DARK SEPTATE FUNGAL ENDOPHYTES FROM A TALLGRASS PRAIRIE AND THEIR CONTINUUM OF INTERACTIONS WITH HOST PLANTS

by

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B.Sc., Bangalore University, 1997
M.Sc., Bangalore University, 1999
M.S., Kansas State University, 2004

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

Dark septate endophytes (DSE) are darkly pigmented microfungal ascomycetes commonly observed in the healthy plant roots. Studying the functional roles of DSE is challenging as fundamental information about their identity, nutritional requirements, host range or host preference are lacking. Objective 1: root colonizing fungi were isolated from Konza plants roots and DSE fungi were identified by testing Koch's postulates using leek plants. *Periconia macrospinosa* and *Microdochium* sp., were identified as DSE as they produced microsclerotia and chlamydospores in the root cortex. Select DSE were tested for their enzymatic capabilities and ability to utilize nitrogen sources: fungi tested positive for amylase, cellulase, polyphenol oxidases and gelatinase. *Periconia* isolates utilized organic and inorganic nitrogen suggesting facultative biotrophic and saprotrophic habits. Objective 2: a *Microdochium* isolate and three *Periconia* isolates were screened on 16 plant species (six native grasses and forbs, four crops) in a resynthesis system to test host range. DSE colonized all plant species, albeit to varying degrees. Host biomass and nutritional levels to DSE colonization varied within and among host species confirming the broad host range. Based on % responsiveness to DSE colonization, a metric similar to 'mycorrhizal dependency', grasses responded positively, while forbs and crops responded negatively. To test this observed 'host preference' under natural conditions, Konza roots from seven grass and nine forb species were surveyed for DSE colonization. Grasses hosted 50% greater DSE than forbs, supporting the broad host range and host preference of DSE fungi. Objective 3: three conspecific *Arabidopsis* ecotypes, Col-0, Cvi-0 and Kin-1 were inoculated with 25 *P. macrospinosa* isolates in resynthesis system. The three

ecotypes responded differently to inoculation: Col-0 and Cvi-0 responded negatively, while Kin-1 response was neutral. Despite the negative or neutral response, each ecotype responded positively to one or two isolates. The outcomes were along the mutualism-parasitism continuum precluding an unambiguous assignment to any particular life-style. This study shows that the outcomes along this continuum are dictated by host and fungal genotypes. However, the more important question about their function remains. Additional studies with *Arabidopsis* microarrays are likely to provide unique insights into the potential roles of DSE.

Key words: *Arabidopsis thaliana*, *Microdochium* sp., mutualism-parasitism continuum, *Periconia macrospinosa*, tallgrass prairie

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Approved by:

Major Professor
Ari Jumpponen

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Dedication

This is dedicated to Amma, Ayya, Thata, Ananda and Kiran.

CHAPTER 1 - Seeking the elusive function of the root colonizing dark septate endophytic fungi

ABSTRACT

A comparison of published estimates of mycorrhizal and dark septate endophyte (DSE) colonization from various ecosystems suggests that DSE may be as abundant as mycorrhizal fungi as judged by the proportion of host plants colonized in mixed plant communities, or by the extent of colonization in sampled root systems. While many strides have been made in understanding the ecological significance of mycorrhizal fungi, our knowledge about the role of DSE fungi is in its infancy. In order to provide a framework of testable hypotheses, we review and discuss the most likely functions of this poorly understood group of root-associated fungi. We propose that, like mycorrhizal symbioses, DSE-plant symbioses should be considered multifunctional and not limited to nutrient acquisition and resultant positive host growth responses. Admittedly, many mycorrhizal and endophyte functions, (e.g. stress tolerance, pathogen or herbivore deterrence) are likely to be mediated by improved nutritional status and increased fitness of the host. Accordingly, it is pivotal to establish whether or not the DSE fungi are involved in host nutrient acquisition, either from inorganic and readily soluble sources, or from organic and recalcitrant sources. Facilitation by DSE of the use of organic nitrogen, phosphorus and sulphur sources by plants is a topic that warrants further attention and research. Even in the absence of a clear nutrient uptake function, the observed extensive DSE colonization is likely to pre-emptively or competitively deter pathogens by minimizing the carbon available in host rhizosphere environment. The DSEs' high melanin levels and their potential production of secondary metabolites toxic or inhibitory to herbivores are also likely to be factors influencing

host performance. Finally, the broad host ranges speculated for most DSE fungi thus far suggest that they are candidates for controlling plant community dynamics via differential host responses to colonization. We emphasize the need for simple experiments that allow unraveling of the basic biological functions of DSE fungi when they colonize their hosts.

Key words: Abundance, dark septate endophytes (DSE), multifunctional symbioses, mutualism, mycorrhiza.

INTRODUCTION

Vascular plants host a great variety of fungi. In addition to being susceptible to soil-borne pathogens, plant roots are also colonized by non-pathogenic or mutualistic fungi like arbuscular mycorrhizae (AM), ectomycorrhizae (EM) and dark septate endophytes (DSE). A vast majority of terrestrial plant species form mycorrhizal associations (Harley and Smith 1983, Smith and Read 1997). The AM fungi comprise about 150 species of zygomyceteous fungi, while EM fungi include about 6000 species that are primarily basidiomycetes, along with a few ascomycetes and zygomycetes. The AM fungi are associated with most herbaceous plants and with various woody plant families, while the EM fungi are confined chiefly to a limited number of woody plant families. It is now evident that the mycorrhizal fungi have many significant functions in ecosystems. To list a few important functions for which there is convincing evidence, they absorb non-mobile nutrients from the soil and translocate them to host plants, sequester potentially harmful heavy metal ions, facilitate interplant transfer of nutrients, and beneficially modify plant water relations (Smith and Read 1997).

In contrast to the plethora of knowledge about the EM and AM fungi, very little is known about the DSE fungi. The DSE are broadly classified as conidial or sterile septate fungal endophytes that form melanized structures such as inter- and intracellular hyphae and

microsclerotia in the plant roots and that have known or likely affinities within ascomycetes (Jumpponen and Trappe 1998). It has also been suggested that hyaline septate hyphae may be associated with DSE colonization (Haselwandter and Read 1982, Newsham 1999, Yu *et al.* 2001).

DSE are found worldwide and coexist often with different mycorrhizal fungi. They have been reported from 600 plant species including plants that have been considered non-mycorrhizal (Jumpponen and Trappe 1998). In this paper, we review recent literature to evaluate the abundance of DSE across various ecosystems. Based on available information, we conclude that DSE colonize a great diversity of plant species and parallel mycorrhizal fungi, AM fungi in particular, in the proportion of plant species they colonize as well as in their frequency of occurrence in root systems. We then discuss the possible functions of DSE. Admittedly, the available data are scanty at best. However, rather than to provide a comprehensive review, our goal is to present a framework of testable hypotheses that may serve as a starting point for experimental testing of possible DSE functions.

ABUNDANCE OF DSE IN VARIOUS ECOSYSTEMS

In this section we review studies that have quantified both mycorrhizal and DSE colonization and infer the potential global abundance of the latter (Table 1.1). The DSE fungi have been reported from various habitats the world over. They do not seem to exhibit any host specificity and have been isolated from plants that are non-mycorrhizal or that form well-defined mycorrhizal associations, including arbuscular, ericoid, orchid and ectomycorrhizal associations (Jumpponen and Trappe 1998, Addy *et al.* 2000). While many reports on the abundance of mycorrhizal fungi from different habitats exist, only a few studies have quantified root colonization or systematically recorded the proportion of taxa hosting DSE. Jumpponen and

Trappe (1998) emphasised that detection of DSE colonization in most studies was incidental. Most of the available DSE abundance data have been collected from the arctic, alpine, antarctic, and temperate habitats, while next to nothing is known about the abundance of DSE in boreal and tropical ecosystems.

Alpine habitats. Read and Haselwandter (1981) studied mycorrhizal fungi of the dominant and sub-dominant plants in the Central and Northern Calcareous Alps of Austria and recorded the colonization of typical AM fungi, fine endophyte, DSE, and EM fungi at two different sampling times at five Austrian alpine sites located at a range of altitudes. They concluded that more than half of the observed plant species had typical AM colonization, with colonization rates ranging up to 100% (Table 1.1). Nearly one third of the plants were colonized by DSE, with colonization frequencies ranging from non-existent to very high within a root system. Interestingly, these authors were able to infer that AM and DSE colonization were correlated with altitude. The lowest AM colonization was recorded at the highest altitudes and in fertilised meadows, whereas the most intense colonization was found in the low-elevation species-rich grasslands. In contrast, although plants through the entire range of altitudes were colonized by DSE, the most intensive colonization was recorded on mountain peaks at an altitude of 3100-3200m. This study suggested that DSE are more prevalent than AM in high-elevation, stressed environments.

In a broad study focusing on five ecoregions in Alberta, Currah and van Dyk (1986) confirmed that DSE did indeed occur mainly in alpine areas (see Table 1.1). Similarly, Treu *et al.* (1996) examined the mycorrhizal status of 40 taxa of vascular plants in montane interior Alaska within the Denali National Park and Reserve. The AM fungi were least common while

ecto- and ericoid mycorrhizae as well as DSE occurred relatively frequently (Table 1.1). Treu *et al.* (1996) concluded that their results on the common occurrence of DSE agreed with Read and Haselwandter (1981): DSE appeared more frequently in stressed environments. However, the elevation-related patterns proposed by Read and Haselwandter (1981) were not observed in Finnish oroarctic and subalpine regions (Ruotsalainen 2003).

Trowbridge and Jumpponen (2004) recorded shrub willow (*Salix* spp.) colonization by AM, EM and DSE fungi on a receding glacier forefront in a subalpine region of the Cascades Mountain Range, Washington, USA. They found that <1% of root length was colonized by AM, 25% of root tips and 19.4% root length by EM while 25.6% of root length was colonized by DSE. DSE colonization varied widely in their study. Melanized hyphae and microsclerotia occurred in up to 80 % of the root length. In comparison, EM structures (Hartig net or pseudoparenchymatous tissue) were observed never to exceed 40% of the root length.

Arctic habitats. AM colonization in arctic and alpine regions is highly variable (Gardes and Dahlberg 1996b). In arctic tundra, it is negligible in some cases (Bledsoe *et al.* 1990, Kohn and Stasovski 1990, Väre *et al.* 1992), while in others it is more common, with studies of some areas showing frequent colonization of a large proportion of any root system observed (Strelkova 1956, Katenin 1962, 1972 in Bledsoe *et al.* 1990). Kohn and Stasovski (1990) concluded that AM fungi were mainly absent in an arctic oasis at Ellesmere Island, Canada, as only one plant species (the fragrant wood fern *Dryopteris fragrans* [L.] Schott.) of the 24 observed was colonized by aseptate hyphae (see Table 1.1). This study also detected septate fungal symbionts in some plant species but they were not considered to be DSE. Similarly, Bledsoe *et al.* (1990) examined 55 herbaceous and woody plant species in the Canadian high arctic for mycorrhizae.

These authors concluded that AM associations at their sites were absent as neither vesicles nor arbuscules were observed. They further confirmed the absence of AM by showing that spore isolation attempts were unsuccessful and that bioassay seedlings produced no AM structures in greenhouse when soils from the test sites were used as the source of AM inoculum. Nonetheless, both of these reports concluded that EM and ericoid mycorrhizas (ERM) were present in plants with such known affiliations.

Väre *et al.* (1992) studied the root colonization of 76 different plant species from 19 families in west Spitsbergen in the middle-northern arctic zone. They found that the DSE were the predominant fungi while EM and ERM fungi were seen in a minuscule number of plant species (Table 1.1). AM fungi were not observed in the examined roots, although 11 soil samples together yielded one AM spore. DSE fungi produced microsclerotia and hyaline hyphae in the roots. Väre *et al.* (1992), however, considered the hyaline septate hyphae to represent a group of root endophytes separate from the DSE (see discussion on melanized and hyaline structures in the "*Abundance of DSE – global inference*" section below). These non-melanized endophytic structures were recorded in seven plant species.

Ruotsalainen (2003) studied AM and DSE colonization over one growing season across an altitudinal gradient in the subarctic meadows in northern Finland. The vegetation supported colonization by AM and DSE fungi. Consistent with the observations of Read and Haselwandter (1981), Ruotsalainen found that DSE were common in the plant species studied. They colonized the host plants simultaneously with mycorrhizal fungi. Contrary to her initial hypotheses that DSE colonization would increase and AM colonization would decrease at higher altitudes, Ruotsalainen (2003) found neither positive correlation between altitude and DSE colonization

nor negative correlation between altitude and AM colonization. The fungal colonization rates and patterns appeared species-specific during the growing season at a given altitude.

Antarctic habitats. AM have been suggested to be absent in the Antarctic, although many plant species that occur in the Antarctic form abundant AM when they grow in cool temperate and subantarctic regions (Christie and Nicholson 1983). In an antarctic study by Christie and Nicholson (1983), DSE were observed in only a limited number of samples. Subsequent studies gave contradictory results and even suggested that AM fungi are commonly present in Antarctica. Laursen *et al.* (1997) found that 18 of the 40 plant species studied in Antarctica harboured AM fungi. The majority of the sampled plants contained only vesicles; only three species were recorded to have both vesicles and arbuscules in their roots. In addition, Laursen *et al.* (1997) rated DSE as frequent in occurrence (Table 1.1). Of the 40 plant species studied, 21 possessed melanized septate hyphae and microsclerotia in addition to co-occurring AM colonization. Laursen *et al.* (1997) found no EM, even though some plant species present belonged to the family *Rosaceae*, which have known EM representatives in the Northern Hemisphere.

Boreal habitats. Currah and van Dyk (1986) conducted a survey of fungal root colonization over five ecoregions and 179 plant species. Four of the six species from the boreal sites were colonized by AM, whereas only two harboured DSE. Most studies conducted in boreal forest habitats tend to focus on EM colonization. Therefore, studies reporting DSE from these habitats are rare or confined to marginal habitats. Thormann *et al*. (1999), for example, studied fungal colonization of 25 plant species in boreal peatlands. Nearly half of the studied species contained melanized structures typical of DSE, whereas AM, EM, and ERM were less frequent (Table 1.1). Although it may be difficult to make conclusions about the relative

abundance of the mycorrhizal and DSE fungi in boreal regions based on the limited available data, it appears that DSE can be commonly observed and may be frequent in some habitats.

Temperate habitats. Horton *et al.* (1998) did a five-month study of fungal colonization of young post-fire seedlings of bishop pine, *Pinus muricata* D. Don, in scrub and forest sites. In the forest site, the frequency of seedlings with DSE colonization was consistently greater than that of seedlings with either EM or AM colonization except during the fourth month, when the number of seedlings with DSE and EM were comparable. In fact, mycorrhizal fungi were absent in the seedlings during the first two months of the study, while DSE were well represented. In the scrub site, the frequency of seedlings colonized by DSE was greater than that colonized by either AM or EM for the first three months. During the last two study months, DSE and EM fungi were found to colonize a similar proportion of the seedlings. Horton *et al.* (1998) suggested that DSE may be pioneering colonizers of young tree seedlings in such secondary successional environments. However, other research indicates that it is not only young seedlings that host DSE. Ahlich and Sieber (1996) estimated the frequency of DSE by isolating them from non-ectomycorrhizal roots of adult EM plants (*Abies alba* Miller, *Fagus sylvatica* L., *Picea abies* [L.] H. Karst. and *Pinus sylvestris* L.) at various temperate forest sites in Europe. They also considered additional EM host species from Asia and the USA. They estimated that about 70-100% of the fine roots of individual plants from Europe were colonized by DSE while 20-100% of the additional samples were infected by these fungi.

In addition to occurring in temperate forests, DSE seem to occur frequently in temperate grassland ecosystems. In a recent year-long study of root endophytes in sandy grasslands of the Great Hungarian Plain, Kovács and Szigetvári (2002) found 67% of the studied plant species to

be colonized by AM. Most of these AM-colonized plants (78%) had high colonization levels ranging from 50 to 100%. Kovács and Szigetvári (2002) also confirmed five plant species normally considered ectomycorrhizal hosts to be colonized by EM fungi. On the whole, plants were colonized by DSE as frequently as by mycorrhizal fungi (Table 1.1). A total of 63 plant species supported DSE colonization while only 60 plant species were mycorrhizal. Within the mycorrhizal group, 56 species harboured only AM and four species had both AM and EM. Colonization by DSE hyphae was found in about 75% of the mycorrhizal plants, while one third of these plants also had microsclerotia. Interestingly, of the 29 non-mycorrhizal plant species, 18 were colonized by intra- and intercellular septate hyphae or by microsclerotia. This study was the first to suggest that DSE colonization may be as abundant as AM colonization in the types of habitats occurring in sandy Hungarian grasslands.

Barrow and Aaltonen (2001) conducted an intense sampling of fungal colonization in the roots of native plants in temperate semi-arid rangelands of New Mexico, USA. Their study was conducted over a span of one year and plants were sampled two or three times per month. The native vegetation was dominated by the four-wing saltbush, *Atriplex canescens* (Pursh) Nutt. Although *A. canescens* is known to form AM (Barrow *et al.* 1997), Barrow and Aaltonen (2001) concluded that the root systems were nearly exclusively colonized by DSE. They attributed the low AM colonization and the prevalence of DSE to extended drought in the region, thus supporting the hypothesis (Read and Haselwandter 1981) that DSE occur most frequently in extreme environments and stressed conditions. In a later study involving a weekly sampling of native grama grasses in the genus *Bouteloua* Lag., Barrow (2003) concluded, similarly, that DSE colonization exceeded that of AM.

These observations from temperate grass- and rangelands are supported by our own unpublished results and may indicate that DSE are widely prevalent in temperate grassland ecosystems. In a sampling of mixed tallgrass prairie plant communities at a mesic prairie site at Konza Prairie Long Term Ecological Research site in Kansas, USA, we observed that DSE colonization exceeded that of AM (Fig. 1.1). In our study, however, a statistically significant interaction involving the type of colonization (AM vs. DSE) established that whilst DSE colonization remained rather stable throughout the growing season, AM colonization was lower than DSE colonization early in the season but reached comparable levels later. Clearly, temporal dynamics need to be taken into account in studies of root colonization, particularly when conclusions are made about patterns of fungal abundance.

Tropical habitats. Tropical ecosystems may be the least well understood in terms of the status of fungal root endophytes. We are aware of only one study that systematically quantified DSE and mycorrhizal colonization. Rains *et al.* (2003) assessed the mycorrhizal status of epiphytes and terrestrial plants in neotropical rain forests in Costa Rica. They surveyed 18 species based on a total sample size of 43 plants, including 23 canopy epiphytes, 16 terrestrial plants and four *Disterigma humboldtii* (Klotzsch) Nied. (*Ericaceae*) plants rooted in coarse woody debris. AM colonization was recorded in only a few plant species while the ericaceous plants possessed typical ERM structures (hyphal coils). Nearly all the species observed were colonized by DSE (Table 1.1). However the extent of DSE colonization was highly variable ranging from a low or moderate to a high level of occurrence (<25-75%) of melanized hyphae or microsclerotia. Consistently with Read and Haselwandter (1981), Rains *et al.* (2003) attributed the frequent occurrence of DSE to the stressful habitat occupied by the epiphytic plants.

Abundance of DSE – global inference. Only a very limited number of studies so far have attempted to evaluate and quantify root colonization by both mycorrhizal and DSE fungi. Based on the information available, however, it appears that DSE may be as abundant as mycorrhizal fungi. In many studies, comparable proportions of the plants within the communities were observed to be colonized by the non-mycorrhizal DSE as well as by mycorrhizal fungi. The actual rates of colonization, although highly variable, seem to fall into similar ranges.

The DSE fungi, when present, seem to occur in large proportions of any examined root system. Grünig *et al.* (2002) isolated DSE from a small (3 × 3m) field plot in Austria. These fungi were obtained from over 80% of root segments. Studies by Grünig *et al.* (2002) and Ahlich and Sieber (1996) clearly indicate that these fungi occur very frequently (see above). If we presume that most roots of EM plants are colonized by EM, DSE colonization in those studies appears to be at nearly comparable levels. Similarly, in a study relying on direct PCR amplification from roots of EM nursery trees, a DSE fungus, *Phialocephala fortinii* Wang and Wilcox, or closely related taxa, were among the three most commonly found sequence types (Kernaghan *et al.* 2003). Clearly, the jury is still out on determining DSE abundance across different ecosystems, since ecosystems and habitats show differing patterns of root colonization (see Read and Haselwandter 1981). We must emphasise the need for further studies with a focus not limited just to mycorrhizal fungi, but rather broadened to include consideration of the overall pattern of root colonization by various typically root-inhabiting fungi.

DSE colonization may be even more abundant than is reported in the studies cited above, since these mainly relied on observing melanized intra- and intercellular hyphae and microsclerotia. Firstly, as has been known now for several decades (Girlanda *et al.* 2002), DSE frequently co-occur with mycorrhizal fungi. Girlanda *et al.* (2002) designed a study focused on

isolating the dark, sterile mycelia from ecto- and endomycorrhizal roots. Up to nearly 60% of the isolates obtained belonged to the target group and possessed melanized hyphae. Observing DSE colonization may be difficult when it occurs in ectomycorrhizal roots, since many inter- and intracellular structures may be hidden under an ectomycorrhizal mantle or a Hartig net. Also, Haselwandter and Read (1982), Newsham (1999) and Yu *et al.* (2001) reported the non-melanized, hyaline hyphae by these fungi in the plant host. The hyaline structures were continuous with melanized hyphae and were clearly produced by the same DSE fungus. Yu *et al.* (2001) suggested that the hyaline hyphae produced by melanized DSE fungi often went unnoticed in microscopic studies and that this resulted in an underestimation of the true abundance of DSE. Supporting these observations, Barrow and Aaltonen (2001) and Barrow (2003) found that hyaline hyphae were extremely common in *A. canescens* and *Bouteloua* spp., but were usually not visible with ordinary light microscopy or staining. They suggested that the hyaline hyphae could only be visualised by careful observation with differential interference contrast (DIC) microscopy at high magnification (400-1000X). The hyaline hyphae did not stain with Trypan blue often used in root studies, suggesting poor chitinization or poor development of the fungal cell wall during host colonization (Barrow and Aaltonen 2001). Staining with Sudan IV, a lipid-specific stain, followed by DIC microscopy was shown to be necessary for visualising the hyaline component of fungal colonization. Although further confirmation based on inoculated plant growth in well-controlled aseptic conditions is required, it can be preliminarily stated that DSE produce a variety of morphological structures, including not just melanized hyphae and microsclerotia but also hyaline hyphae and vesicles, when occupying host tissues.

We concur with Yu *et al.* (2001) that the accurate observation and quantification of non-melanized DSE structures may be impaired by the poor visibility of these structures and their

low affinity for chitin-targeting stains such as Trypan blue. In addition, we have observed that the lipid bodies stained efficiently by Sudan IV stains are highly variable in their occurrence in field-collected samples (unpublished data). Barrow and Aaltonen (2001) suggested that the occurrence of these lipids within the hyaline fungal tissues varies seasonally and may be associated in resource translocation in the host plant. Given the unknown seasonal dynamics affecting the chemistry of the hyaline structures, their true frequency remains difficult to estimate. However, we observe that when DSE colonization is high, the melanized and hyaline structures co-occur and often occupy the same tissues (unpublished data). Under such conditions, the risk of serious underestimation of DSE occurrence is very limited.

In summary, it is emphasised that DSE fungi a) are ubiquitous in occurrence; b) co-occur with different types of mycorrhizae; c) are most prevalent in stressed environments; d) can be as abundant as mycorrhizal fungi, and e) may be underestimated when hyaline structures are formed in the absence of melanized structures.

POTENTIAL FUNCTIONS OF DSE IN NATURAL ECOSYSTEMS

In this section we briefly review the commonly proposed functions for mycorrhizal fungi and hypothesise which are most likely to be performed by DSE. The dependence of plants on their mycorrhizal symbionts is fairly well known. Mycorrhizal fungi confer several benefits on their host plants, and these are especially significant in stressed environments (Smith and Read 1997). While mycorrhizal functions may be relatively well understood, very little is known about the function of DSE. We concluded above that in ecosystems where DSE fungi have been studied, they have generally been found to colonize a high proportion of the plants present. Often this colonization occurs at fairly high density. The great abundance and the apparent

broad host ranges (Jumpponen and Trappe 1998) of DSE suggest that they have an important, albeit unknown, function in ecosystems.

While it may be difficult to estimate the abundance of DSE (see above), understanding the relevant ecological roles of these fungi is even more time-consuming and difficult. The role of DSE in nutrient capture has been studied to a limited extent. However, in discussing the positive contributions of DSE to plant vitality, we cannot limit ourselves to consideration only of the nutritional effects of the symbioses. Mycorrhizal fungi have been shown to fulfil a variety of different types of functions (Newsham *et al*. 1995, Smith and Read 1997). DSE may also have various functions within plant communities. Although many of the mycorrhizal functions are unquestionably related to improved host nutrient acquisition, some rely on the production of inhibitory metabolites or on exploitation competition exerted against rhizosphere-inhabiting microorganisms. The non-nutritional effects of the symbioses, such as protection from soil-borne pathogens or herbivores, modification of environmental tolerance, and involvement in plant community dynamics, can also be of great relevance.

The current understanding of the abundance of DSE and their co-existence with conventional mycorrhizas raises interesting questions. Are DSE more efficient than mycorrhizal fungi in foraging for nutrients from organic sources? Do the functions of DSE complement those of the mycorrhizal fungi? Before we can answer such questions, we must gather experimental evidence. It is our intention to give these investigations some direction by putting forth proposals for the most likely functions of DSE. We argue that the host responses to these fungi fall within the range of the mutualism-parasitism continuum proposed for the mycorrhizal fungi (Johnson *et al*. 1997). Currently, only limited evidence exists for many of the functions discussed here. Consequently, we use examples from studies in mycorrhizal systems, and

propose a framework that can assist in further development of hypotheses and future experiments.

Facilitation of host mineral nutrient uptake by DSE. The involvement of mycorrhizal fungi in plant nutrition has been the most studied function of mycorrhizae. In particular, a great many reports deal with mineral nutrient uptake (see Smith and Read 1997, Allen *et al.* 2003). Mycorrhizal roots are capable of uptake of P, N, Zn, Cu, Ni, S, Mn, B, Fe, Ca, and K (Marschner 1994, Smith and Read 1997, Clark 2000, Liu et al 2000). The improved nutrient acquisition often leads to improved host growth relative to the extent of mycorrhizal colonization, though this does not occur in all cases.

