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Evaluation of the internal colonization of *Atriplex canescens* (Pursh) Nutt. roots by dark septate fungi and the influence of host physiological activity

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Abstract Dark septate (DS) fungal endophytes are the primary root colonizers of fourwing saltbush, *Atriplex canescens* (Pursh) Nutt, a dominant and ecologically important shrub in southwestern USA rangelands. These fungi are characteristically identified as stained or pigmented hyphae and microsclerotia in the root cortex using conventional fungus staining methods. *A. canescens* roots colonized by DS fungi were stained with sudan IV and analyzed with differential interference microscopy. This method revealed substantial internal colonization of the cortex and vascular cylinder by vacuolated hyaline hyphae that were not evident when stained with trypan blue. Hyaline hyphae were internal extensions of melanized DS hyphae and microsclerotia. Sudan IV intensely stained lipids in fungal vacuoles, further enhancing visibility of internal hyaline hyphae. Melanized hyphae and microsclerotia were more abundant in roots sampled from dormant and relatively inactive plants, while hyaline hyphae and lipid accumulation were most prevalent in roots of physiologically active plants. The polymorphic nature of DS fungal endophytes, their dynamic response to metabolic activity, and their similarities and differences relative to aseptate fungal colonization in *A. canescens* roots are discussed.

Keywords Endophyte · Lipid · Method · Mycorrhiza · Fungus stain

Introduction

Mycorrhizal fungi form intimate symbiotic associations with the roots of most vascular plants and contribute to their nutrition and survival. Plant-fungal interfaces are routinely studied microscopically by clearing and staining roots with biological stains such as trypan blue, acid fuchsin, and chlorozol black, which bind specifically to chitin, a component of most fungal walls (Bevege 1968; Kormanik et al. 1980; Phillips and Hayman 1970). The transient nature of internal mycorrhizal structures, such as arbuscules, is variable and can be affected by plant growth and soil conditions (Gange et al. 1999), which may also affect their response to fungus stains (Vierheilig et al. 1998). Sudan IV has been used as a histochemical to specifically stain internal fungal lipids instead of wall components (Gaspar et al. 1997; Jansa et al. 1999; Nemec 1981). Innovative microscopic and histochemical methods such as confocal laser scanning microscopy and epi-fluorescence have improved the definition of internal structures of both endo- and ectomycorrhizal colonization (Gange et al. 1999; Schelkle et al. 1996; Vierheilig et al. 1999).

Plant roots are colonized by many different kinds of fungi, including saprophytic or weakly pathogenic fungi that may have symptomless endophytic or biotrophic phases in their life cycles which are not apparent to casual observers (Parbery 1996). Non-mycorrhizal plants are frequently colonized by septate fungi that may function like mycorrhizal fungi, but their study and importance has been minimized because they do not conform to established mycorrhizal morphology (Trappe 1981). Jumpponen and Trappe (1998) have reviewed reports of dark septate (DS) fungal endophytes which colonize a wide range of plant species in stressed ecosystems and include common soil, saprotrophic, and rhizoplane fungi, as well as known pathogens. They are often mitotic or sterile, differ morphologically from conventional mycorrhizal symbiosis, and are identified primarily as stained or pigmented hyphae and microsclerotia that grow inter- and intracellularly within the cortex. Their characteristic dark color results from the incorporation of melanin, a natural dark pigment and a common fungal wall component. Their ubiquitous presence suggests that they perform a significant but currently unresolved ecological function.

Haselwandter and Read (1982) examined DS colonization of *Carex* sp. in an alpine habitat and found that

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weakly staining, hyaline hyphae extended into the root cortex from dark septate surface hyphae. Newsham (1999) also reported that *Philophora graminicola*, with dark septate runner hyphae on the root surface of *Vulpia ciliata* ssp. *ambigua*, penetrated the cortex as hyaline hyphae which formed melanized hyphopodia (microsclerotia) in the cortex and on the root surface. Väre et al. (1992) found that 40% of 76 plant species in a Norwegian arctic zone were colonized by both dark and hyaline septate fungi that were believed to be functionally mutualistic rather than saprophytic or pathogenic. Williams et al. (1994) reported that a hyaline septate fungus formed mycorrhiza-like associations with a liverwort, *Cephaloziella exiliflora*, in Antarctica. These findings indicate that DS fungi are polymorphic and produce structurally different stained, melanized, and hyaline tissue. Consequently, internal hyaline hyphae which fail to stain could easily be overlooked in roots prepared with fungus-specific stains.

