

A SYMBIOSIS BETWEEN A DARK SEPTATE FUNGUS, AN ARBUSCULAR
MYCORRHIZA, AND TWO PLANTS NATIVE TO THE SAGEBRUSH STEPPE

by

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DEDICATION

I dedicate this work to my parents, Tommy and Juliana Carpenter, for their love and support during the completion of this work, for my life as well as the Great Basin Desert for all its inspiration and lessons.

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ABSTRACT

Plant roots form symbioses with various fungi, including arbuscular mycorrhizae (AMFs) and dark septate endophytes (DSEs). The symbiosis between plants and AMFs has been extensively studied and is generally considered to be mutualistic. Much less is known about the symbiosis between plants and DSE. In sagebrush habitats, DSEs are common, but their effects on the vegetation are unclear. As a first step to study these effects, I isolated and cultured a DSE from the roots of the shrub *Artemisia tridentata*. Based on partial sequences of five genes and phylogenetic analyses, the isolated fungus was a non-described species within the *Darksidea* or a closely related sister group. Subsequently, I performed experiments *in vitro* and in potted plants to determine the effect of the isolated DSE on root tissue integrity, colonization by the AMF *Rhizophagus irregularis*, and plant biomass. These experiments were conducted in two plant species, *A. tridentata* and the native grass *Poa secunda*. Plants were exposed to one of four treatments: no inoculation (-AMF-DSE), inoculation with the DSE isolate (-AMF+DSE), inoculation with *R. irregularis* (+AMF-DSE), and inoculation with both fungi (+AMF+DSE). Microscopic observations revealed that the DSE hyphae grew along the root surface and penetrated epidermal and cortical cells without damage to them. In *A. tridentata*, the hyphae also reached the stele. For both species, total DSE colonization in the -AMF+DSE treatment was similar to that in the +AMF+DSE treatment, indicating the presence of AMF did not alter DSE colonization. Inoculation with DSE did not affect total AMF colonization of *A. tridentata*; however, it increased total colonization of *P.*

secunda from 16.9 ($\pm 5.6\%$) in the +AMF-DSE treatment to 42.6 ($\pm 2.9\%$) in the +AMF+DSE treatment. Also, in both species, the presence of the DSE more than doubled the frequency of AMF intraradical storage structures, which consisted of vesicles plus intraradical spores. These results suggest that via increases in AMF colonization, DSE could lead to a beneficial effect on the host plants. However, neither on its own nor through co-inoculation with AMF, did the DSE isolate affect plant biomass. Thus, under the two conditions tested, the symbiosis was commensalistic. Further work is needed to evaluate the symbiosis in settings that better mimic the natural environment.

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LIST OF ABBREVIATIONS

488-WGA	Alexa Fluor 488 Wheat Germ agglutinin
AMF	Arbuscular Mycorrhizal Fungi
BSU	Boise State University
BS	Bootstrap Support
C	Carbon
cm	Centimeter(s)
CI	Consistency Index
DSE	Dark Septate Endophytic Fungi
DI	Deionized
EGTA	Ethylene glycol tetraacetic acid
g	Gram(s)
h	Hour(s)
ITS	Internal Transcribed spacer
LSU	Large Subunit ribosomal ribonucleic acid
L	Liter(s)
µg	Microgram(s)
ml	Milliliter(s)
min.	Minute(s)
MS	Murashige and Skoog medium
N	Nitrogen

nrRNA	Nuclear-retained Regulatory RNA(s)
PDA	Potato Dextrose Agar medium
pH	Measure of acidity
KOH	Potassium hydroxide
ROS	Reactive Oxygen Species
RC	Rescaled Consistency Index
RI	Retention Index
RNA	Ribonucleic Acid
SSU	Small Subunit ribosomal ribonucleic acid
TEF	Transcription Elongation Factor
UV	Ultraviolet light

INTRODUCTION

Soil microbial communities play critical roles in terrestrial ecosystems through various interactions with other organisms (Lindow and Brandl, 2003; Harris, 2009; Fitzsimons and Miller, 2010; Hassani et al., 2018). Microbes in the rhizosphere have a range of beneficial effects on host plants including recycling of nutrients, enhancement of nutrient and water uptake (Cameron et al., 2013; Ruiz- Lozano and Azcón, 1995; Sharma et al., 1992), and disease suppression (Xavier and Boyetchko, 2003; Laliberté et al., 2015; Yang et al., 2014).

A well-studied group of soil microorganisms is the arbuscular mycorrhizal fungi (AMF). These fungi are members of the phylum Glomeromycota, and they are ubiquitous obligate symbionts found in over 80% of all vascular plants (Bago and Bécard, 2002; Schussler et al., 2001; Wang and Qiu, 2006). The symbiosis between plants and AMF dates back 410 million years to the early Devonian (Remy et al., 1994). Plant root fossils from this period show arbuscules, which are distinctive structures found in AMF. The fossil evidence supports the idea that AMF played a critical role in plant colonization of terrestrial habitats (Helgason and Fitter, 2005).

Plants provide carbon to the AMF in the form of sugars and lipids and, in return, these fungi facilitate plant uptake of mineral nutrients, particularly those with low mobility in the soil, such as phosphorus and zinc (Keymer et al., 2017; Smith et al., 2011; Wang et al., 2017). Other effects of AMF on plants have received less attention. However, there is evidence that AMF can enhance water uptake (Augé, 2004; Khalvati et

al., 2005), reduce pathogen invasion (Newsham et al., 1995; Sikes, 2010), and limit heavy-metal absorption (Liao et al., 2003; Citterio et al., 2005; Ferrol et al., 2016). Also, the extraradical hyphae of AMF enhance soil aggregate formation, increase the water-holding capacity of the soil, and alter the soil microbial community (Rillig et al. 2002; Rillig and Mummey, 2006; Bedini et al., 2009). Through their various effects on plants and other microorganisms, AMF can affect the functionality and stability of ecosystems (Van Der Heijden, 2004).

Another ubiquitous group of fungal symbionts is the dark septate endophytes (DSEs) (Jumpponen and Trappe, 1998b). These facultative organisms are particularly abundant in arid and cold environments, where they may be the most common root fungal symbionts (Porrás-Alfaro et al., 2011). DSE fungi are a paraphyletic group within the Ascomycota. Two typical features found in DSEs are hyphal cell walls with melanin and sclerotia (Jumpponen and Trappe, 1998b; Knapp et al., 2012). The latter are compact masses of fungal hyphae, which in the case of DSEs form inside the host's roots (Jumpponen and Trappe, 1998b). In addition, the categorization of a fungus as a DSE is often limited to fungi that do not cause tissue breakdown or other noticeable pathogenic effects (Mandyam and Jumpponen, 2005). Some variation, however, exists on DSE effects on plant cells and the extent of spread through root cells and tissues. Some DSEs have a predominantly intercellular location, while others are primary intracellular (Yu et al., 2001; Su et al., 2013). Furthermore, during the intracellular growth of the hyphae, the plant cells can remain viable (Barrow and Aaltonen, 2001; Su et al., 2013), or cell death can occur, particularly as colonization progresses or the hyphae become melanized (Su et al., 2013). The extent that hyphae penetrate root tissues can also vary. In asparagus and

rice roots, colonization by *Phalocephala. fortinii* and *Harpophora oryzae*, respectively, occurred predominantly outside the endodermis (Yu et al., 2001; Su et al., 2013). In contrast, colonization of *Bouteloua* spp. grasses and *Atriplex canescens* by an unidentified DSE fungus was more prevalent within sieve elements (Barrow and Aaltonen, 2001; Barrow, 2003). The effect of differences in the distribution of fungal hyphae on the symbiosis is unclear. Barrow (2003) suggested that hyaline hyphae present within the phloem may be an important site of nutrient exchange that contributes to a mutualism. This effect, however, is likely to depend on the degree that the plant controls fungal growth because colonization of the vascular cylinder can also lead to systemic spread and disease (Su et al., 2013).

Associations with DSE are widespread throughout the plant kingdom. They have been reported in at least 600 species and 144 families ranging from seedless vascular plants to angiosperms (Jumpponen and Trappe, 1998; Mandyam and Jumpponen, 2005). The most commonly studied DSE, such as *P. fortinii* and *Cadophora finlandica*, have low host specificity (Jumpponen and Trappe, 1998a; Yu et al., 2001). The low specificity may be a general characteristic of DSE, but the information in this area is presently limited to a few DSE fungal taxa (Knapp et al., 2015).

Notwithstanding the widespread occurrence of the plant-DSE symbiosis, the functional and ecological roles of DSE are still poorly understood. Similar to AMF, the plant-DSE symbiosis can range from mutualistic to parasitic (Smith and Smith, 2013; Mandyam and Jumpponen, 2015). However, for AMF, meta-analyses of plant responses to these fungi tend to show mutualism (Hoeksema et al., 2010; Worchel et al., 2012; Jayne and Quigley, 2014). This trend is not as consistent for DSE (Hoeksema et al.,

2010; Newsham, 2011; Mayerhofer et al., 2013). For example, a meta-analysis conducted by Newsham (2011) revealed positive effects of DSEs on nutrient content and plant biomass. These results contrast with more recent data where the effects of inoculation with DSE were predominantly neutral or negative (Mayerhofer et al., 2013). Nevertheless, the latter meta-analysis also revealed that when the inoculated isolate originated in the same host species from where it was tested, growth was promoted (Mayerhofer et al., 2013). This observation is consistent with the notion that the nature of the symbiosis is partially determined by the particular plant-DSE genotypes involved in the association (Mandyam and Jumpponen, 2005; Reininger and Sieber, 2012).

As noted, DSE are abundant in arid and semiarid environments, such as the sagebrush steppe habitat in western North America. This habitat covers approximately 450,000 km² and is characterized by a vegetative community of perennial grasses, forbs, biological soil crusts, and several subspecies of the shrub *Artemisia tridentata* Nutt (big sagebrush) (Noss, 1996; Anderson and Inouye, 2001). *Artemisia tridentata* is a dominant shrub that contributes to the development of a heterogeneous landscape (Charley and West, 1977; Ryel and Caldwell, 1998; Davies et al., 2007) and provides habitat and forage for local animals (Aldridge and Boyce, 2007; Larrucea and Brussard, 2008). Due to invasion by exotic annual grasses and an associated increase in the frequency of wildfires, the habitat occupied by big sagebrush has declined, as well as its natural reestablishment (Brooks et al., 2004; Baker, 2006; Dettweiler-Robinson et al., 2013). This has prompted efforts to gain a better understanding of the biotic and abiotic factors that affect the survival and growth of big sagebrush seedlings (Lambrecht et al., 2007; Davidson et al., 2016).

Although the presence of DSE in sagebrush habitats has not been extensively examined, two recent studies involving next-generation sequencing approaches suggest that DSE are significant components of the soil microbiota (Weber et al., 2015; Gehring et al., 2016). Many DSE are within the order Pleosporales (Knapp et al., 2015). In sagebrush steppe of eastern Idaho, members of the Pleosporales were the most abundant fungi in the soil (Weber et al., 2015). DSE were also observed in the roots of native and exotic grasses and *A. tridentata* (Gehring et al., 2016). In northern Arizona, colonization of *A. tridentata* roots by DSE ranged from 20 to 30% (Gehring et al., 2016); these rates are similar to those often observed for AMF colonization (Busby et al., 2013; Davidson et al., 2016).

DSEs are present in *A. tridentata* roots, and to my knowledge, these fungi have not been isolated from this plant (Gehring et al., 2016). Reasoning that the isolation of at least one DSE would be a necessary first step to then conduct manipulative experiments aimed at studying the symbiosis, the first goal of my research was to isolate and culture a DSE from *Artemisia tridentata ssp. wyomingensis* (Wyoming big sagebrush). This subspecies grows in xeric locations and is difficult to reestablish (Davies et al., 2011). By isolating endophytes from Wyoming big sagebrush, I hoped to identify microorganisms that could affect the functioning and reestablishment of this subspecies.