Our knowledge of DSE involvement in host nutrient acquisition is limited, and existing reports have concluded variously that DSE exert positive, negative or negligible effects on host performance (see Jumpponen 2001). Haselwandter and Read (1982) isolated DSE fungi from *Carex* species from the European Alps. When inoculated on the same species of *Carex*, these fungi resulted in increased dry weight of roots, shoots and whole plants along with an increase in shoot P content. Jumpponen *et al.* (1998) evaluated the role of the common DSE fungus *P. fortinii* in nutrient uptake by *Pinus contorta*. Inoculation with the endophyte alone did not enhance growth but increased the foliar P concentration. A combination of N amendment and fungal inoculation increased host biomass by more than 50% beyond that obtained via N amendment alone. Jumpponen *et al.* (1998) speculated that the removal of N limitation allowed *P. fortinii* to exhibit mycorrhizal behaviour, *i.e.*, enhancement of plant growth and nutrient uptake.

Newsham (1999) found that the DSE fungus *Phialophora graminicola* (Deacon) J. Walker, currently called *Harpophora radicicola* [Deacon] W. Gams, was beneficial to the grass

Vulpia ciliata Dumort. ssp. *ambigua* (Le Gall) Stace and Auquier. Grasses had more tillers as well as greater root, shoot and total biomass than uninoculated controls. The inoculated seedlings also had increased root length and root N content plus increased root, shoot and total P compared to controls, but had reduced shoot N. The mechanisms underlying these growth responses remained unknown. Barrow and Osuna (2002) concluded that *Aspergillus ustus* (Bainier) Thom and Church, considered a DSE fungus, had a mutualistic association with fourwing saltbrush (*A. canescens*). In pure culture, the fungus was shown to be able to hydrolyse P sources unavailable to the plant, like rock- and tricalcium phosphates. It also improved seedling nutrition by supplying P from these sources. The improved P nutrition derived from the recalcitrant sources resulted in a typical mycorrhizal response, that is, increased shoot and root biomass. It was emphasised that the plants colonized by *A. ustus*, with their access to immobile P sources, were more efficient in P use than were uninoculated plants that could only obtain P from readily soluble sources.

Although the examples cited here seem to support DSE involvement in plant nutrient acquisition, a great number of studies have failed to show positive effects from DSE inoculation (see Table 1 in Jumpponen, 2001). The lack of any growth or nutritional benefits under experimental conditions does not negate the possibility of important functions in natural systems. It is important to note that colonization by fungal hyphae is likely to allow access to soluble nutrient sources otherwise unavailable to the host plant. The relatively small diameter of DSE hyphae, especially when compared to root diameter, allows penetration of soil micropores and acquisition of resources from a soil volume impenetrable to the plant roots. The fulfilment of such functions by DSE under natural conditions remains open to speculation, as many aspects of the basic biology of DSE fungi are still unknown. For example, we are unaware of any reports

on extramatrical DSE mycelium and soil volumes occupied by such mycelia, although Barrow and Osuna (2002) were able to demonstrate that *A. ustus* mycelium extended into the root exclusion chamber in their experiment.

Overall, the range of the observed DSE associations falls within a continuum ranging from mutualism to parasitism, similarly to that of AM fungi (Johnson *et al.* 1997). Although the DSE involvement in host nutrient acquisition appears unclear, innovative studies such as that of Barrow and Osuna (2002) suggest that DSE function may be more complex than simple enhancement of foraging for nutrients in soluble soil pools. For example, use of organic nutrient sources (see below) is one of the areas where DSE may complement the functions of mycorrhizal fungi.

Utilisation of organic nutrient pools by DSE. In most terrestrial ecosystems N is the nutrient most limiting for plant growth (Aerts and Chapin 2000). Current data suggest that in many soils organic N is in greater abundance than inorganic forms (Aerts 2002 and references therein). The most common available forms of organic N in the soil are various simple amino acids. A proportion of the total amino acid content present is readily available in the soil solution (Lipson and Näsholm 2001). Similarly, less than 1% of soil P is in solution in soil, while large pools of organic P are found in the form of inositol phosphates, phospholipids and nucleic acids. Conversion of these organic nutrient forms into inorganic forms for easy uptake by plants is dependent on extracellular enzymes derived from microbes, including fungi and bacteria (Smith and Read 1997).

The nutrient foraging strategies utilized by mycorrhizal fungi differ. While AM fungi may mainly absorb soluble sources of P and N, EM and ERM fungi have an array of extracellular enzymes available for the degradation of complex organic material (Olsson 2002).

DSE fungi, like EM and ERM fungi, produce arrays of hydrolytic enzymes and can access sources of C, N and P in detritus. Table 1.2 lists the hydrolytic capabilities reported so far in the DSE fungi.

One promising avenue of research yet to be explored is the question of whether DSE can mobilize nutrients from the amino acids that are abundant in the soil environment (Lipson and Näsholm 2001). The exploitation of soil amino acids by EM fungi is well known (Read *et al.* 1989, Finlay *et al.* 1992). Smith and Read (1997) suggest that mycorrhizal fungi are more efficient than saprobic fungi in obtaining N from organic sources, since they are not dependant on soil organic C but have access to recently fixed photosynthate C. Our preliminary experiments suggested that most DSE isolates from Konza Prairie in Kansas utilise a great variety of N sources (Mandyam and Jumpponen, unpublished). In a liquid culture system similar to that of Finlay *et al.* (1992), amino acids including alanine, glycine and arginine were utilised as efficiently as NH_4^+ when they were provided to the DSE isolates as the sole source of N.

Caldwell and Jumpponen (2003a) have also investigated the ability of DSE fungi and EM fungi to utilise heterocyclic organic N. The heterocyclic compounds used were guanine and uric acid, which are excretion products of mites and many other invertebrates commonly found in soil. DSE and ERM fungi were capable of utilising guanine and uric acid as sole source of N in pure culture. Interestingly these fungi attained greater growth yields on heterocyclic N than on NH_4^+.

The enzymatic capabilities discussed here demonstrate the potential of DSE fungi to access detrital C, N and P. Caldwell and Jumpponen (2003b) have also established the ability of DSE fungi to hydrolyse organic sulphate. Organic sulphur compounds may be important sources of sulphur for many mycorrhizal plants. Ester-sulphate is an organic form of sulphur and may

contribute significantly to the total soil sulphur (Autry and Fitzgerald 1990). *Phialocephala fortinii* and other DSE species along with ERM fungi were able to produce aryl sulphatase and to hydrolyse aryl sulphate esters. In contrast, the EM fungi studied seemed unable to hydrolyse these organic sulphur compounds. This suggests a potential for DSE fungi to transfer sulphur from organic pools to their host plants.

As a next step, it is necessary to determine if the nutrient use in vitro will prove to be significant when DSE fungi occur in symbiosis. Simple experiments can be designed to show whether complex organic molecules provided as a sole source are made available by DSE, for example when roots are excluded from a soil compartment and only fungal access to the nutrient sources is allowed. We find it very likely that DSE will allow direct host access to recalcitrant nutrients. Barrow and Osuna (2002) have already confirmed that root-inhabiting *A. ustus* improved plant growth when insoluble P sources were placed in a root exclusion compartment. Similar experiments are required to determine whether fungi known to solubilize other complex nutrient sources *in vitro* will do so in a way that improves plant growth *in vivo*.

Alteration of host water uptake and environmental tolerance by DSE. Mycorrhizal fungi can alter the environmental tolerance of host plants in various ways. Inoculation with heavy-metal-tolerant mycorrhizal fungi often improves survival and longevity of hosts in contaminated sites (Jones and Hutchinson 1986, Meharg and Cairney 2000, Sharples *et al.* 2000, Cairney *et al.* 2001, Malcová *et al.* 2001, Hall 2002, Turnau and Haselwandter 2002, Cairney and Meharg 2003). Similarly, colonization by well-adapted mycorrhizal strains on saline sites often improves host performance (Hirrel and Gerdemann 1980, Al-Karaki and Hammand 2001, Feng *et al.* 2002,). Although such functions clearly are critical in stressed environments, we focus here

mainly on another topic, the moderation of host water relations and drought tolerance. We consider these to be the functions most likely to be altered by DSE fungi.

Host water uptake. Augé (2001) comprehensively reviewed the potential mechanisms involved in mycorrhizal modification of plant water relations and drought tolerance. Many of the proposed mechanisms were related to the size of the host plant and the nutrition of the host. Since the jury is still out on determining whether or not DSE enhance host growth and improve host nutritional status, as discussed above, we must limit our discussion of parallel mechanisms here. Clearly, if DSE colonization improves plant nutrient status, the same uptake mechanisms may also affect water relations. Here, however, we mainly concentrate on mechanisms not related to nutrient uptake, as water relations have been shown to be affected by mycorrhizal colonization independent of nutritional changes (Bethlenfalvay *et al.* 1988, Davies *et al.* 1993).

Factors that can affect water absorption by mycorrhizal or DSE-colonized roots tend to be features that affect water movement into the plant (Hardie and Leyton 1981, Allen 1982, Brownlee *et al.* 1983, Landhäusser *et al.* 2002) or through the plant (Johnson *et al.* 1982, Kucey and Paul 1982). Hormonal control of host physiology by root-colonizing fungi may also be a factor (Allen *et al.* 1980, Levy and Krikun 1980). The extent to which plant water absorption may be mediated by DSE hyphae is uncertain. As stressed earlier, many aspects of the basic biology of the DSE fungi are unknown. For example, efficient water scavenging from soil matrix and transportation into the host roots would require extramatrical mycelium. Although soil is likely to contain vast quantities of melanized fungal hyphae, distinction of the extramatrical mycelium of root-inhabiting melanized fungi from melanized hyphae of saprobic soil fungi is almost impossible. Even if plentiful mycelium can be observed in aseptic

resynthesis experiments (Mandyam and Jumpponen, unpublished), it is uncertain whether such structures extend into natural systems.

It is likely that extensive DSE colonization of host plants under field conditions (see Barrow and Aaltonen 2001 and Fig. 1.1) is indicative of altered water conductance within host tissues. According to Boyer (1971) and Black (1979), root system resistance accounts for most of the total resistance to water flow through the plant. It has been suspected that mycorrhizal colonization alters radial or axial resistance to water flow in roots (Safir *et al.* 1972). Similarly, extensive DSE colonization may alter root water dynamics.

In addition to experiencing altered resistance of roots to water flow, mycorrhizal and non-mycorrhizal plants often exhibit altered transpiration rates and stomatal conductances (for reviews see e.g. Allen and Allen 1986, Koide 1993, Smith and Read 1997, Augé 2001). Although the primary physiological drivers for mycorrhizal control of host stomatal conductance have not been identified, hormonal effects seem most likely (Augé 2001). Since the growth promotion of hosts by mycorrhizae often involves production of hormones and since growth promotion by DSE fungi is not consistently observed under experimental conditions, it is unlikely – yet still possible – that DSE could hormonally control host stomatal conductance. Overall, comparisons of stomatal conductances and transpiration rates between inoculated and DSE-free plants would be likely to provide cues to whether or not DSE fungi are involved in host water uptake. Although such experiments are easy to perform, they have not been conducted for the DSE fungi in spite of their prevalence in stressed conditions.

Host drought and heat tolerance. Augé (2001) classified drought tolerance mechanisms as nutritional or non-nutritional. Clearly, DSE involvement in host drought tolerance and the control of water dynamics would depend on the overall fitness of the host plant and on the

growth or nutritional benefits that DSE colonization may confer. Again, we underline that basic physiological questions about DSE need to be addressed before the involvement of these fungi in plant water relations can be assessed. For example, it would be essential to conduct experiments assessing whether DSE fungi are involved in host water acquisition or involved in the control of stomatal conductance or of transpiration rates. At present, however, there are two lines of evidence suggesting DSE may be involved in modifying host environmental tolerance, especially in relation to drought and heat tolerance.

Firstly, the nearly exclusive DSE colonization seen in native plants in an arid ecosystem of New Mexico has been suggested to help plants overcome the severe drought conditions typical of that ecosystem. This was studied by Barrow (2003), who suggested that nearly systemic root colonization by septate endophytes, along with the presence of abundant mucilaginous hyphae extending over 300 μm from the root matrix, aid nutrient and water transport under extended drought conditions. He also proposed that the continuous fungal network linking the vascular sieve elements to the root surface and rhizosphere is probably linked to water uptake.

Second, a fungal endophyte isolated from woolly panic grass, *Dichanthelium lanuginosum* (Elliott) Gould (now generally synonymized with *Dichanthelium acuminatum* [Sm.] Gould and C.A. Clark) in geothermal soils in Lassen Volcanic and Yellowstone National Parks in the USA was shown to increase host thermotolerance (Redman *et al.* 2002). Grass seedlings inoculated with the root- and foliage-inhabiting endophyte, provisionally identified as *Curvularia* sp., were able to withstand constant high soil temperature of 50° C for three days while the non-symbiotic plants shrivelled and were chlorotic. When inoculated and endophyte-free plants were exposed to intermittent soil temperatures of 65°C for ten days, all non-

inoculated seedlings died, whereas the inoculated seedlings survived. Interestingly, neither the fungus nor the grass survived the increased temperature regimen separately. In further inoculation studies, the same fungus conferred improved heat and drought tolerance on various agricultural and horticultural plants (Pennisi 2003). For example, wheat plants were able to withstand substantially longer periods of drought when inoculated with the endophyte. The *Curvularia* endophyte possessed melanized cell walls but was not considered to be a DSE.

The mechanisms for the altered environmental tolerance have only been hypothesised. The endophytes produce melanized cell walls when colonizing the host. Redman *et al.* (2002) suggested that the fungal melanin may play a role in heat dissipation or may form complexes with oxygen radicals formed during stress. If this is true, then the DSE that produce melanized hyphae and microsclerotia, already shown by Read and Haselwandter (1981) and Barrow and Aaltonen (2001) to occur abundantly in stressed environments, may perform similar functions, which may be essential to plant survival and growth in those environments.

Experiments designed to study the role of DSE fungi in drought tolerance are necessary. As mentioned above, preliminary experiments reported by Pennisi (2003) suggest that endophyte colonization can increase drought tolerance of various agricultural cultivars. As many of the experiments required to confirm such important functions are fairly simple, a project to screen multiple hosts and DSE strains would seem to be timely. If the DSE do assist plants in water uptake, or to increase drought-tolerance and thermal resistance, the agricultural implications could be far-reaching. Since these DSE fungi are easily cultured, unlike the obligately symbiotic AM fungi, they may have important uses as biofertilizers, and may allow us to manage agricultural systems more efficiently.

Protection from herbivores. Mycorrhizal fungi can mitigate the effects of herbivory on the host plants (Gehring and Whitham 2002). Although the effects of mycorrhizas on herbivores are highly variable and no consensus on overall trends has been reached, we assume that mycorrhizal and DSE colonization mainly act to reduce rather than increase the negative impacts of herbivores on plant fitness and performance. We propose three possible mechanisms that may limit herbivory or decrease its impact. First, by improving overall plant performance, fungal symbionts may improve plant tolerance of herbivory and thus increase the plant's ability to sustain herbivore damage without incurring visibly reduced productivity (Borowicz 1997, Gehring and Whitham 2002). Second, fungal symbionts can alter carbon-to-(mineral)-nutrient ratios, thus allowing an increased investment in carbon-based antiherbivore (Jones and Last 1991). Third, symbiotic grass endophytic fungi themselves may produce antiherbivore compounds, thus reducing the overall herbivory (Clay 1990, Clay 2001). Because the first mechanism we have listed depends on the improvement of host growth or nutrient uptake by the symbiont, and because those effects are uncertain in the DSE-host association, we will only consider the last two possibilities, i.e., those based on production of anti-herbivory compounds by plants or DSE.

No experimental evidence exists for induction of strong plant defences by DSE colonization. The sole piece of potential direct evidence to date was provided by Yu *et al.* (2001), who observed irregular wall thickening in asparagus cells colonized by *P. fortinii*. Apart from such physical changes, biochemical changes also need to be investigated in connection with DSE. It is possible that DSE may produce or induce production of herbivore feeding deterrents that are unrelated to the compounds hosts produce in conventional pathogen resistance mechanisms. With AM fungi, Gange and West (1994) have shown that colonization of *Plantago*

lanceolata L. increased tissue concentrations of iridoid glycosides, an insect feeding deterrent. As a result, the performance of a generalist herbivorous insect, the garden tiger moth *Arctia caja* L., was negatively affected. EM plants can also differ from non-mycorrhizal plants in their chemical composition. However, changes in host chemical composition as a result of colonization by EM fungi may be minimal, and the effects of EM on insect herbivores are highly variable. For example, only one of several herbivorous insect species was found to be affected by EM colonization of Scots pine (Manninen *et al.* 1998, Manninen *et al.* 1999a, Manninen *et al.* 1999b). Similar to mycorrhizal fungal colonization, colonization by DSE may alter host metabolism and increase plant production of general deterrents against herbivores, although the effects of these deterrents may depend on the individual herbivore species and their sensitivity.

Another interesting question is whether or not the DSE fungi themselves can produce herbivore deterrents. Foliar clavicipitaceous endophytes such as the *Neotyphodium* inhabitants of certain grasses have been shown to inhibit herbivory by producing toxic secondary metabolites (Clay 1988, 1990, 2001). Systemic endophyte colonization and resultant herbivore resistance was suggested to represent a new type of defensive mutualism (Clay 1988). What is the likelihood of DSE fungi producing compounds that inhibit herbivory? There are two main lines of reason that lead us to consider it likely that the DSE chemically inhibit herbivory, even though the compounds involved may not be similar to those reported for clavicipitaceous fungi.

Firstly, the DSE fungi produce large amounts of melanin in their cell walls. The melanins are known to provide rigidity to the cell wall, resistance to microbial grazing, and protection from desiccation and radiation damage (Kuo and Alexander 1967, Bell and Wheeler 1986, Griffith 1994). Typical DSE root colonization, featuring the extensive presence of melanized superficial, intercellular and intracellular hyphae (Currah and Van Dyke 1986, O'Dell

et al 1993, Newsham 1999), may protect the belowground tissues from foraging insects. Curiously, recent observations have shown that structures suggestive of melanized microsclerotia and variously pigmented and stained hyphae are present in the apoplastic spaces in leaves of black grama grass, *Bouteloua eriopoda* (Torr.) Torr., a native grass in arid southwestern rangelands of USA (Aaltonen and Barrow 2003). Similarly, *Periconia* spp. isolated from roots of mixed grassland communities at the Konza Prairie Biological Station, a native tallgrass prairie reserve in northwestern Kansas, were also able to produce melanized microsclerotia in the leaves of *Allium porrum* L. in an *in vitro* resynthesis system. However, this occurred only when multiple strains were inoculated on the host roots (Mandyam and Jumpponen, unpublished). When only a single strain of *Periconia* was inoculated, colonization was confined to the root tissues. It remains to be seen the pattern seen with these *Periconia* isolates is widespread. The contribution of foliar colonization by root-associated fungi herbivore resistance requires further study.

Some species of *Periconia*, presumably conspecific with endophytes commonly isolated from roots in the tallgrass prairie ecosystem (Mandyam and Jumpponen, unpublished), are able to produce toxic chemical compounds. Giles and Turner (1969) showed that *Periconia macrospinosa* Lefebvre and Johnson was able to produce a chlorine-containing compound. Such compounds have been shown to be bioactive (McGahren *et al.* 1969), although the biocidal properties of the *P. macrospinosa* metabolite have not been investigated (Giles and Turner 1969).

The involvement of DSE in controlling herbivory, whether attributable to induction of host metabolites or to toxic molecules and melanins produced by DSE themselves, can be tested in simple feeding trials. For example, preliminary studies could assess herbivore performance

when only DSE-colonized and endophyte-free tissues are made available. Alternatively, herbivore preferences for DSE-colonized vs. uncolonized plants can be evaluated by measuring plant tissue losses in both conditions.

Protection from plant pathogens. AM associations have long been thought to play a role in control of root pathogens. Azcón-Aguilar and Barea (1996) reviewed this topic and outlined the possible mechanisms of root pathogen control by AM fungi. These included general improvement of plant nutritional status, compensatory supply of materials to damaged roots, activation of plant defences, modification of microbial communities in the rhizosphere, promotion of morphological or anatomical changes in roots, and competition with pathogens for host photosynthates. It is very likely that multiple mechanisms are involved in the reduction of pathogen impact on mycorrhizal plants. Borowicz (2001), in a meta-analysis of data collected over 30 years on AM-pathogen interactions, concluded that both non-nutritional mechanisms and facilitation of phosphorus uptake contributed to the observed inhibition of pathogens. As in our section above on protection from herbivory, we will concentrate here on non-nutritional mechanisms.

There are three particularly likely mechanisms through which DSE may inhibit pathogens, or minimise their impacts on plant growth and performance. Firstly, mycorrhizal fungi and rhizosphere-inhabiting pathogens may compete for the plant photosynthates or for sites of colonization. Secondly, compounds inhibitory to pathogens may be produced. Finally, the DSE colonization may have prophylactic value by inducing plant defence responses to subsequent pathogen infection. These topics are detailed below.

Competition between symbiotic fungi and pathogens may be localised in small spatial scales within root systems (Dehne 1982, Linderman 1994). If DSE fungi and root pathogens

depend on similar mechanisms of accessing host photosynthates or on similar host entry and colonization sites, the presence of one or the other fungal type would result in pre-emptive resource utilisation, a form of exploitation competition (Lockwood 1992). Our data from Konza Prairie (Fig. 1.1) shows a great abundance of DSE in mixed grassland communities. The sheer abundance of DSE fungi colonizing the root tissue is likely to consume significant amounts of available carbohydrates, limiting their availability to pathogens and thereby inhibiting pathogen establishment.

With regard to the chemical inhibition of pathogens, we have already mentioned that *Periconia* strains possibly conspecific with those common in tallgrass prairie grasses have been shown to produce chlorine-containing compounds (Giles and Turner 1969) that may be biocidal (McGahren *et al.* 1969). Similarly, *Periconia* sp. from *Taxus cuspidata* Sieb. and Zucc. in Korea produced two compounds (Periconicins A and B), which were antibacterial against *Bacillus subtilis* (Ehrenberg) Cohn, *Staphylococcus aureus* Rosenbach, *Klebsiella pneumoniae* (Schroeter) Trevisan and *Salmonella typhimurium* (Loeffler) Castellani and Chalmers with a minimum inhibitory concentration (MIC) in the range of the well-known antibiotic gentamicin (Kim *et al.* 2004). Although it remains open to discussion whether or not DSE fungi are capable of production of antibacterial or antifungal compounds, the presence of such compounds in congeneric strains warrants further investigation.

With regard to induced plant defenses, it has generally been concluded that AM colonization results in only weak and localised induction of these mechanisms (Koide and Schreiner 1992). However, transient activation of plant defences during early mycorrhizal formation (Gianinazzi-Pearson *et al*. 1996) or induction of low levels of defences (Benhamou *et al*. 1994) may occur. As mentioned above, Yu *et al.* (2001), when studying asparagus roots

during colonization by *P. fortinii*, observed irregular wall thickening adjacent to *P. fortinii* hyphae in certain root cells, specifically, long exodermal cells. They suggested that this was a weak defence reaction. These weak host responses to DSE colonization are somewhat similar to those attributed to AM colonization and suggest that the DSE fungi may be able to effectively induce a degree of host defence.

There are several ways in which DSE fungi may inhibit root-associated pathogens. As a first step, we suggest that simple experiments based on exposing strongly DSE-colonized host plants to an array of pathogens may be most profitable. Selection of an array of root endophytes and pathogens is necessary in such studies. Arnold *et al.* (2003) showed that many antagonistic effects among foliar endophytes and pathogens of a tropical tree (*Theobroma cacao* L.) were direct and appeared to be species-specific. Similarly, competitive and antagonistic interactions among non-pathogenic root endophytes and root pathogens must be analysed within a relevant ecological context: the niche requirements among the component organisms assessed must be sufficiently similar (for discussion see Janisiewicz 1996). Whether the mechanism involved is competitive exclusion, induction of plant defences, or DSE production of biocides, experiments can readily be designed to show whether or not pathogen control by DSE is likely to occur under natural conditions.

Impact on plant community dynamics. AM fungi can influence plant community composition and control plant diversity. Van der Heijden *et al.* (1998) showed that AM fungi enhanced plant diversity in European calcareous grasslands. They also showed for the first time that AM species richness and community composition were very important in determining the primary productivity in mixed grassland plant communities. In contrast, Hartnett and Wilson (1999) concluded that AM fungi actually reduced plant species diversity while bringing about no

significant change in productivity. They suggested that a fungicidal treatment reducing AM fungal colonization allowed dominance by weakly mycotrophic plant species while causing no decrease in the productivity of the system. Van der Heijden (2002) suggested that the conflicting effects of AM fungi on plant diversity depend upon the degree of mycorrhizal dependency of the plants involved, as well as the diversity of AM fungi and the nutritional status of the ecosystem. We concur that plant species must express differing responses to colonizing fungi before any changes will be seen in community composition in experiments manipulating symbiotic fungal diversity or abundance.

Although studies evaluating the responses of different hosts to DSE are few, we propose that DSE can impact plant community composition if the plant species in the community respond differently to DSE colonization. In fact, there is some variation in plant responses to DSE colonization (see Jumpponen and Trappe 1998, Jumpponen 2001). Fernando and Currah (1996) conducted inoculation experiments with several hosts and DSE strains. They concluded that the observed responses were specific to the individual hosts and fungi involved. Although determining the net effects of multiple inocula in soils of mixed plant communities may be a nearly impossible task, it appears that DSE fungi with broad host ranges (see Jumpponen and Trappe 1998) and differing impacts on host performance would be able to have impacts on community composition. Clearly, to investigate our predictions about the role of DSE fungi in structuring plant communities, more experiments are needed. Microcosm studies need to be designed to evaluate the role of various DSE fungi after the appropriate fungi and host plants have been carefully selected. Such studies would allow direct experimental assessment of the effects of single DSE strains in mixed plant communities.

