Fungal Ecology 24 (2016) 53–60

Contents lists available at ScienceDirect

Fungal Ecology

journal homepage: www.elsevier.com/locate/funeco

Seed endophytic microbiota in a coastal plant and phytobeneficial properties of the fungus *Cladosporium cladosporioides*

Yuan Qin, Xueyu Pan, Zhilin Yuan*

Institute of Subtropical Forestry, Chinese Academy of Forestry, Hangzhou, China

ARTICLE INFO

Article history:
Received 22 June 2016
Received in revised form
9 August 2016
Accepted 22 August 2016

Corresponding Editor: James White Jr.

Keywords:
Seed-borne endophytes
Epiphytic fungi
Vertical transmission
Spermosphere

ABSTRACT

While the structure and function of the microbial communities associated with the rhizosphere and phyllosphere have received broad attention, the knowledge of seed-associated microbiota still remains limited. In this work, we characterized the seed fungal endophytes present in a coastal plant *Suaeda salsa* using 454 pyrosequencing. The fungal community present in its seeds exhibited extremely low species richness. Very few genera were identified, and the genus *Cladosporium* dominated. In parallel, we recovered one isolate of *Cladosporium cladosporioides* from the seeds. Hence, we hypothesized that the endophytic *C. cladosporioides* would exert a beneficial effect on plant fitness. Subsequent inoculation test revealed the role of *C. cladosporioides* in improving host seed germination rates. Re-isolation assay and scanning electronic microscopy analysis of its colonization pattern during seed germination, coupled with our previous observation that *C. cladosporioides* was also present in the phyllosphere, rhizosphere and root endosphere of *S. salsa*, supported the evidence of its primary soil-borne origin and both epiphytic and endophytic infection of host tissues. Furthermore, significant growth enhancement was recorded in American sweetgum seedlings inoculated with an optimal conidia concentration of *C. cladosporioides*. In conclusion, our work underscores the ecological significance of seed-borne endophytes and the system presented can be developed into a tool for understanding endophytic associations in the costal environments.

1. Introduction

The community structure and diversity of plant-associated microbiota are crucial determinants of plant performance and productivity (Berendsen et al., 2012; Schlaeppi and Bulgarelli, 2015; van der Heijden and Schlaeppi, 2015). With the rapid progress in high-throughput DNA sequencing technology, microbes thriving in the phyllosphere, rhizosphere and root endosphere of major crops and model plant species have been extensively investigated and shown to have distinct community structures depending on different tissues and plant habitats (Redford and Fierer, 2009; Bulgarelli et al., 2012; Bodenhausen et al., 2013; Shakya et al., 2013; Lebeis, 2014). Moreover, multi-omics approaches (such as metagenomics and whole-genome analysis) have dramatically revolutionized our understanding of the role of the plant microbiome (Bai et al., 2015; Bulgarelli et al., 2015).

While the structure and function of the microbial communities associated with the rhizosphere and phyllosphere have received broad attention, the knowledge of microbiota dwelling in other niches (for example, a host's reproductive structures) is poorly understood. The seed is an important microbial habitat that sustains a variety of beneficial and pathogenic microbes (Nelson, 2004). Much like the rhizosphere, the spermosphere is a zone surrounding seeds, wherein soil microbes, seed microbes and germinating seeds may interact (Nelson, 2004). The microbiota of the spermosphere is usually short-lived but has a long-lasting impact on seed vitality and seedling growth (Nelson, 2004; Sánchez-Coronado et al., 2011; Chen et al., 2012; Schiltz et al., 2015). A recent study revealed that microorganisms from both the seed endosphere and spermosphere, possibly the least-studied group of plant symbionts, are capable of promoting seed germination and providing benefits to plants during exposure to biotic and abiotic stress (Truyens et al., 2014). For example, the seed-borne fungal endophyte *Stagonospora* sp. (Pleosporales, Ascomycota) can enhance reed (*Phragmites australis*) biomass and facilitate the establishment of seedlings in novel habitats (Ernst et al., 2003). Similarly, fungi isolated from the seeds of *Opuntia* spp. (*Phoma* sp., *Penicillium chrysogenum*, and *Trichoderma koningii*) are involved in

* Corresponding author.
E-mail addresses: zlyuanmycology@gmail.com, yuanzl@caf.ac.cn (Z. Yuan).

http://dx.doi.org/10.1016/j.funeco.2016.08.011
1754-5048/

disrupting seed dormancy and promoting a higher rate of seed germination in semi-arid lands (Delgado-Sánchez et al., 2010, 2011, 2013). In addition, the effects of seed microbiota on plant fitness are not specific to plant-fungal interactions. There are also numerous reports of seed-associated bacteria having the same functions in relation to plant fitness (Morpeth and Hall, 2000; Mastretta et al., 2009; Hardoim et al., 2012; Xu et al., 2014; Pitzschke, 2015). It is, thus, reasonable to speculate that seed-associated microbes, including endophytes and epiphytes, may play a larger role in modulating their host plants than previously thought.

In this work, we characterized the seed-borne fungal endophyte community associated with *Suaeda salsa*, a typical coastal plant with superior halo-tolerance, using 454 pyrosequencing targeting the nuclear ribosomal DNA internal transcribed spacers 1 and 2 (ITS1 and ITS2). We first aimed to reveal the diversity and structure of the fungal endophyte community. A culture-based approach was also adopted to recover the endophytes from seeds. Based on these data, we hypothesize that certain culturable endophytes may provide some kinds of benefit to the host and even to other plants. Thus, it is hoped that this study will gain important insight into the ecology and symbiotic potential of the seed-borne endophytes.

2. Methods

2.1. Plant materials and seed surface sterilization

S. salsa is widespread in the eastern coastal regions of China and grows in hypersaline soils. In December 2013, we collected mature seeds of *S. salsa* in Dongying (Shandong province, 37°23′43″N, 118°55′25″E), where *S. salsa* was one of the dominant halophytes. Three sampling plots were selected (around 3–5 m apart), and three to five plants per pot were sampled. Seeds of all these plants were then thoroughly mixed and divided into three homogeneous sub-samples that were used for pyrosequencing analysis. The seeds were excised from the utricles and surface sterilized by immersion in ethanol (75%, v/v) for 30 s, followed by a solution of 2.0% NaClO (v/v) for 8 min, and then transferred to 90% ethanol (v/v) for 30 s to remove the remaining NaClO. Finally, the seeds were rinsed at least five times with sterilized distilled water.

2.2. DNA extraction from the seed-associated microbiome

A DNeasy Plant Mini Kit (QIAGEN Inc.) was used to extract total genomic DNA from the surface-sterilized seeds. The samples were ground in liquid nitrogen using a sterile frozen mortar and pestle. Approximately 100 mg of frozen seed material powder was used for DNA extraction. To obtain high yields of DNA, we processed five subsamples in parallel and used 120 μl of washing buffer to elute all of the collection tubes twice during the final step of the kit protocol.

