strains. These wells were filled with 1 ml of MEA medium and inoculated with 4-mm-diameter fungal plugs. Plates were prepared in triplicate per fungal strain. Controls consisted of plates inoculated with fungus-free MEA plugs. The plates were hermetically sealed and placed in a growth chamber with a photoperiod of 14 h light (660 μmol m<sup>-2</sup> s<sup>-1</sup>) and 10 h dark, and a temperature of 23°C/18°C (day/night). After 1 month of growth, shoot fresh weights were measured.

Twenty-milliliter glass vials filled with 7 ml of solid MEA medium were used to study VOC production by two selected DSE strains (Pr30 and Fe06). Each vial was inoculated with one 8-mm fungal plug, whereas control vials were inoculated with fungus-free MEA plugs. After inoculation, each vial was hermetically sealed and incubated for 1 month at 24°C in the dark. Each treatment was performed in six biological replicates. The volatile metabolites were extracted by headspace SPME using a 100-μM polydimethylsiloxane fiber (SUPELCO) for 15 min at 25°C, followed by 2 min desorption in the GC injector. GC/MS analyses were performed with an Agilent 6890 GC Plus coupled to a quadrupole mass spectrometer 5973 MSD (Agilent, Santa Clara, USA), and equipped with an HP-5 MS capillary column (30 m × 250 μm i.d., 0.5-μm thick film). Working conditions were as follows: injector at 250°C and transfer line to MS system at 250°C. The oven temperature was programmed as follows: initial temperature at 38°C for 4 min, then from 38°C to 100°C at a rate of 7°C min<sup>-1</sup>, from 100°C to 200°C at a rate of 23°C min<sup>-1</sup> and 200°C for 2 min. The carrier gas was He at 1.0 ml min<sup>-1</sup>. Injection was performed in splitless mode and scan mode. Electron impact ionization was set to 70 eV, and the acquisition parameters (scanned m/z) were 50/300 with a threshold of 150. The fungal metabolites were identified by comparing their mass spectra and Kováts retention indices with the US National Institute of Standards and Technology (NIST) Mass Spectral Library.

### Plant growth promotion experiment

Fungal inocula were prepared by aseptically growing DSE strains in flasks containing 500 ml of perlite moistened with 250 ml of MEA medium, according to Likar and Regvar (2013). The flasks were incubated at 24°C in the dark for 2 weeks. They were shaken manually twice a week to allow for homogenous fungal growth. Controls contained fungus-free perlite. *Betula pendula*, *Eucalyptus globulus* and *Lolium perenne* were used as model plants. Seeds were surface-sterilized (5 min in 70% ethanol; 20 min in 5% NaClO) and carefully rinsed five times with sterile distilled water. Birch and eucalyptus seedlings were initially aseptically pre-grown in sterilized perlite for 2 weeks, whereas ryegrass seeds were directly sown in pots. Plants were grown in a sterilized mixture of 90%/10% (v/v) perlite/fungal inoculum for 2 weeks for ryegrass and 8 weeks for birch and eucalyptus. Plants were irrigated alternatively with water and half-strength Ingestad solution. Fourteen independent replicates were performed for each plant/fungus combination. Plants were placed in a growth chamber with a 16/8 h photoperiod (260 μmol m<sup>-2</sup> s<sup>-1</sup>) and a temperature of 23°C/18°C (day/night). At the end of the experiment, the plants were harvested, and roots were analyzed for

fungal colonization. Finally shoots and roots were dried at 50°C for 2 days and weighed.

### Microscopic observation of root colonization

Root colonization by the DSE strains was assessed by using two protocols. Sub-samples of root systems were cleared in 5% KOH at 90°C for 15 min, neutralized in 1% HCl at 20°C overnight and stained with trypan blue. The frequency of root colonization (F%), the intensity of root colonization by intraradical hyphae (H%) and intracellular microsclerotia (MS%) were determined under a microscope by examining 30 1-cm long root fragments per sample, according to Trouvelot, Kough and Gianinazzi-Pearson (1986) and Likar and Regvar (2013).

Roots were also analyzed by confocal laser microscopy. Selected root segments were first cut into pieces of ~5–10 mm in length and were labeled with 5  $\mu\text{g ml}^{-1}$  WGA-AF<sup>®</sup>488 (Invitrogen, Cergy pontoise, France) for 10 min to visualize fungal structures. The autofluorescence of root cell walls was detected between 420 and 470 nm after UV laser irradiation at 405 nm. An argon laser was used to excite Alexa Fluor<sup>®</sup> 488, and fluorescence was detected between 500 and 550 nm. Image stacks were obtained using a Nikon eclipse TE2000U equipped with a confocal scanning head (AGR-3Q-BLD, Rainbow, Radiance Biorad) and a Plan APO x20/0.75 objective. All samples were scanned at an x/y scanning resolution of 512  $\times$  512 pixels; the z direction step size was 2.2  $\mu\text{m}$ . The image stacks were visualized and processed using the software program Image J (v.1.43u). The two detection channels of plant and fungal cell fluorescence were assigned different colors using RGB color tables to discriminate between the two organisms. Mycelia from 2-week-old pure cultures were also stained with WGA-AF488(R) and similarly studied by fluorescence microscopy.

### Statistical analyses

Statistical analyses were performed with R 3.1.3 software (R Core Team 2013). Normality of the data was tested using a Shapiro–Wilk test. Data were then analyzed using one-way analysis of variance (ANOVA) or a one-way non-parametric Kruskal–Wallis test with an alpha level of 0.05. The effect of plants, strains and the interaction between these two factors on root colonization by DSEs was tested with a two-way ANOVA. Pearson correlation coefficients were calculated to determine the relationship between root colonization by DSEs and plant growth. Percentage values were arcsin-transformed before statistical analyses.