CONCLUSIONS

Information on the abundance and possible function of DSE is scanty at best. Based on the limited number of studies available, we conclude that DSE fungi are prevalent in various habitats and colonize a substantial proportion of the species present in mixed plant communities. This group of fungi cannot be overlooked while assessing the fungal communities of any ecosystem, as their abundance may equal or even exceed that of the AM fungi.

There is a conspicuous gap in our understanding of the ecological relevance of these root-associated endophytes. We propose that DSE, like many mycorrhizal fungi, are multifunctional. Even in the absence of clear consensus about positive impacts on host fitness, growth or performance, DSE may perform functions similar to those attributed to mycorrhizal fungi. Although experimental evidence is limited and experimental results may conflict, DSE are likely to be involved in host nutrient uptake, especially from recalcitrant or complex organic sources. Ample production of melanized tissues may indicate a function of altering environmental tolerances, or deterring insect and mammalian herbivores. Simple pre-emptive resource use and competition for host root exudates may be adequate for reducing host susceptibility to soil-borne pathogens. Finally, broad DSE host ranges and the reported diversity of host responses to DSE colonization suggest that these fungi may drive plant community dynamics via differential host responses and resource capture. Determining the impact of these functions under natural conditions is desirable but may be exceedingly difficult. We propose that simple, controlled preliminary experiments may be the most efficient way to obtain clues to the functions of DSE. Our intent in this contribution was to provide a framework of testable hypotheses to be used in designing such experiments. The bottom line is that the DSE fungi are abundant and their ecological significance needs to be understood.

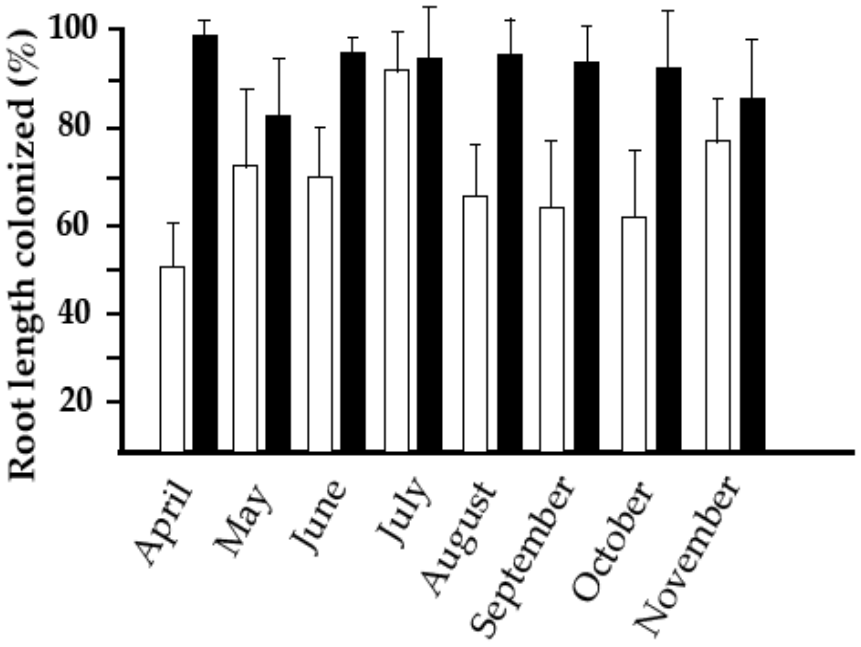


Figure 1.1 Root colonization of AM and DSE in mesic mixed grassland communities at Konza Prairie.

Arbuscular mycorrhizal (AM) fungi (open bars) and dark septate endophytes (DSE; filled bars), DSE had higher colonization levels than AM ($F_{1,80}$=132.7, P<0.001). Colonization varied significantly among months ($F_{7,80}$=3.5, P=0.002). AM and DSE had different seasonal dynamics ($F_{7,80}$=6.5, P<0.001 for the interaction term fungus * time).

Table 1.1 Abundance of mycorrhizal and dark septate endophytes in various ecosystems.

Number of species with fungal colonization over the total number of species examined, followed by the percentage of root length colonized (range in parentheses).

AM = Arbuscular mycorrhiza, EM = Ectomycorrhiza, ERM = Ericoid mycorrhiza, DSE = Dark septate endophytes

Ecosystem type	*AM*	*EM*	*ERM*	*DSE*	*Other*	*Reference*
Alpine						
	63/89 (1-100)	12/89 (NA)[a]	–	33/89 (1-100)	15/89 (FE)	Read and Haselwandter (1981)
	2/40	6/40	7/40	11/40	1/40 (ArM)	Treu *et al.* (1996)
	5/35	–	–	31/35	1/35 (Orc)	Currah and Van Dyk (1986)
Arctic						
	-/55 (NA)[a,b]	3/55 (NA)[a]	2/55 (NA)[a]	Present [c]		Bledsoe *et al.* (1990)
	1/24 (+)[d]	6/24 (+)[d]	2/24 (+)[d]	–		Kohn and Stasovski (1990)
	5/6 (0-100)	–	–	6/6 (10-50)	5/6 (FE)	Ruotsalainen *et al.* (2002)

	-/76	3/76	2/76	30/76			Väre *et al.* (1996)
Antarctic							
	18/40 (NA)[a]	0/40 (NA)[a]		21/40 (NA)[a]			Laursen *et al.* (1997)
Boreal forest							
	4/6	–	–	2/6[e]	–		Currah and Van Dyk (1986)
	4/25	6/25	5/25	14/25	–		Thormann *et al*. (1999)
Temperate grassland							
Sandy grassland	60/89 (0-100)	4/89 (NA)[a]	–	63/89 (NA)[a]	–		Kovács and Szigetvári (2002)
Short grass prairie	77/85 (NA)[a]	–	–	0/85 (NA)[a]	–		Currah and Van Dyk (1986)
Tropical rain forest	5/18 (26-75)	–	12/18 (<25-100)	16/18 (<25-75)	–		Rains *et al.* (2003)

Footnotes:

a = NA = data not available

b = although fine endophyte may have been observed, the absence or arbuscules and vesicles was determined to mean lack of AM associations

c = the taxa colonized were not listed

d = + = high in most replicates; ± colonization of few cells in less than 50% of the replicates

e = DSE was recorded only as superficial melanized hyphae

Arm = Arbutoid mycorrhiza

FE = Fine endophyte

Orc = Orchid mycorrhiza

Table 1.2 Reported enzymatic capabilities of DSE fungi

Enzyme	DSE fungus	References
Cellulases	Unidentified DSE isolates	Bååth and Söderström (1980)
	Cadophora (*Phialophora*) *finlandia* (Wang and Wilcox) Harrington and McNew and isolates similar to *Phialocephala fortinii*	Caldwell *et al.* (2000)
	Periconia species and other DSE isolates	Mandyam and Jumpponen, unpublished
Laccases	*P. fortinii*	Currah and Tsuneda (1993)
Amylases	*C. finlandia* and isolates similar to *P. fortinii*	Caldwell *et al.* (2000)
Lipases	*C. finlandia* and isolates similar to *P. fortinii*	Caldwell *et al.* (2000)
Pectinases	*C. finlandia* and isolates similar to *P. fortinii*	Caldwell *et al.* (2000)
Xylanases	*C. finlandia* and isolates similar to *P. fortinii*	Caldwell *et al.* (2000)
Proteolytic enzymes	Unidentified DSE isolates	Bååth and Söderström (1980)
	C. finlandia and isolates similar to *P. fortinii*	Caldwell *et al.* (2000)
Tyrosinases	*Periconia* ssp. and other DSE isolates	Mandyam and Jumpponen, unpublished
Polyphenol-oxidases	*P. fortinii* strains	Currah and Tsuneda (1993)
	Leptodontidium orchidichola	Ferrnando and Currah (1995)
	Periconia ssp. and other DSE isolates	Mandyam and Jumpponen, unpublished

CHAPTER 2 - Isolation and morphological and metabolic characterization of common endophytes in annually burned tallgrass prairie

ABSTRACT

Dark septate endophytes (DSE) are common and abundant root-colonizing fungi in native tallgrass prairie. To characterize the DSE, fungi were isolated from roots from mixed tallgrass prairie plant communities. The fungal isolates were grouped according to morphology and the grouping was confirmed by ITS-RFLP and/or sequencing. Sporulating species of *Periconia*, *Fusarium*, *Microdochium* and *Aspergillus* were isolated along with many sterile fungi. *Periconia macrospinosa* Lefevbre and Johnson accounted for about 45% of the isolates. In a sterile resynthesis system, leek (*Allium porrum* L.) roots were inoculated with the isolated fungi. After six week incubation, *Aspergillus* and *Fusarium* sp. were clearly pathogenic. *Periconia macrospinosa* and *Microdochium* sp. were endophytic: *Periconia* produced melanized intracellular microsclerotia in host root cortex, whereas *Microdochium* produced abundant melanized inter- and intracellular chlamydospores. Host growth responses to endophytes were variable: strains of *Periconia* increased, decreased or had no effect on leek total biomass, whereas *Microdochium* was neutral. Select *Periconia* and *Microdochium* strains were tested for their enzymatic capabilities and their ability to use organic and inorganic nitrogen. These fungi tested positive for amylase, cellulase, polyphenol oxidases and gelatinase. *Periconia* isolates utilized both organic and inorganic nitrogen sources. Our study identified distinct endophytes in

a tallgrass prairie ecosystem and indicated that these endophytes can utilize a variety of complex nutrient sources suggesting facultative biotrophic and saprotrophic habits.

***Key words*:** enzymes, dark septate endophytes (DSE), *Microdochium*, *Periconia macrospinosa*, sterile fungi

INTRODUCTION

Dark septate endophytes (DSE) are a miscellaneous group of ascomyceteous root-colonizing microfungi characterized by melanized cell walls and intracellular colonization of healthy plants (Jumpponen and Trappe 1998; Addy et al 2005). Although DSE fungi are taxonomically unrelated and vary in ecological or physiological functions (Addy et al 2005), many of these fungi form similar morphological structures in the host roots (Jumpponen and Trappe 1998). Irrespective of the host plant species, the characteristics include superficial mycelium, hyphal penetration into the cortex and formation of melanized microsclerotia (Jumpponen and Trappe 1998; Yu et al 2001).

DSE fungi colonize a variety of host plant species and appear to have a global distribution (Jumpponen and Trappe 1998). To evaluate the abundance of DSE fungi across ecosystems, Mandyam and Jumpponen (2005) compared the published estimates of the proportion of plant species colonized by different mycorrhizal and DSE fungi and concluded that the DSE and mycorrhizal fungi were equally abundant. A two year study on the seasonal variation in the root colonization by arbuscular mycorrhizas (AM) and DSE fungi at Konza Prairie Biological Station, a Long-Term Ecological Research (LTER) site showed that DSE colonization in a tallgrass prairie was as high as AM colonization and, occasionally, even exceeded AM colonization (Mandyam and Jumpponen 2008).

So far, only a limited number of DSE fungi have been identified. These fungi are mainly conidial ascomycetes with varying phylogenetic affinities (Jumpponen and Trappe 1998; Addy et al 2005). In a recent review, Addy et al. (2005) list the most common DSE fungi from different habitats: *Cadophora finlandica* (Wang and Wilcox) Harrington and McNew, *Cryptosporiopsis rhizophila* Verkley and Zijlstra, *Exophiala* sp., *Heteroconium chaetospira* (Grove) Ellis, *Leptodontidium orchidicola* Sigler and Currah, *Oidiodendron maius* Barron, *Phialocephala dimorphospora* Kendrick, *Phialocephala fortinii* Wang and Wilcox, *Phialocephala sphaeroides* Wilson, *Phialophora graminicola* (Deacon) J. Walker, *Rhizoscyphus ericae* (anamorph *Scytalidium vaccinii* Dalpé), Litten and Sigler and *Trichocladium opacum* (Corda) Hughes.

Not only is the diversity of DSE fungi poorly understood, but only few of these taxa have been metabolically characterized (Caldwell and Jumpponen 2003a, b; Caldwell et al 2000; Currah and Tsuneda 1993; Wilson et al 2004). As reviewed in Chapter 1, DSE fungi are thought to utilize organic and inorganic nutrient pools. DSE fungi are especially thought to be involved in N uptake: Mullen et al. (1998) and Mandyam and Jumpponen (2008) suggest DSE role in N uptake during spring. Recently, Green et al (2008) provided indirect evidence in support of C and N exchange between a patch mosaic of grasses and biological crust dominated by a fungal network of symbiotic DSE fungi in a semi-arid ecosystem. Nitrogen is the most limiting nutrient in a tallgrass prairie and its availability can vary with fire, grazing, soil texture, topography (Blair et al 1998). Since DSE are abundant at Konza Prairie where N is a limiting nutrient, it is imperative to understand DSE role in uptake of organic and inorganic N and its subsequent facilitation. DSE fungi are able to produce a variety of extracellular enzymes and some of DSE fungi tested for their enzymatic capabilities are listed in Chapter 1 (Table 1.1). These enzymatic

capabilities suggest the DSE potential to access detrital C, N and P with possible role in nutrient facilitation.

Much of the present understanding on the diversity and function of the root-associated endophytes is based on a limited number of taxa and strains from alpine, arctic and Antarctic environments. Among these fungi, *P. fortinii* is the most studied DSE taxon. In contrast, studies on endophytes from grasslands are few (but see Kovács and Szigetvári 2002). This chapter aims to address three main questions: 1) what are the common DSE taxa in a mesic tallgrass prairie, 2) what are their impacts on a colonized host, host biomass and the fungal morphological manifestations, and 3) what is the range of metabolic and enzymatic capabilities of the isolated endophytic fungi.

MATERIALS AND METHODS

Site description

Konza Prairie Biological Station (KPBS, 39˚05' N, 96˚35' W) represents a native mesic tallgrass prairie in the Flint Hills of eastern Kansas, USA. The site spans 3,487 ha and remains undisturbed by agriculture. The vegetation is dominated by C_4 grasses including *Andropogon gerardii* Vitman, *Sorghastrum nutans* (L.) Nash., *Schizachyrium scoparium* (Michx.) Nash., and *Panicum virgatum* L. (see Towne 2002 for a complete list of vascular plants at Konza Prairie). The soil parent material is chert-bearing limestone with the soil bulk density of 1.0 g/cm^3. January mean temperature is –3º C (range –9 to 3º C) and the July mean is 27º C (range 20 to 33º C). Annual precipitation is 835 mm ~75% of which falls in the growing season. Our research site was subjected to an annual spring burning regime, a typical grassland management practice in this area.

Sample collection

Twelve permanent lowland plots ($4m^2$) at Konza were selected for sampling. The isolations were performed twice, in June 2002 and August 2002. Two random soil cores from each plot were pooled and manually homogenized. Roots were immediately washed free of soil under running tap water and processed immediately. A random sub-sample of roots was used for isolating fungi and another stained for microscopic study of typical DSE structures.

Staining of field collected roots

Roots were cleared and stained as described by Barrow and Aaltonen (2001). In brief, roots were cleared in 2.5% KOH, followed by staining with Sudan IV and de-stained overnight in acidified glycerol. Inter- and/ or intracellular melanized hyphae and microsclerotia in the root cortex and hyaline hyphae stained by Sudan IV in the cortex and stele were examined microscopically and recorded.

Isolation of root-inhabiting fungi

Roots were surface sterilized with bleach (50%) for 1 min, washed with sterile water and treated with 70% alcohol for 2 min followed by several washings with sterile distilled water. The roots were plated on water agar (1.5%) and incubated at 25º C in dark. The roots were routinely observed under a dissecting microscope and the emerging fungi were transferred onto corn meal agar and potato dextrose agar (CMA; PDA; Becton Dickinson and Co, Maryland, USA). Colony morphology was recorded and sporulation studied on CMA and PDA. The isolates were maintained by routine subculturing.

Identification of fungal isolates

The sporulating fungi were identified based on colony morphology, conidiospore and conidiophore characteristics. Many fungi remained sterile; they were broadly grouped by colony color. The morphological groups were further refined by Restriction Fragment Length Polymorphism of the Internal Transcribed Spacer (ITS-RFLP; Gardes and Bruns 1996a), after genomic DNA extraction from pure culture from following a protocol by Gardes and Bruns (1993) or with UltraClean^{TM} soil DNA isolation kit (MoBio Laboratories, Inc. Carlsbad, California). ITS-region was amplified with primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al 1990) and digested with restriction enzymes *Hinf* I and *Alu* I (BioLabs, New England, USA) as described in Gardes and Bruns (1996a). Similar RFLP patterns were considered an approximation of conspecific groups.

Resynthesis with leek

Since *Periconia macrospinosa* Lefevbre and Johnson constituted about 45% of the isolates, we chose five representative isolates, and three isolates from the other species or conspecific groups to confirm whether or not they were endophytic (Table 2.1). *Allium porrum* L. seeds (W. Atlee Burpee and Co, Warminster, Pennsylvania, USA), routinely used in preliminary screening of fungal structures were surface sterilized with 30% bleach for 1 min, followed by 3 min in 70% ethanol, washed several times with sterile distilled water and dried on sterile #1 Whatman filter paper. Sterilized seeds were germinated on 1/10 Murashige Skoog (MS) basal salt mixture medium without any organic additives (Sigma, Missouri, USA) for a week in a growth chamber under 12h cycle of light (ca. 250 μmol m^{-2} s^{-1} PAR at 20^{o} C). A small opening in the Petri dish was made by making overlapping cuts both in the lid and the dish

containing the MS (1/10 strength) medium. A sterile seedling was transferred onto this MS plate so the shoot emerged through the opening while the roots were contained within the Petri dish. The dish was sealed with parafilm except at the opening and placed upright in the growth chamber. The seedlings were allowed to stabilize for four days in the growth chamber prior to inoculation with a 6mm fungal plug. The plugs were cored from isolates grown on PDA at 25º C for 15 days. A total of 15 replicate inoculated leeks for each fungal treatment were incubated upright in the growth chamber under the above conditions. Fungus-free control plants were mock-inoculated with sterile PDA plugs. The plants were observed periodically for survival and survivors harvested after six weeks. Shoots of all and roots of 10 replicates were harvested, dried at 60º C and their dry weight recorded. Roots from the remaining five replicates were used for microscopic confirmation of colonization and the fungal morphology within the roots was recorded.

Roots were cleared and stained as previously described. A fungal endophyte was confirmed if the fungus produced melanized microsclerotia and/or chlamydospores in the root cortex. Additionally, the roots were scored for the presence of inter- and/ or intracellular melanized hyphae and hyaline hyphae stained by Sudan IV.

DNA sequencing

Fungi that produced typical DSE structures in leek roots (see below) were selected for sequencing to further confirm their likely taxonomic affinities. The PCR-amplified DNA from cultures of *Microdochium* and *Periconia* isolates were purified using UltraClean™ PCR clean up kit (MoBio Inc. Carlsbad, California) and sequenced using primers ITS1F and ITS4 at the Kansas State University Sequencing facility (GenBank Accession numbers: *P. macrospinosa*

FJ536207-209 and *Microdochium* sp. FJ536210). The sequence similarities within the two taxa were estimated after alignment in the Sequencher (Genecodes Inc. Ann Arbor, MI) and their taxon affinities confirmed by BLAST. All the sequenced *Periconia* isolates were 99% similar to *P. macrospinosa* and *Microdochium* sp. was 93% similar to *Microdochium*.

Tests for enzymatic activities

Eight *P. macrospinosa* (KS0019, KS0025, KS0045, KS0054, KS0060, KS0082, KS0093 and KS0100) and two *Microdochium* (KS0012 and KS0014) isolates were tested for their hydrolytic capabilities. Enzymes hydrolyzing complex carbon molecules – amylase, cellulase, polyphenol oxidases including laccase and tyrosinase were tested. The confirmed endophytes were also tested for gelatinase. Each test medium was inoculated with a 6mm fungal plug cored from CMA and each isolate was incubated in triplicates for two weeks at room temperature. Tyrosinase tests were incubated for three weeks. A basal medium (Caldwell et al 1991) composed of mineral salts was used for amylase and cellulase evaluation.

(i) *Amylase*- the basal medium was amended with starch (1%) as the sole carbon source in the basal medium (Caldwell et al 2000). Iodine (10 %) was used to visualize the zone of hydrolysis and the strength of activity was classified based on the diameter of the hydrolytic zone.

(ii) *Cellulase (Endoglucanase or CMCase)*- the basal medium was amended with carboxy methyl cellulose (CMC, 1%; Caldwell et al 2000). The zone of hydrolysis was visualized by flooding the plate with an aqueous solution of Congo red (1mg/ml) for 15 min. The plate was flooded with 1M NaCl for 15 min after draining the Congo red, followed by

stabilization with 1M HCl (Teather and Wood 1982). The strength of activity was classified based on the diameter of the hydrolytic zone.

(iii*) Laccase spot test*- Fungal isolates were grown on Sabourauds' medium and PDA. Laccase production can be affected by media components and therefore it must be evaluated on different media (Hutchinson 1990). o-dianisidine (0.01%) was added to the growth media. A positive reaction is indicated by a dark brown zone around the colony. The strength of the activity was based on the visibility of the brown zone.

(iv) *Tyrosinase (cresolase) spot test*- Fungal isolates were grown on 2.5% malt extract agar for three weeks. One drop of 0.1M p-cresol dissolved in ethanol was added on the colony margin (Gramss et al 1998). The indicator p-cresol stains red in the presence of tyrosinase.

(v) *Gelatinase liquefaction test*- Fungi were inoculated into gelatin (12%) slants and incubated for two weeks. A positive test is indicated by liquefaction in the tube after chilling for 30 min. The proportion of liquefied medium indicated the strength of the activity.

Nitrogen utilization

Confirmed endophytes (seven *Periconia* isolates and one *Microdochium*) were grown on liquid medium containing either organic (alanine, arginine and glycine) or inorganic (NH_4Cl and NH_4NO_3) nitrogen source (Finlay et al 1992). Media was adjusted to C:N = 39:1. The N sources were omitted from the control treatments. Fungal isolates were inoculated to 20ml liquid medium of each N source in five replicates and incubated at 22°C for three weeks. The fungal biomass was extracted by filtration and dried at 60°C. The pH of the filtrate and fungal biomass were recorded as proxy for N utilization. The pH after N utilization ranged from 2.5 to 7.5. The fungal

biomass on N-free medium was subtracted from the observed biomass on different N media for each of the tested isolates.

Statistical analysis

The comparison of leek biomass for the different treatments failed the assumptions of ANOVA due to unequal sample size, outliers and unequal variance. The unequal sample size resulted from mortality and fungal contamination. Since the resynthesis plates were open-plate systems (see design of this system in materials and methods section) incubating for five weeks, they were susceptible to aerial contamination. Accordingly, non-parametric tests (Higgins 2003) were considered necessary. Analysis of rank based one-way ANOVA for biomass was performed using a median test available at http://www.stat.wmich.edu/slab/RGLM/. Critical value for comparison was set to α=0.05.

The fungal biomass comparison for N uptake fulfilled the assumptions of ANOVA. The different N sources were separately analyzed by one-way ANOVA in SAS (version 9.0). Pair-wise differences were determined by a conservative Bonferroni's test (P<0.05).

RESULTS

The isolates used for the resynthesis, enzymatic characterization, and N utilization do not completely overlap since many isolates perished on repeated subculturing.

DSE structures in field collected roots

Extensive melanized hyphae were abundant in the cortex of field collected roots (Fig. 2.1a). Microsclerotia were found both in the stele (image not shown) and cortex (Fig. 2.1b) with their lipids staining deep red with Sudan IV. Hyaline vesicles (Fig. 2.1c) were also present in the

cortex and could be visualized by Sudan IV. Inter- and intracellular chlamydospores were routinely observed in the field samples (Fig. 2.1d).

Fungal isolates

A total of 113 isolates were obtained with 52 isolates from the June sampling and 61 from August sampling. Table 2.1 lists the isolated fungal taxa or ITS-RFLP conspecific groups. *Periconia macrospinosa* conidiophores and conidia commonly emerged from the surface sterilized field root samples (Fig. 2.2a). Other sporulating microfungi did not produce visible structures on the field-collected roots, but were identified based on conidial morphology and culture characteristics. A majority of the sporulating fungal isolates were *P. macrospinosa* and easily identified by the characteristic large echinulate spores and conidiophores (Ellis 1971).

The RFLP typing of the isolates confirmed the morphological grouping. Our *Periconia* ITS sequences were 98-99% similar to *P. macrospinosa*. However, the culture characteristics and microscopic morphology of these strains were highly variable. For example, 10d old *Periconia* KS0035 and KS0045 appeared significantly different on PDA. KS0035 was white in color (Fig. 2.2b) and was characterized by sparse sporulation that ceased during prolonged preservation. This strain produced thick, bulbous hyaline and melanized hyphae (Fig. 2.2c), along with clusters of chlamydospores (Fig. 2.2d) or chains of chlamydospores (image not shown). In contrast, KS0045 frequently produced patches of green-pigmented hyphae (Fig. 2.2e) and over time, the colony turned green in color (Fig. 2.2f). KS0045 produced typical macronematous echinulate conidia (Figs. 2.2g, 2.2h). Chlamydospores were never observed.

Our *Microdochium* strain was 93% similar to *Microdochium* sp. (GenBank Accession FJ536210). The culture was black in color (Fig. 2.2i) and produced spores in sporodochia with abundant chlamydospores in chains (Fig. 2.2j).

Leek resynthesis

Leek responses to fungal inoculation are summarized in Table 2.1. *Acremonium* sp., *Aspergillus* sp., *Cladosporium* sp., and *Fusarium* sp. were clearly pathogenic and most of the inoculated plants did not survive through the six week incubation. Leek plants inoculated with *Microdochium* sp. (KS0012), sterile dark fungus (KS0001) and *P. macrospinosa* (KS0019, KS0058, KS0060, KS0093 and KS0100) remained healthy and symptomless through the six-week incubation.