The mycorrhizal status of dominant native plants in nutrient and water-stressed arid ecosystems is not well understood. Fourwing saltbush, *Atriplex canescens* (Pursh) Nutt., a dominant, ubiquitous, and ecologically important chenopodiacous shrub in arid southwestern USA rangelands has been considered by some to be non-mycorrhizal (Aguilera et al. 1998). Barrow et al. (1997) examined *A. canescens* roots collected from native populations at arid rangeland sites differing in elevation and soil moisture, using conventional fungus-staining methods. Dark septates were the dominant fungal colonizers at all sites and were expressed as stained and melanized hyphae and microsclerotia in the cortex. Simultaneous colonization by aseptate fungi was observed occasionally at more mesic, high-elevation sites. These fungi were primarily expressed as stained inter- and intracellular hyphae and vesicles, with sporadic coils, while arbuscules were extremely rare.

We hypothesized that DS fungi are polymorphic and colonize roots with non-staining internal hyaline structures not visible using conventional fungus-staining methods. The objectives of this study were to compare *A. canescens* roots stained with sudan IV and analyzed with differential interference microscopy (DIC) with those stained with trypan blue and viewed with bright field microscopy, and to determine whether internal fungal morphology is influenced by the physiological activity of the host.

Materials and methods

Root collection

Roots were sampled two to three times per month during the year from a native population of *A. canescens* on the USDA Agricultural Research Service Jornada Experimental Range in southern New Mexico during 1999. *A. canescens* plants are evergreen in southern New Mexico, but are relatively quiescent in the winter and during summer drought periods. They actively set new leaves and flowers in the spring and in response to summer precipitation events. Data from five different seasonal periods were included in this study. In the winter, when plants were dormant, two sampling periods were included. In the spring just prior to any visual initiation of new leaves, data from one sampling time were included. Data were included for three sampling times from roots collected from quiescent plants during summer drought, and one sample was collected from roots in moist soil following a precipitation event. In the fall, one sample was included from roots collected before plants became dormant. Soil was chronically dry, having <3% moisture at all sampling times, except for the summer moist soil collection, when the soil was near saturation. Fungal colonization was consistent and expression was uniform within the population at each sampling period. For this study, roots from 2–5 plants were collected, bulked, sealed in a plastic bag and taken to the laboratory for preparation and analysis.

Root preparation and clearing

Methods developed by Bevege (1968), Brundrett et al. (1983), Kormanik et al. (1980), and Phillips and Hayman (1970) were modified for optimal visualization of fungi in *A. canescens* roots. Roots were washed in tap water to remove soil and air dried for 24 h at ambient temperature. From each bulked sample, healthy feeder roots of uniform maturity and appearance (approximately 0.25 mm in diameter) were randomly selected and cleared by placing in an autoclave in 2.5% KOH. Temperature was increased to 121°C over 5 min and maintained for 3 min before samples were removed from the autoclave. Roots were rinsed in tap water, bleached in 10% alkaline H_2O_2 for 10–45 min to remove pigmentation, and placed in 1% HCl for 3 min. Decolorized roots were rinsed in tap water, half of each sample was placed in Sudan IV (0.3 g Sudan IV in 74 ml of 95% ETOH plus 24 ml deionized H_2O), half in trypan blue (0.5 g trypan blue in 500 ml glycerol, 450 ml dH_2O and 50 ml HCl) and all were autoclaved at 121°C for 3 min and stored in acidic glycerol (500 ml glycerol, 450 ml H_20 and 50 ml HCl). Ten to 12 2-cm root segments were placed on microscope slides in several drops of permanent mounting medium. A cover slip was placed over the root sections and pressed between two blocks of wood by tightening two 6.3-mm bolts connecting the blocks to facilitate analysis at high magnification. From each sample, 38–86 randomly selected 1-mm segments of root were analyzed for the number of melanized hyphae, microsclerotia, hyaline hyphae minus lipids, and hyaline hyphae with lipids. Analysis was carried out with a Zeiss Axiophot microscope using both conventional and DIC optics at ×400 and ×1000 magnification.