## RESULTS

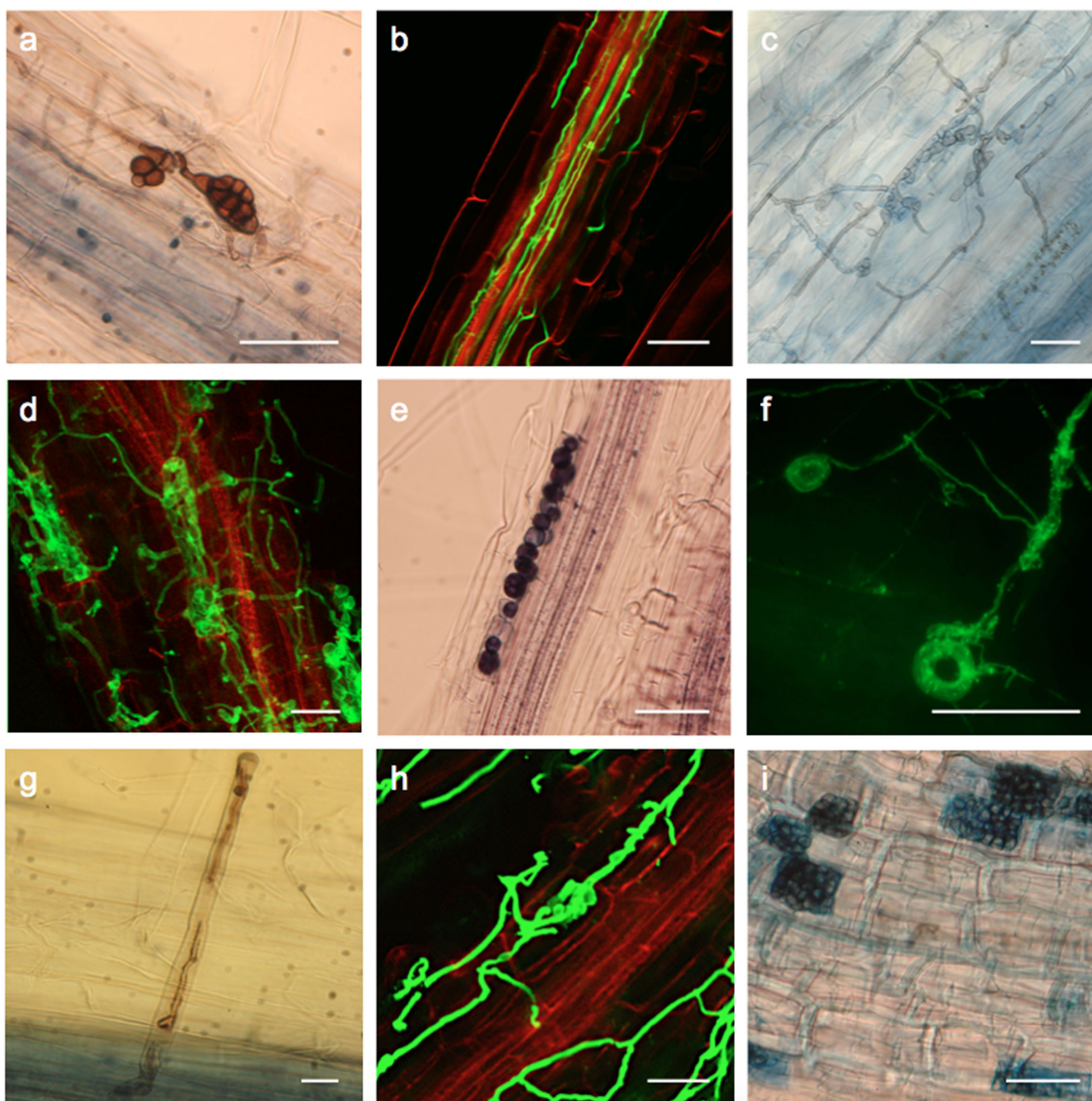
### Isolation and identification of DSE strains

Seven DSE strains were isolated from the roots of poplar trees grown on metal-polluted sites: Me07 and Me10 from the MetalEurop site, Fe06 from the Fresnes-sur-Escaut site and Pr27, Pr28, Pr29, Pr30 from the Pierrelaye site. The prefixes Me, Fe and Pr were respectively assigned to MetalEurop, Fresnes-sur-Escaut and Pierrelaye. All strains produced round-shaped, fast-growing colonies on MEA plates. Pr27 and Pr29 produced yellow to orange colonies, whereas the other colonies were brown to black (Table 1). All strains had typical dark septate hyphae (data not shown). Moreover, looping and twisting hyphae were observed for Pr27, Pr28, Pr30 and Fe06 (Fig. 1f). Me10 sporulated *in vitro* after 1 week of growth.

**Table 1.** Colonization of *B. pendula*, *E. globulus* and *L. perenne* roots by DSEs.

Strains	<i>B. pendula</i> Colonization				<i>E. globulus</i> Colonization				<i>L. perenne</i> Colonization			
	F%	H%	MS%	MS type	F%	H%	MS%	MS type	F%	H%	MS%	MS type
Fe06 (dark brown)	44.4 $\pm$ 8.0 <sup>ab</sup>	7.5 $\pm$ 4.5 <sup>bc</sup>	3.4 $\pm$ 1.0 <sup>ab</sup>	Lp, Fp	68.9 $\pm$ 5.9 <sup>c</sup>	2.7 $\pm$ 0.3 <sup>bc</sup>	1.4 $\pm$ 1.0 <sup>ab</sup>	Fp	70.0 $\pm$ 8.2 <sup>a</sup>	5.0 $\pm$ 3.1 <sup>ab</sup>	11.3 $\pm$ 9.7 <sup>a</sup>	Bl
Me07 (light gray)	44.4 $\pm$ 14.7 <sup>ab</sup>	2.7 $\pm$ 1.2 <sup>ab</sup>	1.5 $\pm$ 1.0 <sup>a</sup>	Lp	56.7 $\pm$ 3.3 <sup>abc</sup>	1.2 $\pm$ 0.2 <sup>a</sup>	1.4 $\pm$ 1.0 <sup>a</sup>	Lp	49.3 $\pm$ 20.3 <sup>a</sup>	5.0 $\pm$ 3.1 <sup>ab</sup>	11.3 $\pm$ 9.7 <sup>a</sup>	Lp
Me10 (dark gray)	60.0 $\pm$ 5.9 <sup>b</sup>	4.5 $\pm$ 1.7 <sup>abc</sup>	4.3 $\pm$ 1.0 <sup>ab</sup>	Fp	60.0 $\pm$ 7.2 <sup>abc</sup>	2.7 $\pm$ 0.4 <sup>bc</sup>	3.6 $\pm$ 1.1 <sup>ab</sup>	Bl	62.2 $\pm$ 3.9 <sup>a</sup>	7.2 $\pm$ 1.9 <sup>ab</sup>	10.0 $\pm$ 4.2 <sup>a</sup>	Lp, Fp
Pr30 (dark gray)	56.7 $\pm$ 8.8 <sup>ab</sup>	2.0 $\pm$ 0.2 <sup>ab</sup>	2.2 $\pm$ 1.1 <sup>a</sup>	Lp	56.7 $\pm$ 8.8 <sup>bc</sup>	6.1 $\pm$ 3.9 <sup>bc</sup>	2.1 $\pm$ 2.1 <sup>a</sup>	Bl	55.0 $\pm$ 12.2 <sup>a</sup>	1.3 $\pm$ 0.2 <sup>a</sup>	3.1 $\pm$ 0.5 <sup>a</sup>	Lp
PML412 (dark gray)	53.3 $\pm$ 6.7 <sup>ab</sup>	12.1 $\pm$ 3.0 <sup>c</sup>	18.3 $\pm$ 11 <sup>b</sup>	Lp	66.7 $\pm$ 6.7 <sup>bc</sup>	1.3 $\pm$ 0.3 <sup>ab</sup>	4.6 $\pm$ 2.5 <sup>ab</sup>	Lp	66.7 $\pm$ 3.3 <sup>a</sup>	7.3 $\pm$ 2.5 <sup>b</sup>	7.0 $\pm$ 2.6 <sup>a</sup>	Lp
Pr28 (black)	66.7 $\pm$ 6.7 <sup>b</sup>	2.8 $\pm$ 0.2 <sup>abc</sup>	4.8 $\pm$ 1.8 <sup>ab</sup>	Lp	55.0 $\pm$ 4.1 <sup>c</sup>	4.5 $\pm$ 0.4 <sup>abc</sup>	9.7 $\pm$ 0.3 <sup>b</sup>	Lp, Fp	56.7 $\pm$ 8.8 <sup>a</sup>	7.1 $\pm$ 2.7 <sup>b</sup>	4.7 $\pm$ 2.5 <sup>a</sup>	Lp, Fp
Pr27 (yellow-red)	33.3 $\pm$ 9.6 <sup>a</sup>	1.7 $\pm$ 0.7 <sup>a</sup>	5.6 $\pm$ 2.4 <sup>ab</sup>	Lp	55.0 $\pm$ 4.1 <sup>abc</sup>	1.3 $\pm$ 0.3 <sup>a</sup>	0.5 $\pm$ 0.4 <sup>a</sup>	Lp, Bl	43.3 $\pm$ 16.7 <sup>a</sup>	1.4 $\pm$ 1 <sup>a</sup>	1.2 $\pm$ 0.2 <sup>a</sup>	Lp
Pr29 (yellow-gray)	36.7 $\pm$ 12.6 <sup>a</sup>	2.3 $\pm$ 0.7 <sup>ab</sup>	5.0 $\pm$ 2.5 <sup>ab</sup>	Lp	53.3 $\pm$ 3.3 <sup>ab</sup>	1.2 $\pm$ 0.2 <sup>a</sup>	1.4 $\pm$ 1.0 <sup>a</sup>	Lp	40.0 $\pm$ 5.8 <sup>a</sup>	1.6 $\pm$ 0.3 <sup>a</sup>	4.2 $\pm$ 1.3 <sup>a</sup>	Lp
DSM 11827 (beige)	41.3 $\pm$ 7.9 <sup>a</sup>	4.1 $\pm$ 3.1 <sup>ab</sup>	NA	Ch	25.0 $\pm$ 4.1 <sup>a</sup>	1.0 $\pm$ 0.0 <sup>a</sup>	NA	Ch	nd	nd	nd	nd