Microscopic observations of roots from resynthesis

Leeks inoculated with *Acremonium* sp., *Aspergillus* sp., *Cladosporium* sp., and *Fusarium* sp. did not produce typical DSE structures. The sterile green fungus (KS0001) produced extensive superficial hyphae around the roots with no visible inter- or intracellular colonization (image not shown). Melanized hyphae were rarely seen in the roots. Melanized microsclerotia were found only in the cortex with lipids staining red with Sudan IV (Fig. 2.3a). Hyaline vesicles occurred frequently in the cortex (Fig. 2.3b). Hyaline vesicles are likely the initial stages of microsclerotia (Mandyam and Jumpponen 2008): we observed a variety of stages ranging from hyaline vesicles to partially melanized microsclerotia. The hyaline hyphae were visible only at the points of their attachment to microsclerotia and hyaline vesicles. Sudan IV stained the lipids faintly in the hyaline hyphae and vesicles. *Periconia* conidiophores and conidia were frequently visible on the colonized leek roots (image not shown). *Microdochium* sp. formed inter- and

intracellular melanized chlamydospores in the cortical cells (Fig. 2.3c). No melanized hyphae were observed in the roots inoculated with the strains we chose.

Leek biomass responses

Leek biomass was highly variable among and within the inoculation treatments (Fig. 2.4). Root biomass was not different among the different fungal treatments (data not shown), while the shoot and total biomass varied among the treatments and compared to the control. Since total and shoot biomass showed a similar trend, only total biomass data is presented. Compared to the control, *Periconia* isolates increased (KS0058, KS0060), decreased (KS0019, KS0100), or had no effect (KS0093) on the total leek biomass. The total biomass of *Microdochium* sp. (KS0012) inoculated plants did not differ from the control. Sterile green fungus (KS0001) increased the total biomass.

Enzymatic capabilities

Results of enzyme hydrolysis for the putative endophytes are presented in Table 2.2. Most isolates tested positive for all the tested extracellular enzymes. The intensity of enzyme hydrolysis varied among the isolates within and between taxa. All the tested fungi hydrolyzed starch and CMC. Laccase and tyrosinase were produced by all the isolates except for one *Periconia* isolate (KS0060) and *Microdochium* (KS0012). Laccases oxidize a variety of organic compounds including diphenols, polyphenols and aromatic amines (Thurston 1994). We used o-dianisidine, an aromatic amine, and syringaldazine (Marr 1979), a commonly used laccase substrate, to test for laccase activity. However, the results were not concordant and only those from o-dianisidine are presented because syringaldazine may serve as a substrate for peroxidase

(Mayer and Staples 2002). Gelatinase was produced by all isolates except one *Periconia* strain (KS0093).

Nitrogen utilization

All the tested nitrogen sources were utilized by the *Periconia* isolates to some degree. Only one *Microdochium* (KS0014) isolate was used. It performed poorly in the liquid media and its ability to use N sources remains uncertain. Biomass and media pH after three weeks of incubation varied substantially among species and strains (Fig. 2.5). *Periconia* isolates did not show any specific trend in N utilization. All the tested *Periconia* isolates were able to utilize both organic and inorganic N. Results are missing for arginine, ammonium and nitrate treatments for some fungal isolates as those substrates were prone to bacterial contamination and accordingly omitted from analyses.

DISCUSSION

Root endophytes

Field collected roots are commonly colonized by melanized hyphae, microsclerotia and chlamydospores (Mandyam and Jumpponen 2008). Similarly, our present study confirmed that these structures are frequent in field collected roots. Our isolation experiment from roots of mixed plant communities at Konza yielded some frequently encountered soil fungi including *Acremonium* sp., *Aspergillus* sp., *Cladosporium* sp., *Curvularia* sp. and *Fusarium* sp. Our resynthesis studies suggest that these fungi were pathogenic in the absence of competition from other root- and rhizosphere-inhabiting organisms. In addition to these common fungi that appeared pathogenic, we isolated two interesting endophytic fungal taxa that were confirmed to

form morphological structures commonly associated with DSE fungi. *Microdochium* sp. was infrequent, whereas *P. macrospinosa* was the most frequent among our isolates. It is possible some additional endophyte taxa may have escaped isolation.

Ellis and Ellis (1970) have described several *Periconia* (Halosphereiales) species based on spore size, morphology and colony characteristics. *Periconia* spp. have been isolated from a wide variety of environments ranging from temperate to tropical areas and from arable to native grassland ecosystems (Ellis 1971; Domsch et al 1980). Most *Periconia* spp. have been considered saprobic (Dunkle 1992), although a few are known pathogens (Ellis 1971). For example, *Periconia circinata* (L. Mangin) Sacc. causes milo disease in Sorghum (Leukel 1948, Leukel and Johnson 1948); *Periconia byssoides* Pers. and *Periconia shyamala* A. K Roy cause leaf spots in legumes and cassava (Ellis 1971); *P. manihoticola* (Vincens) Viégas causes leaf blight of rubber and cassava. *Periconia macrospinosa*, though not usually considered pathogenic to graminoids (Domsch et al 1980; Dunkle 1992), can be pathogenic to wheat under experimental conditions (Carter et al 1999). Our study indicates that *P. macrospinosa* is a common facultative endophyte capable of producing typical DSE structures.

While all our *Periconia* isolates matched *P. macrospinosa* morphologically and molecularly, some did not produce chlamydospores in culture. The colony characteristics of *P. macrospinosa* isolates were highly variable, ranging from white to grey colonies, while some were dark green or pink in color. Colony morphology of many isolates changed with colony age exhibiting a high degree of phenotypic plasticity.

Our *Microdochium* isolates having 93% similarity to *Microdochium* sp., could not be identified to the species level. Genus *Microdochium* (Xylariales) is poorly studied and its species concept poorly defined (Kwaśna and Bateman 2007). Kwaśna and Bateman (2007) list the

morphological characteristics of known *Microdochium* species, among which only four species produce both sporodochia and chlamydospores similarly to our isolates –*Microdochium bolleyi* (R. Sprague) de Hoog and Herm.-Nijh., *Microdochium dimerum* (penz.) Arx, *Microdochium lunatum* (Ellis and Everh.) Arx. and *Microdochium tainanense* (Ts. Watan.) de Hoog and Herm.-Nijh.

DSE fungi and resynthesis

The resynthesis system used in this study did not mimic field conditions, but was adequate to address our main objectives – to identify and confirm root endophytes in a tallgrass prairie ecosystem. In our assays, *Periconia* and *Microdochium* produced typical endophyte structures in leek roots. The plants used in our resynthesis system were juveniles and not native prairie plants. Accordingly, this system may be inaccurate in assessing the long-term impact of *Periconia* and *Microdochium* colonization.

In this study, *Microdochium* sp. produced inter- and intracellular chlamydospores in leek roots but typical microsclerotia and melanized hyphae were lacking. In contrast, *Periconia macrospinosa* formed typical microsclerotia in the leek cortex, confirming it as a septate endophyte of a tallgrass prairie. While melanized hyphae were common in field collected roots, they were lacking in resynthesis studies. If hyaline hyphae were detected in resynthesis, they were visible only at their points of attachment to microsclerotia and hyaline vesicles. These observed differences may be attributed to various factors affecting fungal morphology and development: i) choice of host plant and/ or fungal isolate; ii) incubation conditions (resynthesis did not mimic field conditions); and iii) time of incubation (six weeks may not be sufficient).

Our resynthesis experiments suggest that *P. macrospinosa* and *Microdochium* sp. are unlikely to be harmful to their hosts: hyphae, microsclerotia or chlamydospores were not seen in the vascular cylinder. However, the biomass estimators from the resynthesis studies show that, even in a controlled laboratory study, the host responses can be variable. *Periconia* increased, decreased or had no effect on the total leek biomass and *Microdochium* sp. had no effect on the plant biomass. The observed responses are similar to previous reports of DSE effects on hosts (Jumpponen 2001). While experimental conditions can influence the results (see Addy et al 2005), a study by Fernando and Currah (1996) showed that under similar experimental conditions, the effects of four strains of *L. orchidicola* on a host were variable. Similarly, our results indicate that the different isolates induce variable responses under the same experimental conditions. The outcome of the symbiosis is probably controlled both by the host and the fungus genotypes as postulated by Schulz and Boyle (2005).

Enzymatic capabilities

DSE fungi produce a variety of extracellular enzymes. Mandyam and Jumpponen (2005) summarized enzymatic capabilities of DSE fungi. The reported activities included amylase, cellulases, lipase, pectinase, polyphenol oxidases (laccase and tyrosinase), protease and xylanase. In our studies *Periconia* and *Microdochium* isolates produced hydrolytic enzymes suggesting facultative saprobic capabilities.

The hydrolytic capabilities of facultative endophytes are interesting. These enzymes can break down detritus or may aid in penetration into the plant tissues. Unlike mycorrhizal fungi, there is no experimental evidence to support transfer of nutrients from hydrolysis to the host plant (Addy et al 2005). In our study, most isolates produced laccase. Burke and Cairney (2002)

discuss many functions for laccase in non-mycorrhizal fungi including lignin degradation, a role in mycelial growth and hyphal cross-linking, fruiting body differentiation, detoxification of phenolics and melanin production. A role in melanin production seems appropriate given that DSE fungi often produce abundant melanized tissues. A role in lignin degradation must be confirmed via lignin hydrolysis assays. We did not test for lignolytic activity, but we expect it to be highly variable as determined for other assayed enzymatic activities. Ultimately, the importance of these observed enzymatic activities must be confirmed *in planta* to assess their relevance for plant nutrient acquisition.

Nitrogen utilization

The *Microdochium* isolate we tested was unable to grow in liquid medium; its ability to utilize organic and inorganic N sources could not be tested. On the other hand, *P. macrospinosa* isolates utilized both organic (amino acids) and inorganic N sources. Additionally, the tested isolates produced a proteolytic enzyme, gelatinase. Caldwell and Jumpponen (2003a) showed that DSE fungi can utilize heterocyclic N sources, guanine and uric acid. All these studies have used fungal pure cultures to establish N use *in vitro*. Experimental verification of the uptake during symbiosis and its relevance to plant nutrient uptake is lacking. Our results confirm that DSE from a tallgrass prairie utilize complex organic and inorganic N compounds. Accordingly, N transfer from complex sources to host plants is possible. Mullen et al. (1998) hypothesized that endophytic fungi are important in N uptake in an alpine environment during snowmelt. Green et al (2008) provide indirect evidence in support of C and N exchange between a patch mosaic of grasses and biological crust dominated by a fungal network of symbiotic DSE fungi in a semi-arid ecosystem. Mandyam and Jumpponen (2008) also suggest a role of DSE fungi in N uptake based on the greater DSE colonization during early spring compared to mycorrhizal fungi at

Konza Prairie. A role of DSE fungi in N uptake and transfer to host plant can be confirmed only when there is sufficient experimental evidence (Addy et al 2005; Govindarajulu et al 2005) for, i) presence of extraradical DSE hyphae from well colonized hosts, ii) N uptake from complex sources by the extraradical DSE hyphae, iii) N transfer from either organic and/or inorganic sources to the host plant with subsequent incorporation of N into host amino acid and, iv) presence of exchange interface formed by the DSE fungi inside the host.

CONCLUSIONS

In this study, many fungal isolates obtained from roots of tallgrass prairie plants were detrimental to the test plants and are suspected to be common soil-borne pathogens or antagonists. However, *Periconia macrospinosa* and *Microdochium* sp. were commonly isolated and produced typical microsclerotia or intra- and intercellular chlamydospores. Thus, they are root endophytes in this ecosystem. Their effect on leek growth was variable and ranged from positive to neutral and negative. Most of the putative endophyte strains produced extracellular enzymes for hydrolyzing complex C and N compounds indicative of a facultative saprobic nutrition. These endophytes also utilized organic and inorganic N sources. The ecological importance of this symbiosis among the root associated endophytes and their hosts remains elusive. Our future experiments aim to determine the impacts of the endophyte colonization on host physiology, nutrient metabolism and photosynthetic rates.

Table 2.1 List of fungal strains isolated from Konza Prairie Biological Station and their effect on leek in resynthesis experiments.

Fungal isolates	Total number of isolates	Leek resynthesis
Acremonium sp.	5	Pathogenic *
Aspergillus sp.	4	Pathogenic *
Cladosporium sp.	2	Pathogenic *
Curvularia sp.	1	—
Fusarium sp.	11	Pathogenic *
Microdochium sp.	6	Non-pathogenic, Cortical chlamydospores
Papularia sp.	1	—
Periconia macrospinosa	49	Non-pathogenic, Cortical microsclerotia in roots
Green sterile, slow growing fungus	8	Non-pathogenic, copious superficial hyphae
Sterile white, fungus	22	Non-pathogenic, copious superficial hyphae
Sterile dark fungus	18	Non-pathogenic, copious superficial hyphae

* Majority of leek succumbed to inoculation at the end of six week incubation

— A fungal species or conspecific group that had fewer than three isolates were omitted from resynthesis studies

Table 2.2 Hydrolytic capabilities of select *Periconia macrospinosa* (KS0019-KS0100) and *Microdochium* sp. (KS0012 and KS0014).

	Periconia macrospinosa								*Microdochium* sp.	
Enzymes	KS0019	KS0025	KS0045	KS0054	KS0060	KS0082	KS0093	KS0100	KS0012	KS0014
Amylase	+	++	++	++	+++	++	+	+++	++	++
Cellulase	+	+	+	++	+	+	++	+++	++	+
Laccase	++	+	+++	+++	–	+	+++	++	–	++
Tyrosinase	+	+	+	+	–	+	+	+	–	+
Gelatinase	+	++	+	+++	++	+	–	++++	++++	+++

Key:

Amylase test:

–, Absence of clearing around fungal mat, negative for amylase

+, Clearing 1-3cm diameter

++, Clearing 3-6cm diameter

+++, Clearing >6cm dimeter

Cellulase test:

–, Absence of clearing, negative for cellulose

+, Clearing <2cm diameter

++, Clearing about 2cm diameter

+++, Clearing >2cm diameter

Laccase test:

–, Absence of brown color under or around fungal mat, negative for Laccase

+, Dark brown color under fungal mat at the center, visible only on the underside of the plate

++, Dark brown color formed under most of mat but not extending to margin, seen from under side of the plate

+++, Dark brown color extending beyond margin of fungal colony and visible from the topside of the plate

Tyrosinase test:

–, Absence of orange-brown color, negative for cresolase

+, Presence of orange-brown color, positive for cresolase

Gelatinase test:

–, Absence of liquefaction at 4ºC, negative for gelatinase

+, Liquefaction < 25% of medium

++, Liquefaction 26-50% of medium

+++, Liquefaction 51-75% of medium

++++, Liquefaction 76-100% of medium

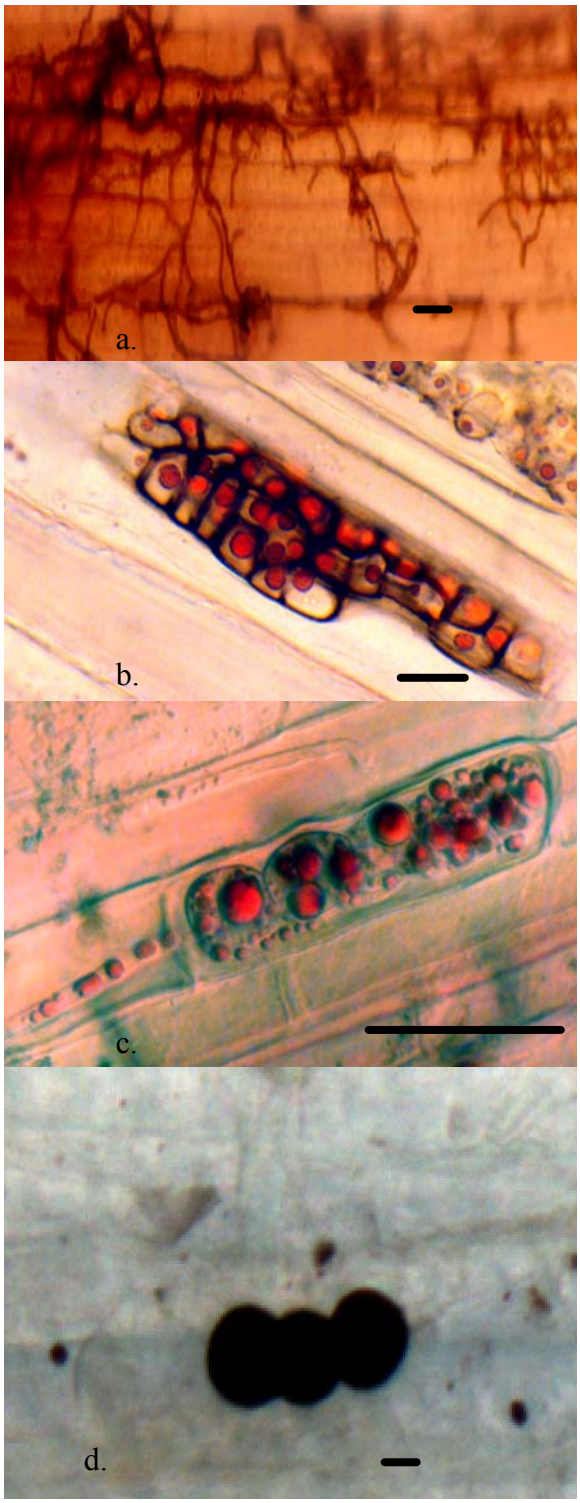


Figure 2.1 DSE structures from mixed samples of KPBS roots.
a) Melanized hyphae b) Microsclerotia in root cortex c) Hyaline vesicle with lipids stained red with Sudan IV d) Chlamydospores. Bar =10µm

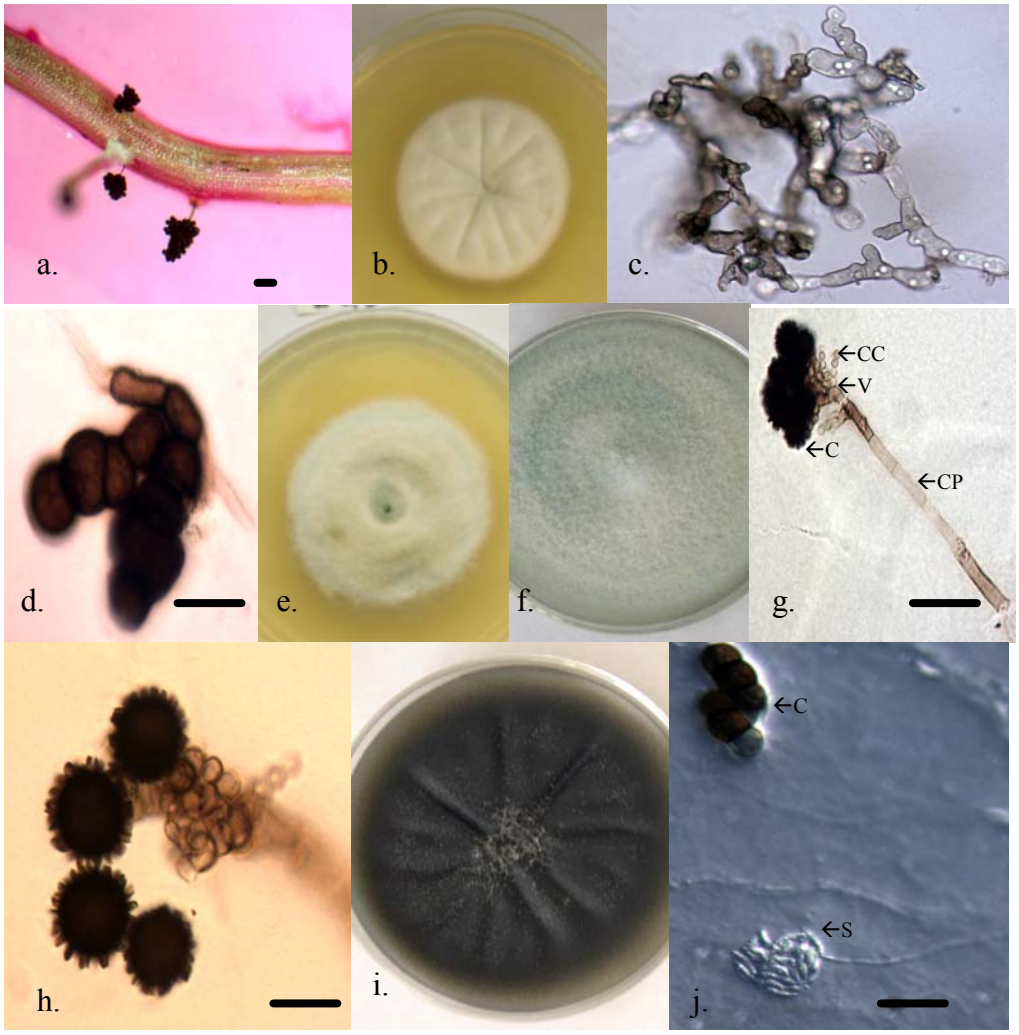


Figure 2.2 DSE fungal morphology.

a) *Periconia* conidiophores and conidia emerging from field collected root b) 10d old culture of KS0035 c) Bulbous, hyaline and melanized hyphae of KS0035 d) Chlamydospores of KS0035 e) 10d old culture of KS0045 f) Month old culture of KS0045 g) Typical conidial apparatus of *Periconia* with partially melanized conidiophore (CP), vesicle (V), conidigenous cell (CC) and conidia (C) h) Typical echinulate conidia of *P. macrospinosa* i) Month old culture of *Microdochium* sp. j) Sporodochium (S) and chlamydospores (C) of *Microdochium* Bar=10µm

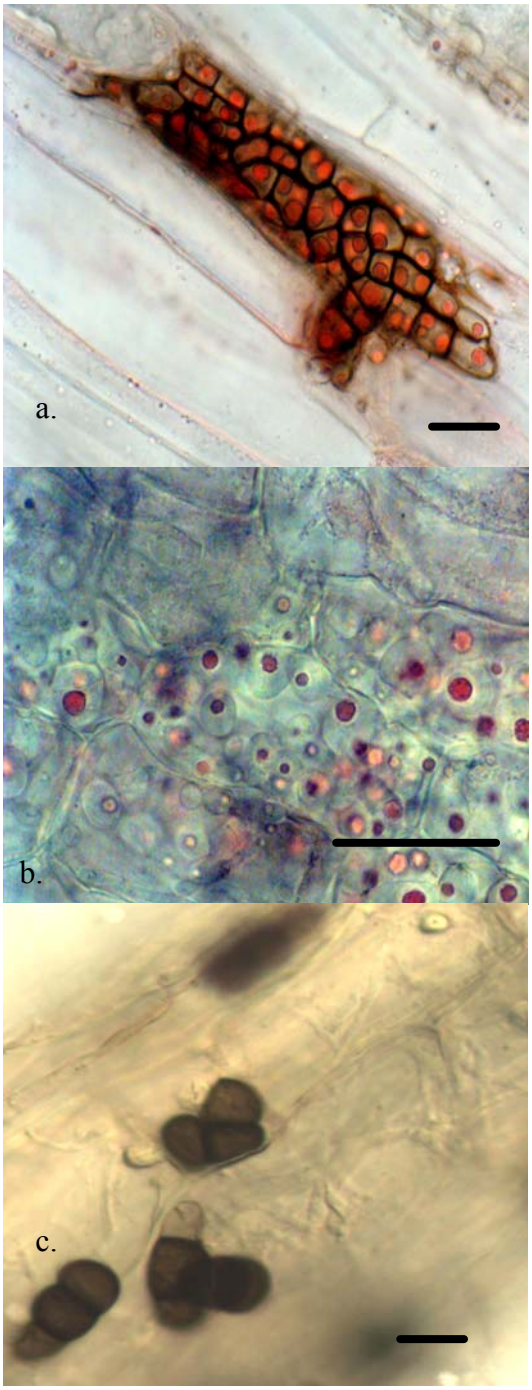


Figure 2.3 DSE morphology in resynthesis leek roots.
a) *P. macrospinosa* microsclerotium with its lipids stained red by Sudan IV b) *P. macrospinosa* hyaline vesicle with lipids stained by Sudan IV c)*Microdochium* chlamydospores