After isolating and culturing a DSE, my second goal was to investigate the effects of this fungus at the tissue and whole plant level. I conducted these studies in seedlings of Wyoming big sagebrush and the native perennial grass *Poa secunda* (Sandberg bluegrass). Two species were used to ascertain whether the symbiosis varies between different hosts, and to gain a broader understanding of possible effects of the isolated

fungus on plants. Furthermore, the restoration of sagebrush habitats often involves seeding with *P. secunda* (Raymondi, 2017). Thus, microorganisms present in this plant and the surrounding soil could potentially spread to and alter the microbiome and performance of *A. tridentata* (Serpe et al., 2020).

Typically, the effects of DSEs on plants are first studied through re-synthesis experiments, where plants grown *in vitro* are inoculated with a fungal isolate (Knapp et al., 2012; Mandyam and Jumpponen, 2014). I followed this approach to ascertain that the DSE isolate was not pathogenic and to obtain highly colonized roots. Roots with a high density of hyphae facilitate the study of DSE effects at the cell and tissue level (Peterson et al., 2008).

At the whole plant level, a common approach to assess the impacts of DSEs and other symbionts on plants is to determine their effects on plant growth (Mandyam and Jumpponen, 2015; Vergara et al., 2018; Yakti et al., 2018). Negative, neutral, and positive growth responses are indicative of parasitic, commensalistic, and mutualistic symbioses, respectively (Johnson and Graham, 2013; Mandyam and Jumpponen, 2015). Measurements of growth responses in plants grown *in vitro* have the benefit that the response is free from the influence of other microorganisms. On the other hand, *in vitro* conditions represent a very artificial environment, where the effect of the fungus on the plant may differ from that in soil (Mandyam and Jumpponen, 2014). A complementary scheme to correct this limitation is to study the symbiosis in potted plants with inoculum applied to sterilized soil (Newsham, 2011). While not equivalent, this is closer to a natural setting than *in vitro* and provides some control of the microorganisms present.

Based on these considerations, I investigated the effect of the isolated DSE on plant biomass both *in vitro* and in potted plants.

Apart from potential direct effects on plant biomass, DSEs may affect plants by altering the abundance of other microorganisms. As mentioned earlier, AMFs are common root endophytes that can play a critical role in nutrient uptake. Prior work indicated that increases in survival of Wyoming sagebrush seedlings were associated with increases in AMF colonization (Davidson et al., 2016). Based on this and similar results (Stahl et al., 1998), I was intrigued by the possibility that DSEs could affect sagebrush through changes in AMF colonization. AMF and DSE often co-occur in the roots of the same plant (Lingfei et al., 2005; Chaudhry et al., 2009; Seerangan and Thangavelu, 2014). The co-occurrence indicates that colonization by DSE does not fully inhibit AMF colonization. On the other hand, DSE may affect the extent of AMF colonization or somewhat alter the benefits of the plant-AMF symbiosis. Studies that have directly investigated these questions are rare and have yielded conflicting results. In *Populus*, Gehring et al. (2013) observed that an increase in DSE fungal colonization was correlated with a decrease in AMF colonization. In contrast, Della Monica et al. (2015) and Wężowicz et al. (2017) showed similar rates of AMF colonization in plants co-inoculated with AMF and DSE than those solely inoculated with AMF. Given these different results, it is not clear how the presence of DSE may affect AMF colonization of Wyoming big sagebrush and Sandburg bluegrass seedlings. Thus, an additional motivation for the present study was to investigate this question.

Again, the first goal was to isolate and identify a DSE from *A. tridentata* ssp. *wyomingensis*. Subsequently, experiments were designed to investigate the effects of the

isolated fungus in two plant species, to answer the following questions. How does DSE penetration affect the integrity of root cells and tissues? Is the nature of the plant-DSE symbiosis parasitic, commensalistic, or mutualistic? Does the presence of the DSE alter AMF colonization? Are the effects of the DSE similar in *A. tridentata* and *P. secunda*? Overall, answers to these would increase our understanding of symbiotic interactions between DSEs, AMFs, and plants. Furthermore, knowledge in these areas may help to identify biotic factors that affect the performance of *A. tridentata*, which ultimately could be used to improve the reestablishment of this shrub.

MATERIALS AND METHODS

Fungal Isolation

Roots for the isolation of DSE were collected from one to two-year-old seedlings of Wyoming Big Sagebrush (henceforth referred to as *A. tridentata*) growing at the Morley Nelson Snake River Birds of Prey National Conservation Area in southwestern Idaho, USA (43°19.272'N, 116°23.643'W; 873 m). The sample site appeared to be in relatively pristine condition as judged by the low abundance of annual exotic grasses and the presence of perennial native grasses, native forbs, and biological soil crusts.

Roots were cut in approximately 5 cm segments and were extensively washed in running tap water to remove all soil particles. Subsequently, the roots were surface sterilized by soaking them in 70% ethanol for 1 min and 0.5% sodium hypochlorite for 30 min, followed by five rinses with sterile water. After surface sterilization, the root segments were further cut into 0.5 cm fragments and placed in water agar plates, which were inspected every 48 h for two weeks. Hyphae growing from the cut ends were transferred to potato dextrose agar for further analysis. From the various fungi collected using this method, 8-10 Petri dishes were obtained hosting mostly Mucoraceae and one sterile but melanized and septate species.

Sporulation Attempts

I decided to test whether the sterile melanized and septate fungus could be induced into forming reproductive structures. Various cryptic species of fungi form asexual and sexual spore structures when exposed to abiotic and biotic factors (Su et al.,

2012). To test this, the isolate was exposed to some of the conditions used by Knapp (2015) seeking both the anamorphic and teleomorphic reproductive stages, which together would give the holomorph for this fungal species.

The DSE isolate was incubated in four culture media: full strength PDA, 1% PDA, 1/2 MS medium with Gamborg's vitamins at pH 5.7 (hereafter referred to as 1/2 MS) with 1% sucrose, and 1/2 MS without sucrose. The two MS media had 0.3% phytagel as the gelling agent. The use of 1% PDA and the 1/2 MS medium without sucrose was aimed at simulating a limited nutrient environment. A 5 mm plug of DSE colonized PDA was taken from the growing front of the fungus and used to inoculate each of the media, with four Petri dishes per medium type. Cultures were grown in the dark at room temperature.

Apart from the different culture media, I also tested whether putative stress conditions and dead plant material could induce the formation of asexual or sexual spores. In terms of stress conditions, I exposed DSE growing in full strength or 1% PDA to slow drying of the Petri dishes, 4 °C for two weeks, or exposure to UV light for 15 minutes. Mandyam and Jumpponen (2005) suggested that N derived from the breakdown of dead plant material may facilitate the formation of asci and ascospores. To test this, I also added to some Petri dishes with 1/2 MS plus 1% sucrose autoclaved stems of various plant species. The species tested were *Achillea millefolium* (Western Yarrow), *A. tridentata sp. wyomingensis* (Wyoming Big Sagebrush), *Ericameria nauseosa* (Rubber Rabbitbrush), *Leymus cinereus* (Great Basin Wildrye), *Mentha arvensis* (Mint), *Oryzopsis hymenoides* (Indian Ricegrass), *Poa secunda* (Sandbergs Bluegrass), *Urtica dioica* (Stinging Nettle).

Molecular identification

To identify the isolated DSE fungus, it was initially grown in 1/2 MS containing 1% sucrose and 0.3% phytigel. Subsequently, the phytigel was dissolved with a sterile solution of 10 mM citric acid (pH 6.0) (Doner and Bécard, 1991) and the hyphae used for DNA extraction with the FastDNA® Green Spin Kit (MP Biochemicals). For molecular identification, the ITS region and part of the LSU region of genes for ribosomal RNA were amplified using the primer pairs ITS1F-ITS4 and LR1-FLR2, respectively. Amplification products were cleaned using ExoSAP-IT and sequenced in both directions at a commercial facility (Genewiz, Inc). The consensus sequences for the ITS and LSU regions were compared to available GenBank sequences using the BLAST tool to ascertain that they were from Ascomycota, and to identify closely matching sequences. The sequences from the isolated fungus plus closely related sequences from known taxa were downloaded from GenBank, initially aligned with ClustalW2, and then manually reviewed and adjusted using Phyde® v0.9971.

Further taxonomic analysis of the DSE isolate using the partial sequences of the 18S nrRNA (SSU), the 28S nrRNA (LSU), and the transcription-elongation factor 1- α (TEF) genes, which were amplified and sequenced with the NS1/NS4, LR0R/LR5, and EF1-728F/EF2Rd primer pairs, respectively. These sequences were combined with closely related sequences of known taxa (Knapp et al., 2015) and used to conduct a three-loci phylogenetic analysis. Sequences were first aligned with MUSCLE (Edgar, 2004) and then manually reviewed and adjusted using MEGA v.7 (Kumar et al., 2016).

Maximum parsimony analyses were performed using PRAP2 (Muller, 2004) in conjunction with PAUP*4.0b10 (Swofford, 2002). Bootstrap support (BS) for nodes was

estimated with 1,000 heuristic replicates using PRAP2 (Felsenstein, 1985). Descriptive statistics reflecting the amount of phylogenetic signal in the parsimony analysis were given by the consistency index (CI), retention index (RI), and the resulting rescaled consistency index (RC) (Kluge and Farris, 1969; Farris et al., 1994). Bayesian analyses were run with MrBayes 3.2. Molecular evolutionary models for Bayesian analysis were estimated with jModelTest (Guindon and Gascuel, 2003; Posada, 2008). The best fit model was GTR+G for SSU, and GTR+I+G for LSU and TEF. Bayesian analyses were performed using four-to-one heated chains for ten million generations. Convergence was determined by viewing in Tracer v1.3 (Drummond et al., 2005), and burn-in of 50,000 generations was discarded prior to sampling the posterior distribution.

Plant Material and in Vitro Tests

To ascertain that the DSE isolate was a root endophyte, I conducted *in vitro* tests starting with seeds of *A. tridentata ssp. wyomingensis* harvested near Big Foot Butte, Idaho (43° 18' 48.43" N, 116° 21' 48.57" W). The seeds were washed under running tap water for 30 minutes, and subsequently, surface-sterilized by soaking them in 70% ethanol for 15 seconds, followed by soaking in 0.5% sodium hypochlorite with 1% Triton X-100 for 15 min, and five final rinses with sterile water. These surface-sterilized seeds were placed in magenta vessels (GA-7) containing 100 ml ½ MS with 0.3% phytagel. Four weeks after germination, five vessels were inoculated, each with approximately ten seedlings, with PDA plugs (5 mm diameter) containing the isolated DSE hyphae from the growing front of the culture. Each plug was placed in a 1 cm deep hole punched into the phytagel next to the roots of the seedling. Similar procedures were done for *P. secunda*, except that the seedlings were inoculated two weeks after germination.

Eight weeks after inoculation, seedlings from both species were harvested, weighed, and examined for necrotic areas or other apparent signs of damage. Additionally, the distribution of the hyphae and microsclerotia were examined in root whole mounts and tissue sections. Before observation of whole mounts, roots were cleared in 5% KOH for 3 min at 121 °C. Subsequently, the roots were rinsed in water and incubated with Alexa Fluor 488 wheat germ agglutinin (488-WGA) ($10 \mu\text{g ml}^{-1}$ in PBS for 1h). Samples were then rinsed in water, mounted on 50% glycerol, and observed under bright-field microscopy to identify melanized hyphae and microsclerotia, and fluorescence microscopy to identify hyaline-septate hyphae. These kinds of hypha represent an active growth stage in DSE that eventually progresses to melanized hyphae (Barrow, 2003).