Inoculated plants were grown for 2 weeks (ryegrass) or 2 months (birch and eucalyptus) on perlite substrate. Colonization frequency (F%) and microsclerotia (MS%) are given as means ( $\pm$ SE) of 30 root fragments. Between brackets: color of fungal colonies growing on MEA plates; Bl: Brain-like microsclerotia; Fp: Fully packed microsclerotia; Lp: Loosely packed microsclerotia; Ch: Chlamydozooids; NA: not applicable; nd: not determined. Statistically significant differences between DSE strains ( $P < 0.05$ , Kruskal–Wallis test) are indicated by different letters.



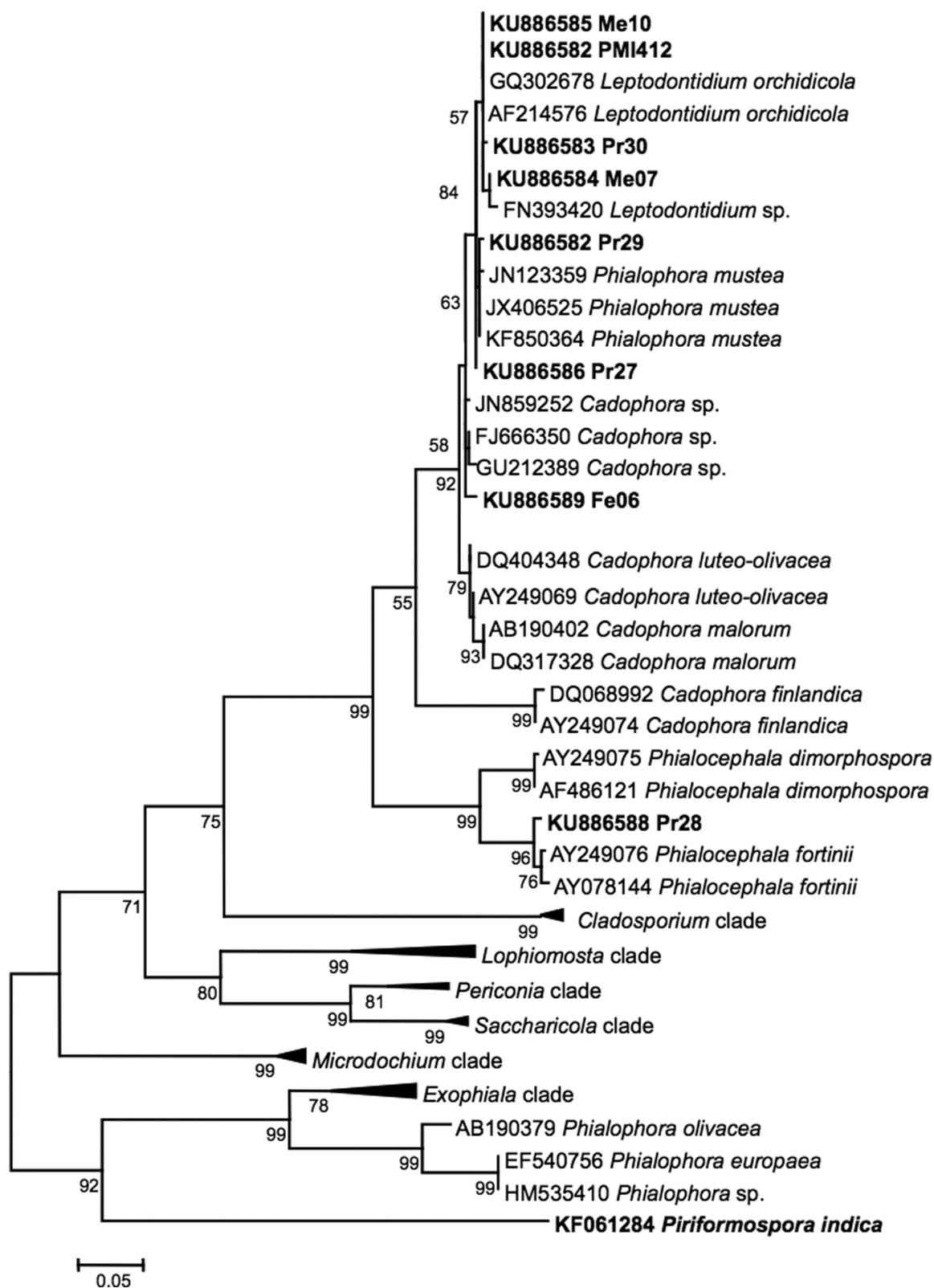
**Figure 1.** Morphological characteristics of DSEs in roots or in pure cultures. Fungal structures presented in c-d-h, a-e-i and b-g were observed in roots of *E. globulus*, *B. pendula* and *L. perenne*, respectively. Trypan blue-stained roots were observed by optical microscopy (a-c-e-g-i), whereas WGA-AF488-stained roots and mycelia were observed by confocal microscopy. Green: fungal material; red: autofluorescence of root cells. Typical brain-like (a), loosely packed (c and h) and fully packed (d and i) microsclerotia were observed in the cortical cells of plants inoculated with Fe06, PMI.412 and Pr27, Pr27 and Me10, respectively. Vascular cylinder colonized by hyphae of Pr30 (b). Chlamydospores (e) of *P. indica* DSM 11827 localized in cortical cells. Root hair colonized by Pr28 (g). Asexual looping structures (f) observed in Pr27 colonies growing on MEA plates. Scale bars = 25  $\mu$ m.