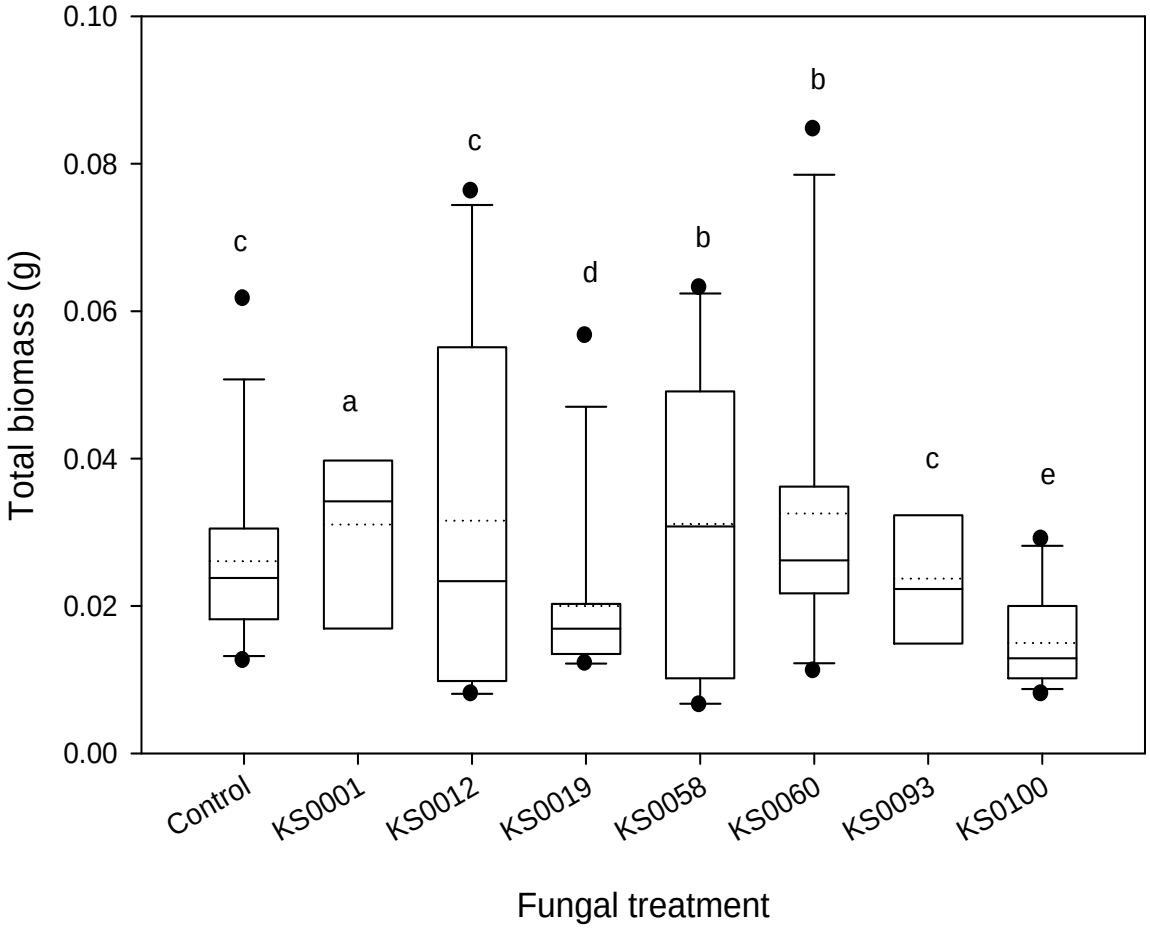
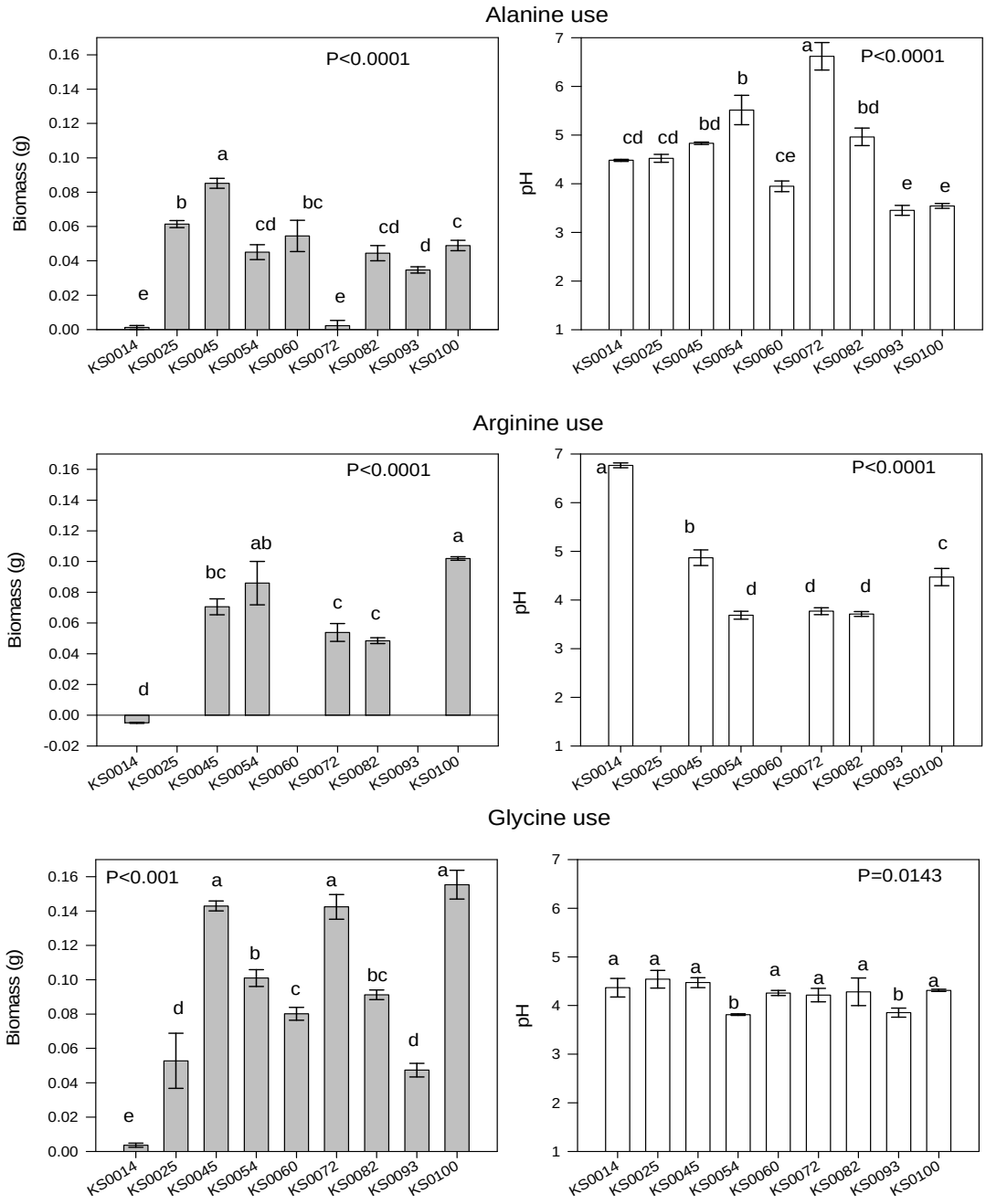


Figure 2.4 Effects of fungal inoculation on leek total biomass.

Non-parametric median test was used for pair-wise comparison of treatment differences established at α=0.05. The boxes indicate 75th and 25th percentile. Bars above and below the box indicate 90th and 10th percentile respectively. Median and mean are indicated by the solid and dotted lines respectively. Outliers are indicated by the closed black circles. Treatments with similar letters are not statistically different. Fungal treatments include uninoculated control, sterile green fungus (KS0001), Microdochium sp. (KS0012), and P. macrospinosa(KS0019-KS0100).


Alanine use
Biomass (g)
P<0.0001
e b a cd bc e cd d c
pH
P<0.0001
cd cd bd b ce a bd e e
Arginine use
Biomass (g)
P<0.0001
d bc ab c c a
pH
P<0.0001
a b d d d c
Glycine use
Biomass (g)
P<0.001
e d a b c a bc d a
pH
P=0.0143
a a a b a a a b a
KS0014 KS0025 KS0045 KS0054 KS0060 KS0072 KS0082 KS0093 KS0100

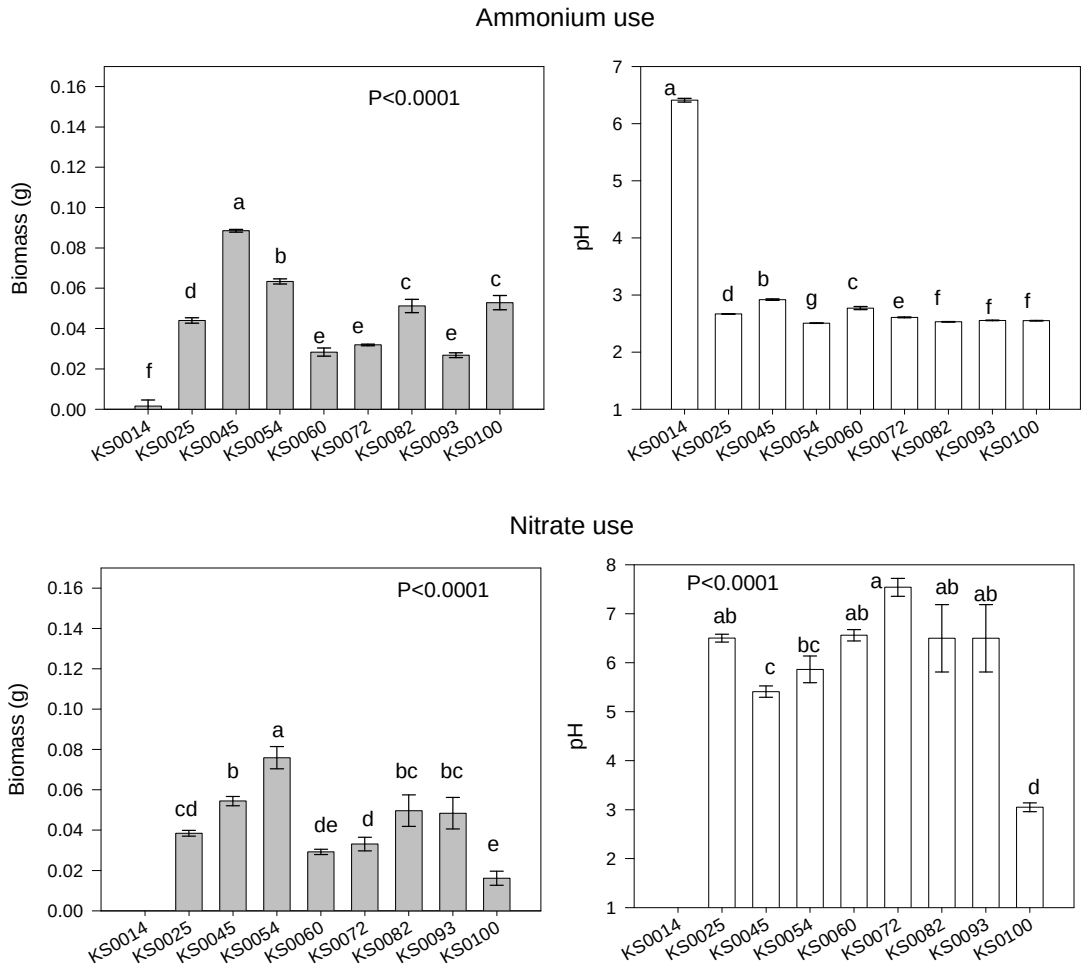


Figure 2.5 Organic and inorganic nitrogen use by fungal endophytes.

Gray bars indicate biomass of the tested fungal isolates and open bars indicate pH of the medium after fungal incubation for 20 days. Control biomass was subtracted from each treatment biomass. The tested isolates include *Microdochium* sp. (KS0014), and *P. macrospinosa* (KS0025-KS0100). Pair-wise comparison was conducted using Bonferroni test (P=0.05). Treatments sharing a letter are not significantly different from each other.
Some treatments are missing due to bacterial contamination of N media.

CHAPTER 3 - Host preference of septate endophytes *Periconia macrospinosa* and *Microdochium* sp. from a native tallgrass prairie

ABSTRACT

Konza Prairie, a native tallgrass prairie in north-eastern Kansas supports unique DSE populations: *Periconia macrospinosa* and *Microdochium* sp. are the most commonly isolated dark septate endophytes (DSE). Since they were isolated repeatedly from grasses and forbs, our main objective was to test their host ranges. One strain of *Microdochium* and three of *Periconia* were screened in sixteen plant species (six native grasses and forbs plus four domesticated crops) in a resynthesis system for colonization and growth responses. All plant species were colonized by the DSE isolates albeit to varying degrees. The host biomass and nutritional levels were variable within and between host species. The outcomes of the host-fungus interactions were along a mutualism-parasitism continuum. Percent responsiveness to DSE colonization, a metric similar to 'mycorrhizal dependency' was calculated for each of the species. Overall, the grasses tended to respond positively to DSE colonization, while forbs and crops responded negatively. Based on the results from the resynthesis study and therein observed responsiveness, we predicted that grasses in the tallgrass prairie ecosystem would support greater DSE colonization than forbs. A survey of field-collected roots from seven grass and nine forb species supported our hypothesis: grasses hosted 50% greater DSE colonization than forbs. Our study lends support to the 'broad host range' of DSE fungi. However, the results from the resynthesis and field studies strongly suggest a possible 'host preference'. Furthermore, different DSE isolates of a species elicit different host responses along the mutualism-parasitism continuum, suggesting an

interplay among the fungal and host genotypes that determines the outcome of the DSE symbiosis.

Key words: dark septate endophytes (DSE), host range, host preference, *Microdochium* sp., mycorrhizal dependency, mutualism-parasitism continuum, *Periconia macrospinosa*

INTRODUCTION

Dark septate endophytes (DSE) are darkly pigmented microfungal ascomycetes commonly observed and isolated in the roots of healthy plants (Jumpponen and Trappe 1998; Addy et al 2005). They are ubiquitous and coexist with mycorrhizal and other root colonizing fungi (Jumpponen and Trappe 1998; Mandyam and Jumpponen 2005). DSE fungi have been observed in 600 plant species across 114 plant families from diverse habitats (Jumpponen and Trappe 1998) and the list of host plants is increasing as more studies specifically survey plant roots for DSE (Ruotsalainen et al 2002; Thormann et al 1999; Kovács and Szigetvári 2002; Rains et al 2003). These fungi have been proposed to possess a broad host range based on the number of hosts they can colonize in resynthesis experiments and on the number of plant species from which a given DSE fungus has been isolated from. For example, *Phialocephala fortinii* Wang and Wilcox, colonizes at least eight plant species and has been isolated from as many as 29 plant species (Jumpponen and Trappe 1998). Similarly, Fernando and Currah (1996) isolated *Leptodontidium orchidicola* Sigler and Currah from 10 species at a Canadian site. Currently, we know of at least eight DSE fungi that are frequently isolated (Addy et al 2005). The host specificity, or host preference, among most of these DSE species has not been explicitly tested in resynthesis studies and remains thus subject to more detailed examination.

The list of DSE fungi is unlikely to be comprehensive and many details of their basic ecology remain unknown. A two-year survey of mixed plant communities at Konza Prairie, a

native tallgrass prairie in mid-western Kansas, showed that DSE fungi were equally abundant as the AM fungi (Mandyam and Jumpponen 2008). [1]Mandyam et al (*In press*) have repeatedly isolated *Periconia macrospinosa* Lefevbre and Johnson and *Microdochium* sp. from grasses and forbs at this native tallgrass prairie site. In resynthesis assays to confirm their endophytic nature, these fungi consistently produced typical DSE structures including microsclerotia and chlamydospores respectively in leek roots.

In this study, we used resynthesis experiments to explore the host specificity and host range of *P. macrospinosa* and *Microdochium* sp. that were isolated from a tallgrass prairie ecosystem. We inoculated a total of 16 plant species including six native grasses and forbs plus four crop species and microscopically established the presence/absence of root colonization by the DSE fungi. The effects of these DSE fungi on the host growth were also evaluated. Led by the findings of these resynthesis experiments, we additionally validated our laboratory observations and specifically tested hypotheses on whether or not the observed affinity of the DSE fungi to native grasses could be confirmed in the field collected material from Konza prairie.

MATERIALS AND METHODS

Fungal strains

One *Microdochium* sp. (KS0014) and three *Periconia macrospinosa* strains (KS0018, KS0045 and KS0100) previously isolated from Konza Prairie Biological Station were used for resynthesis with native prairie and crop plants.

Resynthesis with native prairie and crop plants.— Six grass species (*Andropogon gerardii* Vitman, *Bouteloua gracilis* (Willd. Ex Kunth) Lag, *Elymus canadensis* L., *Panicum*

virgatum L., *Schizachyrium scoparium* (Michx.) Nash, *Sorgastrum nutans* (L.) Nash.) and forbs (*Asclepias syriaca* L., *Baptisia australis* (L.) Br. ex Aiton, *Echinacea angustifolia* DC, *Dalea purpurea* Vent, *Helianthus maximilianii* Schrad, *Viola* sp.), and four crop species (*Cucumis sativus* L., *Solanum lycopersicum* L., *Triticum aestivum* L. and *Zea mays* L.) were selected for the resynthesis experiments (Table 3.1). Grass seeds (USDA-NRCS) were surface sterilized in 70% alcohol for 30min followed by 30% bleach for 20min. Forbs and crop seeds (W. Atlee Burpee and Co. Warminster, Pennsylvania, USA) were sterilized in alcohol for 10min followed by 30% bleach for 10min. Sterilized seeds were germinated on $1/10^{th}$ strength Murashige Skoog (MS) medium (Sigma, Missouri, USA) for a week in a growth chamber under 12h cycle of light (ca. 250 µmol quanta m^{-2} s^{-1} PAR at 20°C). Seeds of some plant species were repeatedly contaminated by seed-borne fungal endophytes. Due to the unavailability of a large number of sterile seeds, some fungal treatments were omitted. A small opening in the Petri dish was made by making overlapping V-shaped cuts, both in the lid and the dish containing the MS (1/10 strength) medium. A sterile seedling was transferred onto this MS plate so that the shoot emerged through the opening while the roots were contained within the Petri dish. The dish was sealed with parafilm except at the opening and placed upright in the growth chamber. The seedlings were allowed to stabilize for four days in the growth chamber prior to inoculation with a 6mm fungal plug. The plugs were cored from isolates grown on Difco ™ PDA (Becton Dickinson and Co, Maryland, USA) at 25°C for 15 days. A total of 15 replicates for each fungal treatment of inoculated plants were incubated upright in the growth chamber under the above conditions. Fungus-free control plants were inoculated with sterile PDA plugs. The plants were harvested after six-week incubation. Shoots of all 15 replicates and roots of 10 replicates were harvested, dried at 50°C and their dry weight recorded. Roots from the remaining five replicates

were used for microscopic observation. Total biomass and root:shoot ratios were calculated. Root mass of crop plants was so extensive and embedded into the growth medium to a degree that it could not be separated from the medium. Accordingly, the root biomass data are unavailable for the crop species.

Confirmation of root colonization in resynthesis roots

Root samples from five replicates were used for screening presence/absence of DSE structures. Microsclerotia and melanized hyphae were recorded in *Periconia* treatments, and chlamydospores in the *Microdochium* treatment ([1]Mandyam et al, *in press*). Because the fungal structures were melanized, the roots were observed without any staining. The agar medium dried in some treatments with fast growing host species after six weeks of incubation rendering the determination of percent root length colonized (%RLC) impossible.

Plant responses to DSE colonization

To estimate the host responses, we used a metric modified from the 'mycorrhizal dependency' (%MD; Hetrick et al 1988; Wilson and Hartnett 1997).

%MD = [Dry weight of mycorrhizal plant — Dry weight of non-mycorrhizal plant/ Dry weight of mycorrhizal plant] * 100.

Because of non-normal distribution of our observations, we used median dry weights instead of mean dry weight to estimate the host responsiveness to DSE.

N, P, K analyses

Table 3.1 lists the plant species used for the N, P and K analyses. Some slow-growing native species failed to produce adequate biomass for nutrient analyses even after pooling. As a

result, only those species that provided in excess of 200mg of shoot biomass were used for the analyses. A total of 3-5 oven dried individual plant shoots were pooled into 3-5 pools to acquire a pool biomass (approx. 200mg) adequate for the analyses. These samples were analyzed for percent N, P and K at the Soil Testing facility, Dept. of Agronomy, Kansas State University. The samples were sulfuric acid/hydrogen peroxide digested, analyzed for N and P using Technicon AAII auto analyzer with colorimetric industrial method 334-74W/B using separate channels; K was analyzed using a flame atomic absorption.

Site description

Konza Prairie Biological Research Station (KPBS, 39˚05' N, 96˚35' W) represents a mesic native tallgrass prairie in the Flint Hills of eastern Kansas, USA. This site spans 3,487 ha and has remained undisturbed by agriculture. The vegetation is dominated by *Andropogon gerardii, Sorghastrum nutans, Schizachyrium scoparium, and Panicum virgatum* (see Towne 2002 for a complete list of vascular plants at Konza Prairie). The soil parent material is chert-bearing limestone and the soil bulk density is 1.0 g/cm^3. January mean temperature is –3°C (range –9 to 3°C) and the July mean is 27°C (range 20 to 33°C). Annual precipitation is 835 mm, of which about 75% occurs in the growing season. Samples were collected from two annually spring burned lowlands and two infrequently (every 20 year burn) burned watersheds to account for management and geographic variability in fungal colonization.

Sample collection

Roots from three individuals of select, commonly-occurring grasses (Andropogon gerardii, Bouteloua curtipendula (Michx.) Torr., Bouteloua gracilis, Buchloe dactyloides (Nutt.) Engelm, Poa pratensis L., Schizachyrium scoparium, Sorgastrum nutans) and forbs (Achillea

millefolium L., Ambrosia artemesifolia L., Artemesia ludoviciana Nutt., Asclepias syriaca, Lespadeza capitata Michx., Plantago patagonia Jacq., Spharalcea sp., Solidago missouriensis Nutt.) were sampled in spring and summer of 2004 from Konza Prairie (Table 3.1). Two species (A. gerardii and A. ludoviciana) were also sampled in fall for a total of three separate sampling occasions. A whole plant individual was collected to assure sampling of the correctly identified roots attached to the shoots. Roots were washed free of soil under running tap water and processed immediately.

Staining and microscopy of field-collected roots

Roots adhering to the shoots were cut into 1cm fragments and cleared by autoclaving (121˚C) in 2.5% potassium hydroxide for 5 min followed by several washes with water and neutralization with acetic acid. To observe arbuscular mycorrhizal (AM) and DSE colonization in the sampled roots, one random half of the cleared roots was soaked in Trypan blue (Philips and Hayman 1970) whereas another half was immersed in Sudan IV (Barrow and Aaltonen 2001) and autoclaved for 4 min followed by washing in several changes of water. The stained roots were allowed to de-stain in acidic glycerol (50%) overnight.

The colonization in the stained roots was estimated by magnified intersections method (McGonigle et al. 1990) at 200× magnification for total %RLC by AM and DSE fungi. In each case, ten randomly selected roots (1cm) were used for quantification. Overall percent colonization of AM (*i.e.* presence of any AM structure – AM hyphae, vesicles, arbuscules, or coils) and DSE (*i.e.* presence of any DSE structure – melanized septate hyphae and microsclerotia) were recorded.

Statistical analysis of resynthesis

The comparison of total biomass and root:shoot ratio for the different treatments failed the assumptions of ANOVA due to unequal sample size, outliers and unequal variance. The unequal sample size resulted from mortality and fungal contamination. Since the resynthesis plates were open-plate systems (see design of this system in materials and methods section) incubating for five weeks, they were susceptible to aerial contamination. Accordingly, non-parametric tests (Higgins 2003) were considered necessary. Analysis of rank based one-way ANOVA for biomass was performed using a median test available at http://www.stat.wmich.edu/slab/RGLM/. Critical value for comparisons was set to P=0.05.

Statistical analysis of plant % responsiveness to DSE under resynthesis conditions

The mean % responsiveness of grasses, forbs and crops were analyzed using ANOVA in PROC GLM in SAS (Version 9.1). Pair-wise differences, where necessary, were determined by Tukey- Kramer test.

Statistical analysis of shoot %N, P, K content

The effect of different fungal treatments on %N, P and K in shoot tissue were analyzed separately for each plant species using SAS (Version 9.1). The data were normally distributed and did not violate the assumptions of ANOVA. Accordingly these data were analyzed with ANOVA in PROC GLM as above.

Statistical analysis of AM and DSE colonization at Konza Prairie

Statistical analyses were performed using SAS (Version 9.1). The DSE data were not normally distributed and variances were not homogeneous, whereas the AM data were normally

distributed and the variances were homogeneous. To correct for these violations of the assumptions for ANOVA, all data were arcsine square root transformed and analyzed with ANOVA in PROC GLM in SAS. Pair-wise differences, when necessary, were determined by a conservative *Bonferroni* test.

RESULTS

Root colonization in the resynthesis study

All tested hosts were colonized by *Microdochium* sp. (KS0014) and two of the *P. macrospinosa* strains (KS0045 and KS0100). The third *P. macrospinosa* strain (KS0018) colonized the hosts very sparsely or not at all. *Microdochium* sp. produced abundant chlamydospores in the cortex and root hairs in all native and crop plants. *Periconia macrospinosa* (KS0045 and KS0100) produced melanized microsclerotia and intercellular hyphae in the grasses, but colonized the forbs only sparsely. Additionally, *P. macrospinosa* KS0045 sporulated frequently on the roots and produced melanized, septate conidiophores with black and echinulate spores characteristic to the taxon. In contrast to native grasses, in the crop plants, *P. macrospinosa* isolates (KS0045 and KS0100) formed primarily intercellular hyphae while microsclerotia were rare. Regrettably, %RLC could not be determined and more detailed analyses of these data were omitted.

Host responses to inoculation in the resynthesis study

All the tested native grasses responded to fungal inoculation one way or another (Fig. 3.1a, b): Two *P. macrospinosa* isolates (KS0045 and KS0100) and *Microdochium* sp. significantly improved total biomass in *A. gerardii* while *P. macrospinosa* (KS0018) had no effect. *Periconia macrospinosa* (KS0045) significantly improved the root:shoot ratio, *P.*

macrospinosa (KS0100) reduced root:shoot ratio while the other two treatments had no effect. All fungal treatments increased the total biomass in *B. gracilis*. *Microdochium* sp. lowered the root:shoot ratio while the *P. macrospinosa* treatments had no effect. In *E. canadensis*, *Microdochium* reduced the total biomass, while the *Periconia* isolates had no effect. Root:shoot ratio was significantly lowered by *Microdochium* sp. and *P. macrospinosa* KS0100, whereas the other two *Periconia* isolates had no effect. In *P. virgatum*, the total biomass was unaffected by *Microdochium* sp. and significantly reduced by all the *Periconia* isolates. However, root:shoot ratio was not affected by the fungal inoculation. All the tested isolates improved total biomass in *S. nutans*. Similarly, all the isolates improved the root:shoot except for *P. macrospinosa* KS0100 which had a neutral effect. In *S. scoparium*, the total biomass was unaffected by *Microdochium* sp., and *P. macrospinosa* (KS0018 and KS0045) but was increased significantly by *P. macrospinosa* KS0100. Root:shoot ratio was unaffected by fungal inoculation in *S. scoparium*.

None of the fungal treatments affected any of the forbs. Their biomass as well as root:shoot ratios remained similar to those in control treatments (Fig. 3.1c, d). Among the crop plants, fungal inoculations did not significantly affect the *C. sativus*, *S. lycopersicum* or *Z. mays* shoot biomass (Fig. 3.1e). However, *Microdochium* sp. and *P. macrospinosa* KS0045 inhibited *T. aestivum* growth while *P. macrospinosa* KS0100 did not alter the biomass compared to the control.

Plant % responsiveness to DSE colonization

The % plant responsiveness to DSE colonization for the plant species are listed in Table 3.2. Among the six grasses, four responded positively, with *B. gracilis* being the most responsive. In contrast, four forbs responded negatively to DSE inoculation, while only two species, *D. purpurea* and *E. angustifolia* responded positively. Of the four crop species, only *Z.*

mays exhibited a weak positive response to DSE. Overall, the grasses responded positively to DSE colonization. This response was significantly greater than the forbs and crops, both of which greatly exhibited a negative response (Table 3.2).