The distribution of the fungus within the roots was analyzed in plastic-embedded root sections. Prior to embedding, root segments were fixed in 4% (w/v) paraformaldehyde in 50 mM Pipes (pH 6.9) containing 5 mM MgSO_4 and 5 mM EGTA. Tissue was fixed for 3 h at 4 °C. The samples were then rinsed twice in the same buffer, twice in deionized water, and dehydrated in a graded ethanol series. After dehydration, the samples were infiltrated and embedded in JB-4 resin (Polysciences) and sectioned at a thickness of 5 μm . Sections were incubated with 488-WGA to detect fungal hyphae and subsequently stained with propidium iodide or calcofluor-white to visualize plant cells. Observations were made using an Olympus BX-60 fluorescence microscope, and images were taken with a Micropublisher QImaging 5.0 camera.

Preparation of additional inoculum

To obtain inoculum that could be more readily used to inoculate seedlings both *in vitro* and soil, a suspension of chlamydospores as well as a fungal spawn was prepared. While growing the DSE isolate in Petri dishes containing ½ MS without sucrose, it was noticed that the radiating hyphae changed from a cottony to ropy growth form and it also developed chlamydospores. The chlamydospores were harvested and tested for their viability. For this purpose, the phytagel was digested with a sterile solution of 10 mM citric acid at pH 6.0. Subsequently, the fungal tissue was collected through a 50 µm mesh and finely chopped and resuspended in sterile deionized (DI) water. This suspension was plated on PDA, and growth was observed from both hyphal fragments and chlamydospores. Suspensions prepared similarly were later used to inoculate *A. tridentata* or *P. secunda* seedlings *in vitro*. This approach resulted in more uniform colonization than that obtained with gel plugs.

Another approach that can be used to produce fungal inoculum is via the production of a fungal spawn. Spawn is the term used when a fungus is grown through a carrier medium, such as seeds or sawdust. This spawn can be applied to inoculate larger volumes of soil or compost and represent a more convenient and less costly approach than the multiplication of fungi in PDA or other media. I prepared the spawn by bringing a mixture of Sudan grass seeds and water to a boil for 5-10 minutes and then reducing the heat to a simmer for an hour. The waterlogged seeds were allowed to cool, strained, and autoclaved for 60 minutes in jars that were ¾ full with Sudan grass seeds. When the jars reached room temperature, a half plate of PDA colonized with DSE was cut into smaller pieces and added to each spawn jar. The jars were shaken once a week until most grains

showed signs of infection by the DSE isolate. To ascertain that the spawn contained the fungus of interest, I extracted DNA from two to three infected grains and tested for the presence of the isolated DSE by PCR. Using the *Darksidea* specific primers DSE7F and DSE7R (Knapp et al., 2015), amplicons of the expected size (330 bp) were recovered, while no band was observed in autoclaved Sudan grass seeds that were not inoculated.

Tests of interactions between DSE and AMF

The effect of the isolated DSE on AMF colonization as well as the effect of AMF on DSE colonization was tested in two experiments. Both experiments had four inoculation treatments: -DSE-AMF, +DSE-AMF, -DSE+AMF, +DSE+AMF. However, the experiments differed in the manner in which the seedlings were initially grown and the method of inoculation. In one experiment, I initially grew and inoculated the seedlings with DSE *in vitro*. In the other experiment, I planted the seeds directly in the soil, which for the inoculation treatments also contained the inoculum. The details of these two experiments are described below.

Initial growth and inoculation in vitro

Before transplanting, seedlings of both species were grown aseptically on 1/2 MS with 1% sucrose, as described earlier. Half of the Magenta boxes were controls, and the rest were inoculated with a suspension of the DSE chlamydospores. This suspension was added approximately two weeks after germination at 2 cm from the bottom of the Magenta vessel. Seedlings were grown *in vitro* until the roots of the host plants and the isolated DSE fungi had grown together, and melanized hyphae were visible on the roots under the dissecting microscope. At this time, control and inoculated seedlings were randomly assigned to either of two AMF treatments, -AMF or +AMF. Those assigned to

the AMF treatment were inoculated with a suspension of *Rhizophagus irregularis* spores, which I extracted from *in vitro* cultures growing in Ri T-DNA transformed carrot roots. Seedlings for the –AMF treatment received 5 ml of water which was similar to the volume of the applied suspension. The two AMF inoculation treatments were applied when the seedlings were placed in 3.8 x 20.3 cm cone-tainers, which were partially filled with a 3:1 mixture of washed sand and native soil. Subsequently, the cone-tainers were filled with this mixture, and covered with a clear plastic cup to reduce transplanting shock. I collected the soil used in this experiment at a sagebrush steppe community in Dedication Point, Idaho (43°16'36" N, 116°23'38" W). This silty-loam soil was screened through a 1 mm mesh to remove leaf litter and roots, and after mixing with sand, the mixture was autoclaved twice, for 1 h each time. After transplanting to the sand:soil mixture, the seedlings were grown at the Boise State greenhouse, which had a 15- hour photoperiod and day/night conditions of 23/18 ±3°C.

Seedlings were grown for about two months and subsequently analyze for DSE and AMF colonization. For this purpose, the roots were cleared and then stained with Alexa Fluor 488 wheat germ agglutinin, as described earlier. Fluorescence microscopy was used to identify arbuscular mycorrhizal structures and hyaline-septate hyphae of DSE fungi, and the bright field to identify melanized hyphae and microsclerotia of DSE fungi. I identified and tallied the different mycorrhizal and DSE fungal structures using the intersection method of McGonigle (1990) with about 150 intersections per sample. These data were used to calculate the percent colonization for each structure of both AMF and DSE fungus.

Planting of seeds and inoculation in soil

Due to the limited survival of aseptically-grown seedlings upon transplanting to the soil-sand mix, I planned a different experiment, which started with seeds planted in soil. For each plant species, I conducted a completely randomized factorial combination experiment consisting of two AMF inoculation treatments (-AMF and +AMF) and two DSE inoculation treatments (-DSE and +DSE). As in the previous experiment, the AMF used in this study was *Rhizophagus irregularis*, which was grown *in vitro* in Ri T-DNA transformed carrot roots. The spores and Ri T-DNA carrot root fragments were extracted from these cultures and used for inoculating seedlings. The DSE inoculum was the spawn in Sudan grass seeds described earlier, whereas batches of Sudan grass seeds without inoculation were prepared as controls.

The inoculum was placed within 3.8 x 20.3 cm cone-tainers filled with the same 3:1 autoclaved soil:sand mixture used for the experiment with transplanted seedlings. The cone-tainers for the -DSE treatments received 5-6 control grains of uninfected spawn and the +DSE treatment 5-6 grains of living spawn infected with the isolated DSE fungus, which were placed at a depth of approximately 5 cm from the surface. Similarly, the cone-tainers for -AMF treatment received 2000 dead spores and one gram of sterile root fragments containing *R. irregularis* that were autoclaved twice for one hour. The cone-tainers for the +AMF treatments received live spores and root fragments added at a depth of about 2 cm. After adding the inoculum, the cone-tainers were topped off with the soil mixture, and seeds were planted just below the surface and covered with soil. When the seeds had germinated, they were thinned to 3 seedlings per cone-tainer. Ten cone-tainers for each plant species and inoculation treatment were prepared for a total of

totaling 80 cone-tainers. The seedlings were grown in the greenhouse under a 15- hour photoperiod with day/night conditions of 23/18 \pm 3°C.

One cone-tainer of each plant species and treatment was harvested 5 to 8 months after planting at two-week intervals. A third of the fresh weight of the root system from each seedling to measure AMF and DSE fungal colonization, and the remaining roots for estimating dry root and shoot weights. Colonization analysis was conducted as in the previous experiment. The shoots and remaining roots were dried in an oven at 120° C for 24 hours, the weight was recorded, and checked every 12 hours until no change in weight was detected. This final weight was recorded for both shoots and roots. The percent dry weight over fresh weight in each sample was estimated from the root dry weight. This percent was multiplied by the fresh weight of the roots used for colonization to estimate the dry weight of the latter ones. This value was then added to the previously measured root dry weight to approximate the total root dry weight in each sample.

STATISTICAL ANALYSES

To assess the effectiveness of inoculation with the DSE isolate on causing colonization by this fungus, I compared the colonization of -DSE and +DSE seedlings. Similarly, the effectiveness of inoculation with *R. irregularis* on AMF colonization was determined by comparing the colonization of the -AMF and +AMF treatments. A Krustal-Wallis test was used rather than a parametric analysis because the data were not normally distributed.

The effect of DSE on AMF colonization was determined using a linear mixed model with the package nlme in R (Pinheiro et al., 2019). Separate analyses for *A. tridentata* and *P. secunda* was conducted, and in both cases, sampling time was treated as a random factor. For these analyses, only the +AMF seedlings were included. In -AMF seedlings, AMF colonization was minimal, and the inclusion of these values resulted in a lack of normality of residuals. A similar approach to analyze the effect of AMF on DSE colonization was used. In this case, AMF inoculation was treated as the fixed factor, and only +DSE seedlings were included in the analysis. The effects of the inoculation treatments on plant biomass were analyzed with a model that had DSE inoculation, AMF inoculation, and their interactions as fixed factors and sampling time as a random factor. When necessary, different variances were modeled in the nmle procedure to allow for unequal variances between treatments. Significant differences between treatments were estimated using the emmeans function (emmeans package in R) with *p*-values adjusted

for multiple comparisons by the Tukey method. All estimates of treatment variability are reported as standard errors.

RESULTS

Sporulation Tests

The nutrient limitation tests were ineffective in inducing sporulation of the isolated DSE except for the 1/2 MS medium without sucrose. In this medium, the DSE changed from a cottony to a ropy growth form, and chlamydospores abundantly developed. The slow drying, refrigeration, and UV light treatments and the autoclaved stem tests all failed to induce sporulation.

Molecular Identification

The BLAST results from partial sequences obtained from the ITS and LSU genes revealed a 98 to 99% identity with sequences of several *Darksidea* taxa. *Darksidea* is within the Lentitheciaceae, which is in the Pleosporales that resides in the phylum Ascomycota. The phylogenetic relationship of the isolated DSE fungus with *Darksidea* and other Lentitheciaceae taxa was further investigated using the partial sequences of the SSU, LSU, and TEF genes from our sample plus similar sequences from Knapp et al. (2015). The maximum parsimony and Bayesian analyses of the concatenated sequences produced trees with similar structure, and only the latter is shown (Fig. 1). The isolated DSE was more closely related to *Darksidea* than to other genera. However, the isolated DSE was not nested within the *Darksidea*, but it appeared as a strongly supported sister taxon.

In Vitro Tests and Anatomical Observations

Although colonization of seedlings grown *in vitro* was not quantified, hyphae were copious along the length of the roots. Despite high level of hyphae, no significant differences in fresh weight were detected between non-inoculated and inoculated seedlings. The average weight of non-inoculated and inoculated seedlings of *A. tridentata* was 40.5 and 30.2 mg per seedling, respectively ($p = 0.39$). Similarly, the average weight of non-inoculated and inoculated seedlings of *P. secunda* was 38.6 and 29.1 mg per seedling, respectively ($p = 0.39$). There was no sign of damage apparent to the inoculated seedlings.

In *A. tridentata*, the hyphae were found from the differentiation to the mature zone. Some of the hyphae appeared attached to the outer walls of epidermal cells, where they tended to run parallel to the long axis of the roots (Fig. 2A). The hyphae also penetrated the epidermis, cortex, and vascular cylinder (Fig. 2B), showing predominately an intracellular location (Fig. 2D and E). In the young seedlings, where we conducted the anatomical analysis, most of the hyphae were hyaline. However, dark hyphae and sclerotia were also present (Fig. 2C). In *P. secunda*, the distribution of hyphae was similar to that in *A. tridentata*. However, the extent of penetration inside the root varied between the two species. In *P. secunda*, the hyphae penetrated the root epidermis and cortex, but not in the vascular cylinder (Fig. 2F).