The taxonomic identification of the strains was performed by analyzing their ITS1-5.8S rDNA-ITS2 regions. A ML phylogenetic tree was drawn, including sequences from previously reported DSE strains as well as other endophytic strains (Fig. 2). Me07, Me10, Pr30 clustered together with sequences of the previously described *Leptodontidium orchidicola* and the reference *Leptodontidium* sp. PMI.412 strain. Pr27 and Pr29 had a very close evolutionary relationship with *Phialophora mustea* (JN123359). Pr28 matched with *Phialocephala fortinii* (AY078144) with 98% identity of their respective ITS sequences. Fe06 was assigned to the genus *Cadophora* (99% identity with *Cadophora* sp. JN859254). Additional phylogenetic analysis using the NJ method was performed and gave congruent conclusions that confirmed the confidence level of the clustering (data not shown). Sequence data were deposited in GenBank under the following accession numbers: KU886589 (Fe06), KU886584 (Me07), KU886585

(Me10), KU886587 (PMI.412), KU886586 (Pr27), KU886588 (Pr28), KU886582 (Pr29) and KU886583 (Pr30).

### Impacts of the carbon source and metal on the growth of DSE isolates

DSEs colonize root tissues, so they have access to a variety of carbon sources. We therefore investigated the use of various carbon compounds by the different strains. All strains produced measurable biomass on the soluble carbohydrates (Table 2). However, their degradation ability was both carbon source- and strain-dependent. We used the *Piriformospora indica* DSM 11827 strain as a reference of endophytic fungi. Its growth was the highest on glucose and sucrose, but lower on the other carbon sources, especially on mannitol. The DSE strains displayed three distinct patterns. First, the carbon source did not significantly affect the



**Figure 2.** ML phylogram showing the position of strains based on ITS1-5.8S-ITS2 sequences. The evolutionary history was inferred using the ML method based on the Tamura-Nei model. The percentage of trees (>50%) in which the associated taxa clustered together is shown below the branches. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013). The strains used in the present study (in bold) were compared with known and selected fungal species from GenBank with high sequence similarities. The different clades are represented and are compressed for the sake of simplicity. The sequences contained within each genus are provided in Table S1, Supporting Information.

Table 2. Utilization of different carbon substrates by the fungal strains.

Carbon sources	Strains									
	Fe06	Me07	Me10	PML412	Pr30	Pr28	Pr27	Pr29	DSM 11827	
Cellulose <sup>1</sup>	+	-	+	+	+	+	+	+	+	
Lignin <sup>1</sup>	-	-	-	-	-	-	-	-	-	
Starch <sup>1</sup>	+	+	+	+	+	+	+	+	+	
Xylan	231 ± 34 <sup>e</sup>	100 ± 9 <sup>a,b</sup>	108 ± 11 <sup>a,b</sup>	96 ± 3 <sup>a</sup>	95 ± 8 <sup>a</sup>	49 ± 8 <sup>a</sup>	73 ± 14 <sup>a,b,c</sup>	75 ± 12 <sup>a,b</sup>	73 ± 13 <sup>a,b</sup>	
Cellobiose	107 ± 5 <sup>b,c</sup>	75 ± 15 <sup>a</sup>	69 ± 10 <sup>a</sup>	102 ± 17 <sup>a</sup>	81 ± 5 <sup>a</sup>	74 ± 5 <sup>b</sup>	73 ± 19 <sup>a,b,c</sup>	73 ± 17 <sup>a,b</sup>	60 ± 4 <sup>b,c</sup>	
Maltose	116 ± 5 <sup>a,b</sup>	109 ± 9 <sup>a,b</sup>	166 ± 25 <sup>c</sup>	124 ± 9 <sup>a</sup>	112 ± 9 <sup>a</sup>	85 ± 2 <sup>b,c</sup>	89 ± 18 <sup>b,c</sup>	86 ± 2 <sup>a,b</sup>	91 ± 4 <sup>d</sup>	
Sucrose	131 ± 36 <sup>b</sup>	190 ± 30 <sup>c</sup>	99 ± 15 <sup>a,b</sup>	110 ± 11 <sup>a</sup>	112 ± 11 <sup>a</sup>	114 ± 7 <sup>c</sup>	100 ± 9 <sup>c</sup>	114 ± 15 <sup>b</sup>	103 ± 5 <sup>e</sup>	
Fructose	74 ± 2 <sup>a</sup>	125 ± 4 <sup>b,c</sup>	96 ± 22 <sup>a,b</sup>	103 ± 12 <sup>a</sup>	95 ± 5 <sup>a</sup>	82 ± 8 <sup>b</sup>	47 ± 2 <sup>a</sup>	69 ± 10 <sup>a,b</sup>	84 ± 4 <sup>d</sup>	
Glucose	100 ± 3 <sup>a,b</sup>	100 ± 19 <sup>a,b</sup>	100 ± 15 <sup>a,b</sup>	100 ± 3 <sup>a</sup>	100 ± 26 <sup>a</sup>	100 ± 19 <sup>b,c</sup>	100 ± 11 <sup>c</sup>	100 ± 21 <sup>a,b</sup>	100 ± 4.8 <sup>e</sup>	
	(55.5)	(79.3)	(101.3)	(92.3)	(67.1)	(86.9)	(111.1)	(130.6)	(71.5)	
Mannitol	188 ± 19 <sup>d,e</sup>	91 ± 2 <sup>a,b</sup>	121 ± 1 <sup>b,c</sup>	110 ± 11 <sup>a</sup>	90 ± 9 <sup>a</sup>	46 ± 5 <sup>a</sup>	55 ± 7 <sup>a,b</sup>	64 ± 2 <sup>a</sup>	25 ± 2 <sup>a</sup>	
Xylose	188 ± 24 <sup>d,e</sup>	101 ± 9 <sup>a,b</sup>	81 ± 3 <sup>a,b</sup>	106 ± 6 <sup>a</sup>	93 ± 5 <sup>a</sup>	73 ± 5 <sup>b</sup>	94 ± 6 <sup>c</sup>	90 ± 11 <sup>a,b</sup>	65 ± 2 <sup>c</sup>	
CMC <sup>2</sup>	3.1 ± 0.12	3.1 ± 0.05	3.0 ± 0.10	4.8 ± 0.10	2.1 ± 0.47	3.6 ± 0.03	3.7 ± 0.1	3.5 ± 0.5	4.1 ± 0.27	
Halo	4.9 ± 0.20	3.0 ± 0.03	3.4 ± 0.15	5.6 ± 0.03	2.2 ± 0.49	5.0 ± 0.13	4.3 ± 0.19	5.2 ± 0.29	5.3 ± 0.18	
Ratio	1.6 ± 0.05 <sup>f</sup>	1.0 ± 0.01 <sup>a</sup>	1.1 ± 0.01 <sup>a,b,c</sup>	1.3 ± 0.02 <sup>b,c</sup>	1.1 ± 0.05 <sup>a,b</sup>	1.4 ± 0.02 <sup>d,e</sup>	1.2 ± 0.06 <sup>b,c</sup>	1.5 ± 0.15 <sup>e,f</sup>	1.3 ± 0.05 <sup>c,d</sup>	

Values (expressed as %) represent relative fungal growth after 2 weeks of culture on a specific carbon source as compared to growth on glucose used as a control. Values between brackets represent fungal dry biomass (mg) in minimum liquid medium supplemented with glucose. Statistically significant differences between carbon sources are indicated by different letters ( $P < 0.05$ , Kruskal-Wallis test). <sup>1</sup>Due to the non-solubility of the compounds, the degradation ability was considered as positive when the strain displayed more than 5 mm mycelial development around the initial plugs and formation of new small fungal pellets as compared to the negative control (glucose) (+; growth, -; absence of growth).