2.3. PCR amplification and amplicon sequencing

Because there is no universal primer pair that can target all microbial groups, primer selection has a great impact on the characterization of a microbial community. Small subunit (SSU) and large subunit (LSU) ribosomal DNA in fungi, which are less informative for making precise identifications, were not used in this work. Therefore, we adapted the two primer pairs ITS1F-ITS2 (targeting the ITS1 region; rapidly evolving) and fITS7-ITS4 (targeting the ITS2 region; moderately evolving) to conduct a parallel pyrosequencing analysis of the seed endophytic fungal community (Cordier et al., 2012; Ihrmark et al., 2012; Zimmerman and Vitousek, 2012). We concur with other researchers that one of the most challenging technical issues faced when characterizing the structure of plant microbiomes is the amplification of plant DNA with primers used for fungi (Lucero et al., 2011; e.g., Dr. Petr Baldrian, Academy of Sciences of the Czech Republic, personal communication), and the degree of contamination can vary between plant species. Preliminary analyses have revealed that plant-derived ITS sequences can contaminate amplicon libraries when using the direct PCR method. To reduce the occurrence of non-specific amplification, a nested PCR method was developed. Briefly, NSA3 and NLC2, which are considered to be Dikarya-specific, were used as outer primers for the first PCR run to amplify the complete ITS region (Martin and Rygiewicz, 2005). The second amplification cycle was performed using the two primer sets mentioned above. The primer sequences, primer pairs, and annealing temperatures used in the nested PCRs are provided in Table S1. All positive amplicons obtained from the three replicate PCRs per sample were pooled, gel purified, and sequenced with the Roche 454 platform (GS FLX + System) at the Beijing Genomics Institute. For this process, ITS fusion primers containing template-specific primers, adapters, a sample-specific 10-bp MID (Multiplex Identifier) barcode, and a 454 key were designed for the construction of amplicon libraries. All qualified fungal amplicon libraries were clonally amplified using emulsion PCR.

2.4. Processing pyrosequencing data

Multiple levels of sequence processing and quality filtering were performed using Mothur (v1.31.2) (Schloss et al., 2009). The trim.seqs command was used to trim the sequence when the average quality score over a 50 bp sliding window dropped below 25. Sequences with homopolymers with more than eight nucleotides, those containing ambiguous base calls, and those less than 100 bps in length were removed. The sequences were clustered and assigned to operational taxonomic units (OTUs) using the average neighbor clustering algorithm implemented in Mothur, with a threshold of 97% pairwise identity. Chimeras were identified and removed using UCHIME (v4.2, http://drive5.com/uchime). One representative sequence from each OTU was subjected to a BLAST search against GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for taxonomic assignment.

2.5. Isolation and identification of seed culturable fungal endophytes

Endophytic fungi were isolated from healthy and mature *S. salsa* seeds. Surface-sterilized seeds (as described above) were dried on sterile filter paper, and cut into two halves. A total of 70 segments (10 segments per plates) were transferred to 2% malt extract agar (MEA) plates containing streptomycin sulfate (50 mg l^{-1}) and tetracycline hydrochloride (20 mg l^{-1}) to suppress bacterial growth. Although some workers have used the media supplemented with salt for isolating endophytes from halophytes (Lucero et al., 2011), the authors found that the recovered fungi grew satisfactorily on the common MEA. This indicates that the endophytes from halophytes are most likely halotolerant but not halophilic, which was also supported by previous studies (Rodriguez et al., 2008; Maciá-Vicente et al., 2012). Second, unlike the phylloplane environments, in which salt secretion sometimes occurs on leaves, the interior of the seed does not contain high salt content. Third, most plates used in this isolation experiment were nearly absent of any fungal growth so that there is no issue of halophilic fungi being overgrown by less specialized, but faster growing fungi on plain MEA. Therefore, we still consider that the widely used MEA without additional salt is a suitable medium for isolating fungi from halophyte seeds. After incubation for up to1 week in the dark at 20 °C, fungal hyphae developed from the tissue fragments were transferred to fresh potato dextrose agar (PDA) for purification.

Fungal DNA was also extracted using DNeasy Plant Mini Kit according to the manufacturer's protocol. The internal transcribed spacer (ITS) as a barcode for molecular identification, which was amplified using the universal primer set ITS-1F (5′-CTTGGTCAT TTTAGAGGA AGTAA-3′) and ITS4 (5′- TCCTCCGCTTAT TGATATGC -3′). 50 μl of PCR mixture contained 10–50 ng of the DNA template, 50 μM of each primer, and 25 μl 2 × Taq MasterMix (Cwbio, Beijing) and nuclease-free H_2O. The PCR reaction consisted of initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C for 50 s and extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. PCR products were purified and Sanger-sequenced in both directions. After removing the ambiguous bases, sequences were subjected to the BLAST search in the UNITE (https://unite.ut.ee/) and NCBI (www.ncbi.nlm.nih.gov/) database to obtain the top ten hits for each BLAST query. The hits including a number of uncultured or unidentified taxa were not considered. Given some degree of unreliability for submitted sequences (Hawksworth, 2004; Holst-Jensen et al., 2004), authentic data from well annotated fungal materials deposited in public culture collections were preferred.

2.6. Morphological description of the Cladosporium isolate

Morphological characteristics of the *Cladosporium* isolate seed4 were recorded on PDA, oatmeal agar (OA, Difco), synthetic nutrient agar (SNA, KH_2PO_4 1.0 g, KNO_3 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g, glucose 0.2 g, sucrose 0.2 g, agar 20 g, and 1.0 l distilled water), and MEA after14 d at 25 °C in the dark, and we chose the fungal colony on SNA to observe the conidial development and branching patterns of conidial chains (Schubert et al., 2009). Squares of transparent adhesive tape were placed on conidiophores growing in the zone between the colony margin and 2 cm inwards, and mounted between two drops of Shear's solution (consisting of 2 g potassium acetate, 40 ml glycerol, 60 ml 95% ethanol, 100 ml distilled water, and 0.4 g blue ink) under a glass cover slip (Crous et al., 2009). Conidiophores and conidia were observed with differential interference contrast (DIC) microscopy (Zeiss, Axio Scope A1).

2.7. Effects of Cladosporium cladosporioides on S. salsa seed germination

To study the effects of *Cladosporium cladosporioides* on host seed viability, we compared the germination rates of *S. salsa* seeds with and without inoculation. To prepare conidia suspensions for inoculation, approximately 20 ml of sterile water was added onto the surface of actively growing colonies. These conidia were then spread with a sterile glass rod, followed by filtration with two layers of gauze to remove the mycelia. The spore suspensions were then adjusted to the desired concentrations (1×10^5 ml^{-1} and 1×10^6 ml^{-1}) by counting spore density on a counting chamber. A total of 810 seeds of high quality and uniform size were selected, divided into three groups and then surface sterilized as described above. Two groups of seeds were soaked in the two concentrations of conidia suspension for 30 min, while the remaining seeds in sterilized water were used as a control group. The seeds were then sown in pots (11.5 cm length × 10 cm width × 6.5 cm height) at a uniform depth in a soil substrate (saline soil: organic fertilizer: vermiculite: perlite = 4:1:1:1, autoclaved at 121 °C for 10 min) to guarantee uniform emergence. This also created an environment that mimics the natural soil conditions. There were three replicates (3 pots per replicate, 30 seeds per pot) for each treatment (inoculated vs. control). A total of 27 pots were randomly placed in a growth chamber maintained at 25 °C under a 12 h photoperiod with regular watering. Germination rates were measured, and the growth conditions of the seedlings were recorded every day.