Initial growth and inoculation in vitro

Transplanting from *in vitro* conditions to cone-tainers in the greenhouse resulted in many losses of *A. tridentata* and *P. secunda* seedlings. Despite that, I was able to grow successfully after transplanting 4 to 8 seedlings per species and treatment. These

seedlings were analyzed for DSE and AMF colonization, but overall the extent of colonization observed was low. For *A. tridentata*, the DSE colonization started *in vitro* did not persist after transplanting to soil; there were no differences in DSE colonization between –DSE and +DSE treatments (Fig. 3A, $p = 0.62$). Similarly, only a few of the AMF inoculated seedlings showed high levels of AMF colonization, and I did not detect differences between the –AMF and + AMF treatments (Fig. 3B, $p = 0.72$).

For *P. secunda*, DSE colonization in the +DSE treatments was overall higher than in the –DSE treatments (Fig. 4A). However, this difference depended on the AMF inoculation treatment. Only the -AMF+DSE had higher DSE colonization than either of the –DSE treatments. The +AMF+DSE treatment had higher DSE colonization than the +AMF-DSE treatment ($p = 0.004$), but similar colonization to the –AMF-DSE treatment ($p = 0.6$, Fig 4A). Inoculation of *P. secunda* with *R. irregularis* increased AMF colonization compared to the non-inoculated seedlings, but only in the +AMF+DSE treatment ($p = 0.02$, Fig. 4B). However, AMF colonization in this treatment was only 4%, indicating that the method used for AMF inoculation was not effective.

A central goal of this experiment was to analyze the effect of DSE on AMF colonization and vice versa. However, the low and variable rates of DSE and AMF colonization achieved in +DSE and +AMF treatments, prevented such analyses.

Planting of seeds and inoculation in soil

In contrast to the previous experiment, inoculation in soil resulted in clear differences in colonization between non-inoculated and inoculated seedlings. For *A. tridentata* the median value for DSE colonization was less than 5% in the –DSE treatments, and above 20% in +DSE treatments (Fig. 5A). Differences in colonization

were greater between the AMF treatments. AMF colonization in the –AMF treatments was negligible, while median values for the +AMF treatments were above 50%. (Fig. 5B). *Poa secunda* showed similar results, except for a more marked effect of DSE on increasing AMF colonization (Fig. 6).

Effects of DSE inoculation on AMF colonization

To analyze the impact of DSE inoculation on AMF colonization, I conducted statistical analyses after excluding the –AMF treatments. In *A. tridentata*, total AMF colonization was 44.6 (± 7.5) and 56.6 (± 7.8) % for the –DSE and +DSE treatment, respectively ($p = 0.09$, Fig. 7A). Although DSE inoculation did not have a significant effect on total AMF colonization, it affected arbuscular colonization and the occurrence of intraradical storage structures, such as vesicles and intraradical spores. The +DSE treatment had lower arbuscular colonization compared to the -DSE treatment ($p = 0.005$, Fig. 7A), while the opposite was observed for storage structures. The abundance of intraradical storage structures in the +DSE treatment was twice the level found in the -DSE treatment ($p = 0.01$, Fig. 7A).

For *P. secunda*, colonization by DSE increased total AMF colonization, which was 16.9 (± 5.6) % for the –DSE treatment and 42.6 (± 2.90) % for the +DSE treatment ($p = 0.003$, Fig. 7B). Co-inoculation with AMF and DSE was also associated with an increase in AMF storage structures, which had average values of 7.8 (± 3.6) and 26.0 (± 3.6) % for the –DSE and +DSE treatment, respectively ($p = 0.004$, Fig. 7B). In contrast to total AMF and storage structures, DSE inoculation did not have a significant effect on arbuscular colonization, which was 1.7 (± 1.7) and 6.5 (± 1.7) % without and with DSE, respectively ($p = 0.08$, Fig. 7B).

Effect of AMF inoculation on DSE colonization

In seedlings inoculated with DSE, AMF inoculation did not affect the DSE colonization, with total DSE at 25.2 (± 2.6) % for the $-AMF+DSE$ treatment and 20.4 (± 2.7) % for seedlings co-inoculated with DSE and AMF (Fig. 8A, $p = 0.25$). Similarly, inoculation with AMF did not alter the occurrence of sclerotia (Fig. 8A). Overall, the presence of these resting structures was low, averaging 2.5 (± 0.8) and 2.8 (± 0.8) % for the $-AMF$ and $+AMF$ treatments, respectively ($p = 0.96$).

The results for *P. secunda* were similar to those with *A. tridentata*. Total DSE colonization was 24.7 (± 3.9) % for the $-AMF+DSE$ treatment and 16.1 (± 4.1) % for seedlings co-inoculated with DSE and AMF (Fig. 8B, $p = 0.2$). Similarly, AMF inoculation did not affect the occurrence of sclerotia (Fig. 8B), with an incidence of 1.00 (± 0.35) and 1.05 (± 0.69) % in $-AMF$ and $+AMF$ inoculated seedlings, respectively ($p = 0.45$).

Plant biomass response to inoculation with AMF and DSE

The dry weight of *A. tridentata* was not affected by AMF ($p = 0.31$) or DSE ($p = 0.91$) inoculation and the interaction between these treatments was not significant ($p = 0.52$) (Fig. 9A). In contrast, for *P. secunda*, there was an interaction between the AMF and DSE treatments ($p = 0.03$); inoculation with AMF decreased the dry weight of *P. secunda* seedlings ($p = 0.01$), but only in the $-DSE$ treatments (Fig. 9B).

DISCUSSION

Taxonomic identification

This study demonstrates the challenges and rewards of an exploratory research agenda in a complex symbiotic system. First, a DSE fungus colonizes local *A. tridentata* roots under natural conditions. While other studies have reported the presence of DSE in *A. tridentata* (Gehring et al., 2016), to my knowledge, this is the first attempt to isolate a DSE from this shrub and ascertain its endophytic nature through an *in vitro* resynthesis assay. DSE are a paraphyletic group found within several orders within the Pezizomycotina and are among the most common fungi colonizing plant roots (Grünig et al., 2011; Knapp et al., 2015). Based on partial sequences of five genes, the isolated DSE from Idaho is within the *Darksidea* or a closely related sister group to known species that have been sequenced. Knapp et al. (2015) established the *Darksidea* genus based on phylogenetic analyses of DSE isolates from grass roots collected at the Great Hungarian Plain. According to the phylogenetic tree in Fig. 1, the *Darksidea* species reported by Knapp et al. (2015) are more closely related to each other than they are to the DSE that I isolated from *A. tridentata* roots. Such results would be expected given the large geographic distance between Knapp et al. (2012) and my collection sites. This distance is likely linked to a longer separation time from a common ancestor and different selection pressures, both of which increase the possibilities for genetic divergence (Cabej, 2012).

Attempts to obtain holotype material for the DSE were challenging. This is not surprising and traditionally have placed DSE in the Deuteromycota due to the lack or

evidence of sexual spore formation. The absence of ascospore, sexual spore, development in my isolate is also in general agreement with the results observed with *Darksidea* species by Knapp et al. (2015). Most of the *Darksidea* species that they tested did not form ascocarp with the exception of *Darksidea alpha* (Knapp et al., 2015). Some isolates of *D. alpha* developed ascocarps; however, subsequent attempts to induce sporulation were unsuccessful, indicating the rarity of sexual reproduction in this group of fungi (Knapp et al., 2015).

Species of *Darksidea* are within the Lentitheciaceae in the Pleosporales (Knapp et al., 2015). Members of the Lentitheciaceae are root endophytes in a wide range of habitats (Green et al., 2008; Knapp et al., 2012; Loro et al., 2012; Porrás-Alfaro et al., 2011b). However, thus far, *Darksidea* species have only been found in semiarid and arid environments. Apart from the Great Hungarian Plain, analyses of the fungal community of the desert grass *Bouteloua gracilis* from a semiarid grassland in New Mexico and of the grass *Stippa grandis* from the Inner Steppe of Mongolia revealed ITS sequences that exhibit high similarity with those obtained from *Darksidea* isolates (Porrás-Alfaro et al., 2008; Su et al., 2010). The finding that *Darksidea* or a very closely related sister group is present in sagebrush steppes of southern Idaho adds to the notion that this genus is common in arid lands and has a wide geographical distribution (Knapp et al., 2015). In addition, my finding indicates that species within this genus are not limited to grasses and that they may have low host specificity. At least, that seems to be the case for my isolate, which readily colonized both *A. tridentata* and *P. secunda*.

Nutritional characteristics and nature of the symbiosis

The DSE isolated in this study grew in autoclaved grains suggesting that it can live as a saprophyte. However, further work is needed to ascertain that it can grow in other materials such as dead leaves or roots. As an endophyte, the DSE isolated was symptomless, with no obvious cell or tissue damage in the roots colonized by this fungus. Furthermore, for both species, differences in dry weight between the –DSE and +DSE treatments were not significant, and that was true for seedlings growing *in vitro* as well as in soil. Thus, under the two conditions tested, the relationship between the seedlings and DSE was commensalistic.

Symbiotic associations between plants and fungi can vary from parasitic to mutualistic depending on environmental conditions (Allen et al., 1993; Johnson and Graham, 2013; Konvalinková and Jansa, 2016; Mandyam and Jumpponen, 2015). For fungi such as DSE and AMF that can improve plant-nutrient uptake, the benefit of the association may not occur if plants are growing in small pots that restrict the fungal ability to search for nutrients (Allen et al., 2003; Jumpponen, 2001; Poorter et al., 2012). A meta-analysis by Poorter et al. (2012) indicated that a plant-biomass to pot volume ratio higher than 1 g L⁻¹ leads to growth limitations due to the small size of the pot. For the cone-tainer experiment, the proportion of *A. tridentata* biomass to pot volume at the time of harvest was approximately 1 g L⁻¹, suggesting that the pot size was adequate for the analysis of growth responses. In contrast for *P. secunda*, the biomass to pot volume ratio was about 2.4 g L⁻¹. Thus, for *P. secunda* the small soil volume may have prevented a potential positive response to DSE.

Another environmental factor that can affect the nature of plant-fungal symbiosis is light intensity. In the *in vitro* and soil tests, the seedlings were exposed to lower light intensities than those typically experienced in the field. As light intensity decreases, the C gain associated with the symbiosis tends to decline and eventually be similar or lower than the cost of maintaining the fungus. This can lead to neutral and parasitic associations (Konvalinková and Jansa, 2016).

Limitations to carbon gain due to the size of the pot or low light may also explain the observed response to AMF colonization. The symbiosis between these fungi and *A. tridentata* has led to enhancements in P uptake and plant biomass, and is, in general, considered mutualistic (Allen et al., 1993; Stahl et al., 1998). However, I did not detect differences in *A. tridentata* biomass between the –AMF and +AMF treatments, and *P. secunda* showed a negative growth response to AMF. Busby et al. (2011) reported similar results for potted *A. tridentata* and *P. secunda* seedlings grown in a greenhouse. Thus, there are conflicting reports about the growth response of *A. tridentata* to AMF colonization, which suggests that further work is needed to characterize the environmental conditions that result in mutualistic effects (Hovland et al., 2019).

At the anatomical level, the interaction between the DSE isolate and the roots was somewhat similar for the two host species. Along the root surface and inside the roots both melanized and hyaline hyphae were observed. In contrast, in the soil, the fungus produced melanized hyphae primarily. Barrow and Aaltonen (2001) indicated that hyaline hyphae represent an active stage in DSE that eventually progresses to melanized hyphae. Melanization may also be triggered by stressful environmental conditions such as large temperature fluctuations, high light intensity, reactive oxygen species (ROS), and

low water or nutrient availability (Cordero and Casadevall, 2017). An increase in the production of ROS is a common plant defense to fungal infection (Segal and Wilson, 2018). Melanin can neutralize ROS, suppressing the effectiveness of these plant defenses, which facilitates fungal access to the plant host cells (Jacobson et al., 1995; Segal and Wilson, 2018). Based on these considerations, it seems possible that some degree of melanization by the DSE contributed to colonization. However, and particularly *in vitro*, very extensive colonization with little Melanization was observed, suggesting that melanin synthesis is not a significant factor contributing to infection.