N, P, K concentrations in the resynthesis study

Only those plant species with at least three pools of plants with each pool weighing about 200mg were used for nutrient analyses. Because of the limited shoot biomass among many species, only three grass species, one forb and four crop species were analyzed for N, P and K (Table 3.3).

The hosts responded variably to inoculation (Fig. 3.2). Only *T. aestivum* shoot N content responded to endophyte colonization; compared to the uninoculated control, *Microdochium* and *P. macrospinosa* KS0045 inoculation lowered shoot %N while *P. macrospinosa* KS0100 had no effect (Fig. 3.2a). The fungal colonization altered P content in three host species. First, in *P. virgatum*, all *Periconia* isolates significantly improved shoot %P compared to the control; *Microdochium* sp. had no effect. Second, in *H. maximilianii*, only *Periconia* KS0018 improved %P while other fungal treatments did not differ from the control. Third, in *S. lycopersicum*, *P. macrospinosa* inoculations did not alter %P, while *Microdochium* sp. clearly lowered the shoot %P compared to the fungus-free control (Fig. 3.2b). The shoot %K was significantly lowered by *Periconia* sp. in *P. virgatum*; all other treatments were similar to the control. In *T. aestivum*, *Microdochium* sp. increased %K while the two *Periconia* treatments were similar to the control (Fig. 3.2c).

Endophyte colonization in field collected grasses and forbs at Konza Prairie

The comparison of overall abundance of endophytes is presented in Table 3.4. The overall colonization by AM fungi was greater than DSE colonization as indicated by the significant 'type' effect. Grasses hosted greater fungal colonization compared to forbs as indicated by the significant 'plant' effect. Abundance of AM and DSE fungi were dissimilar in grasses and forbs indicted by the significant 'type*plant' interaction term. The DSE and AM colonization in grasses were similar, whereas in forbs, DSE colonization was significantly lower than that of AM fungi (Fig. 3.3a). While the AM colonization was similar in grasses and forbs, the DSE colonization in forbs was half of that in grasses (Fig. 3.3a). The DSE and AM colonization among the grass and forb species were dissimilar as indicated by a significant 'type*species (plant)' effect. DSE and AM abundance were similar in the seven grass species (Fig. 3.3b), while the endophyte colonization differed among the forb species (Fig. 3.3c). *Achillea millefolium*, *A. artemisifolia*, *A. syriacea*, *P. patagonia* and *Spharalcea* sp. had significantly lesser DSE colonization than AM colonization, while there were no significant differences in *A. ludoviciana*, *B. australis*, *L. capitata* and *S. missouriensis* (Fig. 3.3c). Overall, as suggested by the resynthesis study, grasses supported greater DSE colonization than the forbs. Furthermore, the forbs tended to be more variable in their susceptibility to the DSE colonization than the grasses.

DISCUSSION

This study used DSE fungi isolated from a tallgrass prairie and tested their host range on native prairie plants, clearly demonstrating the 'broad host range' of DSE fungi in resynthesis system and confirming the same in the field.

The native grasses and forbs as well as crop plants were variably colonized by three isolates of *P. macrospinosa* and one isolate of *Microdochium* sp. supporting our hypotheses on a broad host range of DSE fungi but variable susceptibility among the hosts. *Microdochium* sp. colonized all the plant species and produced abundant chlamydospores in the root cortex of all the included host plants. The colonization patterns of the *Periconia* isolates were variable: two strains (KS0045 and KS0100) colonized all the plant species and produced microsclerotia and intercellular hyphae in the grasses but colonized the forbs sparingly. The third *Periconia* isolate, KS0018 did not colonize many plants, and if it did, did so sparingly without any microsclerotia.

To better understand the relative host responses to DSE fungi, we modified the 'mycorrhizal dependency', a metric occasionally used in determining plant responsiveness to mycorrhizal symbiosis (Hetrick et al 1992; Wilson and Hartnett 1997). Since use of the term 'dependency' for DSE symbiosis was deemed premature, perhaps even inaccurate, we chose 'responsiveness' to DSE colonization as a means to assist in explaining the variable plant growth responses. Of the six native grasses, four (*A. gerardii*, *B. gracilis*, *S. scoparium* and *S. nutans*) responded positively to DSE inoculation. All native forbs, except *D. purpurea* and *E. angustifolia*, responded negatively. Similarly to forbs, most crop species responded negatively. A notable exception among the crop species is the warm-season Poaceae *Z. mays*. Based on these results, we hypothesized that native tallgrass DSE fungi exhibit 'host preference' wherein the warm-season grasses more frequently form mutualisms with the DSE fungi, while they are less common among the forbs.

To test the hypothesis of 'host preference', we sampled an assembly of native hosts from the tallgrass prairie ecosystem where the hosts and the fungi co-occur. The results of this field survey were similar to our observations in the resynthesis experiments: all native hosts were

colonized by DSE fungi to some degree. More importantly, as predicted from the observations in the resynthesis experiment, grasses hosted nearly twice the DSE colonization as the forbs did. The field experiments validate the results from the resynthesis trials and strongly indicate that the laboratory findings can be extrapolated into the field. Ultimately, our results, for the first time, suggest that there are predictable patterns in root colonization by the DSE fungi and that these fungi may indeed show preferences among the available hosts.

In this study, growth and nutritional status were used to screen for symbiotic lifestyle expression of DSE fungi under resynthesis conditions. DSE colonization increased, decreased or had no effect on the total biomass, root:shoot ratio and shoot nutrients, indicating a range of potential and variable interactions. *Microdochium* induced positive growth responses in three native grasses (*A. gerardii*, *B. gracilis* and *S. nutans*), while two of the monocotyledonous hosts (*E. canadensis* and *T. aestivum*) responded negatively. All the forbs and two grasses (*P. virgatum* and *S. scoparium*) were unresponsive to *Microdochium*. Similarly to *Microdochium*, *P. macrospinosa* induced different responses among the hosts. Many native grasses appeared to mainly benefit from *P. macrospinosa* inoculation. In contrast, the forbs did not respond to inoculation. Additionally, the three *Periconia* isolates elicited different responses among the grass hosts. For example, *P. macrospinosa* KS0018 and KS0045 increased the biomass of *S. nutans,* whereas KS0100 had a neutral effect. In most cases, *P. macrospinosa* KS0018 had no effect on the host plants. Overall, our DSE fungi exhibited a spectrum of growth responses along the 'mutualism-parasitism or symbiotic continuum.'

It is notable that most of our responses were neutral, some were mutualistic and fewer parasitic. While it has been hypothesized that mutualistic interactions are more frequently developed between microbes and roots (Schulz and Boyle 2005), only a fraction of these

endophytes interact positively with their hosts (Schulz 2006). Kageyama et al (2008) also note that mutualisms in these symbioses are rare or infrequent. None of our DSE isolates were pathogenic as all the tested plants appeared to be visibly healthy and without any colonization in the root vascular cylinder, suggesting that disease is the exception in endophyte interactions, an unbalanced status of symbiosis (Schulz et al 1999; Kogel et al 2006). Redman et al (2001) suggest that a single fungal isolate from a specific geographical site may be pathogenic in one host, mutualistic in another or colonize some plants as a commensal. Even in mycorrhizal symbioses, neutral and negative responses are commonly encountered (Johnson et al 1997). This leads us to a pertinent question: what factors determine the symbiotic lifestyle expression of endophytic fungi? For example, Redman et al (2001) showed that plant physiology and plant genetic differences among conspecific cultivars vastly altered the outcome of the symbiosis. They were also able to show that pathogenic *Colletotrichum* spp. were mutualistic or had a commensal lifestyle in non-host plants. Similarly, the fungal genotype may affect the outcome of the symbiosis: Tanaka et al (2006) selected a *noxA* mutant of *Epichloë festuca* Leuchtm., Schardl and Siegel, a claviceptaceous endophyte of ryegrass, *Lolium perenne* L., altering the interaction from mutualism to parasitism. In summary, whether the interaction of plant-fungal endophyte is balanced (mutualism or commensalism) or imbalanced (parasitism or pathogenicity) depends on the virulence of the fungus and host plant susceptibility, both of which are affected by plant and fungal genotype, plant physiology, nutritional status, developmental stages of the partners and environmental factors (Schulz 2006; Schulz and Boyle 2005, 2006).

According to Schulz and Boyle (2005), the plant-endophyte interactions fall within the symbiotic continuum, precluding the assignment of a particular life-history strategy to a given endophyte. Phenotypic plasticity of endophytes both as a group and individually result in the

continuum of life-history strategies. The outcomes of plant-endophyte interactions depend on a 'balance of antagonisms' and the plasticity can result from variable factors affecting the continuum (see above). One is also cautioned about the bias in the screening method (growth or nutrition enhancement, stress tolerance, etc) employed for ascertaining the outcome of the symbiosis (Redman et al 2001). It is possible that DSE isolates in this study that exhibited negative or neutral effects on some hosts, could confer other positive traits including enhanced water uptake or improved pathogen resistance or resistance to adverse conditions that could not be observed in our broad but simple screen of endophyte interactions across a variety of plants. Interestingly, Rodriguez and Redman (2008) suggest that changing life-history strategies in endophytes may signify evolutionary transitions or that the fungi have achieved a greater ecological flexibility ensuring optimal growth and reproduction in different hosts.

The symbiotic continuum expressed in this study by the DSE isolates in different hosts may be akin to the free movement of *Colletotrichum* species between life styles and hosts, possibly expanding their bio-geographic distribution (Freeman et al 2001).

CONCLUSIONS

To our knowledge this is the first research effort to characterize interactions among DSE isolates, grasses and forbs native to a tallgrass prairie ecosystem. The results supported our initial hypotheses that these DSE fungi possess a broad host range. However, the combination of resynthesis experiments in the laboratory and microscopic observations of field-collected plant material indicated that the DSE fungi preferentially colonized native grasses, colonizing forbs to a lesser degree. Domesticated crop plants also hosted DSE colonization. However, the level of colonization was comparable to that observed in native forbs, regardless of whether the crop species they were monocotyledonous or dicotyledonous. DSE effects on host growth and

nutrition were variable and represented responses along the mutualism-parasitism continuum. Host growth responses were not correlated with the nutritional levels precluding any generalizations about a possible DSE role in nutrient uptake. Based on our results and other published reports, inoculation experiments yield variable results that depend on choices of host species, endophyte taxa or strains, their genetic makeup, extent of fungal colonization and experimental conditions.

Table 3.1 List of plant species used for testing host ranges of DSE

Family	Species	Photo synthesis	Field study	Resynthesis	N, P, K analyses
Asclepiadaceae					
	Asclepias syriacea	C_3	+	+	–
Asteraceae					
	Achillea millefolium	C_3	+	–	–
	Ambrosia artemesifolia	C_3	+	–	–
	Artemesia ludoviciana	C_3	+	–	–
	Echinacea angustifolia	C_3	–	+	–
	Helianthus maxmilianii	C_3	–	+	+
	Solidago missouriensis	C_3	+	–	–
Cucurbitaceae					
	Cucumis sativus	C_3	NA	+	+
Fabaceae					
	Dalea purpurea	C_3	–	+	–
	Lespedeza capitata	C_3	+	–	–
Malvaceae					
	Spharalcea sp.	C_3	+	–	–
Plantaginaceae					
	Plantago patagonia	C_3	+	–	–
Poaceae					
	Andropogon gerardii	C_4	+	+	–
	Bouteloua curtipendula	C_4	+	–	–
	Bouteloua gracilis	C_4	+	+	+
	Buchloe dactyloides	C_4	+	–	–
	Elymus canadensis	C_3	–	+	–
	Panicum virgatum	C_4	–	+	+

	Poa pratensis	C_3	+	–	–
	Schizachyrium scoparium	C_4	+	+	+
	Sorgastrum nutans	C_4	+	+	–
	Triticum aestivum	C_3	NA	+	+
	Zea mays	C_4	NA	+	+
Solanaceae					
	Solanum lycopersicum	C_3	NA	+	+
Violaceae					
	Viola sp.	C_3	–	+	–

+ Plant species used in the study
– Plant species not used in the study
NA Crop plants used only in resynthesis

Table 3.2 Plant response to DSE colonization

Plant species	*Microdochium* sp.	*Periconia macrospinosa* KS0018	*Periconia macrospinosa* KS0045	*Periconia macrospinosa* KS0100	Mean response
Grasses					
Andropogon gerardii	53.10	12.10	54.60	57.40	44.30
Bouteloua gracilis	75.40	79.70	72.30	79.00	76.57
Elymus canadensis	-44.20	-34.20	-76.90	-48.10	-50.85
Panicum virgatum	-2.20	-72.00	-81.30	-51.40	-51.72
Schizachyrium scoparium	27.20	25.90	-6.60	36.00	20.63
Sorgastrum nutans	61.20	53.50	42.20	37.80	48.67
Forbs					
Asclepias syriacea	-	-	-18.70	-11.00	-14.85
Baptisia australis	-	-5.10	-17.70	-49.70	-24.16
Dalea purpurea	-7.80	3.90	18.40	18.90	8.35
Echinacea angustifolia	28.30	42.90	31.60	23.50	31.60
Helianthus maximilianii	-24.90	-44.50	-3.90	-	-24.40
Viola sp.	-	-18.50	-15.70	11.00	-7.70
Crops					
Cucumis sativus	-	-	-36.30	-11.90	-24.10
Solanum lycopersicum	-2.20	-	-2.70	1.45	-1.13
Triticum aestivum	-18.90	-	-34.40	3.40	-16.63
Zea mays	6.90	8.50	-4.40	-	3.66

- Missing data

% response to DSE = [Median dry weight of DSE inoculated plant – Median dry weight of DSE uninoculated plant/ Median dry weight of DSE inoculated plant]*100

Positive responses are bold faced.

Table 3.3 *F*-values from ANOVA for response variables N, P, K of select plant species following fungal treatment

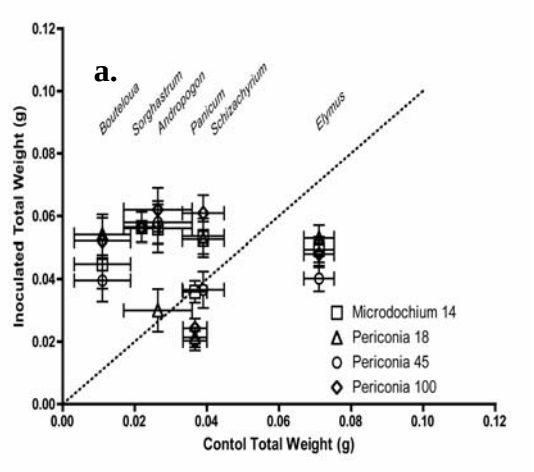
Plant type	Species	%N	%P	%K
Grass	*Bouteloua gracilis*	1.19 n.s	2.9 *	1.24 n.s
	Panicum virgatum	1.26 n.s	4.84 **	8.74 ***
	Schizachyrium scoparium	0.84 n.s	0.91 n.s	2.78 *
Forb	*Helianthus maxmilianii*	3.39 *	4.13 **	2.31 n.s
Others	*Cucumis sativus*	0.85 n.s	3.22 n.s	0.19 n.s
	Solanum lycopersicum	2.18 n.s	5.56 **	3.42 *
	Triticum aestivum	13.7 ***	1.8 n.s	8.19 ***
	Zea mays	2.43 n.s	3.2 *	2.23 n.s

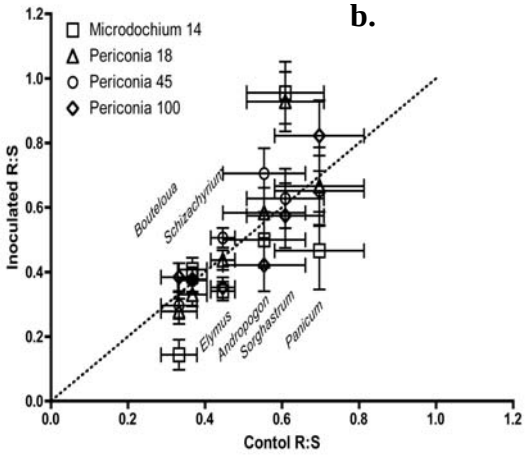
* *P<0.1*, ** *P<0.05*, *** *P<0.01*, n.s non-significant *P>0.1*

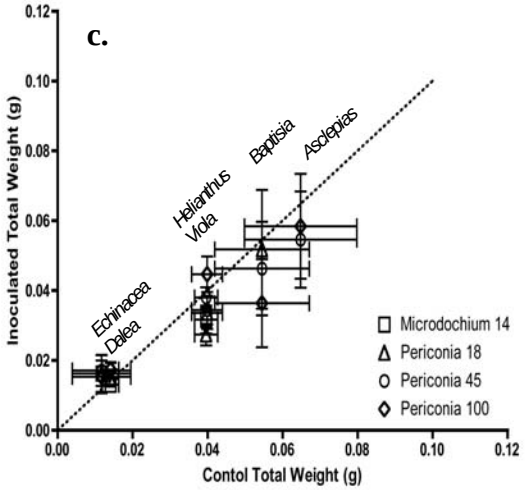
Table 3.4 ANOVA results for the overall colonization of AM and DSE fungi from 16 plant species at Konza Prairie.

Effect	df	F	*P*
Type	1	120.1	<0.0001
Plant	1	45.35	<0.0001
Species(Plant)	15	5.25	<0.0001
Type*Plant	1	43.74	<0.0001
Type*Species(Plant)	15	2.47	0.002

Type compares the AM vs. DSE colonization, Plant compares the colonization in forbs vs. grasses; Species(Plant) is a nested variable


a.
Bouteloua
Sorghastrum
Andropogon
Panicum
Schizachyrium
Elymus
Microdochium 14
Periconia 18
Periconia 45
Periconia 100
Inoculated Total Weight (g)
Contol Total Weight (g)
0.00
0.02
0.04
0.06
0.08
0.10
0.12


b.
Microdochium 14
Periconia 18
Periconia 45
Periconia 100
Bouteloua
Schizachyrium
Elymus
Andropogon
Sorghastrum
Panicum
Inoculated R:S
Contol R:S
0.0
0.2
0.4
0.6
0.8
1.0
1.2


c.
Echinacea
Dalea
Helianthus
Viola
Baptisia
Asclepias
Microdochium 14
Periconia 18
Periconia 45
Periconia 100
Inoculated Total Weight (g)
Contol Total Weight (g)
0.00
0.02
0.04
0.06
0.08
0.10
0.12

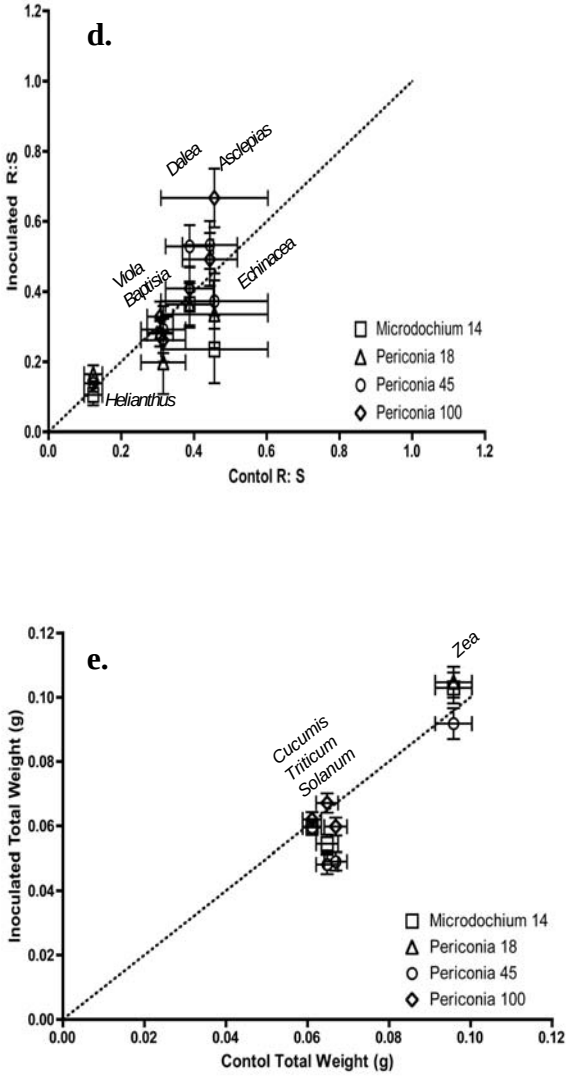
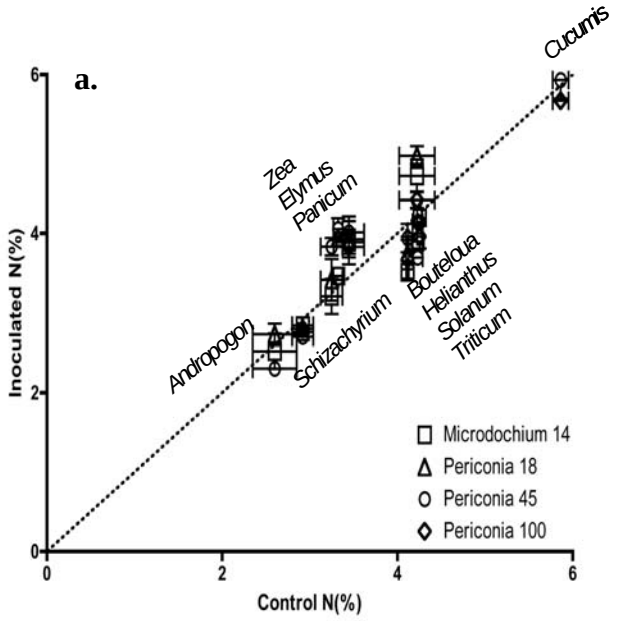


Figure 3.1 Effect of fungal inoculation on a) Grass total biomass b) Grass root:shoot c) Forb total biomass d) Forb root:shoot e) Crop shoot biomass.

The dotted line represents the 1:1 of the control and inoculated biomass. Data points above and below the line are different from their controls. Since many treatments overlap, pair-wise differences are not shown. They are listed in the results.


a.
Cucumis
Zea
Elymus
Panicum
Bouteloua
Helianthus
Solanum
Triticum
Andropogon
Schizachyrium
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Control N(%)
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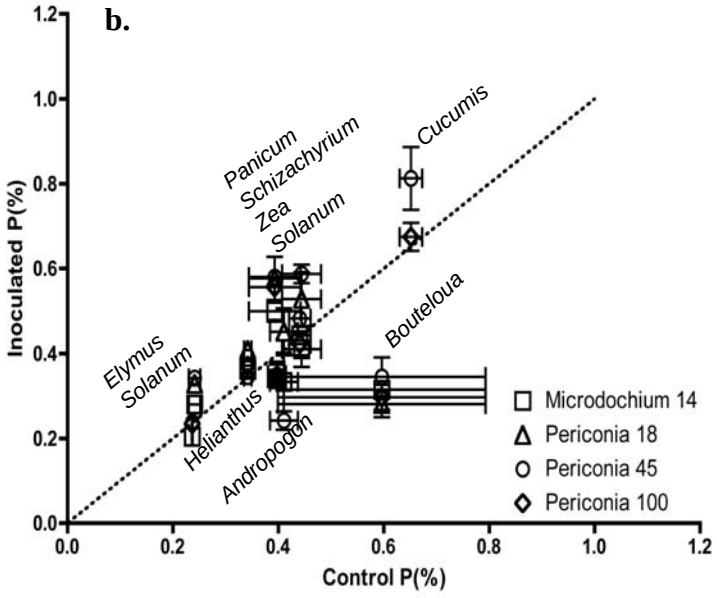
Periconia 18
Periconia 45
Periconia 100
0
2
4
6


b.
Panicum
Schizachyrium
Cucumis
Zea
Solanum
Bouteloua
Elymus
Solanum
Helianthus
Andropogon
Inoculated P(%)
Control P(%)
Microdochium 14
Periconia 18
Periconia 45
Periconia 100
0.0
0.2
0.4
0.6
0.8
1.0
1.2

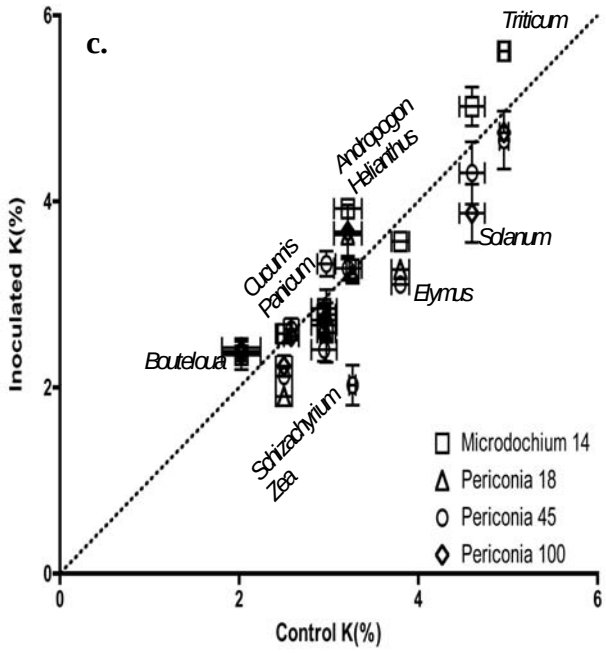
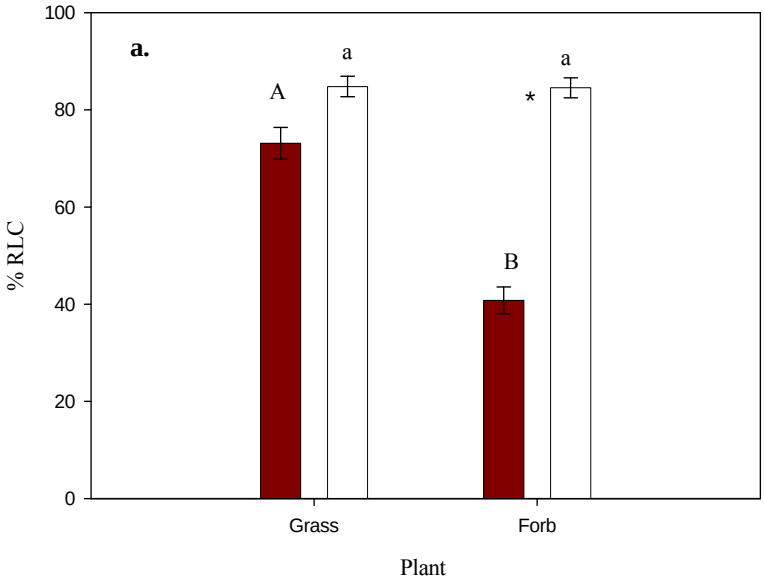
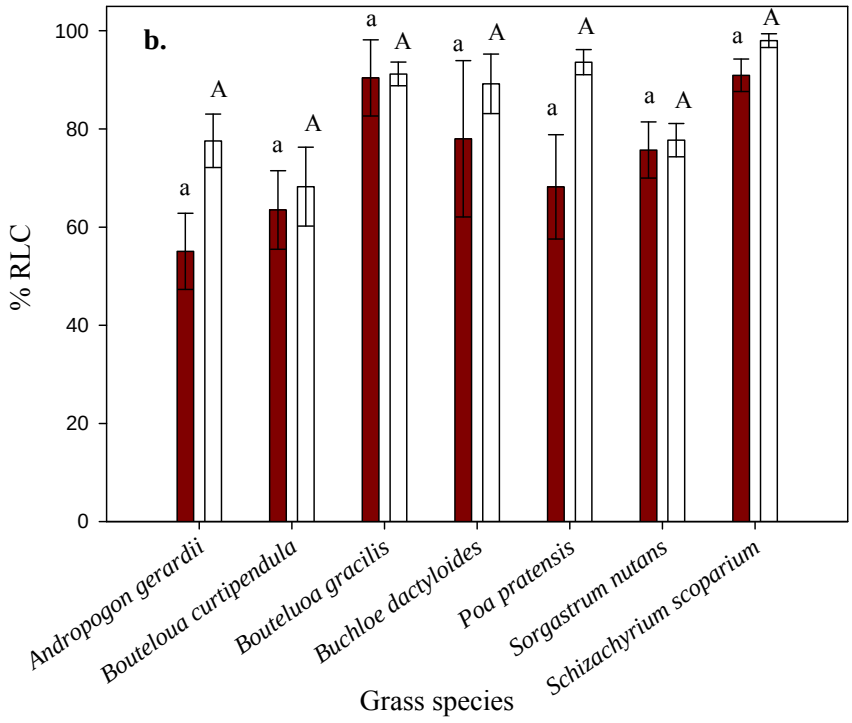


Figure 3.2 Effect of fungal inoculation on shoot a) % nitrogen b) % Phosphorous and c) % Potassium in the host plants.