2.8. Investigation of the colonization pattern of C. cladosporioides in S. salsa seedlings

To investigate the *ex* and *in planta* colonization patterns of *C. cladosporioides*, we re-isolated the fungi from the belowground (roots) and aboveground (stems and leaves) tissues of *S. salsa* seedlings. The roots were washed with sterile water three times to recover the fungi from either the rhizoplane or endosphere, and young leaves containing stem tissues were subjected to slight surface disinfection with $HgCl_2$ (0.1%, v/v) for 2 min. The tissue fragments were incubated on MEA, and purification and identification of fungi was the same as those described above.

Scanning electron microscopy (SEM) method was employed to further observe the fungal growth pattern in the process of seed germination and seedling emergence in comparison to seeds that were not inoculated with *C. cladosporioides*. Seeds inoculated with conidia (1×10^5 ml^{-1}) were transferred to 1.5% (w/v) water agar. Embryo bud and seed coat were taken at respectively12 h, 24 h, 36 h, 48 h after the start of the experiment. The samples were first fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, at 4 °C, and then dehydrated through a graded series of ethanol (30%, 50%, 70%, 80%, 90%, 100%), 40 min per change. Samples were dried to a critical point by carbon dioxide with K850 critical points dryers (Quorum/Emitech, Ashford, UK). Subsequently, the samples were mounted on SEM conductive tapes and coated with gold (20 s, 50 mA) using an SBC-12 ion sputter coater (KYKY Technology Co., Beijing, China). Images were produced using a Phenom ProX desktop scanning electron microscope (Phenom-World, Eindhoven, The Netherlands) with an accelerating voltage of 5 kV.

2.9. Effects of C. cladosporioides on the growth of other plants

We further investigated whether *C. cladosporioides* is beneficial for the growth of other plants. American sweetgum (*Liquidambar styraciflua*) was selected for inoculation experiments because it is a popular ornamental tree and is easily grown under controlled laboratory conditions. Fresh seedling biomass was measured to compare the treatment and control groups.

For inoculation, seeds were surface sterilized with 0.1% (v/v) $HgCl_2$ for 5 min, rinsed five times with sterilized distilled water, sown on an autoclaved soil substrate (clay soil: organic fertilizer: vermiculite: perlite = 3:1:1:1), and then incubated in a growth chamber at 25 °C for 20 days, with a photoperiod of 12 h light/12 h darkness. Prior to inoculation, seedlings of uniform size were selected. Conidia were harvested by rinsing PDA plates with sterile saline solution (0.85% NaCl). The roots were immersed in three spore concentrations (1×10^5, 1×10^6, and 1×10^7 ml^{-1}) for at least 30 min. For the control groups, the roots were treated with sterile 0.85% NaCl without conidia. All seedlings were planted in pots supplemented with the same substrate and were supplied with conidia of the corresponding concentration near the rhizosphere (100 μl per seedling) to ensure effective infection. Each treatment consisted of four pots and 13 seedlings per pot. In total, 16 pots were used and 208 seedlings were planted. The pots were maintained under the same environmental conditions and placed randomly in a growth chamber. The fresh weight of the seedlings was measured and recorded after 2 weeks.

2.10. Data accessibility and statistical analyses

All the original *sff* (Standard Flowgram Format) files were deposited in the European Nucleotide Archive (ENA) under study accession number: PRJEB8726 (www.ebi.ac.uk/ena/data/view/PRJEB8726). R software (version 2.15.3) was used to plot the rarefaction curve based on the indices of the observed species. To test

for sampling effectiveness, rarefaction curves based on the resulting OTU tables rarefied to the lowest numbers of reads obtained for any single sample were generated using the vegan R package.

The raw data generated from inoculation experiments were exported to Excel, and statistical analysis was conducted by GraphPad Prism v 6.0 (GraphPad, San Diego, CA, USA). The statistical significance of the differences between the treated and control groups was analyzed using a two sample *t*-test or one-way ANOVA.

3. Results

3.1. Pyrosequencing data and taxonomic classification of sequence reads

After filtering, denoising, chimera removal, quality control, and discarding plant-derived sequences, a total of 5492 and 9349 effective reads were obtained for the fungal ITS1 and ITS2 data sets (Tables S2 and S3). The mean numbers of effective reads per sample were 3699 and 4623 for ITS1 and ITS2, respectively. The average lengths of the trimmed sequences (following the removal of adapters, barcodes, and primers) were 190 bp (ITS1) and 150 bp (ITS2).

Rarefaction analysis showed asymptotic or nearly asymptotic rarefaction curves across all samples (Fig. 1A and C), suggesting that the sequencing depth was adequate to capture the true diversity of the fungal endophytes in the seeds. In some rare cases, the rarefaction curves failed to reach a plateau.

3.2. Endophytic fungal community and the dominant species in S. salsa seeds

The endophytic fungal community in the seeds was of extremely low diversity, with only a few identified genera (Fig. 1B and D). The genera *Cladosporium* and *Alternaria* were detected in both the ITS1 and the ITS2 amplicon libraries. The ITS1F-ITS2 primer pair identified more endophytic fungal 97% OTUs than fITS7-ITS4.

The genus *Cladosporium* was the most common fungus in the seeds, as inferred from both ITS1 (19 OTUs) and ITS2 (9 OTUs) pyrosequencing data. Moreover, based on the calculation of the mean p-distance and on a nucleotide diversity analysis, the genus *Cladosporium* showed a relatively high level of infrageneric diversity, indicating some degree of phylogenetic redundancy at the genus level (see more details in Figure S1). Based on BLAST annotation, the highest abundance of pyrosequencing reads to the OTU was classified as *C. cladosporioides.*

3.3. The culturable fungal microbiota in S. salsa seeds

Instead of a large scale of fungal isolation from seeds, in this work, we intended to obtain the culturable isolates for a plant inoculation test rather than comparing the species diversity with pyrosequencing approach. A total of six isolates were recovered