For seedlings in cone-tainers, the high proportion of melanized extraradical hyphae is consistent with a function of melanin in protecting against dry conditions (Cantrell et al., 2011; Cordero and Casadevall, 2017). Even though I watered the seedlings daily, the small size of the pots combined with the high proportion of sand could have resulted in dry areas within the cone-tainers. Melanin is hygroscopic and can also reduce cell wall porosity (Kogej et al., 2007). These characteristics could have made the hyphae more hypertonic and minimize water loss from the fungus to dry areas of the sand: soil mix (Cordero and Casadevall, 2017; Kogej et al., 2007). Clearly, the inside of the roots and the tissue culture medium provided more constant moist conditions, and if so, the need for melanization to cope with desiccation would be less critical.

For both *A. tridentata* and *P. secunda* roots melanized hyphae, microsclerotia, and hyaline hyphae were observed in the cortex, with the latter also in the vascular cylinder of *A. tridentata* roots. While colonization of the epidermis and cortex is typical for DSEs, penetration to the vascular cylinder seems to be less frequent (Peterson et al., 2008). In some cases, such penetration has been associated with the breakdown of cells within the

stele and therefore considered pathogenic (Fernando and Currah, 1996; Wilcox and Wang, 1987). In contrast, anatomical studies by Barrow and Aaltonen (2001) and Barrow (2003) in the shrub *Atriplex canescens* and the grass *Bouteloua* sp. showed extensive colonization of the vascular cylinder by an unknown DSE without apparent structural damage to the colonized cells. These observations are in agreement with those of my own embedded cross-sections of *A. tridentata* roots. Hyaline hyphae were abundant in the vascular cylinder, but their presence was not associated with noticeable structural changes or breakdown of the plant cells. These results, combined with the lack of adverse effects on growth, suggest that the presence of hyphae in the vascular cylinder is not necessarily indicative of pathogenicity. On the contrary, the proximity to the vascular tissues might facilitate nutrient exchange between the partners and contribute to mutualism (Barrow, 2003).

Interestingly, *P. secunda* was not observed with hyphae in the vascular cylinder. The extent to which a particular DSE colonizes roots can vary between different plant species. For example, *P. fortinii* hyphae were present within the stele of *Pinus resinosa*, but the hyphae did not reach the vascular cylinder of *Pinus strobus* despite colonization of epidermal and cortical cells (Peterson et al., 2008; Wilcox and Wang, 1987). A similar situation may be occurring between the isolated DSE and the two native plants tested. However, I cannot dismiss the possibility that the difference between the two species reflects incomplete anatomical analysis. The root cross-sections where I detected hyphae in *P. secunda* had a well-developed endodermis, which may have restricted penetration. The endodermis is less developed or fractured in growing regions of the roots and at sites

of lateral root formation. Anatomical studies in these regions would help to ascertain whether hyphae are excluded throughout the vascular cylinder or only in some areas.

Comparison of inoculation methods

The two attempts to inoculate seedlings with the DSE isolate and *R. irregularis* yielded different results. Even though seedlings were colonized by the DSE isolate *in vitro*, roots that developed after transplanting showed little colonization. Similarly, inoculation with *R. irregularis* during transplanting resulted in negligible levels of colonization. One possible explanation is that transplanting reduced the signals that mediate plant-fungal symbioses. Strigolactones, in particular, are known to play a critical role in the establishment of plant-AMF associations (Sbrana et al., 2015). As judged by the high mortality of seedlings after transplanting, the plants experienced severe stresses during this period. Perhaps, stresses associated with the handling of seedlings and changes in humidity and temperature reduced strigolactone production and AMF colonization (Bhojwani and Dhawan, 1989; Kapulnik and Koltai, 2014; Liu et al., 2013). Upon transplanting seedlings were kept in shaded areas of the greenhouse to help them adapt to the new environment. This placement may have resulted in a low red/far-red ratio, which can also lead to reductions in strigolactone synthesis (Nagata et al., 2015).

Little is known about signals that mediate interactions between plants and dark septate fungi (Mandyam and Jumpponen, 2015). However, studies of the symbiosis between *Arabidopsis thaliana* and the endophytic *Falciphora oryzae* indicate that root exudates promote the growth of this fungus and suggest that exchange of signals initiate the symbiosis (Sun et al., 2020). Thus, it is plausible that changes in the composition or

amount of root exudates associated with transplanting may have prevented continued colonization of the seedlings by the DSE isolate.

Seedlings planted in soil did not suffer from transplanting stress. As the seeds germinated and the seedlings grew, they may have been in better conditions to initiate symbioses than transplanted seedlings. Also, the type and position of the inoculum may have favored fungal colonization in the former. For seed planted in soil, the AMF inoculum consisted of spores and colonized root fragments containing vesicles. Intraradical vesicles are active AMF propagules, which could have increased colonization (Biermann and Linderman, 1983; Klironomos and Hart, 2002). Further, the AMF inoculum was placed in a small area of the soil, which contrasts with the more dispersed application of spores over the roots of transplanted seedlings. Based on simulations of hyphal growth conducted by Schnepf (2016), placement of concentrated AMF propagules increases the chances of colonization compared to a dispersed distribution.

Concerning DSE, a difference between transplanted seedlings and seeds directly planted in soil was that in the latter, the inoculum was the spawn in Sudan grass grains. As indicated earlier, DSEs are not obligate biotrophs and can have a saprophytic lifestyle (Schlegel et al., 2016). The presence of dead organic matter not only can sustain the DSEs but also improve the benefits of the symbiosis (Green et al., 2008; Mahmoud and Narisawa, 2013). For the transplanted seedlings, the possibility of maintaining a saprophytic lifestyle was probably minimal because I removed most of the organic litter from the soil: sand mix. For the seeds placed in the soil: sand mix, I also removed the litter. However, the added grains may have facilitated the growth of the DSE inoculum, thus resulting in more consistent colonization of the host roots.

Effects of DSE inoculation on AMF colonization

The results of this study indicate that inoculation with the DSE isolate increased total AMF colonization in one of the plants tested, *P. secunda*, and enhanced the formation of storage structures in both *P. secunda* and *A. tridentata*. Many studies have reported the co-occurrence of DSE and AMF in the roots of the same plant (e.g., Chaudhry et al., 2009; Lingfei et al., 2005; Seerangan and Thangavelu, 2014). However, few have analyzed how the presence of one type of fungus affects the other. The studies that have investigated the effect of DSE on AMF colonization have revealed two general patterns. Saravesi (2014) and Berthelot et al. (2018) reported no effect of DSE on total AMF colonization of *Medicago sativa* (alfalfa) and *Lolium perenne* (Ryegrass), respectively. In contrast, Arriagada et al. (2012) saw an increase in total AMF colonization when the roots of *Vaccinium corymbosum* (Blueberry) were co-inoculated with several different saprophytic fungi. Similarly, Sabra et al. (2018) recorded increases in total colonization of *Ocimum basilicum* (sweet basil) by *R. irregularis* with the addition of the basidiomycete *Serendipita indica*. They attributed this increase in AMF colonization to immune suppression response within the host promoted by *S. indica*. A similar increase in *R. irregularis* was also observed on *Lactuca serriola* (Prickle Lettuce) grown with *Mucor* species in uncontaminated soils (Wazny et al., 2018). Thus, the reports available suggest that endophytic fungi either have no effect or promote total AMF colonization, which is in agreement with the results I obtained for *A. tridentata* and *P. secunda*, respectively.

The most consistent effect of DSE inoculation was increasing the abundance of AMF intraradical storage structures, mainly vesicles. I could not find any report of DSE

effects on specific AMF structures, but Scervino et al. (2009) described results that may explain the changes observed. They extracted exudates from a DSE, *Dreschlera sp.*, that increased spore germination and hyphal branching of the glomeromycete *Gigaspora rosea*, both are responses that will tend to promote colonization. However, after the initial colonization, the exudate inhibited the growth of extraradical hyphae (Scervino et al., 2009). If the presence of the DSE isolate inhibited the growth of extraradical hyphae, this will reduce the AMF ability to explore the soil and thus, the benefits to the plant. Under such circumstances, the plant may provide less carbon to the AMF, triggering in the fungi the formation of storage structures and an overall switch to a resting state (Hawkes et al., 2008; Kiers et al., 2011; Mendoza et al., 2005; Müller et al., 2017). Experiments *in vitro* to assess the effect of my DSE isolate on AMF extraradical hyphae may provide support to this explanation or give insights into other mechanisms by which the isolate enhanced the development of storage structures. In this regard, a possibility is that as AMF colonization increases, the marginal benefit of the association decreases, which could also trigger the formation of storage structures and a shift to a resting state (Biermann and Linderman, 1983; Sylvia and Jarstfer, 1992).

Although DSE inoculation caused some changes in AMF colonization, these changes did not appear to affect the plant response to AMF. The higher density of storage structures in the + DSE treatment seems to require an increase in the allocation of carbon to these structures. Such an increase may be at the expense of AMF structures more actively involved in the symbiosis or reflect an overall higher transfer of carbon from the plant to the AMF. Also, in *A. tridentata*, DSE inoculation decreases the density of arbuscules, which are the primary sites of nutrient exchange. Based on these

considerations, either of these changes could have reduced the benefits of the symbiosis. However, if this occurred, it did not result in a decrease in plant growth.

Effects of *R. irregularis* inoculation on DSE colonization

Information about the effect of AMF on DSE colonization is rare. Of the studies mentioned above, two investigated this question. Berthelot et al. (2018) reported that AMF slightly decreased DSE colonization, while Saravesi et al. (2014) did not observe any effect. The results with *A. tridentata* and *P. secunda* suggests that *R. irregularis* had no impact on colonization by the DSE isolate. Inoculation with *R. irregularis* shows, in both species, a small but not significant decrease in DSE colonization in Figure 8. Both plants were grown together and exposed to the same treatments. Thus, the results of the two species can be combined into a single statistical analysis to increase the number of replications per inoculation treatment. I conducted a statistical analysis using this approach. While the *p*-value decreased compared to that obtained for each species, still the difference was not significant ($p = 0.11$). To different extents, both AMF and DSE depend on the plant host for carbohydrates (Mandyam and Jumpponen, 2015; Smith and Smith, 2011). Given this dependency, it seems counterintuitive that the presence of one fungus did not decrease the abundance of the other. Such results suggest that the cost of maintaining the DSE was low or that this fungus provided additional benefits to those offered by AMF that compensated for the carbon drain.

CONCLUSIONS

The dark septate fungus isolated from *A. tridentata* roots is a non-described, possibly a new species within the *Darksidea* or a closely related sister group. The DSE isolate readily colonized *A. tridentata* and *P. secunda* *in vitro* and soil, indicating that this DSE has low host specificity. The association of the DSE isolate with the plants included hyphae running along the root surface as well as hyphae that penetrated epidermal and cortical cells. Also, in *A. tridentata* the hyphae reached into the stele. The penetration of DSE hyphae through root tissues did not cause damage to the host cells and did not affect plant growth. These observations indicate that the symbiosis was commensalistic.

The effects of the DSE isolate on colonization by *R. irregularis* somewhat differed between the two host plants tested. In *A. tridentata*, DSE inoculation did not affect total AMF colonization but reduced arbuscular colonization. In contrast, in *P. secunda*, DSE inoculation increased total AMF colonization and did not affect arbuscular colonization. A common effect of DSE on AMF was on the density of intraradical storage structures. The frequency of these structures was more than doubled in the +DSE treatment compared to the –DSE.

Neither on its own nor through co-inoculation with AMF, was there an effect of the DSE isolate on plant biomass. These results are in agreement with several studies that have reported neutral plant responses to the presence of DSE (Mandyam and Jumpponen, 2005, 2015). However, to conclude that the isolate is only commensalistic seems premature at this time. Outdoor experiments with large pots or involving direct planting

in the field may yield a different outcome. In comparison to the situation in my research, a larger soil volume would increase the pool of water and mineral nutrients that the fungus can transfer to the plant. Furthermore, the light intensity outdoors would tend to increase the carbon gain associated with the additional mineral brought by the fungus (Konvalinková and Jansa, 2016). Thus, under such conditions, the symbiosis may shift from commensalistic to mutualistic. Independent of whether the symbiosis is always commensalistic or not, it would be valuable to estimate the carbon cost of maintaining the DSE fungus. Such estimates may help to explain the lack of effects on plant growth as well as the apparent absence of negative impacts on AMF observed with this and other studies (Arriagada et al., 2012; Berthelot et al., 2018).