<sup>2</sup>CMC solubilization by the endophytic strains was recorded on agar plates after 2 weeks of growth. The solubilization index was calculated as the 'diameter of the solubilization halo' over 'diameter of the colony' ratio. Values are the means of three replicates ±SE. Statistically significant differences ( $P < 0.05$ , Kruskal-Wallis test) between strains are indicated by different letters.



**Table 3.** Metal tolerance of the strains.

Strains	MIC (mM)			
	As	Cd	Mn	Zn
Fe06	10	0.6	9	9
Me07	25	1.5	9	7
Me10	6	0.5	6	5
PMI.412	4	0.2	12	>12
Pr30	6	0.3	12	10
Pr28	14	0.5	12	12
Pr27	16	0.4	5	10
Pr29	14	0.5	3	3
DSM 11827	>30	0.3	9	9

MIC is the minimum inhibitory concentration of metal that completely inhibited fungal growth.

growth of PMI.412, Pr30 and Pr29. Second, growth of Pr28 on xylan and mannitol, as well as of Pr27 on fructose and mannitol was reduced by ~50% as compared to glucose. Finally, growth of Fe06, Me07 and Me10 was increased 1.7–2.3-fold by different carbon compounds. Fe06 growth was the highest on xylan, xylose and mannitol, whereas sucrose and maltose treatments significantly enhanced biomass production by Me07 and Me10, respectively.

The cellulose (Avicel), starch and lignin substrates were insoluble and adhered to mycelial pellets, artificially increasing mycelium weight at harvest. Therefore, qualitative growth on cellulose, starch and lignin was solely evaluated, and was not compared to growth on other carbon sources (Table 2). Whatever the strain, no growth was noted on lignin. Mycelial growth was evident for all strains on cellulose and starch, except Me07 on cellulose. To confirm the cellulolytic activity of the strains, an agar test was performed on plates containing CMC. We measured the ratio of the diameter of the hydrolytic zone (degradation halo) over the diameter of the colony (Table 2). Fe06 exhibited the highest cellulolytic ability with a ratio of 1.6, followed by Pr29, Pr28, PMI.412 and DSM 11827 (1.3–1.5). No degradation halo was noted for Me07. Very low ratios (1.1) were recorded for Me10 and Pr30.

Since the DSE fungi were isolated from metal-contaminated soils, their metal tolerance was further assessed by determining MIC values for As, Cd, Mn and Zn. Metal tolerance varied among strains and metal species (Table 3). Me10, PMI.412 and Pr30 were the least tolerant to As, with MICs ranging between 4 and 6 mM, whereas Me07 and DSM 11827 were the most tolerant with MICs of 25 and >30 mM, respectively. Me07 was also the most tolerant strain to Cd (MIC = 1.5 mM), and PMI.412, Pr30 and DSM 11827 were the least tolerant ones. PMI.412, Pr30, Pr28 were the most tolerant to Mn, and PMI.412, Pr30, Pr28 and Pr27 were the most tolerant to Zn. Conversely, Pr27 and Pr29 were the least tolerant strains to Mn, and Pr29 the least tolerant to Zn.

### PGP properties

IAA production by DSEs grown in liquid CM medium with or without tryptophan, the IAA precursor, was examined by colorimetric assays (Table 4). No IAA was recovered in the tryptophan-free treatments. Conversely, all strains produced IAA in medium supplemented with tryptophan. Most of the strains produced similar concentrations of IAA. IAA production ranged between 106  $\mu\text{g}$  (g dry wt.)<sup>-1</sup> for Pr27 and 581  $\mu\text{g}$  (g dry wt.)<sup>-1</sup> for PMI.412. However, statistically significant differences were only found for Pr30 and PMI.412 relative to Pr27.

**Table 4.** PGP properties of DSEs.

Strains	IAA production <sup>1</sup> ( $\mu\text{M g}^{-1}$ dry wt.)	Tobacco SFW <sup>2</sup> (mg)
Control	–	75 $\pm$ 2 <sup>a</sup>
Fe06	277 $\pm$ 35 <sup>a,b</sup>	95 $\pm$ 3 <sup>f</sup>
Me07	245 $\pm$ 9 <sup>a,b</sup>	80 $\pm$ 5 <sup>a,b</sup>
Me10	262 $\pm$ 34 <sup>a,b</sup>	87 $\pm$ 2 <sup>d-f</sup>
PMI.412	581 $\pm$ 266 <sup>b</sup>	90 $\pm$ 3 <sup>e,f</sup>
Pr30	401 $\pm$ 64 <sup>b</sup>	91 $\pm$ 2 <sup>e,f</sup>
Pr28	296 $\pm$ 166 <sup>a,b</sup>	78 $\pm$ 2 <sup>a</sup>
Pr27	106 $\pm$ 35 <sup>a</sup>	81 $\pm$ 2 <sup>a-c</sup>
Pr29	271 $\pm$ 23 <sup>a,b</sup>	88 $\pm$ 5 <sup>c-e</sup>
DSM 11827	nd	91 $\pm$ 4 <sup>e,f</sup>

<sup>1</sup>IAA production was measured in liquid growth medium after 2 weeks of culture in minimal medium supplemented with tryptophan (n = 3).

<sup>2</sup>Production of VOCs was determined by measuring the shoot fresh weight (SFW) of 1-month-old tobacco seedlings in the absence or presence of DSEs (n = 36). Values are the means ( $\pm$ SE). Statistically significant differences ( $P < 0.05$ , Kruskal-Wallis test) between strains are indicated by different letters. nd: not determined.

Nine strains were screened for their growth promotion effect on *Nicotiana tabacum* seedlings (Table 4). The partners were grown for 1 month in 24-well microplates without any physical contact. No strain showed deleterious effects on tobacco growth. Tobacco biomass was not affected by Pr28, Pr27 or Me07, but was significantly increased by the other strains (Table 4). The highest growth promotion was recorded with Fe06 co-cultures, with a 21% increase in shoot biomass as compared to the fungus-free treatment.

To determine the nature of the VOCs emitted by Pr30 and Fe06, SPME-GC/MS analyses were performed with 1 month-old DSE cultures. The major VOCs (i.e. those with a relative proportion >1%) are given in Table 5. These compounds were absent from mock-inoculated vials. Altogether, seven major VOCs were identified by comparison with the NIST database. Ethyl acetate, 3-methyl-1-butanol, 2-methyl-1-butanol and styrene were emitted by both strains. The relative proportion of each compound was similar between the two strains. Among these four compounds, ethyl acetate was the most produced. Pr30 emitted two other compounds. 1,3-Cyclopentadiene, 1-3 bis was produced in the same proportion as ethyl acetate, whereas  $\beta$ -chamigrene was present in a similar proportion to styrene. These two compounds were not synthesized by Fe06, but Fe06 emitted a fifth compound (cyclohexene, 1-ethyl) in a similar proportion to *n*-methyl-1-butanol (n = 2 or 3).