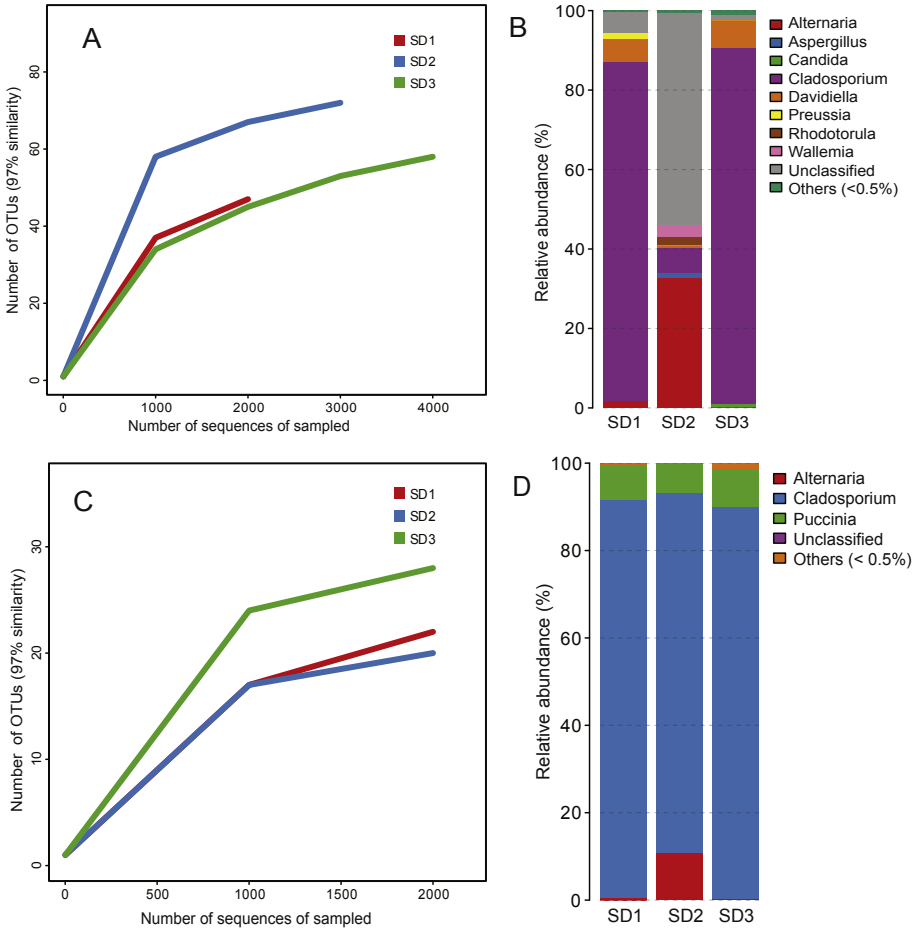

Fig. 1. Rarefaction analyses of *S. salsa* seed fungal libraries inferred from ITS1 (A) and ITS2 (C) pyrosequencing data and the relative abundance of fungal communities (at the genus level) in the seeds, represented by bar charts. Each curve illustrates the cumulative number of operational taxonomic units (OTUs) at a phylogenetic distance of 0.03. The replicated libraries are indicated by '1', '2' and '3'.

from 70 seed fragments, indicating a low frequency of colonization. Based on simple BLAST searches of ITS sequences, their tentative taxonomic placement was determined (Table 1). Briefly, they belonged to three ascomycetous orders (Capnodiales, Pleosporales, and Sordariales) and four genera (*Chaetomium*, *Cladosporium*, *Leptosphaeria*, and *Preussia*). Except for *Cladosporium*, the remaining three genera were not detected in pyrosequencing analysis. Since the pyrosequencing data suggested *Cladosporium* as the dominant genus, we focused on morphological description of the *Cladosporium* sp. isolate. After 2 weeks of cultivation on SNA, the isolate was identified as *C. cladosporioides* according to the long unbranched chains with small, smooth and ovoid terminal conidia, aseptate secondary ramoconidia as well as the macronematous and narrower conidiophores (Figure S2) (Bensch et al., 2010).

3.4. Effects of C. cladosporioides on seed germination

Germination rates of seeds pre-inoculated with *C. cladosporioides* were significantly increased relative to the control group (Student's two-sample *t*-test; for the treatment of 1×10^5 conidia ml^{-1}, $t = 4.949$, $df = 6$, $P = 0.0026$; for the treatment of 1×10^6 conidia ml^{-1}, $t = 4.869$, $df = 6$, $P = 0.0028$) (Fig. 2A–B). However, we observed no significant difference in the biomass and height of the resultant seedlings between the treatment and control groups (data not shown). It can also be concluded that the high concentration of conidia promoted seed germination after 24 h of inoculation, especially after 4 and 5 d. A significant difference among the two treatments was also recorded (Student's two-sample *t*-test, $t = 2.962$, $df = 6$, $P = 0.0252$).

Efforts were also made to re-isolate the fungi from roots, as well as from above-ground tissues of the resultant seedlings. A total of seven *Cladosporium*-like colonies were picked up and purified for identification (Fig. 2C and D). Their ITS sequences shared 100% similarity with the sequences of the original *C. cladosporioides* isolate. This indicates that *C. cladosporioides* may exhibit both endophytic and epiphytic infection of the host seedlings. SEM analysis clearly showed that the hyphae grew rapidly and spread across the seed coat at 12 h after inoculation. Subsequently, the seeds became extensively colonized, and the hyphae further proliferated on the buds, as well as on the younger emerging roots and leaves (Fig. 3). Unfortunately, it is relatively difficult to observe the endophytic growth of *C. cladosporioides*, mainly due to its failure to enter into the host at the time of sampling as well as the disadvantage of a destructive sample preparation of this general SEM method. The scanning electron micrographs of uninoculated *S. salsa* seeds are presented in Figure S3.

3.5. C. cladosporioides colonization benefits the growth of other plants

C. cladosporioides significantly promoted the growth of American sweetgum seedlings after inoculation (Student's two-sample *t*-test, $t = 5.408$, $df = 95$, $P < 0.0001$) with 1×10^5 conidia ml^{-1} (Fig. 4). When using either low or high conidia concentrations as inocula, the total biomass of seedlings increased relative to control groups. However, the differences failed to reach the statistical significance, indicating that 1×10^5 conidia ml^{-1} was the optimum concentration of conidia suspension for the plant growth promotion effect. This data also suggests that either insufficient or excessive fungal colonization will weaken the plant-fungal mutualism.

4. Discussion

In this study, we employed a high-throughput DNA sequencing strategy to reveal the assembly and structure of the endophytic fungal microbiota in *S. salsa* seeds. We also investigated the seed bacterial endophytes, but, the bacterial amplicon libraries of the seeds showed unexpectedly high contamination of plant organelles (mitochondria and plastids), despite the introduction of bacteria-specific primer sets and the use of touch-down PCR (Table S4). This may indicate a lower bacterial load inside the seeds. Although the bacterial members are not considered in this work, their roles in plant fitness warrant further investigation.

Our data suggest that the species richness of the seed endophytic fungal community is very low and consists of a small number of fungal genera. *Cladosporium* is the predominant genus found in seeds, based on either fungal ITS1 or ITS2 pyrosequencing data. Several previous reports have indicated that *Cladosporium* spp. are often isolated from the inner seeds of a wide range of herbaceous plants (Ikeda et al., 2006; Lucero et al., 2011; Hodgson et al., 2014), suggesting that *Cladosporium* spp. serve as the generalist endophytes of seeds and exhibit low host specificity (Hodgson et al., 2014; Parsa et al., 2016). Few studies have reported high frequencies of *Cladosporium* spp. in seeds. In one study, *Cladosporium sphaerospermum* was found to extensively colonize seeds, cotyledons, and the true leaves of six forb species (Hodgson et al., 2014). Furthermore, a relatively high degree of phylogenetic redundancy, reflected by the infrageneric diversity determined by the calculation of mean p-distance and nucleotide diversity, was recorded in the genus *Cladosporium* (Fig. S1). It has long been thought that phylogenetic redundancy creates functional redundancy, which plays an important role in stabilizing microbial communities (Shade and Handelsman, 2012). These preliminary data further underscore the potential functions of *Cladosporium* spp.