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FIGURES

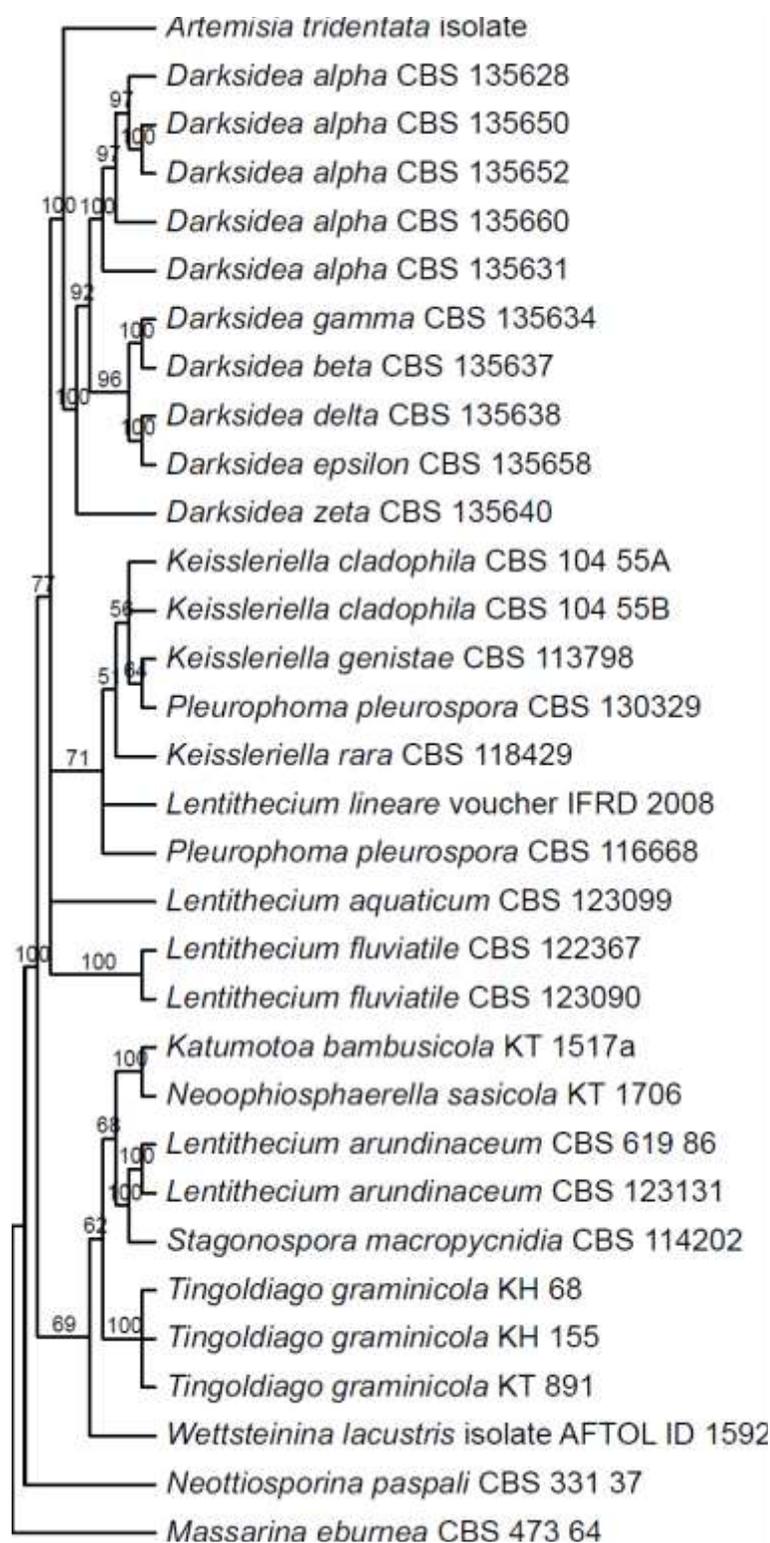


Figure 1. Phylogenetic relationship of the dark septate fungus isolated from *Artemisia tridentata* to other taxa in the Lentitheciaceae. The phylogenetic relationships were inferred from the Bayesian analysis of three loci (LSU, SSU, and TEF). The values above the branches are posterior probabilities.

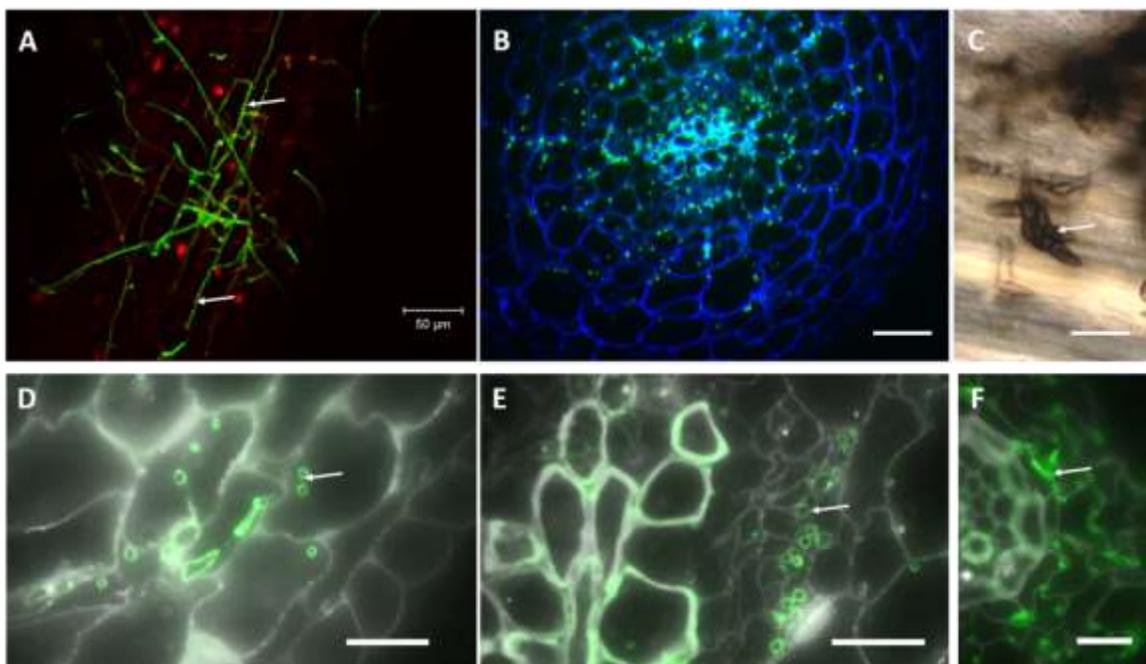


Figure 2. Distribution of the DSE isolate hyphae through root tissues of *Artemisia tridentata* (A to E) and *Poa secunda* (F). A, Root whole mount showing hyphae (green) and plant cell walls and nuclei (red). B, Root cross-section showing hyphae (green) and plant cell walls (blue). C, Whole mounts of roots showing dark hyphae and microsclerotia. D, Higher Magnification of cortical cells with intracellular hyphae (green). E, Higher Magnification of cells in the vascular cylinder with intracellular hyphae (green). F, Cross-section of *P. secunda* root showing hyphae (green) and plant cell walls (grey). some hyphae are pointed with arrows. Sections in B, D, E, and F were labeled with Alexa Fluor 488-wheat germ agglutinin (green fluorescence) and counter stained with calcofluor white (blue in B and grey in D, E, and F). Scales bars: A, B, and C = 50 μm ; D, E, and F = 20 μm

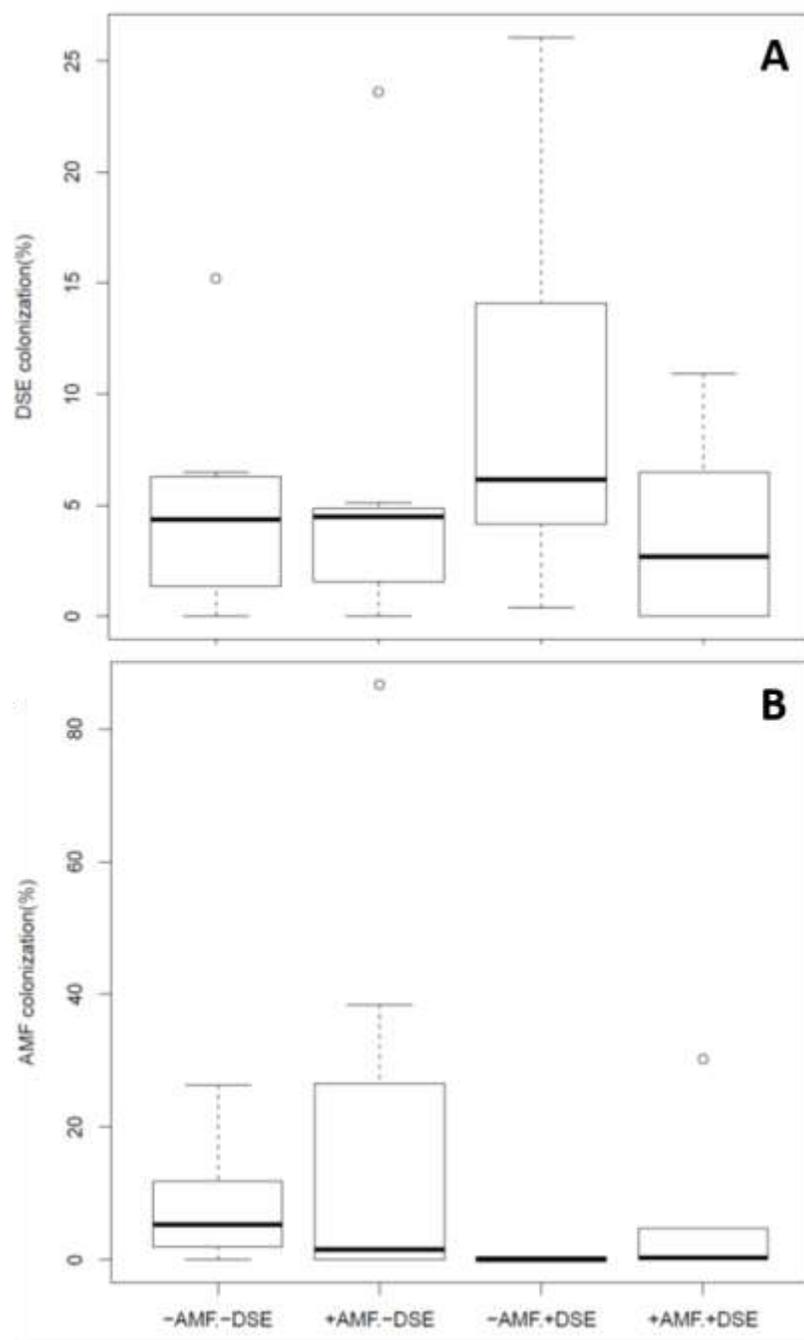


Figure 3. Dark septate and arbuscular mycorrhizal colonization of *Artemisia tridentata* seedlings initial grown in vitro. A, Dark septate colonization. B, AMF colonization. Box plots of 4 to 8 seedlings per treatment. Median values were not significantly different ($p > 0.05$ based on Kruskal-Wallis test).