### Effect of DSEs on plant growth

To assess the effect of DSEs on plant growth, *Betula pendula*, *Eucalyptus globulus* and *Lolium perenne* were inoculated with each of the eight DSE strains and harvested after 2 (*L. perenne*) or 8 weeks (*B. pendula*, *E. globulus*). *Piriformospora indica* was used as an endophytic reference. Microscopic observations of roots revealed the presence of fungal structures (Fig. 1; Table 1), whereas the non-inoculated plants were fungus free. All strains colonized the roots of the different plants (Table 1), formed microsclerotia (Fig. 1; Table 1) and spread in both the root epidermal and cortical cells. Three types of microsclerotia (Table 1), namely brain-like (Fig. 1a), loosely packed (Fig. 1c and h) and fully packed microsclerotia (Fig. 1d and i) were identified in the roots of the different plants. Several strains produced different types of microsclerotia according to the plant species they colonized (Table 1). Hyaline and septate hyphae were also observed in the

**Table 5.** Identification of VOCs synthesized by strains Fe06 and Pr30 by SPME-GC/MS.

Compounds	Pr30			Fe06			Molecular weight
	Retention time	Relative %	Quality	Retention time	Relative %	Quality	
Ethyl acetate (C <sub>4</sub> H <sub>8</sub> O <sub>2</sub> )	1.9	33.6 ± 4.7 <sup>c</sup>	43	1.9	40.0 ± 1.0 <sup>c</sup>	78	88
3-methyl-1-butanol (C <sub>5</sub> H <sub>12</sub> O)	3.6	12.1 ± 2.0 <sup>b</sup>	64	3.6	18.4 ± 5.0 <sup>b</sup>	64	88
2-methyl-1-butanol (C <sub>5</sub> H <sub>12</sub> O)	3.7	13.3 ± 2.2 <sup>b</sup>	83	3.7	19.8 ± 3.7 <sup>b</sup>	83	88
Styrene (C <sub>8</sub> H <sub>8</sub> )	7.4	4.1 ± 1.2 <sup>a</sup>	22	7.7	5.1 ± 1.1 <sup>a</sup>	64	104
β-Chamigrene (C <sub>15</sub> H <sub>24</sub> )	9.7	3.0 ± 0.3 <sup>a</sup>	80	–	–	–	150
1,3-cyclopentadiene 1,3bis (C <sub>13</sub> H <sub>22</sub> )	10.2	33.9 ± 3.1 <sup>c</sup>	58	–	–	–	150
Cyclohexene, 1-ethyl (C <sub>8</sub> H <sub>14</sub> )	–	–	–	12.0	15.4 ± 3.6 <sup>b</sup>	72	110

Fungi were grown for 2 weeks in glass vials containing MEA medium. The compounds corresponding to the major peaks (area > 1%) are presented as relative percentages. Values are means ±SE (n = 9). Statistically significant differences (P < 0.05; Kruskal–Wallis test) between compounds are indicated by different letters.

root hairs of ryegrass inoculated with Pr28 (Fig. 1g) and Me07 (data not shown). Ryegrass roots inoculated with Me07 (data not shown) and Pr30 (Fig. 1b) were also colonized by hyphae in vascular parenchyma tissues, but the cells were not degraded. Moreover, microsclerotia formed by Me07 were also detected in these tissues. *Piriformospora indica* DSM 11827 formed typical chlamydospores in birch and eucalyptus roots (Fig. 1e). Two-way ANOVA results revealed no significant differences among plant species or for the interaction between plant species and fungal strains for any of the three root-colonization parameters (F%, H% and MS%) (Table S2, Supporting Information). However, there were significant differences among fungal strains for H% and MS% (Table S2, Supporting Information). All plants taken together, Pr28 and PMI.412 were the best colonizing strains, while Pr27 was the least effective (Kruskal–Wallis test, P-value < 0.05). Pr28- and Me10-inoculated plants displayed the highest colonization frequencies in birch roots (F% = 66.7% and 60%, respectively), whereas eucalyptus roots displayed the highest colonization frequencies when inoculated with Fe06 (Table 1). Generally, DSE-inoculated plants were more intensively colonized than *P. indica*-inoculated plants. The colonization frequency of ryegrass was similar with all DSEs. Results for microsclerotia indices (MS%) were similar. PMI.412 significantly produced more microsclerotia than Me07 and Pr30 in birch roots. Similarly, more microsclerotia were observed in Pr28-inoculated eucalyptus roots than in Me07-, Pr30-, Pr27- and Pr29-inoculated ones.

Shoot and root dry weights of birch and eucalyptus inoculated with Pr30 and Pr27 were 3–5-fold higher than those of the non-inoculated controls (Fig. 3). PMI.412 also promoted birch and eucalyptus shoot growth, but to a lesser extent. The endophytic reference strain DSM 11827 promoted both shoot and root growth of birch, but did not affect eucalyptus growth. The other strains did not significantly alter tree seedling growth. In *L. perenne*, four strains had a positive effect on root growth, and Pr30 also significantly increased shoot growth. Root dry weight of ryegrass inoculated with Me07 and Me10 reached 160% of the non-inoculated control value set to 100%. Root growth of Fe06-inoculated seedlings was doubled as compared to the control. Conversely, inoculation with PMI.412 significantly decreased root biomass of *L. perenne*. There was no correlation between plant growth and root colonization intensity (Pearson test, P-value = 0.5 for F%; P-value = 0.9 for MS% and P-value = 0.9 for H%).