It has been reported that both the seed-associated epiphytes and endophytes play significant roles in plant growth and health (Ernst et al., 2003; Pitzschke, 2015; Tahtamouni et al., 2016). In the rainy tropics, seed epiphytic fungi (*Fusarium* sp. and *Penicillium* sp.) induced seed germination (Tamura et al., 2008). Under arid climate and drought stress, fungi found in *Opuntia streptacantha* seeds may help the host break seed dormancy and promote germination (Delgado-Sánchez et al., 2011). Consistent with these findings, our work showed another example of how an endophytic fungus affects the seed germination. High colonization frequency of *C. cladosporioides* and its considerable contribution to seed germination efficiency, clearly suggest that it forms a highly integrated

Table 1
Taxonomic placement of fungal isolates obtained in this study inferred from BLAST searches.

Isolate name	Classification	Nearest match	Max identity	Accession number
Seed 1	Ascomycota; Pleosporales	*Preussia minimoides* (KJ471500)	99%	KU869522
Seed 2	Ascomycota; Sordariales	*Chaetomium erectum* CBS140.56 (NR_077201)	100%	KU869523
Seed 3	Ascomycota; Sordariales	*Chaetomium globosporum* CBS108.83 (KC109750)	94%	KU869524
Seed 4	Ascomycota; Capnodiales;	*Cladosporium cladosporioides* (LN835262)	100%	KU869525
Seed 5	Ascomycota; Pleosporales	*Leptosphaeria* sp. (KM979814)	100%	KU869526
Seed 6	Ascomycota; Pleosporales	*Leptosphaeria* sp. (KM979814)	100%	KU869527

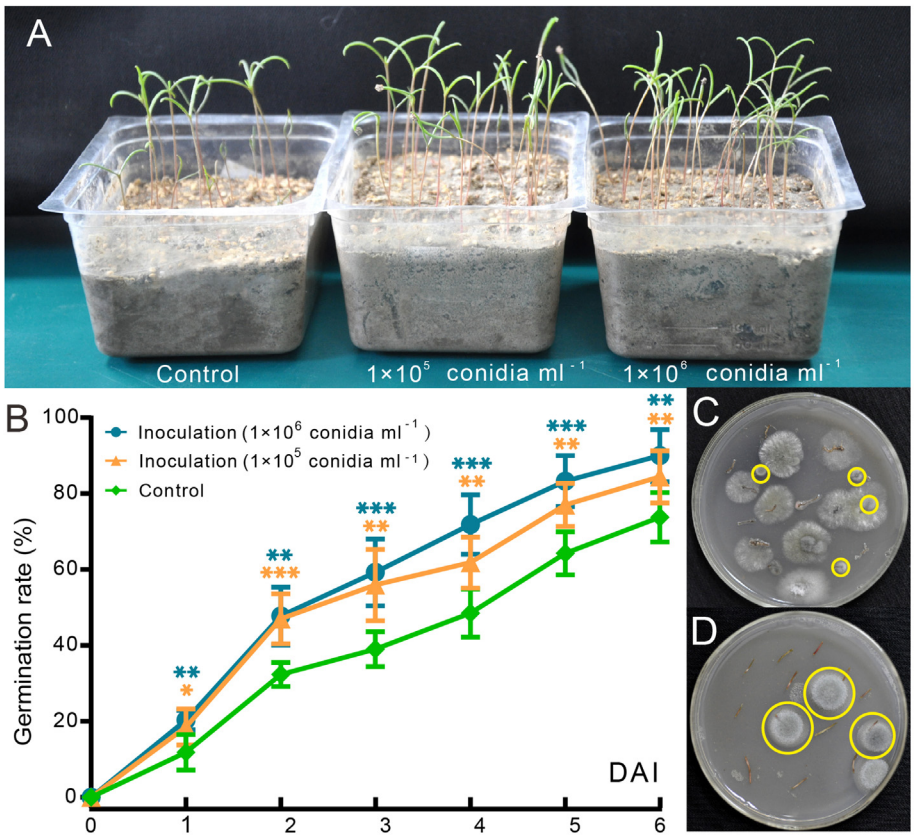

Fig. 2. The beneficial effects of *C. cladosporioides* on *S. salsa* seed germination. (A) comparison of seedling emergence and growth between the control and treatment groups after 5 d of inoculation. (B) effects of fungal inoculation on germination rate of *S. salsa* seeds presented as line graphs. Statistical analysis of the data was performed using the Student's *t*-test to determine significant differences between treatment and control groups. Significance levels of the effects are denoted with (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.0001$. C–D: re-isolation of *Cladosporium*-like fungi from roots (C) and above-ground tissues (D); colonies indicated in yellow were identified as *C. cladosporioides* by ITS sequence analysis. DAI: days after inoculation.

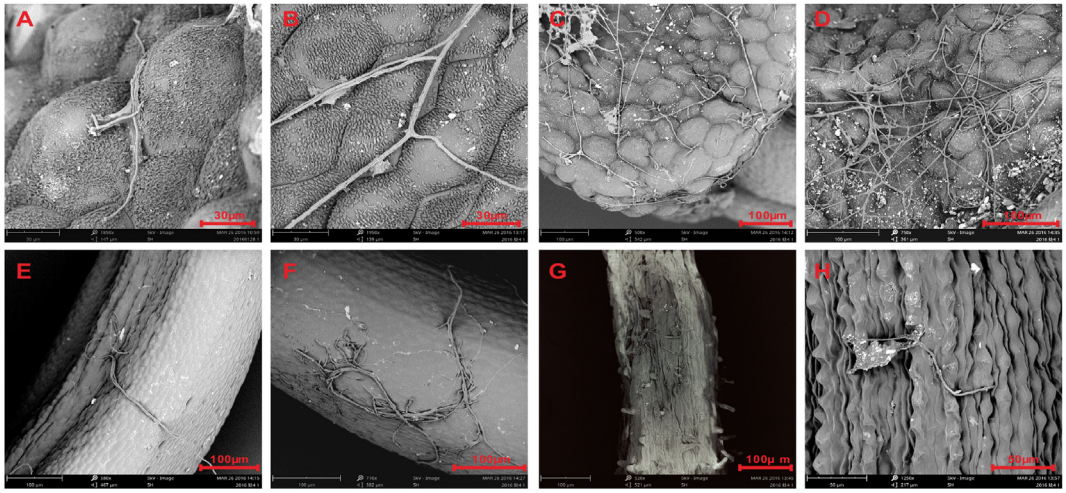

Fig. 3. Scanning electron microscopy analysis of the growth and colonization pattern of *C. cladosporioides* on the surface of the seed coat, emerging buds and whole seedlings of *S. salsa*. The hyphae proliferated on the surface of the seed coat after 12 h (A), 24 h (B), 36 h (C), and 48 h (D) of inoculation. Hyphae extended to the surface of the emerging buds after 24 h (E) and 36 h (F) of inoculation and extensively colonized the root surface (G) and leaf surface (H) after 48 h of inoculation.

and specialized symbiosis with the host seeds. More interestingly, *C. cladosporioides* also shows potential in promoting the growth of other plants in a concentration-dependent manner. However, the physiological and molecular mechanisms underlying *C. cladosporioides*-mediated modulation of plant phenotype are largely unknown. We surmise that the *C. cladosporioides* may secrete signaling molecules that initiate positive seed germination responses and reprogram plant hormone signaling.