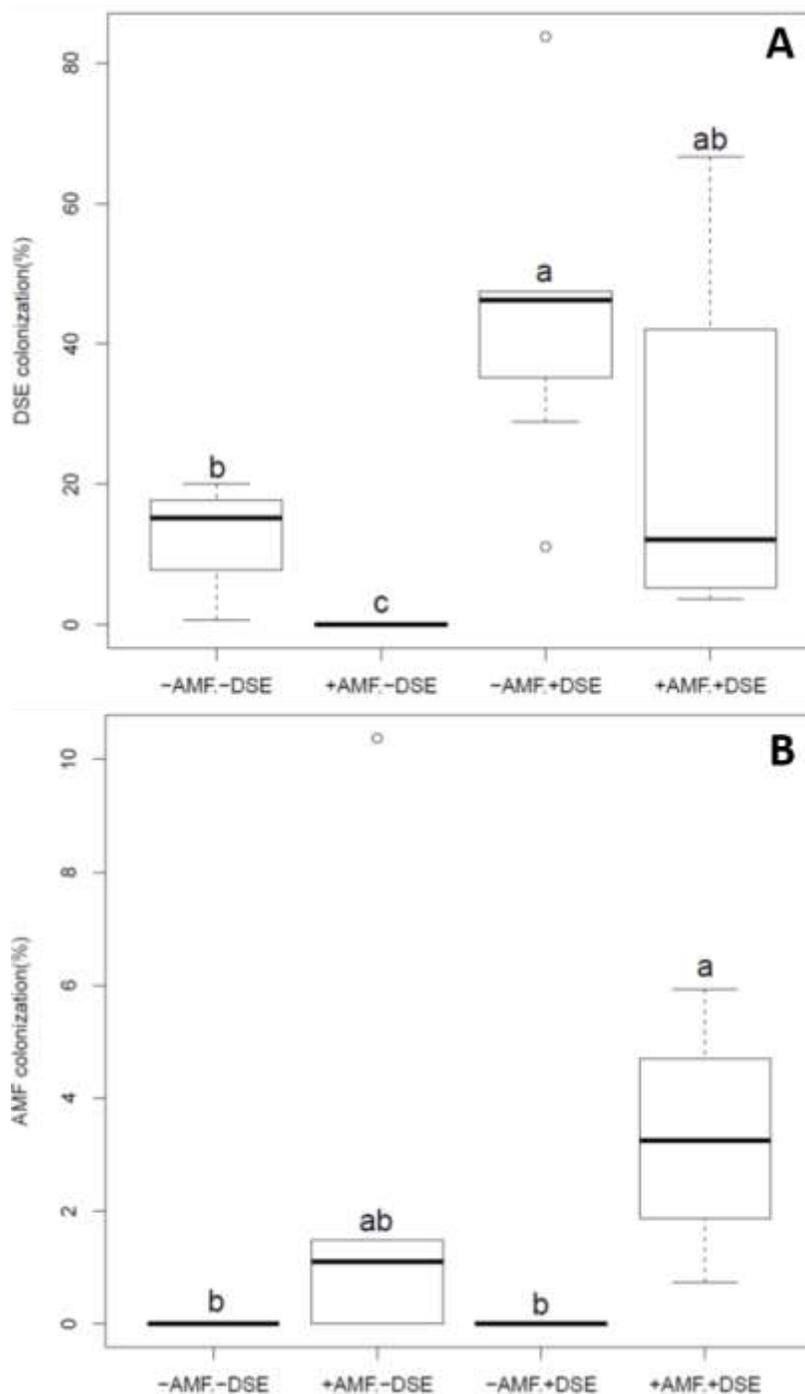


Figure 4. Dark septate and arbuscular mycorrhizal colonization of *Poa secunda* seedlings initial grown in vitro. **A**, Dark septate colonization. **B**, AMF colonization. Box plots of 4 to 8 seedlings per treatment. Medians of boxes not labelled with the same letter are significantly different ($p < 0.05$ based on Kruskal-Wallis test).

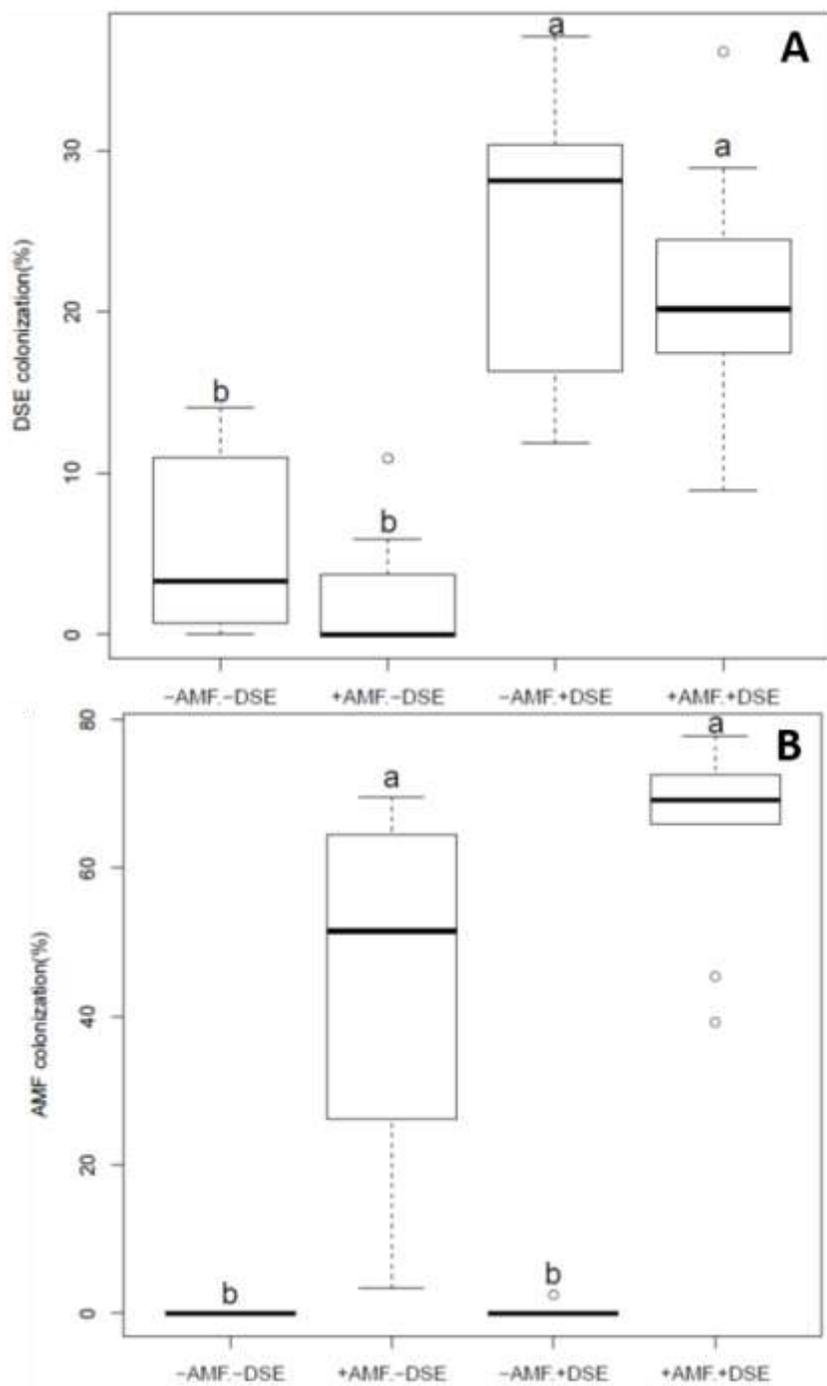


Figure 5. Dark septate and arbuscular mycorrhizal colonization of *Artemisia tridentata*, seeds were planted in soil which contained different inoculum according to the indicated treatments. **A**, Dark septate colonization. **B**, AMF colonization. Box plots of 8 to 9 seedlings per treatment. Medians of boxes not labelled with the same letter are significantly different ($p < 0.05$ based on Kruskal-Wallis test).

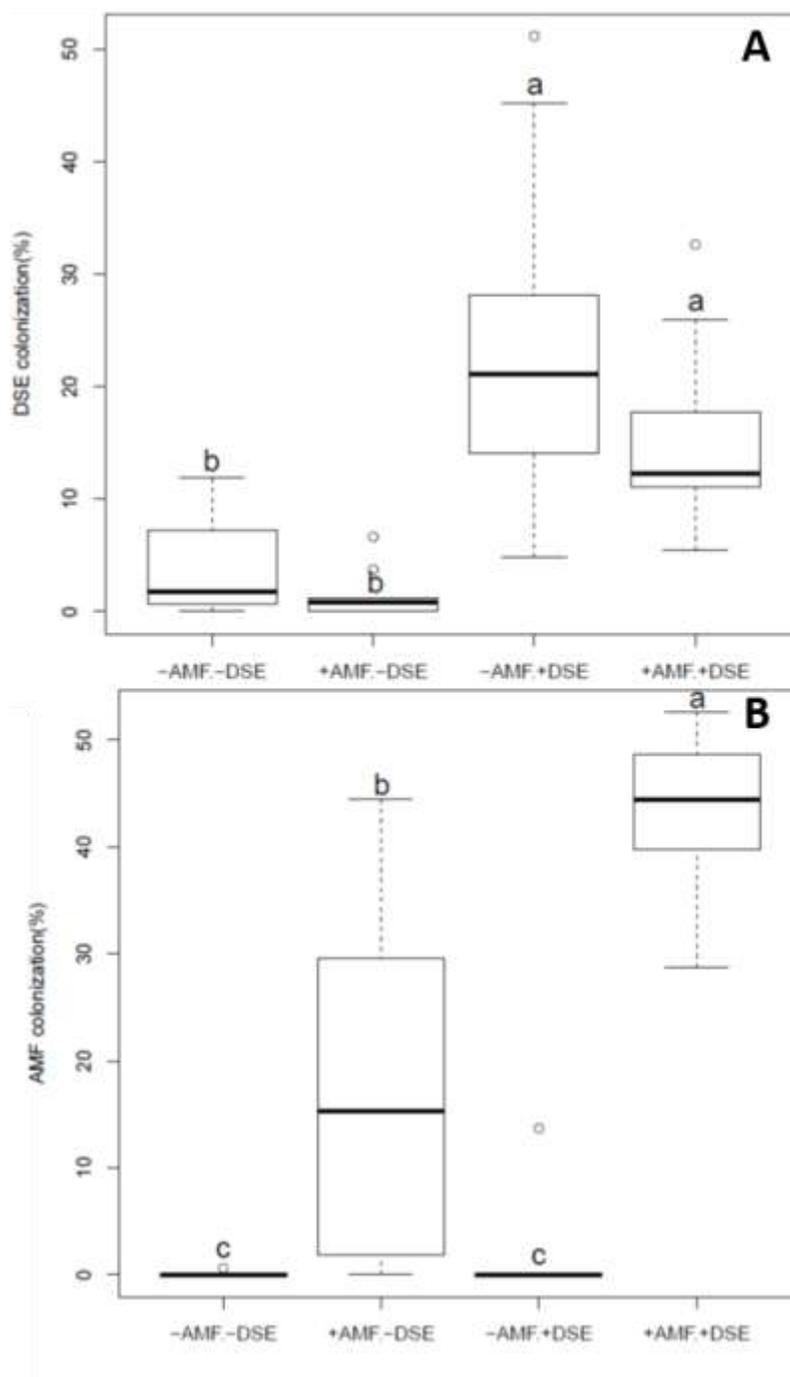


Figure 6. Dark septate and arbuscular mycorrhizal colonization of *Poa secunda*, seeds were planted in soil which contained different inoculum according to the indicated treatments. A, Dark septate colonization. B, AMF colonization. Box plots of 8 to 9 seedlings per treatment. Medians of boxes not labelled with the same letter are significantly different ($p < 0.05$ based on Kruskal-Wallis test).