The dotted line represents the 1:1 of the control and inoculated biomass above and below which the treatments are different from the control. Due to overlap of many treatments, pair-wise differences between fungal treatments within a host species are not depicted in the figure. They are explained in the results section.


a.
100
80
60
40
20
0
% RLC
A
a
B
*
a
Grass
Forb
Plant


b.
100
80
60
40
20
0
% RLC
a
A
a
A
a
A
a
A
a
A
a
A
a
A
Andropogon gerardii
Bouteloua curtipendula
Bouteluoa gracilis
Buchloe dactyloides
Poa pratensis
Sorgastrum nutans
Schizachyrium scoparium
Grass species

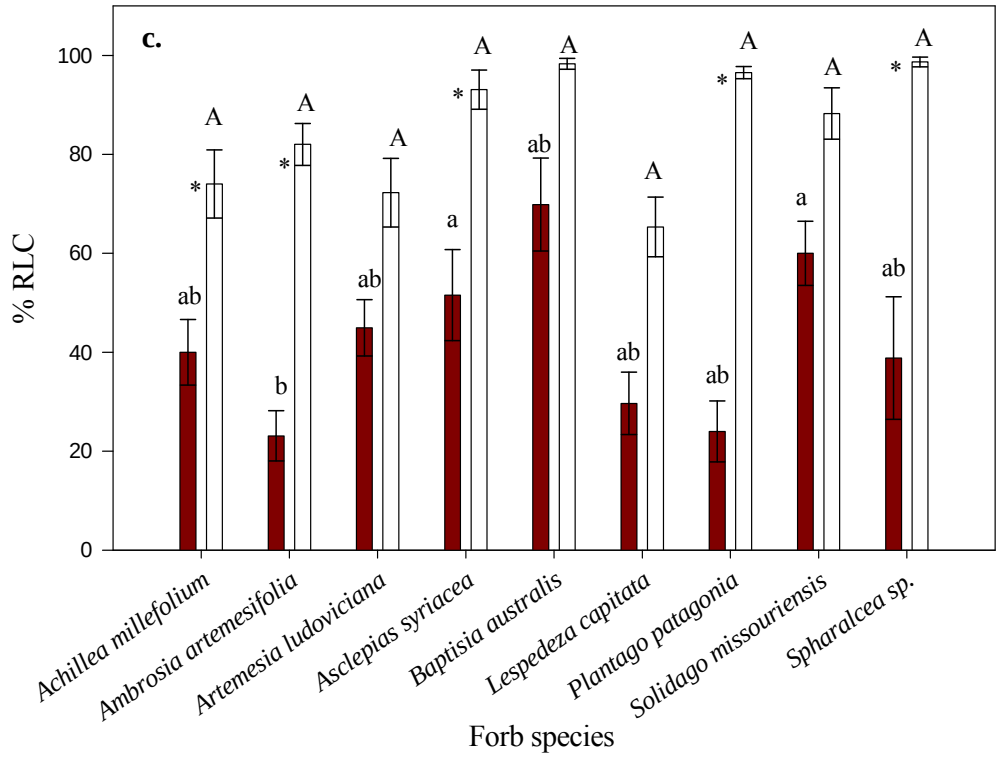


Figure 3.3 Colonization of dark septate endophytic fungi (DSE) and arbuscular mycorrhizal fungi (AM) in field-collected plants from Konza Prairie.

a) Comparison of colonization in grasses and forbs. b) Comparison of colonization in select grasses. c) Comparison of colonization in select forbs. Gray bars=DSE colonization, Open bars=AM colonization. * for a species indicates a statistically significant difference in the overall DSE and AM colonization. Letters in lower-case signify statistically significant variation in DSE colonization and letters in upper-case signify statistically significant variation in AM colonization. Treatments are different if they do not share a letter (*Bonferroni* test, *P=0.05*). Standard error is indicated.

CHAPTER 4 - Symbiosis between *Arabidopsis thaliana* and root endophytes: a continuum of interactions controlled by host and fungal genotype

ABSTRACT

Dark septate endophytes (DSE) are a miscellaneous group of ascomyceteous root-colonizing microfungi, which have a global distribution and have been observed in at least 600 plant species from diverse habitats. Thus far, the non-mycorrhizal model plant *Arabidopsis thaliana* has not been surveyed for its ability to form DSE symbioses. Roots of field-grown *A. thaliana* (Cvi-0 accession) were screened for DSE. The roots were colonized by DSE fungi that formed melanized inter- and intracellular hyphae, microsclerotia and chlamydospores. After confirming that *Arabidopsis*-DSE symbiosis occurs naturally, we screened more than 30 DSE isolates in Col-0, Cvi-0 and Kin-1 accessions. The DSE fungi included 34 conspecific *Periconia macrospinosa* isolates of and four *Microdochium* sp. isolates. DSE colonization and its effect on *A. thaliana* growth were evaluated. All three accessions were colonized and most *P. macrospinosa* formed microsclerotia and *Microdochium* sp. formed chlamydospores similar to those observed in field-collected plants. On a population level, the Col-0 and Cvi-0 responded negatively to *P. macrospinosa* colonization, whereas Kin-1 response was neutral. The responses to strains of conspecific DSE fungi ranged from negative to positive indicating variability along the symbiotic continuum within each accession. Most *A. thaliana* responses to the endophytes

were neutral but examples of parasitic or mutualistic symbioses were observed within each *A. thaliana* accession. Our results provide further support for mutualism-parasitism paradigm and emphasize that the host responses to a fungal symbiont are controlled by the genotypes of both the host and the fungus.

Key words: *Arabidopsis thaliana*, dark septate endophytes (DSE), *Microdochium* sp., mutualism-parasitism continuum, *Periconia macrospinosa*

INTRODUCTION

Dark septate endophytes (DSE) are a miscellaneous group of ascomyceteous root-colonizing microfungi characterized by melanized cell walls and intracellular colonization of healthy plants (Jumpponen and Trappe 1998). Although DSE fungi are taxonomically unrelated and vary in ecological or physiological functions (Addy et al 2005), many of these fungi form similar morphological structures in the host roots (Jumpponen and Trappe 1998).

DSE fungi have a global distribution and have been observed in 600 plant species across 114 plant families from diverse habitats (Jumpponen and Trappe 1998). The list of susceptible host plants is increasing as more studies specifically survey plant roots for DSE. However, thus far the model plant, *Arabidopsis thaliana* (L.) Heynhold., native to Europe and central Asia but now naturalized worldwide (Al-Shehbaz and O'Kane 2002) has not been surveyed or tested for its ability to form these common symbioses. *Arabidopsis* is non-mycorrhizal and no natural root mutualisms have been reported. Recently, *Arabidopsis* was shown to benefit from root association with a soil-inhabiting basidiomycetous fungus, *Piriformospora indica* Verma, Varma, Rexer, Kost and Franken (Peškan-Beghöfer et al 2004). In laboratory resynthesis studies, this fungus colonized a variety of plants often improving their growth or fitness, and inducing

disease resistance in some (Waller et al 2005, Shahollari et al 2007). These results lead to conclusions of *P. indica* mutualisms across a wide range of hosts. However, it remained unknown if this fungus would form symbiosis with *Arabidopsis* or any other plant species under natural conditions.

The *Arabidopsis*-DSE symbiosis can answer many questions about the obscure, but common DSE symbiosis. The practical and scientific merits of Arabidopsis, allows one to test hypotheses quickly. Many tools like whole genome microarrays, ecotypes and/or accessions of *Arabidopsis*, mutants of many physiological pathways, and abundant literature are available that can be exploited to dissect the DSE symbiosis at the whole plant, genetic, molecular or physiological level in a model organism. The *Arabidopsis* Information Resource (TAIR; www.arabidopsis.org) a database for genetic and molecular data of *Arabidopsis*, indicates that over 750 accessions of *A. thaliana* have been identified and collected from the world over. These accessions are variable in form, development and physiology and are routinely used to understand the complex genetic interactions underlying plant responses to pathogens, stress, environment etc.

Mutualism-parasitism continuum, originally described for mycorrhizal fungi to account for an array of outcomes of symbiosis (Johnson et al 1997), is also applied for non-mycorrhizal endophytes including DSE fungi (Schulz and Boyle 2005, Schulz 2006). The outcomes of DSE symbiosis along the continuum, similarly to mycorrhizal symbiosis, are thought to be influenced by biotic (host and fungal identity and genotypes; pathogens, herbivores) and abiotic factors (soil nutrient status, environmental conditions, stress). Unlike the mycorrhizal symbiosis, many influencing factors in DSE symbiosis lack substantial evidence.

The Arabidopsis-DSE symbiosis can help dissect the influence of host and fungal genotype on the outcome of the DSE symbiosis along the mutualism-parasitism continuum by using multiple accessions of *A. thaliana* and many strains of *P. macrospinosa*, a known DSE fungus from a native tallgrass prairie. While some degree of realism is compromised by using native tallgrass endophytes in symbiosis with a non-native plant, *Arabidopsis*, being a model plant, can provide 'proof of concept' for DSE symbiosis.

Our present study aimed to i) microscopically evaluate the field grown *Arabidopsis* roots for the presence of typical DSE structures, ii) evaluate the *Arabidopsis* responses to 38 DSE isolates distributed across two taxa that commonly occur in a tallgrass prairie ecosystem, iii) evaluate the root colonization, shoot biomass and host responsiveness to DSE inoculation, and iv) ascertain if genotypes of conspecific fungi and hosts or combinations thereof controlled the observed mutualism-parasitism continuum in DSE fungi.

MATERIALS AND METHODS

Field-grown Arabidopsis *material*

The field-grown material was acquired from a larger common garden experiment that included a field site in Norwich, England (Wilczek et al *in press*) For planting in September 2006, Cvi-0 seeds were incubated in the dark at 4 °C in 0.1% water agar for four days prior to sowing to stratify them. Seeds were then sown onto peat-based plugits held together by a permeable, biodegradable fabric (Bulrush Horticulture Ltd.; Co. Londonderry, N. Ireland; Recipe 5919). The plugits contained a small amount of slow release fertilizer. Seedlings were germinated on the surface of these moist plugits in the greenhouse under natural photoperiod conditions and thinned to one seedling/plugit. The temperature was set as close to current

outdoor conditions as possible. Within ten days of germination, plugits with seedlings were transplanted to the field and watered for up to a week. From then on, seedlings were left under natural conditions with no further watering and allowed to grow until harvested in February 2007 after all the flowers had opened and formed siliques. After removal of the shoot, soil surrounding the plant was dug up and the roots were removed gently from the soil and placed in water. After rinsing the remaining soil off the roots, they were fixed in 3.7% formaldehyde and 15% methanol in water.

Confirmation of root colonization in field-collected Arabidopsis roots

Presence or absence of DSE structures was recorded in the field grown Cvi-0 roots. Estimation of percent root length colonization was deemed unnecessary as these estimates tend to be inaccurate as a result of abundant hyaline hyphae that are difficult to visualize (Barrow and Aaltonen 2001, [1]Mandyam et al *in press*). Roots were left unstained because the indicative DSE structures are usually melanized and easily detected. The roots were screened for melanized hyphae, microsclerotia and chlamydospores under a light microscope.

Fungal isolates for resynthesis with Arabidopsis

A total of 34 conspecific isolates of *P. macrospinosa* and four conspecific *Microdochium* sp. isolates were used for the laboratory inoculation assays. The fungi originated from native tallgrass prairie (Konza Prairie Biological Station), and were confirmed root-associated endophytes as they fulfilled the Koch's postulates in previous resynthesis assays ([1]Mandyam et al, *in press*). The isolates were cultured on Difco^{TM} Potato Dextrose Agar (PDA; Becton Dickinson and Co, Maryland, USA) at 25º C for 15 days.

Resynthesis with Arabidopsis

Three accessions of *Arabidopsis thaliana* – Columbia (Col-0), Kendallville (Kin-1) and Cape Verde Island (Cvi-0) – were used for resynthesis. Seeds (Lehle Seeds, Round Rock, TX, USA) were surface sterilized in 0.1% Triton-X for 30min, followed by 70% ethylalcohol in 0.1% Triton-X for 5 min and finally in 30% bleach in 0.1% Triton-X for 5min. The seeds were washed 4-5 times with sterile water and stratified for 3 days in 4°C. The sterilized seeds were plated on 1/10 strength Murashige Skoog basal salt mixture (MS; Sigma, Missouri, USA) medium and incubated for one week in the growth chamber under 12h cycle of light (ca. 250 μmol m^{-2} s^{-1} PAR) at 20º C. Petri dishes with 1/10 MS were prepared and after solidification one half of the medium was cut out and placed into another dish, resulting in two half plates. The seedlings were transferred to the center of the half plates and inoculated with a 6mm fungal plug cored from isolates grown on PDA at 25º C for 15 days. The fungus-free control plants were inoculated with identical sterile PDA plugs. The plates were sealed with parafilm resulting in a self-contained closed plate system. A total of ten replicates were assigned to each fungal treatment and its paired control. Some of the original pure cultures failed to revive from repeated subculturing. As a result, the isolates and their numbers varied across the accessions. Of the *Periconia* isolates, 25 were common across all three accessions, and all the accessions were screened with a total of 29 isolates. Of the *Microdochium* isolates, all accessions were screened with two common isolates, but Col-0 was screened with four, Kin-1 with three and Cvi-0 with two isolates. The plants were incubated upright in the growth chamber under the above conditions, their shoots harvested five weeks after inoculation and dried at 50°C for dry weight. Roots were used for microscopic analyses.

Confirmation of colonization in resynthesis roots

The harvested roots were immediately screened for presence or absence of fungal colonization under a light microscope at 200×. Absence of contamination was confirmed for the fungus-free controls. Microsclerotia and melanized hyphae were recorded in *Periconia* treatments, and chlamydospores in the *Microdochium* treatments as was expected for these two endophytes ([1]Mandyam et al, *in press*). Our experiment included nearly one thousand inoculated experimental units and an equal number of fungus-free controls. To expedite the assessment of root colonization and to avoid the time-consuming gridline intersection enumeration (McGonigle et al. 1990), we ranked the colonization on a scale of 0 to 2, with 0 indicating no colonization, 1 indicating at least one to two DSE structures per field and 2 indicating more than two DSE structures per field.

Arabidopsis responsiveness to DSE colonization

To estimate the host responses to inoculation, we used a metric modified from the 'mycorrhizal dependency' (Hetrick et al 1988, Wilson and Hartnett 1997, [2]Mandyam et al, *in press*).

$$\% R_{DSE} = (1-C/I)* 100$$

where R_{DSE} is the responsiveness to DSE fungus, I the mean dry weight of the inoculated plants and C the mean dry eight of the fungus-free control plants. The responsiveness was calculated for each treatment and its paired control for all the fungal isolates used in all the three ecotypes.

Statistical analyses of DSE colonization in resynthesis

To test for the differences in colonization among the isolates and accessions, the fungus-free control plants were omitted from these analyses. To maintain balance and a complete experimental design matrix, colonization data for only those 25 *Periconia* and two *Microdochium* isolates that were common to all accessions were included in these analyses. The two endophyte species were analyzed separately. Treatment (isolate, accession) level differences were determined using a two-way ANOVA in PROC GLM in SAS (Version 9.1). Pair-wise differences, when necessary, were determined by a Tukey test.

Statistical analyses of shoot biomass in resynthesis

We tested the shoot biomass responses to endophyte colonization using two strategies. i) To test whether the shoot biomass responses differed among DSE isolates and *Arabidopsis* accessions, a global ANOVA on shoot biomass was conducted using data from the 25 *Periconia* and two *Microdochium* isolates common to all accessions. Because our main focus in these analyses was to determine differences among isolates and accessions, shoot biomass of only the fungal treatments were included – the paired controls were omitted. PROC GLM was used in SAS (Version 9.1) and pair-wise differences, when necessary, were determined by a Tukey test. These analyses were conducted separately for *Periconia* and *Microdochium*. ii) To test whether there were any biomass differences at the level of an isolate, the fungal treatment was compared with its fungus-free control within each paired experiment. These analyses were conducted separately for each of the three accessions and the biomass data for all the isolates were included. PROC GLM in SAS (Version 9.1) was used to infer differences among the inoculated and fungus-free treatments.

Statistical analyses of the correlation between DSE colonization and shoot biomass

Spearman's rank correlation was used to study the association between DSE colonization and shoot biomass or % responsiveness for each of the accessions using PROC CORR in SAS (SAS version 9.1). The data from the 25 common *Periconia* isolates and two *Microdochium* isolates were used and the data included the shoot biomass of the fungal treatments and their colonization only; fungus-free controls were omitted. *Periconia* and *Microdochium* data were analyzed separately.

Statistical analyses plant responsiveness to DSE colonization

We aimed to address whether or not there was an overall response to a population of fungal isolates in any of the three *A. thaliana* accessions. To do this, the data for overall responses to fungal colonization were analyzed separately for each of the three *Arabidopsis* accessions. Although the data were not normally distributed, we chose to conduct the *t* test since our sample size was relatively large for the *Periconia* treatments, the populations were similarly skewed, and the *t* test is fairly robust against deviations from normality. PROC TTEST was used in SAS (Version 9.1) to test the null hypothesis that the sample was drawn from a population with a mean responsiveness equal to zero. Since the *Microdochium* dataset from the three accessions was very small, they were omitted from the analyses.

RESULTS

DSE colonization of field-collected Arabidopsis roots

A majority of the field-collected *A. thaliana* root samples was colonized by DSE fungi. Of the 18 Cvi-0 samples screened, six did not contain any indicative melanized structures. The

remaining samples were colonized with melanized inter- and intracellular hyphae (Fig. 4.1) and some contained melanized microsclerotia (Fig. 4.1) or chlamydospores (image not shown).

Root colonization in the resynthesis study

Arabidopsis roots, when inoculated with *Microdochium* isolates produced typical intracellular chlamydospores without melanized hyphae (Fig. 4.2a). *Periconia* isolates formed typical melanized microsclerotia in the cortex (Fig. 4.2b); some also produced melanized intercellular hyphae (image not shown). The root colonization varied among the *Periconia* isolates as indicated by the significant 'fungus' term (Table 4.1). Colonization varied also among the *A. thaliana* accessions as indicated by the significant 'accession' term (Table 4.1): Cvi-0 had the highest colonization, followed by Col-0 and Kin-1. A significant interaction term 'fungus*accession' indicated that the different *Periconia* isolates colonized the accessions differently, suggesting that both host and fungal genotype influence the colonization levels. A similar analysis on *Microdochium* colonization was also carried out. The ANOVA terms 'fungus', 'accession' and 'fungus*accession' were not significant at $\alpha = 0.05$ indicating that both common *Microdochium* isolates colonized all accessions equally heavily, without any differences in colonization among the accessions.

Neither *Periconia* nor *Microdochium* colonization was correlated with the shoot biomass in any of the accessions (data not shown).

Shoot biomass in the resynthesis study

The ANOVA for the global shoot biomass is presented in Table 4.1. Similarly to the *Periconia* colonization results, the 'fungus', 'accession' and 'fungus*accession' terms were highly significant implying that the shoot biomass varied among the *Periconia* isolates, varied

also among the *A. thaliana* accessions: Cvi-0 had the highest growth response, followed by Col-0 and Kin-1. Most importantly, a significant interaction term 'fungus*accession' indicated that the different *Periconia* isolates affected the shoot biomass variably in the different accessions, suggesting that both host and fungal genotype influence the shoot biomass. A similar analysis on responses to *Microdochium* colonization was carried out (Table 4.1). All three accessions performed differently as indicated by the significant 'accession' term. Cvi-0 exhibited the greatest shoot biomass, followed by Kin-1 and Col-0 had the least biomass. In contrast to *Periconia*, the two *Microdochium* strains did not affect the *A. thaliana* performance as indicated by the non-significant 'fungus' and 'fungus*accession' terms.

In addition to the overall analyses across all accessions and common isolates, each paired experiment within an accession was analyzed to test for host biomass differences between the inoculation and its fungus-free control treatments at α=0.05. The Kin-1 growth was negatively impacted by only three *Periconia* isolates, 19 were neutral and seven increased the host biomass significantly. One *Microdochium* had a positive effect, while the other two were neutral. In Col-0 accession, 19 *Periconia* isolates negatively influenced shoot biomass, 10 were neutral, and none had a positive effect. Among the *Microdochium* isolates, one had a negative effect while the remaining three were neutral. In the *Periconia*-Cvi-0 interaction, 14 were negative, 14 neutral and one had a positive growth response. Neither of the two *Microdochium* isolates had an effect on Cvi-0 biomass. These observations suggest that different conspecific isolates elicit a continuum of growth responses within an accession and that the host accessions substantially vary in their responses to common fungal strains. Table 4.2 compares the effect of an isolate among the three accessions. For example, *Periconia* KS3055_2 had a positive growth effect in Kin-1, negative in Col-0 and neutral in Cvi-0. Only four isolates of the 25 common *Periconia*

isolates elicited the same response in all accessions. Taken together, this supports the significant 'fungus*accession' interaction in the global shoot biomass analysis: both host and fungal genotype influence the outcome of the symbiotic interaction.

Plant % responsiveness to DSE colonization

The null hypothesis that the mean host responsiveness to a population of root-associated fungal endophytes equals zero was tested to assess the effect of *Periconia* colonization on three accessions of *Arabidopsis*. Distribution of responses to more than two dozen *Periconia* isolates were tested for each accession. The null hypothesis was rejected for Col-0 and Cvi-0, whose responses to *Periconia* colonization were negative (Fig. 4.3a, b). In contrast, on average, Kin-1 did not respond to inoculation with *Periconia, i.e.*, this symbiosis was neutral (Fig. 4.3c). The Cvi-0 and Kin-1 responsiveness were not correlated with the DSE colonization, while it was negatively correlated in Col-0.

DISCUSSION

This unique study showed that *Arabidopsis* roots are colonized by DSE fungi under natural conditions. The *Arabidopsis*-DSE symbiosis was exploited to test where in the mutualism-parasitism continuum the DSE fungi would fall. A selection of conspecific strains of DSE endophytes, *P. macrospinosa* and *Microdochium* sp., native to tallgrass prairie ecosystem were screened with three accessions of *Arabidopsis*. As in the case of mycorrhizal fungi and grass endophytes, the responses elicited by the conspecific DSE fungi were along the symbiotic continuum.

Arabidopsis thaliana is a non-mycorrhizal model plant. Our data show that it is susceptible to DSE fungi and forms DSE symbioses in nature. In field grown *Arabidopsis* Cvi-0

roots, melanized inter- and intracellular hyphae were common and melanized chlamydospores and microsclerotia were present. Our resynthesis studies with *Periconia* and *Microdochium* with five *Arabidopsis* accessions (Columbia, Niederzenz, Kendalville, Cape Verde Island, Estland and Wassilewskija) showed that they were colonized to varying extents (Mandyam et al, unpublished). In the present study with a broader selection of fungal symbionts, Col-0, Kin-1 and Cvi-0 accessions were observed to vary in their susceptibility to *Periconia* colonization. The *Microdochium* isolates colonized the accessions equally, although these conclusions are based on far fewer conspecific strains. Our microscopic observations confirmed that in the resynthesis studies, most *Periconia* isolates formed melanized microsclerotia in the cortex whereas some isolates formed melanized intercellular hyphae only. *Microdochium* isolates invariably produced abundant chlamydospores in the root cortex. The Cvi-0 and Kin-1 responsiveness were not correlated with the DSE colonization, while it was negatively correlated in Col-0. However, the colonization levels in the different accessions were not correlated with the shoot biomass. This is similar to the results from ectomycorrhizal meta-analysis of Karst et al (2008) where colonization did not explain the observed variation in growth responses. ANOVA of the colonization data (Table 4.1) shows that colonization levels are different in the three accessions. More importantly, the significant 'fungus*accession' interaction shows that the colonization susceptibility of different accessions varies in response to different DSE isolates. The fungus* accession interaction is a reflection of the host and fungal genotype influence on the colonization level. In case of arbuscular mycorrhizal fungi, Graham and Eissenstat (1999) hypothesized that colonization of the host is not controlled by the fungal genotype, but only by the host genotype.