Results

Melanized hyphae were readily observed on the root surface with a stereo-microscope prior to clearing and staining but were less abundant on prepared roots. Septate hyphae and microsclerotia that either stained with trypan blue or that were melanized were visible with both bright field and DIC microscopy (Fig. 1). Careful examination of colonized roots by DIC at high magnification revealed extensive internal colonization by vacuolated hyaline hyphae that did not stain with trypan blue and generally were not visible with bright field microscopy. Hyaline hyphae were connected to melanized septate hyphae (Fig. 2) and they also formed melanized microsclerotia (Fig. 3). Septa were generally more frequent in melanized and stained hyphae than in hyaline hyphae. In many cases, sudan IV positively stained lipids within fungal vacuoles, further enhancing fungal visibility. Staining with sudan IV and analysis with DIC microscopy re-

vealed substantial fungi that failed to stain with other techniques and, therefore, was used exclusively for all additional analysis.

Internal hyaline hyphae varied in visibility and grew both inter and intracellularly in the cortex and sieve elements. Staining with sudan IV revealed extensive internal colonization of the cortex with fungal hyphae that were distinguished only as linear and branched lipid-stained vacuoles (Fig. 4). These hyphae were invisible when stained with trypan blue and analyzed with bright field microscopy. Most frequently, fungal hyphae observed in the cortex were 2–3 µm in diameter and their walls were only visible with DIC microscopy (Fig. 5). In the vascular cylinder, hyaline hypha were more exten-

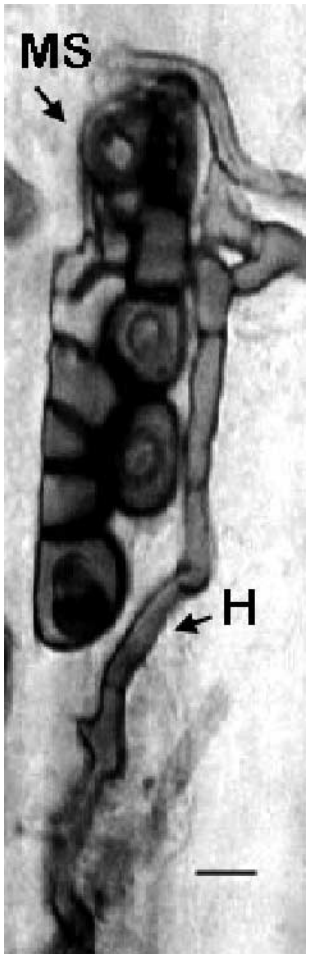

Fig. 1 Melanized hypha (*H*) and microsclerotia (*MS*) of dark septate fungal endophyte (*DS*) in the cortex of *Atriplex canescens* (Pursh) Nutt. Magnification for all figures is ×1000; *bar* 5 µm