Several lines of evidence now reveal that *C. cladosporioides* also prefers to colonize the leaf and seed surfaces of taxonomically

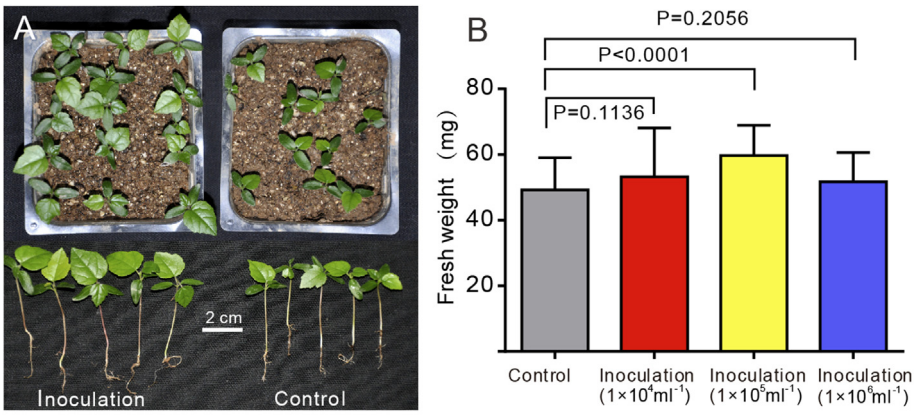

Fig. 4. Growth response of American sweetgum (*Liquidambar styraciflua*) seedlings to inoculation with different *C. cladosporioides* conidia concentrations. (A) seedlings were inoculated with *C. cladosporioides* conidia (1×10^5 ml^{-1}), while the control groups were treated with sterile water. (B) effect of three conidia concentrations (1×10^4 ml^{-1}, 1×10^5 ml^{-1}, and 1×10^6 ml^{-1}) on the fresh weight of the seedlings. P values less than 0.05 were considered statistically significant.

diverse host plant species (Mittal and Wang, 1987; Tokumasu, 1996; Nishikawa et al., 2006; Kharwar et al., 2010), indicating that it also exhibits an epiphytic lifestyle (Osono, 2014; Zambell and White, 2015). More commonly, where it is found in trace endophytic levels, it is found in higher epiphytic levels. Therefore, we asked whether *C. cladosporioides* was also present on the surface of *S. salsa* seeds. Relying solely on a culture-based approach, we found that all seeds were colonized by the *Alternaria* sp. (much like *Alternaria alternata*) as well as very few *Fusarium* sp. isolates, but *Cladosporium*-like colonies did not appear (Figure S4). This finding is not surprising since *Alternaria* spp. are also very common in saline environments or on plant surfaces (Kageyama et al., 2008; Osono, 2014; Okane and Nakagiri, 2015). This data perhaps indicates that in natural conditions, *C. cladosporioides* inhabiting the *S. salsa* seeds would be largely *via* endophytic hyphal extension and its epiphytic growth appears scarce (or even absent). We, thus, hypothesized that infection by *C. cladosporioides* may primarily arise from soil-borne but not airborne spores, as our previous pyrosequencing analysis provided evidence that *C. cladosporioides* occurred in the rhizosphere, endosphere of roots and leaves at relatively low frequency (Yuan et al., 2016). Moreover, in late May 2016, we re-sampled the newly emerged *S. salsa* seedlings (seeds will typically germinate in mid-April each year) at the same sampling site, and the isolation experiment revealed that only *C. cladosporioides* and one unidentified species occurred in roots. While we do not know exactly whether *C. cladosporioides* directly comes from the surrounding soils of *S. salsa* or the germinated seeds, or both, this data may suggest that *C. cladosporioides* acts as a pioneering symbiont. We further asked whether the epiphytic *Alternaria* sp. isolate also plays a similar role in promoting seed germination, but results clearly showed that it had no significant effect on seed germination relative to the control group (Figure S5). It also appears likely that the *Alternaria* sp. may not become the dominant seed endophytic fungus, evidenced by its low pyrosequencing reads (Fig. 1) and absence in our culture collections.

However, the transmission mode of *C. cladosporioides* in the *S. salsa* system still remains elusive. In general, seed-borne fungi and bacteria can be both horizontally and vertically transmitted (Saikkonen et al., 2010; Truyens et al., 2014; Hardoim et al., 2015). Only very few fungi, such as the *Epichloë*/*Neotyphodium* (Clavicipitaceae, Ascomycota) endophytes are strictly vertically transmitted *via* extending hyphae internally into the developing seeds (Clay, 1987; Tadych et al., 2014). To demonstrate vertical transmission experimentally, a mother plant would have to be inoculated or shown to be inhabited by a particular fungus, and the original fungus must be re-isolated from these plants emerging from seeds of the mother plant. We have not yet investigated its possibility, as currently it is difficult to generate strictly gnotobiotic *S. salsa* seedlings for inoculation. In brief, what has been demonstrated in this work is the transmission of *C. cladosporioides* from heavily inoculated seeds to seedlings in artificial systems *via* epiphytic growth shown in SEM images, with perhaps a low rate of subsequent endophytic colonization inferred from the re-isolation test. However, this does not detract from the potential positive effects of *C. cladosporioides*. It may be quite effective in germination and growth enhancement even if it is predominantly spread *via* epiphytic hyphal growth. In contrast, although *Alternaria* sp. was found both as an epiphyte and as an endophyte, it showed no sign of beneficial effects on seed germination. Taken together, it seems plausible that functional divergence has occurred among the two ubiquitous seed-associated fungal groups despite their high niche overlap.

Our ongoing work is addressing whether *C. cladosporioides* confers salt tolerance to host and non-host plants. Although it has been well-acknowledged that plants have readily evolved an array of genetic and epigenetic regulatory systems to respond to abiotic stresses such as salinity and drought, we know little about how much of such plant phenotype is related to the seed-associated microbiome. In conclusion, our work supports the importance of seed-associated fungi in increasing plant fitness. Therefore, a holistic understanding of spermosphere microbiome warrants a large-scale investigation. The system presented is unique and can be developed into a tool for understanding endophytic associations in the marine or coastal environments.