We recorded DSE symbiosis in *Arabidopsis* under natural conditions. However, we did not identify and isolate the fungi from those samples. Instead, we screened DSE fungi isolated

from a native tallgrass prairie ([1]Mandyam et al, *in press*). These isolates have previously demonstrated a broad host range, ability to colonize both native and non-native plants, and an ability to induce host responses along the mutualism-parasitism continuum ([2]Mandyam et al, *in press*).

Our screening of a large number of *Periconia* and few *Microdochium* isolates in three *Arabidopsis* accessions provides further insight into the mutualism-parasitism concept for DSE fungi. DSE fungal symbioses in native grasses, forbs and crop plants have been previously reported to elicit responses along the symbiotic continuum ([2]Mandyam et al, *in press*). The 'responsiveness' to DSE colonization, previously used as a means to assist in explaining the variable plant growth responses ([2]Mandyam et al, *in press*), was used to evaluate the response of the accession to DSE colonization. Our study confirms the mutualism-parasitism continuum of responses at three levels: First, at a population level, the *Arabidopsis*-DSE interaction was either neutral (Kin-1) or negative (Col-0 and Cvi-1; Fig. 4.3). These results suggest that on an average, DSE fungi are likely weak parasites supporting Addy et al (2005), although individual strains may behave as mutualists. Second, at the strain level, growth responses varied along this continuum within each accession. The accessions responded negatively, neutrally or positively to different conspecific isolates (Fig. 4.4). The responses were mainly neutral or negative, with few mutualisms. Col-0 had the most negative responses and no positive response. In contrast, Kin-1 had the fewest negative responses, most positive responses, but was mostly neutral. Third, many conspecific isolates elicited a range of growth responses depending upon the host accession (Table 4.2). As exemplified in our results, one isolate could yield a positive, negative and neutral response, depending on the host accession. Taken together, these observations suggest that the outcome of the host-fungus interactions are highly variable, fall within a mutualism-parasitism

continuum and are governed by both the host and fungal genotypes, even in laboratory experiments where environmental conditions are tightly controlled and nearly invariable.

The DSE isolates used in this study, especially *Periconia* exhibited broad phenotypic plasticity ([1,2]Mandyam et al, in press). The isolates commonly varied morphologically, although they were conspecific strains. The colony morphology and microscopic features were variable from one isolate to the other and often changed within an isolate during development and after repeated subculturing ([1]Mandyam et al, in press). Phenotypic plasticity is one proposed controls of the outcome in a symbiotic interaction (Schulz and Boyle 2005).

Mycorrhizal fungi are usually considered beneficial to their hosts, *i.e.*, they are mutualistic symbionts (Jones and Smith 2004). However, under some environmental conditions the host does not respond positively to colonization. The concept of the symbiotic continuum has been used primarily to describe the range of outcomes in mycorrhizal symbioses (Francis and Read 1995, Johnson et al 1997, Karst et al 2008) or to explain the host responses to foliar, non-mycorrhizal or systemic endophyte symbioses (Saikkonen et al 1998, Redman et al 2001, Müller and Krauss 2005, Schulz and Boyle 2005, Schulz 2006, [2]Mandyam et al, *in press*). The outcome of an interaction depends on the delicate balance between the fungal virulence and host defenses, both of which are affected by plant and fungal genotype, plant physiology, nutritional status, developmental stages of the partners and environmental factors (Saikkonen et al 1998, Redman et al 2001, Faeth and Sullivan 2003, Schulz 2006). Our study emphasizes the contribution of fungal and host genotypes – under controlled laboratory resynthesis conditions with minimal environmental variability, host accessions responded differently to a single fungal strain and fungal strains produced variable responses within and among accessions. Few studies have documented the effect of host and/or fungal genotypic effects on the outcome of the host-

endophyte interaction. Redman et al (2001) showed that fungal lifestyle expression and subsequent outcome of symbiosis was controlled by the plant: *Colletotrichum magna* pathogenic in a susceptible host, was non-pathogenic or even mutualistic in non-hosts; *C. orbiculare* (Berck. and Mont.) Arx exhibited a range of lifestyles depending on the host. Similarly, using a *Neotyphodium*-tall fescue system, Faeth and Sullivan (2003) concluded that host genotype controlled the outcome of host-endophyte interaction. In addition to the host genotype, fungal genotype likely affect the outcome of the symbiosis: Freeman and Rodriguez (1993) converted a pathogenic *C. magna* to a non-pathogenic mutant, Path-1, by UV mutagenesis. This non-pathogenic mutant behaves mutualistically in many hosts (Redman et al 2001). The ability of *Colletotrichum* to switch between symbiotic lifestyles was thought to be controlled by a single locus (Rodriguez and Redman 2008). Similarly, Tanaka et al (2006) selected a *noxA* mutant of *Epichloë festuca* Leuchtm., Schardl and Siegel, a claviceptaceous fungal endophyte of ryegrass, *Lolium perenne* L. This mutation altered the interaction from mutualism to parasitism. Contrary to our findings, a meta-analysis of the symbiotic continuum in ectomycorrhizas concluded that the outcome of the mycorrhizal symbiosis was explained only by the host plant identity and not by fungal identity (Karst et al 2008). However, it should be noted that Karst et al (2008) focused on species level variability, while our study focused on the variability at the strain level.

Finally this brings us to the question, what is the functional classification of DSE fungi? Are they mutualists, weak parasites or commensals? The answer is none and all of the above. The DSE fungi, similarly to mycorrhizal fungi or grass endophytes elicit a range of responses that are controlled by numerous abiotic and biotic factors. Mycorrhizas and grass-*Neotyphodium* interaction are considered mutualisms. However, numerous studies demonstrate a continuum of responses by mycorrhizas when factors controlling the interaction are altered. Therefore, based

on the experimental evidence from laboratory-controlled and field experiments, Jones and Smith (2004) argue that mycorrhizas should be defined structurally or developmentally – not based on demonstrated mutualism. Similarly, Muller and Krauss (2005) propose a mutualism-parasitism continuum of responses for the grass asexual endophyte interaction. Based on published reports, they argue that grass-asexual fungus interactions are not always mutualistic and can be affected by the grass species, cultivar or genotype, genotype of fungal endophyte, environmental factors (fire, soil conditions, CO_2 concentrations), multitrophic interactions (herbivory) and intra- and interspecific competition and soil microbes. While it has been hypothesized that mutualistic interactions are more frequently developed between microbes and roots (Schulz and Boyle 2005), only a fraction of fungal endophytes interact positively with their hosts (Schulz 2006). This is in fact supported by our study. Similarly, Kageyama et al (2008) also note that mutualisms in these symbioses at the taxon level are rare or infrequent. Whether at the level of conspecific strains or of species, it appears that mutualisms are rare among DSE fungi and that these associations are most frequently neutral or weakly parasitic.

CONCLUSIONS

This study established that *Arabidopsis*, a non-mycorrhizal plant, forms DSE symbioses under natural conditions. The use of the well-established model plant system provides a convenient tool for further exploration of the mutualism-parasitism continuum in DSE symbiosis. On average at the population level, accessions Col-0 and Cvi-0 responded negatively to *Periconia* colonization, whereas Kin-1 response was neutral. However, at the strain level, we found that Cvi-0 and Kin-1 responded positively to some isolates, while Col-0 responded mainly negatively and never positively. Our screening of three *A. thaliana* accessions with multiple isolates of a single DSE species, *P. macrospinosa* clearly indicates that the host and fungal

genotypes contribute to the outcome of a symbiosis and that these outcomes may be unpredictable if only the species identities of the host and fungus are known.

Table 4.1 ANOVA results for *Periconia* colonization, and *Periconia* and *Microdochium* effects on shoot biomass

	Periconia colonization			*Periconia* biomass			*Microdochium* biomass		
Effects	**df**	**F**	***P***	**df**	**F**	***P***	**df**	**F**	***P***
Fungus	24	251.53	<0.0001	24	5.8	<0.0001	1	1.55	0.2185
Accession	2	322.53	<0.0001	2	16.32	<0.0001	2	6.02	0.0044
Fungus*Accession	48	91.1	<0.0001	48	4.66	<0.0001	2	0.01	0.9859

All three *Arabidopsis* accessions (Col-0, Kin-1 and Cvi-0) were screened with 25 *Periconia* and two *Microdochium* isolates

Table 4.2 Effect of fungal endophyte on the shoot biomass of three *Arabidopsis* ecotypes. F values are presented from the ANOVA.

DSE isolates	Col-0	Cvi-0	Kin-1
Periconia			
KS3032	4.13 (–)	15.27 (–)	9.22 (–)
KS3037	28.97 (–)	3.41 (0)	0.95 (0)
KS3043	8.38 (–)	1.65 (0)	1.09 (0)
KS3044	1.70 (0)	0.48 (0)	0.36 (0)
KS3046	9.25 (–)	10.10 (–)	0.38 (0)
KS3049	20.56 (–)	7.48 (–)	14.86 (+)
KS3050	2.27 (0)	17.66 (–)	0.04 (0)
KS3052	2.67 (0)	0.33 (0)	9.65 (+)
KS3056	11.25(–)	17.42 (–)	0.79 (0)
KS3057	1.76 (0)	5.27 (–)	0.59 (0)
KS3058	13.99 (–)	. (–)	0.86 (0)
KS3061	4.41 (–)	0.43 (0)	0.02 (0)
KS3069	na	7.99 (+)	na
KS3078	0.0 (0)	3.33 (0)	0.0 (0)
KS3080	1.05 (0)	na	3.13 (0)
KS3083	na	4.67 (–)	na
KS3084	10.96 (–)	3.50 (0)	0.01 (0)
KS3086	5.27 (–)	na	4.33 (0)
KS3087	7.21 (–)	12.39 (–)	6.08 (–)
KS3089	na	8.68 (–)	na
KS3038_1B	na	44.46 (–)	0.26 (0)
KS3041_B	12.69 (–)	4.38 (–)	4.61 (+)
KS3045_2	1.47 (0)	0.08 (0)	0.80 (0)
KS3047_1	9.67 (–)	2.24 (0)	4.36 (–)
KS3047_2	7.01 (–)	0.12 (0)	0.0 (0)
KS3047_2A	3.29 (0)	na	11.23 (+)
KS3047_2B	7.52 (–)	2.89 (0)	4.44 (+)
KS3054_1	7.75 (–)	10.20 (–)	0.29 (0)
KS3054_2	2.32 (0)	6.48 (–)	0.03 (0)
KS3055_1	1.31 (0)	na	0.04 (0)
KS3055_2	17.64 (–)	3.10 (0)	4.66 (+)
KS3067_1	na	0.53 (0)	na
KS3067_2	8.13 (–)	1.14 (0)	4.81 (+)
KS3074_1	19.10 (–)	0.11 (0)	2.78 (0)
Microdochium			
KS3064	9.77 (–)	na	na
KS3072	2.40 (0)	0.50 (0)	10.26 (+)
KS3073	1.20 (0)	0.07 (0)	0.30 (0)
KS3091	4.07 (0)	na	1.89 (0)

(0)=neutral, (–)=parasitic and (+)=mutualistic

na= fungus not used in the interaction

KS3058 killed the Cvi-0 plants and hence is a negative association

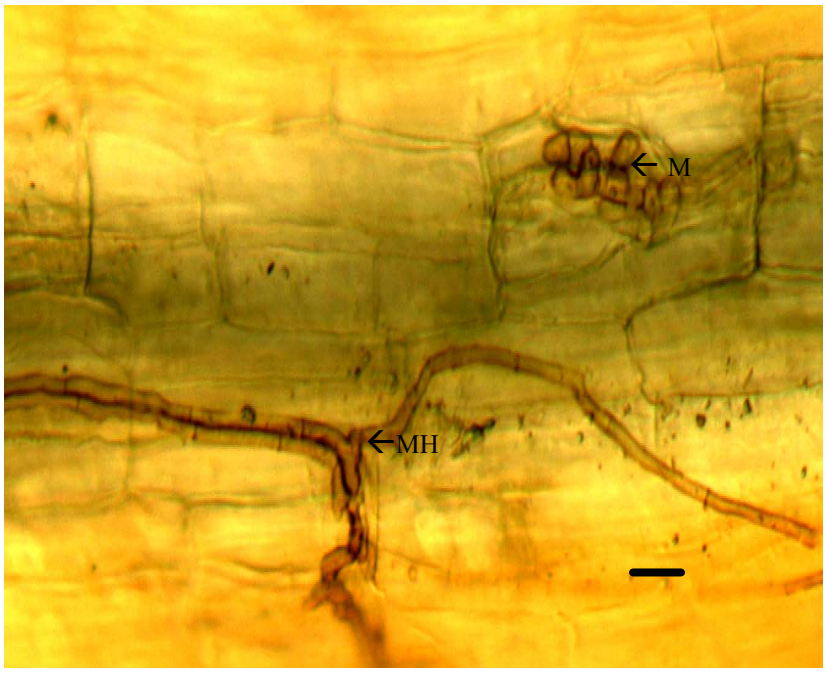


Figure 4.1 Typical DSE morphology in field-collected *Arabidopsis* (Cvi-0 ecotype).
Microsclerotia (M) and melanized hyphae (MH) in root cortex. Identity of DSE fungus is unknown. Bar=10µm

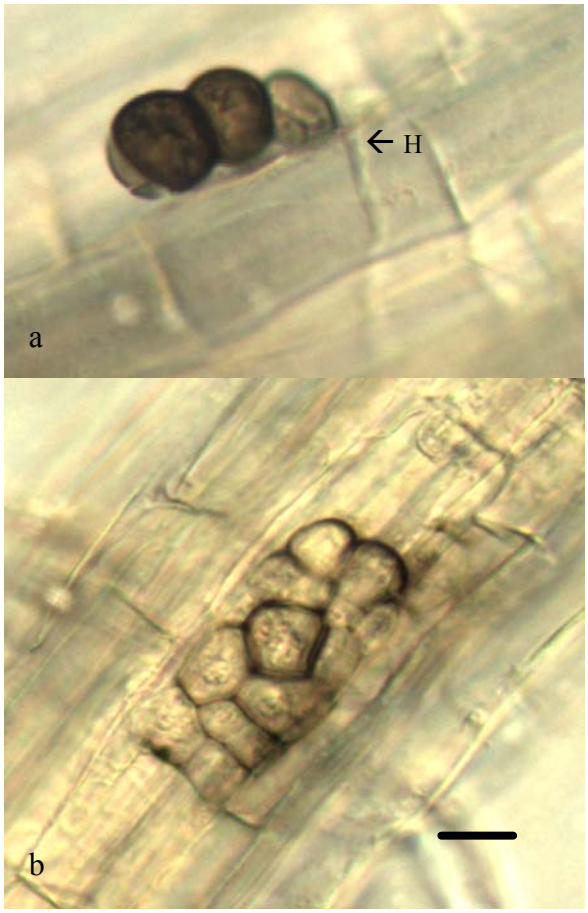


Figure 4.2 DSE fungal colonization of *Arabidopsis* (Col-0) root cortex in resynthesis system.
a) Chlamydospores of *Microdochium* sp. with intracellular hyphae (H). b) *Periconia* melanized microsclerotium. Colonization in Kin-1 and Cvi-0 were similar to that in Col-0. Bar=10μm.

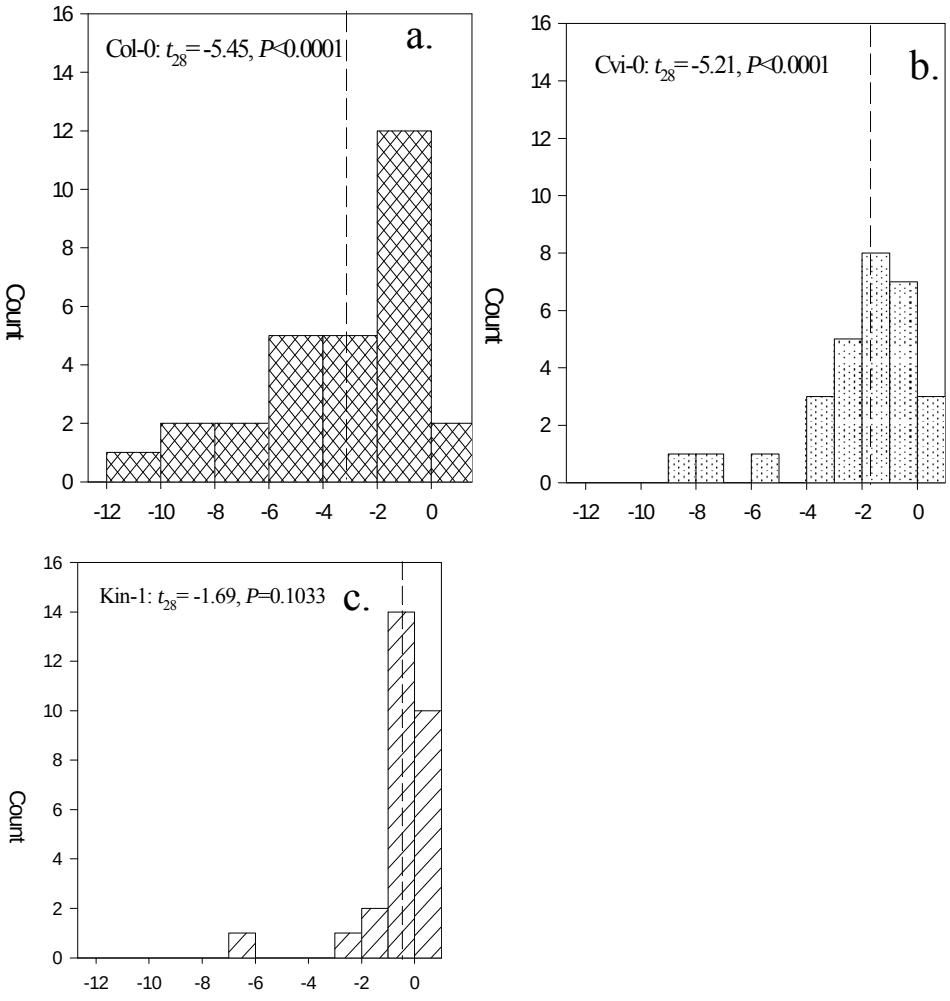


Figure 4.3 Distribution of *Arabidopsis* '% response' to *Periconia macrospinosa* colonization.
Ecotypes a) Columbia b) Cvi c) Kin-1. X-axis refers to the host response to colonization [Response= (Biomass of Inoculated – Biomass of Control/Biomass of Inoculated)*100] and the Y-axis refers to the number of DSE isolates. The broken line indicates the population mean.

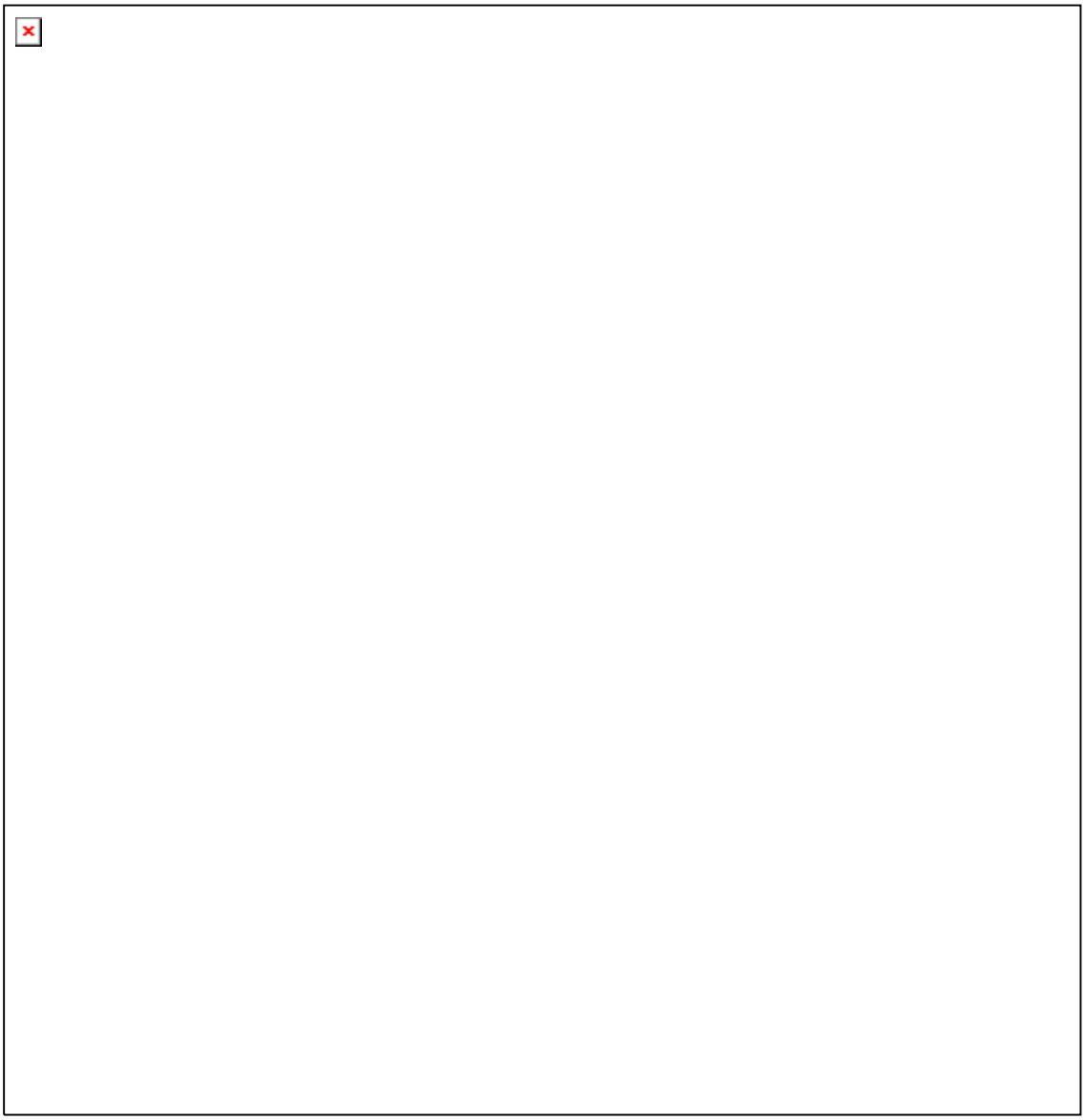


Figure 4.4 Effect of fungal inoculation on host biomass.

The dotted line represents the 1:1 line of the control and inoculated biomass. Data points above the line are greater than their control and points below the line are lesser than the control. The fungal treatments with significantly different biomass from the control are bold faced.

CHAPTER 5 - Future research

This study has addressed some of the fundamental questions about the DSE identity, their nutritional requirements, host range and/or host preference. This study establishes that i) the native tallgrass prairie hosts a unique set of DSE fungi: *Periconia macrospinosa* and *Microdochium* sp., both of which are well known soil saprobes, ii) the DSE fungi produce a variety of extracellular enzymes to hydrolyze complex molecules and have the ability to utilize a range of organic and inorganic nitrogen sources, suggesting that they are facultative saprobes, iii) exhibit a broad host range by colonizing hosts to varying extents, iv) exert a host preference, preferring native grasses to forbs and v) elicit growth responses along the symbiotic continuum, controlled by host and fungal genotype.

DSE fungi are ubiquitous in many habitats (Jumpponen and Trappe 1998). DSE fungi co-occur with mycorrhizal fungi, and are sometimes as abundant as mycorrhizal fungi (Jumpponen and Trappe 1998, Mandyam and Jumpponen 2005, 2008). This brings us back to the most interesting question about the DSE fungi: what are their functional roles in an ecosystem? Many recent reviews have hypothesized many potential functions, and suggest both nutritional and non-nutritional benefits. Similarly to mycorrhizal and grass endophyte functions, the DSE functions include i) improved growth responses accompanied with or without nutrient acquisition, ii) utilization of organic nutrient pools and facilitation of plant use of these pools by DSE, iii) alteration of the host environmental tolerance, for example, increased drought tolerance of plants due to DSE colonization, iv) Protection of host plants from herbivores and plant

pathogens, and v) impact on plant community dynamics (Addy et al 2005, Mandyam and Jumpponen 2005, Schulz 2006, Kageyama et al 2008).

To understand the potential functions of DSE fungi, we can exploit the DSE-host symbiosis at molecular and metabolic levels. In fact the *Arabidopsis*-DSE symbiosis makes a great model. Whole genome Affymetrix microarrays are available for *Arabidopsis*, and they can provide a list of differentially expressed genes in the symbiotic host compared to nonsymbiotic control plants. This research effort has been initiated and our preliminary data show an up-regulation of genes involved in protein and carbohydrate metabolism, hormone signalling, defense responses and cell wall reorganization. The improved defense responses of *Arabidopsis* at the molecular level has been further substantiated: *Arabidopsis-Periconia* symbiotic plants when challenged with a fungal pathogen, *Botrytis cinerea*, have fewer disease symptoms compared to nonsymbiotic *Arabidopsis*. Additionally, the metabolites from symbiotic *Arabidopsis* plants have been analyzed for their metabolite signature. More than 5% of the total polar and non-polar metabolites are differentially expressed in symbiotic plants. This is an ongoing project and the final objective is to ascertain if the levels of phytohormones and defense compounds are also differentially expressed in the symbiotic plants. To understand the abundant DSE symbiosis in a tallgrass prairie, microarray experiments of *Andropogon gerardii-Periconia macrospinosa* symbiosis have also been initiated.

The data emerging from both the molecular and metabolic profiling of the DSE symbiosis should shed more light on the functional roles of DSE fungi.

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Appendix A - Permission Letter

Centraalbureau Voor Schimmelcultures

Fungal Biodiversity Centre

Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW)

Keerthi Mandyam
421 Ackert Hall
Division of Biology
Kansas State University
Manhattan,KS 66502 USA

Utrecht, 14 December 2008

Dear Keerthi Mandyam,

Herewith I grant permission to use the article Mandyam & Jumpponen: "Seeking the elusive function of the root-colonizing dark septate endophytic fungi" published in Studies in Mycology, 53(1):173-189, 2005 to include in your Phd Dissertation.

With kind regards

Dr Robert A Samson
Editor- in Chief of the Studies in Mycology