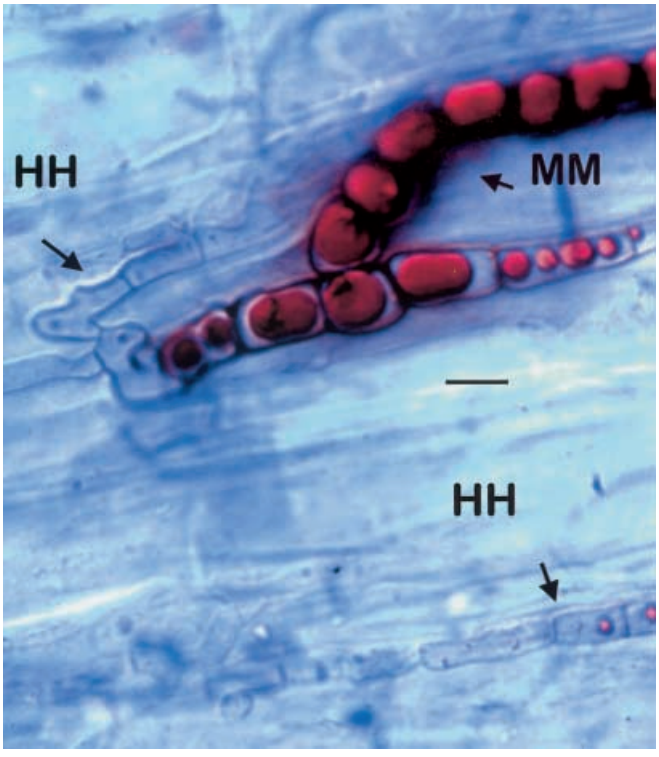

Fig. 3 Melanized microsclerotia (*MM*) developing from hyaline septate hypha (*HH*) in the cortex of *A. canescens* (Pursh) Nutt. (*H*)

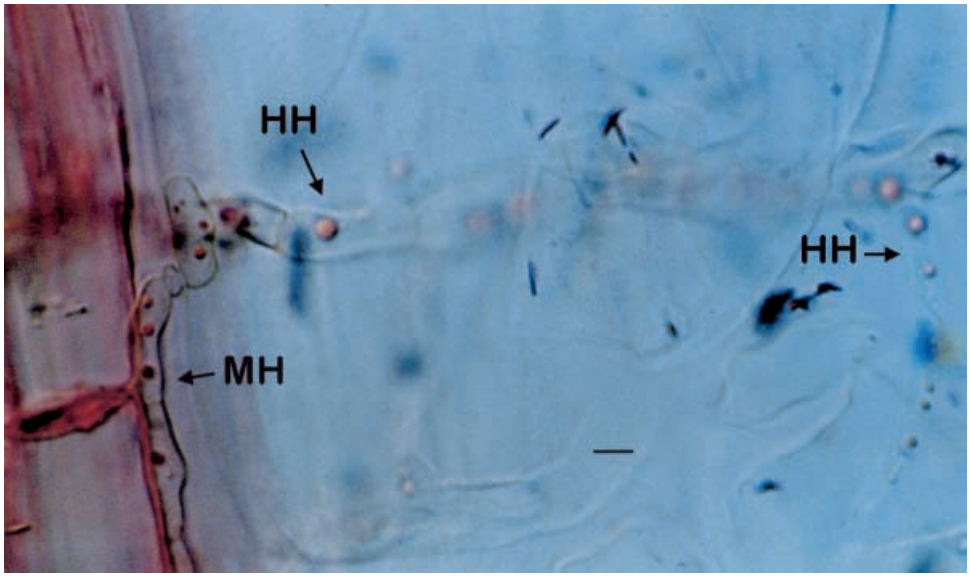

Fig. 2 Melanized hypha (*MH*) continuous with hyaline hyphae (*HH*) in the cortex of *A. canescens* (Pursh) Nutt.

Table 1 Mean number of internal dark septate fungal structures per mm of root length sampled during 1999. Pigmented hyphae refers to internal stained or melanized hyphae (*n* Number of 1-mm root sections analyzed, *sd* standard deviation)

Seasonal conditions	*n*	Pigmented hyphae		Microsclerotia		Hyaline hyphae –lipids		Hyaline hyphae +lipids		Date of collection Month/day
		Mean	sd	Mean	sd	Mean	sd	Mean	sd	
Winter	43	0.37	1.25	3.21	2.60	0.02	0.15	0	0	1/11
Dormant	59	1.24	1.49	2.03	2.81	0	0	0	0	2/22
Spring	65	0.07	0.27	0	0	1.68	1.85	2.51	2.50	4/12
Summer dry soil	86	0.78	1.11	0.07	0.25	1.93	1.11	0.84	1.96	5/10
	60	1.88	1.88	0.18	0.66	2.53	1.64	0.57	1.04	6/7
	38	0.11	0.39	0.37	1.20	0.24	0.54	0.55	1.01	7/9
Summer moist soil	66	0.09	0.24	0	0	0.10	0.29	6.18	2.42	8/9
Fall	72	0	0	0	0	0	0	6.89	2.04	10/28