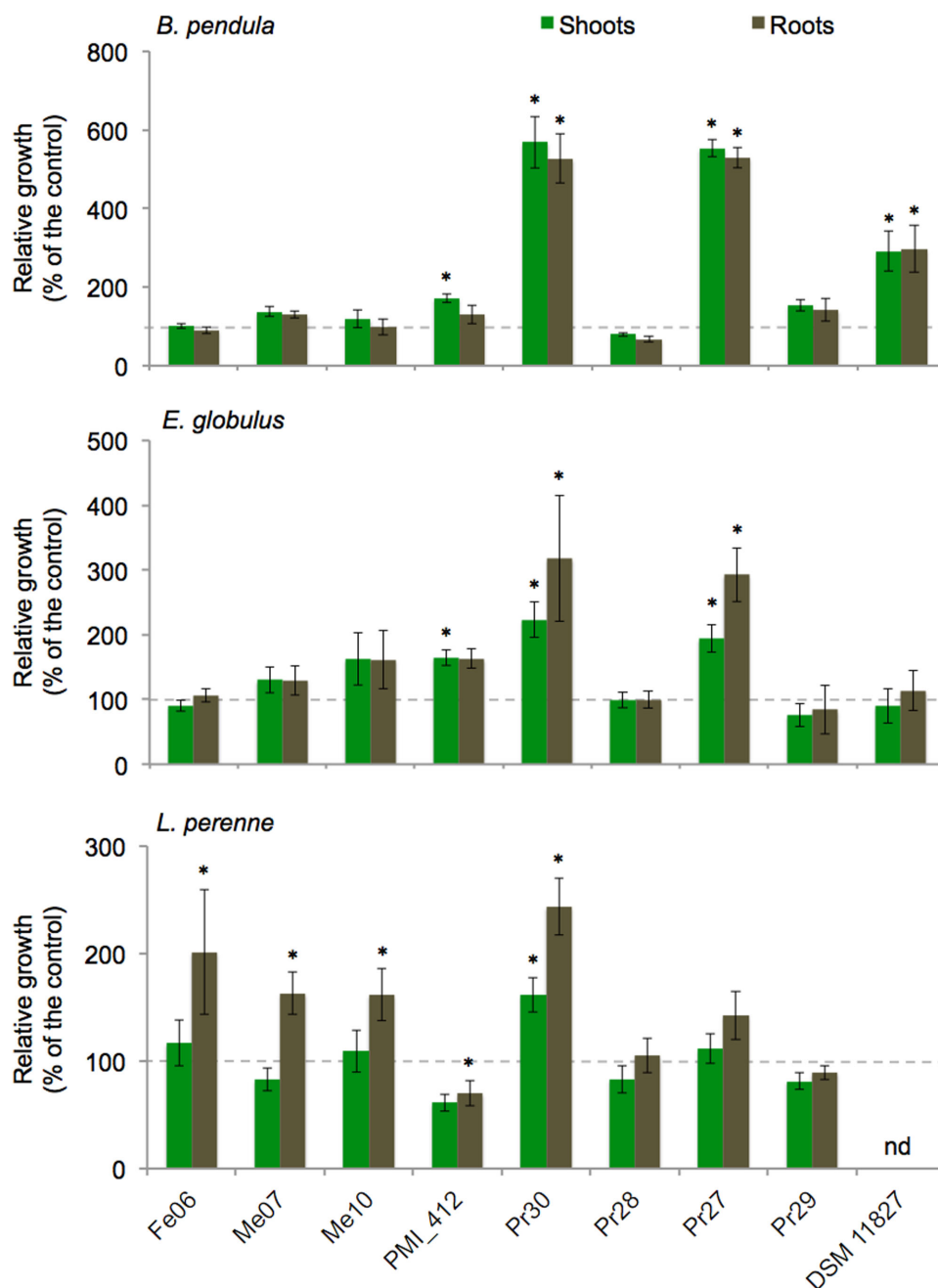
## DISCUSSION

### Isolation of metal-tolerant DSEs from poplar

DSEs represent a polyphyletic group of ascomycetes, with members distributed in the Helotiales, Pleosporales, Sordariales or

Pezizales orders (Grünig, Queloz and Sieber 2011). In the present study, the seven DSE strains isolated from poplar all belonged to the Helotiales order, but to two different groups. *Phialocephala* Pr28 belonged to the *Mollisia*, *Phaeomollisia* and *Vibrissia* group, whereas the other six strains (as well as PMI.412) belonged to another group clustering with the well-known *Leptodontidium orchidicola* and several *Cadophora* species. Whereas strains belonging to the *Leptodontidium*, *Cadophora* and *Phialocephala* genera had a typical DSE gray to black phenotype on agar medium, the *Phialophora* strains had orange to red colors. However, the distinction based on the degree of pigmentation is often arbitrary because pigment strength varies greatly in some species (Hambleton and Sigler 2005), especially in mycelium of colonized roots (Yu, Nassuth and Peterson 2001; Barrow 2003). These four genera are known colonizers of tree roots, and to a lesser extent of herbaceous plants (Jumpponen and Trappe 1998b; Yu, Nassuth and Peterson 2001; Grünig et al. 2008; Peterson, Wagg and Pautler 2008; Likar and Regvar 2009; Knapp, Pintye and Kovács 2012; Zhang, Li and Zhao 2013; Op De Beeck et al. 2015). However, few studies have evidenced the presence of DSEs in poplar roots by microscopy (Beauchamp, Stromberg and Stutz 2005; Krpata et al. 2008; Gehring et al. 2014). Except *Cadophora*, *Exophiala* and *Leptodontidium* isolated from poplar roots (Bonito et al. 2016), molecular identification is still lacking, and DSE strains have not yet been isolated.

DSEs are abundant on highly metal-enriched locations. For example, *Phialophora* sp., *Phialocephala* sp. and *Leptodontidium* sp. were identified and/or isolated from Chinese and European metal-polluted sites (Ban et al. 2012; Likar and Regvar 2013; Zhang, Li and Zhao 2013). Similarly, *Cadophora* sp. was also found in Zn/Cd-polluted soils, in pine roots from a Zn/Cd-polluted site in Belgium and in a metal-polluted soil in Austria (Utmezian et al. 2007; Op De Beeck et al. 2015). We also isolated DSEs from Cd-, Zn- and Pb-contaminated soils and tested their tolerance to these metals as well as to As and Mn. Although our work focused on only a few strains, we noted that metal tolerance was strain- and metal-dependent and did not seem to be related to the metal concentration in the soil of origin. Such variations between DSE strains have been reported in different studies (Zhang et al. 2008; Ban et al. 2012; Likar and Regvar 2013). These differences could be explained by the presence/absence of different tolerance mechanisms, e.g. efflux systems or vacuolar compartmentalization, or by various levels of expression of genes/proteins involved in these mechanisms (Ban et al. 2012; Zhan et al. 2014, 2015; Shen et al. 2015). Several studies report that under severe metal contamination, the rate of root colonization by arbuscular mycorrhizal (AM) fungi decreases but remains unaffected for DSEs (Ruotsalainen, Markkola and Kozlov 2007; Likar and Regvar 2009; Deram, Languereau and Haluwyn 2011).



**Figure 3.** Effect of fungal inoculation on plant growth. Root biomass (brown bars) and shoot biomass (green bars) were measured after 2 weeks (*L. perenne*) or 2 months (*B. pendula* and *E. globulus*) of growth. Values of the controls (non-inoculated plants) were set to 100% (dotted lines). Data of the inoculated plants are given as relative values of the controls. Data are given as means  $\pm$  SE ( $n = 14$ ). Asterisks indicate significant differences as compared to the control ( $P \leq 0.05$ , Kruskal-Wallis test). nd: not determined.

The relatively high metal tolerance of DSEs could be related to the high melanin content in their cell walls. Melanin contains carboxyl, phenolic, hydroxyl and amine groups that provide many potential binding or biosorption sites for metal ions, ensuing immobilization of metals on cell walls, and decreased metal toxicity (Fogarty and Tobin 1996; Bellion et al. 2006; Zhan et al. 2011). However, DSEs and other endophytic strains forming white/cream colonies have comparable MIC values. This was the case for *Piriformospora indica* (this study) and endophytic Helotiales (Lacercat-Didier et al. 2016). Therefore, the role of melanin in the high metal tolerance of dark-pigmented strains

still requires further genetic evidence. Moreover, experiments are also needed to determine whether root colonization by DSEs is less affected by metal stress than AM or ectomycorrhizal (ECM) symbiosis in the same soils.

#### Degradation of cell-wall-related compounds

The isolated DSEs usually grew better on a larger set of C compounds than the endophytic model *P. indica*. The best evidence was with mannitol, where *P. indica* growth was strongly limited as compared to DSEs. We also investigated the ability of the

strains to degrade complex carbon compounds. Laccases are involved in lignin degradation. DSEs, especially *Periconia* and *Microdochium* can display high laccase activity (Mandyam, Loughin and Jumpponen 2010). However, we found no evidence for lignin degradation in any of our strains. This inability could be explained by the lack of one or several enzymes of the lignin degradation pathway. Xylan is a hemicellulose compound that is degraded into xylose by xylanase activity and subsequently into glucose by xylosidase activity. All strains grew on xylan, xylose and glucose as sole carbon sources, suggesting that these strains can entirely degrade xylan. All strains but Me07 degraded Avicel cellulose, CMC and cellobiose. These results suggest the production of cellobiohydrolases, endoglucanases and  $\beta$ -glucosidases enabling the complete hydrolysis of cellulose into glucose. Interestingly, the capacity of *Cadophora*, *Leptodontidium* PMI.412, *Phialophora* and *Phialocephala* to grow on Avicel cellulose or to degrade CMC was similar to that of *P. indica*. However, complete hydrolysis of cellulose is expected, as these enzymes are presumably required for root cell penetration. *Leptodontidium* Me07 did not grow on Avicel cellulose and produced a minute CMC degradation halo. This result may reflect a difference in cellobiohydrolase or cellulase activity as compared to the other DSEs. Another possibility is that the forms of cellulose (Avicel, CMC) were not optimal for this strain. These fungi exclusively colonize root tissues, so their lifestyle requires several enzymes to degrade cell-wall components (Rodriguez et al. 2009). But, as detailed below, re-synthesis experiments can best demonstrate the fungal capacity to enter root cells.