Acknowledgments

This research was supported financially by the National Natural Science Foundation of China (No. 31370704) and the Non-Profit Sector Special Research Fund of the Chinese Academy of Forestry (RISF2013005). We would like to extend our sincerest thanks and great appreciation to Dr. Ryoko Oono, the University of California (Santa Barbara) for her valuable comments.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funeco.2016.08.011.

References

Bai, Y., Müller, D.B., Srinivas, G., Garrido-Oter, R., Potthoff, E., Rott, M., Dombrowski, N., Münch, P.C., Spaepen, S., Remus-Emsermann, M., Hüttel, B., McHardy, A., Vorholt, J.A., Schulze-Lefert, P., 2015. Functional overlap of the *Arabidopsis* leaf and root microbiota. Nature 528, 364–369.

Bensch, K., Groenewald, J.Z., Dijksterhuis, J., Starink-Willemse, M., Andersen, B., Summerell, B.A., Shin, H.D., Dugan, F.M., Schroers, H.J., Braun, U., Crous, P.W., 2010. Species and ecological diversity within the *Cladosporium cladosporioides* complex (Davidiellaceae, Capnodiales). Stud. Mycol. 64, 1–94.

Berendsen, R.L., Pieterse, C.M., Bakker, P.A., 2012. The rhizosphere microbiome and plant health. Trends Plant Sci. 17, 478–486.

Bodenhausen, N., Horton, M.W., Bergelson, J., 2013. Bacterial communities associated with the leaves and the roots of *Arabidopsis thaliana*. PLoS One 8, e56329.

Bulgarelli, D., Rott, M., Schlaeppi, K., Ver, L.v.T.E., Ahmadinejad, N., Assenza, F., Rauf, P., Huettel, B., Reinhardt, R., Schmelzer, E., 2012. Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. Nature 488, 91–95.

Bulgarelli, D., Carrido-Oter, R., Münch, P.C., Weiman, A., Dröge, J., Pan, Y., McHardy, A.C., Schulze-Lefert, P., 2015. Structure and function of the bacterial root microbiota in wild and domesticated barley. Cell Host Microbe 17, 392–403.

Chen, M.H., Jack, A.L., McGuire, I.C., Nelson, E.B., 2012. Seed-colonizing bacterial communities associated with the suppression of *Pythium* seedling disease in a municipal biosolids compost. Phytopathology 102, 478–489.

Clay, K., 1987. Effects of fungal endophytes on the seed and seedling biology of *Lolium perenne* and *Festuca arundinacea*. Oecologia 73, 358–362.

Cordier, T., Robin, C., Capdevielle, X., Fabreguettes, O., Desprez Loustau, M., Vacher, C., 2012. The composition of phyllosphere fungal assemblages of European beech (*Fagus sylvatica*) varies significantly along an elevation gradient. New Phytol. 196, 510–519.

Crous, P.W., Verkley, G.J.M., Groenewald, J.Z., Samson, R.A., 2009. Fungal Biodiversity. CBS Laboratory Manual Series, vol. 1. Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands.

Delgado-Sánchez, P., Ortega-Amaro, M.A., Rodríguez-Hernández, A.A., Jiménez-Bremont, J.F., Flores, J., 2010. Further evidence from the effect of fungi on breaking *Opuntia* seed dormancy. Plant Signal. Behav. 5, 1229–1230.

Delgado-Sánchez, P., Ortega-Amaro, M.A., Jiménez-Bremont, J.F., Flores, J., 2011. Are fungi important for breaking seed dormancy in desert species? Experimental evidence in *Opuntia streptacantha* (Cactaceae). Plant Biol. 13, 154–159.

Delgado-Sánchez, P., Jiménez-Bremont, J.F., Guerrero-González Mde, L., Flores, J., 2013. Effect of fungi and light on seed germination of three *Opuntia* species from semiarid lands of central Mexico. J. Plant Res. 126, 643–649.

Ernst, M., Mendgen, K.W., Wirsel, S.G., 2003. Endophytic fungal mutualists: seed-borne *Stagonospora* spp. enhance reed biomass production in axenic microcosms. Mol. Plant Microbe Interact. 16, 580–587.

Hardoim, P.R., Hardoim, C.C., van Overbeek, L.S., van Elsas, J.D., 2012. Dynamics of seed-borne rice endophytes on early plant growth stages. PLoS One 7, e30438.

Hardoim, P.R., van Overbeek, L.S., Berg, G., Pirttilä, A.M., Compant, S., Campisano, A., Döring, M., Sessitsch, A., 2015. The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiol. Mol. Biol. Rev. 79, 293–320.

Hawksworth, D.L., 2004. "Misidentifications" in fungal DNA sequence databanks. New Phytol. 161, 13–15.

Hodgson, S., Cates, C.D., Hodgson, J., Morley, N.J., Sutton, B.C., Gange, A.C., 2014. Vertical transmission of fungal endophytes is widespread in forbs. Ecol. Evol. 4, 1199–1208.

Holst-Jensen, A., Vealstad, T., Schumacher, T., 2004. On reliability. New Phytol. 161, 11–13.

Ihrmark, K., Bodeker, I., Cruz-Martinez, K., Friberg, H., Kubartova, A., Schenck, J., Strid, Y., Stenlid, J., Brandström-Durling, M., Clemmensen, K., Lindahl, B., 2012. New primers to amplify the fungal ITS2 region-evaluation by 454-sequencing of artificial and natural communities. FEMS Microbiol. Ecol. 82, 666–677.

Ikeda, S., Fuji, S.I., Sato, T., Ytow, N., Ezura, H., Minamisawa, K., Fujimura, T., 2006. Community analysis of seed-associated microbes in forage crops using culture-independent methods. Microb. Environ. 21, 112–121.

Kageyama, S.A., Mandyam, K.G., Jumpponen, A., 2008. Diversity, function and potential applications of the root-associated endophytes. In: Varma, A. (Ed.), Mycorrhiza: State of the Art, Genetics and Molecular Biology, Eco-function, Biotechnology, Eco-physiology, Structure and Systematics. Springer, pp. 29–57.

Kharwar, R.N., Gond, S.K., Kumar, A., Mishra, A., 2010. A comparative study of endophytic and epiphytic fungal association with leaf of *Eucalyptus citriodora* Hook., and their antimicrobial activity. World J. Microbiol. Biotechnol. 26, 1941–1948.

Lebeis, S.L., 2014. The potential for give and take in plant-microbiome relationships. Front. Plant Sci. 5, 287.

Lucero, M.E., Adrian, U., Peter, C., Scot, D., Shulei, S., 2011. Endophyte microbiome diversity in micropropagated *Atriplex* canescens and *Atriplex torreyi* var griffithsii. PLoS One 6, e17693.

Maciá-Vicente, J.G., Ferraro, V., Burruano, S., Lopez-Llorca, L.V., 2012. Fungal assemblages associated with roots of halophytic and non-halophytic plant species vary differentially along a salinity gradient. Microb. Ecol. 64, 668–679.