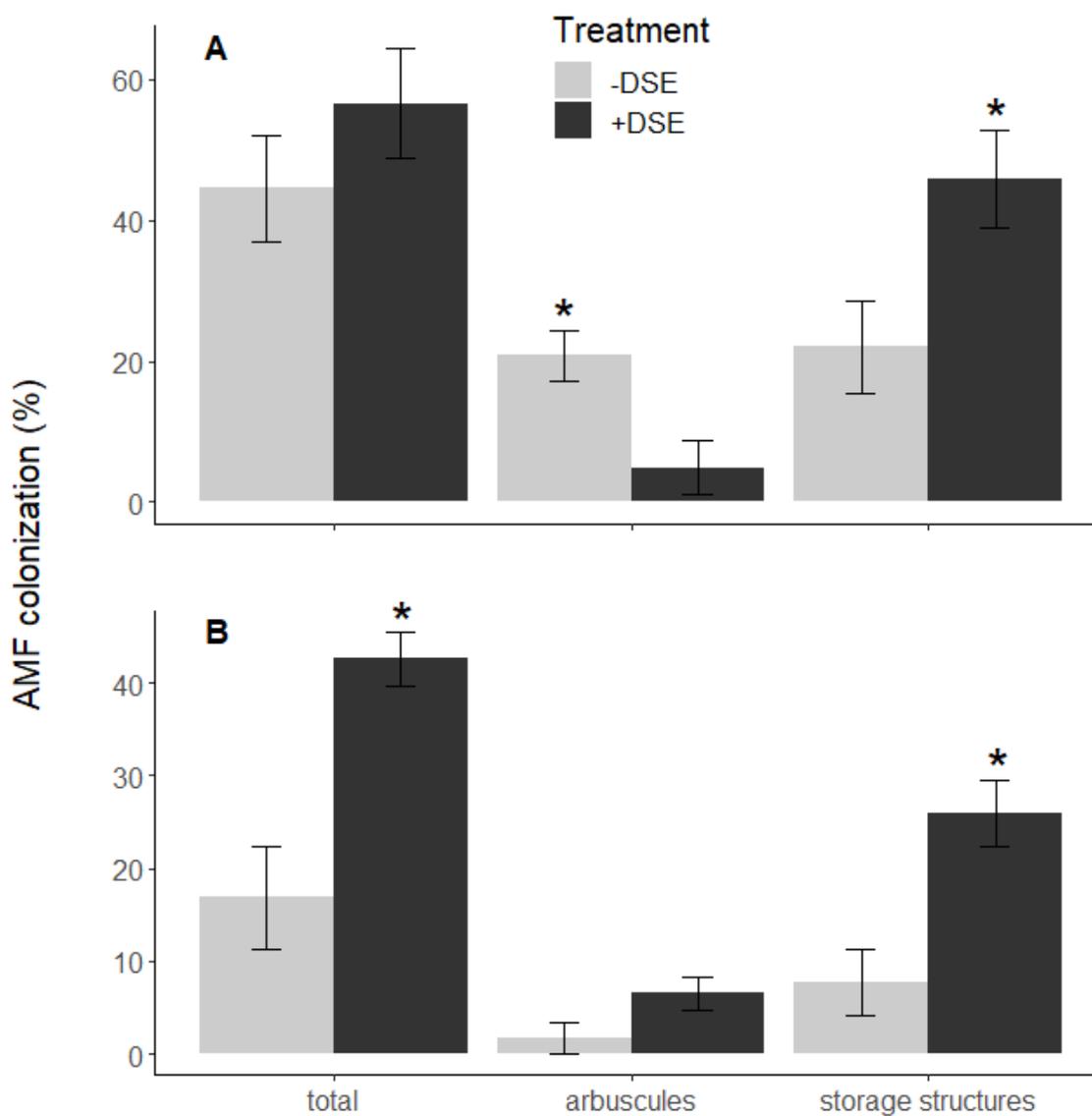


Figure 7. Effect of inoculation with *Darksidea* sp. on arbuscular mycorrhizal colonization of *Artemisia tridentata* (A) and *Poa secunda* (B). Bars represent least square means (\pm SE) of 8 or 9 replications. For a particular variable (total, arbuscules, or storage structures), bars labeled by an asterisk (*) are significantly different ($p < 0.05$).

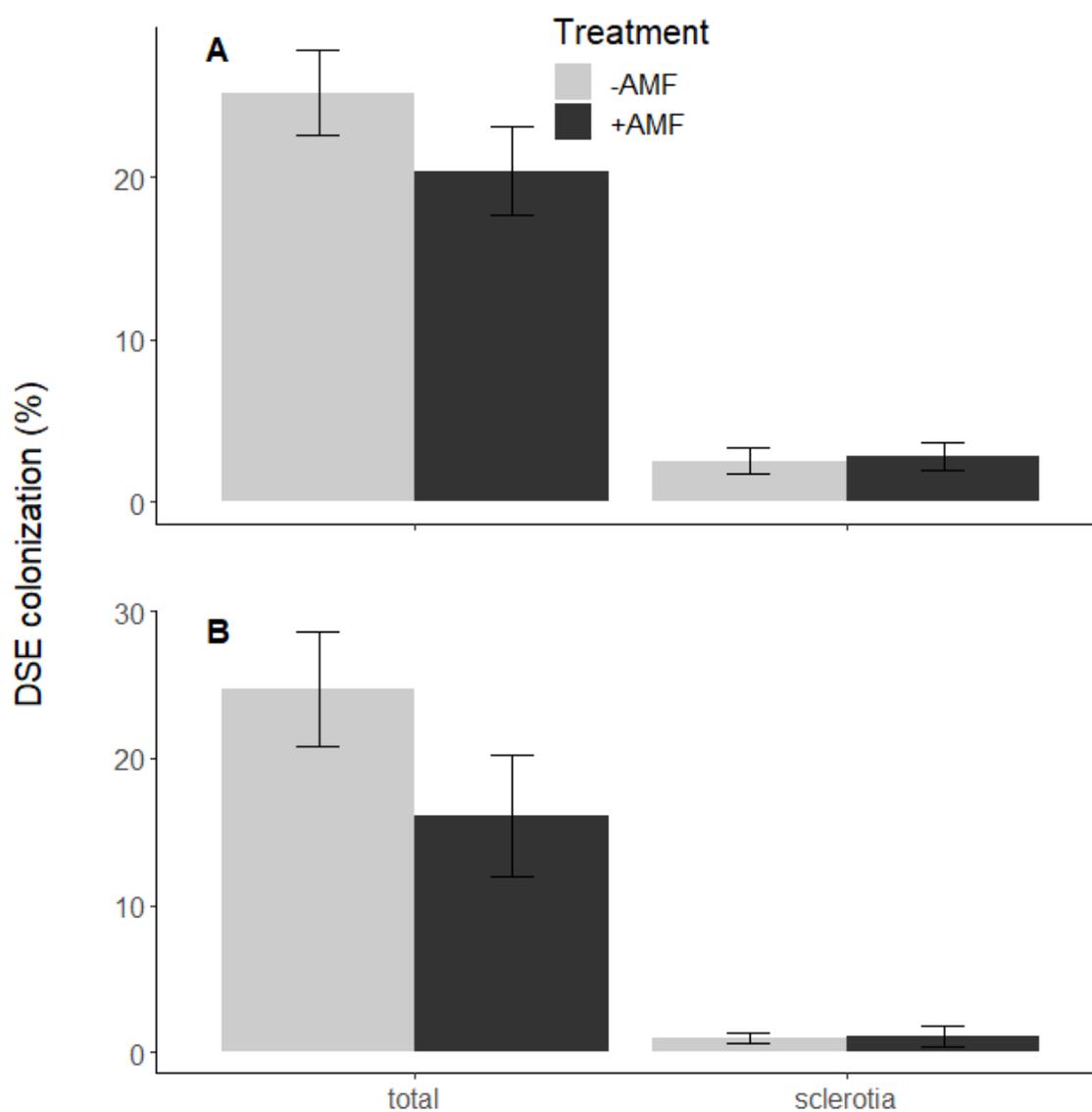


Figure 8. Effect of inoculation with *Rhizophagus irregularis* on colonization of *Artemisia tridentata* (A) and *Poa secunda* (B) roots by septate fungi. Bars represent least square means (\pm SE) of 8 or 9 replications. Differences between the -AMF and +AMF treatments were not significant.

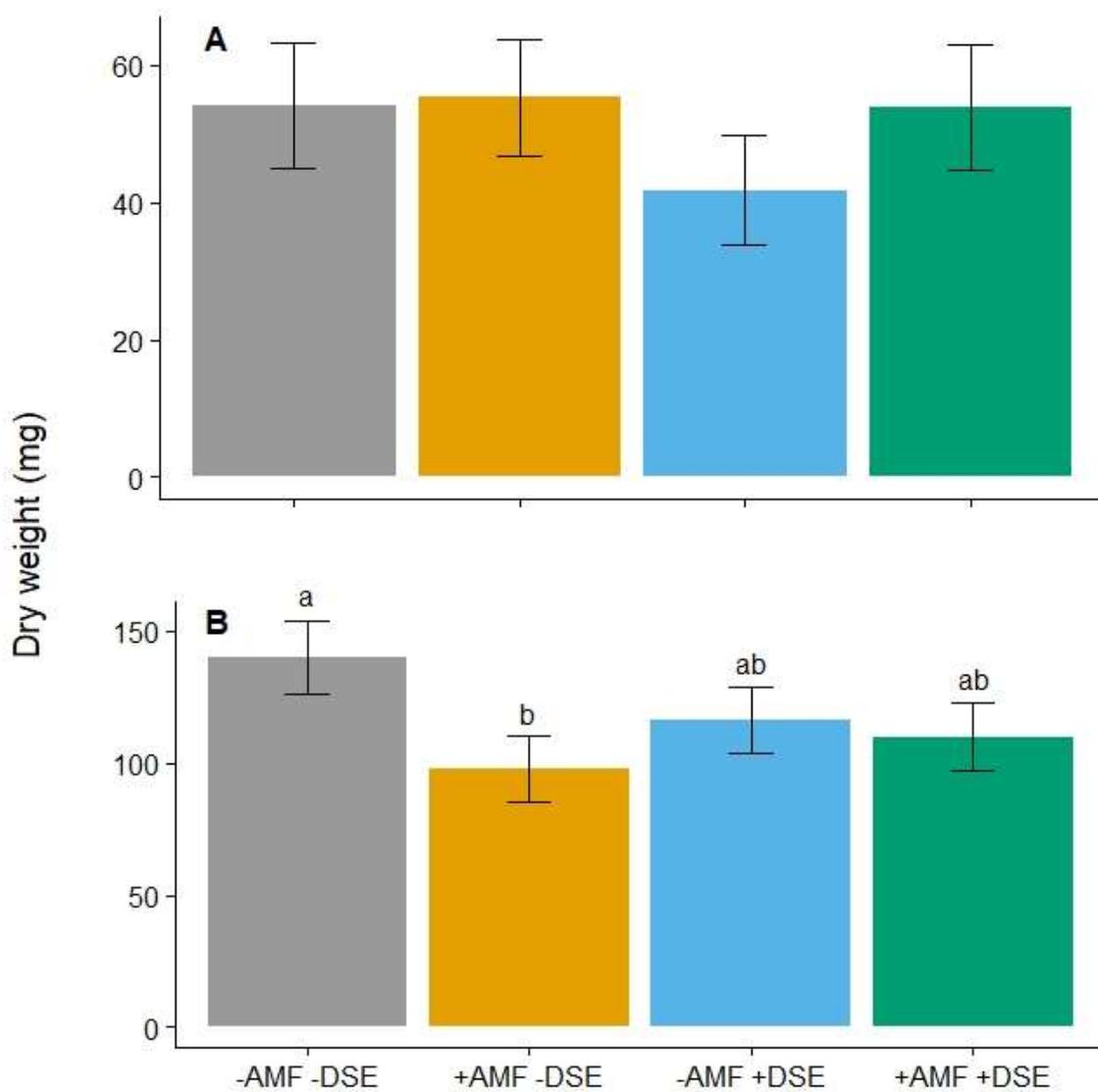


Figure 9. Dry weight per plant of *Artemisia tridentata* (A) and *Poa secunda* (B) under the different inoculation treatments. Bars represent least square means (\pm SE) of 8 or 9 replications. For A. *tridentata*, differences between treatments were not significantly different.