### Root colonization by DSEs and impact on plant growth

Three types of microsclerotia were observed in roots: fully packed, loosely packed and brain-like microsclerotia. Loosely packed microsclerotia were the most abundant structure across all treatments. We therefore report for the first time that a given DSE strain can form various types of microsclerotia when associated to different plant species, but also within a same plant species. Little information is available about the diversity and function of the different types of microsclerotia (Yu, Nassuth and Peterson 2001). Therefore, more experiments are required to state which of the following factors—DSE strain, plant species or environment—is uppermost in shaping microsclerotium type.

Root colonization by DSEs has been reported to cause a variety of host responses ranging from pathogenic to mutualistic interactions (Jumpponen and Trappe 1998a). A few studies report a positive impact of *Leptodontidium*, *Cadophora* and *Phialocephala* on the growth of woody and herbaceous plants (Andrade-Linares et al. 2011; Newsham 2011; Khastini et al. 2014). In our study, several *Leptodontidium* and *Cadophora* strains also enhanced plant growth. Additionally, *Phialophora mustea* Pr27 promoted birch and eucalyptus growth. To our knowledge, this is the first report about plant growth promotion by *Phialophora mustea*.

Surprisingly, two *Leptodontidium* strains (Me07 and Pr30) colonized the vascular stele of ryegrass, as in roots of *Salix glauca* and *Asparagus officinalis* colonized by *Leptodontidium orchidicola* and *Phialocephala fortinii*, respectively (Fernando and Currah 1996; Yu, Nassuth and Peterson 2001). Necrosis of the stele tissues of *Salix* roots was evident, so the authors suggested a parasitic lifestyle for *Leptodontidium* in that association. However, in the present study, we did not notice any cell damage in ryegrass roots, and both strains enhanced ryegrass growth. Cumulatively, these results suggest that, under our experimental conditions, *Leptodontidium* acted more as a mutualistic fungus than as a parasite.

Plant growth promotion was strain-dependent since different host responses were observed as a result of inoculation by different *Leptodontidium* and *Phialophora* strains. Fernando and Currah (1996) also showed that inoculation by four strains of *L. orchidicola* induced variable effects on *Salix* and *Picea* growth. Inoculation of *Arabidopsis thaliana* with different strains of *Periconia* or *Microdochium* yielded similar results (Mandyam, Roe and Jumpponen 2013). We hypothesized that the different strains could colonize roots in quantitatively different manners and consequently produce different responses. This hypothesis prompted us to investigate the levels of root colonization by DSEs in the different plants. The three plant species were colonized whatever the DSE strain used in the inoculum. This experiment therefore demonstrates that DSEs have a broad host specificity spanning monocots and dicots, including woody species. Additionally, as observed previously on AM fungi, we found no correlation between plant growth and the intensity of root colonization by DSEs (Hildebrandt, Regvar and Bothe 2007). This result suggests that growth promotion was more probably strain-dependent. However, additional experiments under different experimental conditions are needed to fully demonstrate this statement. More particularly, soil nutrient levels could influence plant responses when plants are associated to DSEs. DSEs could promote plant growth by enhancing nitrogen, carbon and/or phosphorus nutrition (Caldwell, Jumpponen and Trappe 2000; Barrow and Osuna 2002; Mandyam and Jumpponen 2005; Usuki and Narisawa 2007; Upson, Read and Newsham 2009). For example, in response to N fertilization, *Pinus contorta* growth response to *Phialocephala fortinii* inoculation switched from neutral to positive (Jumpponen, Trappe and Mattson 1998).

### PGP abilities

To gain further insight into DSE-related growth promotion, we analyzed the ability of the strains to secrete IAA and to release VOCs. IAA production was monitored in the culture medium. The results suggested the existence of IAA biosynthesis pathways in all strains. Several studies report that endophytic fungi produce IAA (e.g. *Chaetomium globosum*, *Phoma glomerata*, *Penicillium* sp. and *P. indica*) (Sirrenberg et al. 2007; Khan et al. 2012; Waqas et al. 2012). Secretion of auxin by hyphae could contribute to increasing plant biomass (Khan et al. 2012; Waqas et al. 2012; Sukumar et al. 2013). Synthesis of fungal-derived IAA could play key roles in root and shoot development, increase the root surface absorption area with resulting better water and nutrient uptake, and promote root colonization by other fungi (Hilbert et al. 2012; Sukumar et al. 2013).

VOCs are low-molecular-weight compounds that can easily diffuse through the soil and the atmosphere. Their functions and exact mechanisms of action are poorly understood (Kramer and Abraham 2012; Lemfack et al. 2014). However, several authors suggest a major role of VOCs emitted by rhizospheric and endophytic fungi in plant growth promotion (Hung, Lee and Bennett 2013; Naznin et al. 2013; Paul and Park 2013). That is why we further tested whether DSEs could also promote plant growth by releasing VOCs. A microplate culture system was used to restrict physical contact between fungi and seedlings and only allow for gaseous exchanges. Interestingly, five strains enhanced the growth of tobacco seedlings, demonstrating that DSEs can also promote plant growth without any physical contact. This promoting effect could be explained by the release of phytostimulant VOCs and/or by the presence of additional respiration-derived CO<sub>2</sub> increasing photosynthesis (Jumpponen and Trappe 1998b; Naznin et al. 2013). Although we did not

measure the final CO<sub>2</sub> concentration, it is unlikely that CO<sub>2</sub> concentrations alone accounted for the total increase in biomass accumulation. A more probable explanation is that growth promotion resulted from a combination between the VOCs released by the fungi and the elevated CO<sub>2</sub> concentration (Naznin et al. 2013). VOC nature was characterized for the two strains that best promoted tobacco growth. To our knowledge, this is the first detailed analysis of VOCs produced by DSEs. Both strains produced hydrocarbon-like molecules with C<sub>4</sub> to C<sub>15</sub> skeletons, and with alcohol derivatives such as 2-methyl-1-butanol and 3-methyl-1-butanol. These compounds were identified among the major VOCs emitted by different fungi and likely play key roles in plant growth promotion (Strobel et al. 2001; Naznin et al. 2013; Paul and Park 2013). However, the complete set of VOCs differed between the two strains, suggesting that VOC emission is species- or strain-dependent, as demonstrated before for saprophytic, ECM and endophytic fungi (Bäck et al. 2010).

## CONCLUSIONS

In the context of fungus-assisted phytomanagement of polluted sites, we successfully isolated six PGP DSE strains from metal-contaminated sites. Based on their ability to colonize different plants, to promote plant growth and on their metal tolerance, we suggest that DSE inoculants could be used to sustainably improve growth of trees or other bioenergy crops on rehabilitated brownfields. Further studies using DSE inoculation of birch and poplar in metal-contaminated soils are currently under way. More generally, and due to the broad host specificity of these fungi, they could also be used as plant growth promoting fungi in horticulture or agriculture.

## SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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