Martin, K.J., Rygiewicz, P.T., 2005. Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. BMC Microbiol. 5, 28.

Mastretta, C., Taghavi, S., Lelie, D.V.D., Mengoni, A., Galardi, F., Gonnelli, C., Barac, T., Boulet, J., Weyens, N., Vangronsveld, J., 2009. Endophytic bacteria from seeds of *Nicotiana tabacum* can reduce Cd phytotoxicity. Int. J. Phytorem. 11, 251–267.

Mittal, R.K., Wang, B.S.P., 1987. Fungi associated with seeds of eastern white pine and white spruce during cone processing and seed extraction. Can. J. For. Res. 17, 1026–1034.

Morpeth, D.R., Hall, A.M., 2000. Microbial enhancement of seed germination in *Rosa corymbifera* 'Laxa'. Seed Sci. Res. 10, 489–494.

Nelson, E.B., 2004. Microbial dynamics and interactions in the spermosphere. Annu. Rev. Phytopathol. 42, 271–309.

Nishikawa, J., Kobayashi, T., Shirata, K., Chibana, T., Natsuaki, K.T., 2006. Seed-borne fungi detected on stored solanaceous berry seeds and their biological activities. J. Gen. Plant Pathol. 72, 305–313.

Okane, I., Nakagiri, A., 2015. Assemblages of endophytic fungi on *Salicornia europaea* disjunctively distributed in Japan: towards clarification of the ubiquity of fungal endophytes on halophytes and their ecological roles. Curr. Sci. 109, 62–71.

Osono, T., 2014. Diversity and ecology of endophytic and epiphytic fungi of tree leaves in Japan: a review. In: Verma, V.C., Gange, A.C. (Eds.), Advances in Endophytic Research. Springer, pp. 3–26.

Parsa, S., García-Lemos, A.M., Castillo, K., Ortiz, V., López-Lavalle, L.A.B., Braun, J., Vega, F.E., 2016. Fungal endophytes in germinated seeds of the common bean, *Phaseolus vulgaris*. Fungal Bio 120, 783–790.

Pitzschke, A., 2015. Developmental peculiarities and seed-borne endophytes in quinoa: omnipresent, robust Bacilli contribute to plant fitness. Front. Microbiol. 7, 2.

Redford, A.J., Fierer, N., 2009. Bacterial succession on the leaf surface: a novel system for studying successional dynamics. Microb. Ecol. 58, 189–198.

Rodriguez, R.J., Henson, J., Van Volkenburgh, E., Hoy, M., Wright, L., Beckwith, F., Kim, Y.O., Redman, R.S., 2008. Stress tolerance in plants *via* habitat-adapted symbiosis. ISME J. 2, 404–416.

Saikkonen, K., Saari, S., Helander, M., 2010. Defensive mutualism between plants and endophytic fungi? Fungal Divers. 41, 101–113.

Sánchez-Coronado, M.E., Márquez-Guzmán, J., Rosas-Moreno, J., Vidal-Gaona, G., Villegas, M., Espinosa-Matías, S., Olvera-Carrillo, Y., Orozco-Segovia, A., 2011. Mycoflora in exhumed seeds of *Opuntia tomentosa* and its possible role in seed germination. Appl. Environ. Soil Sci. http://dx.doi.org/10.1155/2011/107159.

Schiltz, S., Gaillard, I., Pawlicki-Jullian, N., Thiombiano, B., Mesnard, F., Gontier, E., 2015. A review: what is the spermosphere and how can it be studied? J. Appl. Microbiol. 119, 1467–1481.

Schlaeppi, K., Bulgarelli, D., 2015. The plant microbiome at work. Mol. Plant Microbe Interact. 28, 212–217.

Schloss, P., Westcott, S., Ryabin, T., Hall, J., Hartmann, M., Hollister, E., Lesniewski, R., Oakley, B., Parks, D., Robinson, C., 2009. Community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75, 7537–7541.

Schubert, K., Greslebin, A., Groenewald, J.Z., Crous, P.W., 2009. New foliicolous species of *cladosporium* from South America. Persoonia 22, 111–122.

Shade, A., Handelsman, J., 2012. Beyond the Venn diagram: the hunt for a core microbiome. Environ. Microbiol. 14, 4–12.

Shakya, M., Gottel, N., Castro, H., Yang, Z.K., Gunter, L., Labbé, J., Muchero, W., Bonito, G., Vilgalys, R., Tuskan, G., Podar, M., Schadt, C.W., 2013. A multifactor analysis of fungal and bacterial community structure in the root microbiome of mature *Populus deltoides* trees. PLoS One 8, e76382.

Tadych, M., Bergen, M.S., White, J.F., 2014. *Epichloë* spp. associated with grasses: new insights on life cycles, dissemination and evolution. Mycologia 106, 181–201.

Tahtamouni, M.E., Khresat, S.E., Lucero, M., Sigala, J., Unc, A., 2016. Diversity of endophytes across the soil-plant continuum for *Atriplex* spp. in arid environments. J. Arid. Land 8, 241–253.

Tamura, R., Hashidoko, Y., Ogita, N., Limin, S.H., Tahara, S., 2008. Requirement for particular seed-borne fungi for seed germination and seedling growth of *Xyris complanata*, a pioneer monocot in topsoil-lost tropical peatland in Central Kalimantan, Indonesia. Ecol. Res. 23, 573–579.

Tokumasu, S., 1996. Mycofloral succession on *Pinus densiflora* needles on a moder site. Mycoscience 37, 313–321.

Truyens, S., Weyens, N., Cuypers, A., Vangronsveld, J., 2014. Bacterial seed endophytes: genera, vertical transmission and interaction with plants. Environ. Microbiol. Rep. 7, 40–50.

van der Heijden, M.G.A., Schlaeppi, K., 2015. Root surface as a frontier for plant microbiome research. Proc. Natl. Acad. Sci. 112, 2299–2300.

Xu, M., Sheng, J., Lin, C., Men, Y., Lin, G., Guo, S., Lin, S., 2014. Bacterial community compositions of tomato (*Lycopersicum esculentum* Mill.) seeds and plant growth promoting activity of ACC deaminase producing *Bacillus subtilis* (HYT-12-1) on tomato seedlings. World J. Microbiol. Biotechnol. 30, 835–845.

Yuan, Z.L., Druzhinina, I.S., Labbé, J., Redman, R.S., Qin, Y., Rodriguez, R., Zhang, C.L., Tuskan, G.A., Lin, F.C., 2016. Specialized microbiome of a halophyte and its role in helping non-host plants to withstand salinity. Sci. Rep. 6, 32467.

Zambell, C.B., White, J.F., 2015. In the forest vine *Smilax rotundifolia*, fungal epiphytes show site-wide spatial correlation, while endophytes show evidence of niche partitioning. Fungal Divers. 75, 279–297.

Zimmerman, N.B., Vitousek, P.M., 2012. Fungal endophyte communities reflect environmental structuring across a Hawaiian landscape. Proc. Natl. Acad. Sci. 109, 13022–13027.