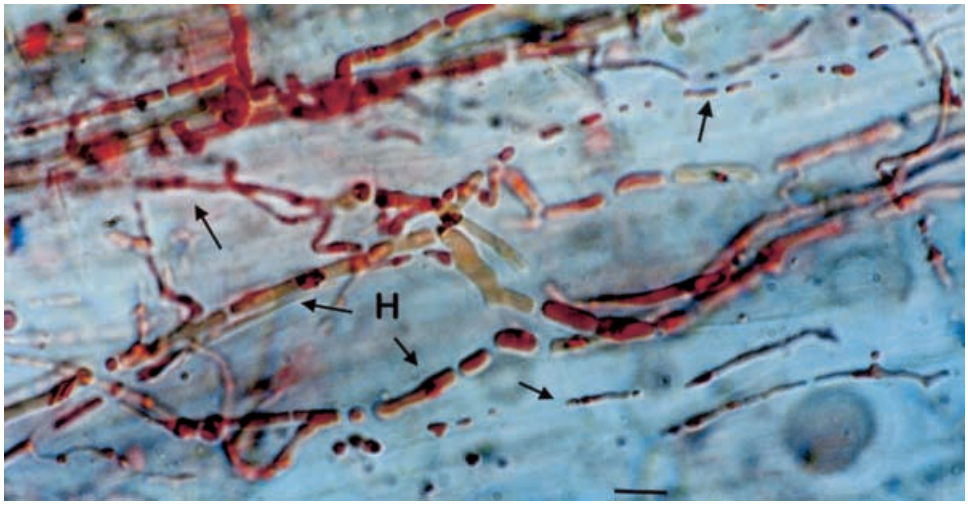

Fig. 4 Hyphae distinguishable only as linear-arranged lipid bodies (*arrows*) in the cortex of *A. canescens* (Pursh) Nutt.

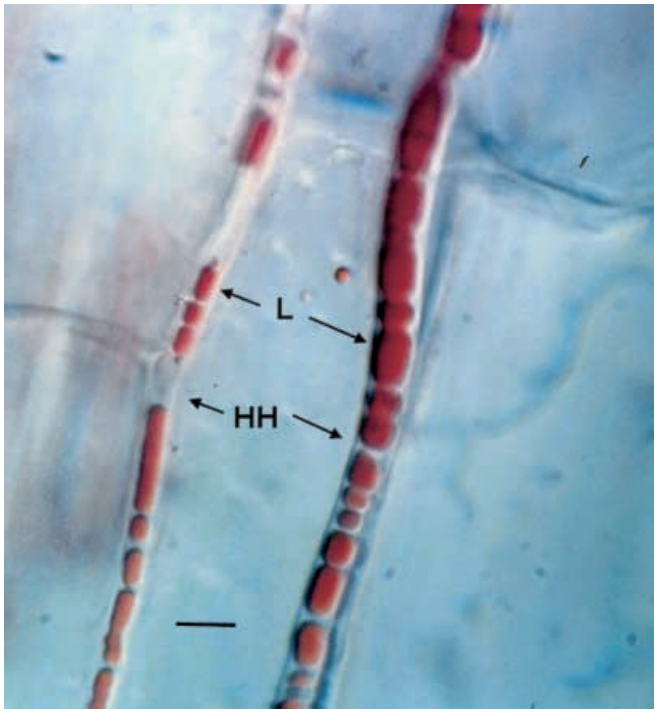

Fig. 5 Hyphae (*HH*) with distinguishable hyaline walls and lipid-filled vacuoles (*L*) in the cortex of *A. canescens* (Pursh) Nutt.

sive and smaller in diameter (1–2 µm). Their walls were less distinguishable and their vacuoles stained positively for lipids in physiologically active roots (Fig. 6). Vesicles were occasionally observed attached to hyaline hyphae. Their internal vacuoles exhibited variable staining for lipids corresponding to the lipid content of the attached hyphae. Figure 7 shows a vesicle with attached hyphae where internal vacuoles failed to stain for lipids with sudan IV. As host physiological activity increased, fungal vacuoles in sieve elements also increased in visibility in response to sudan IV staining as they accumulated lipids.

Four distinct types of internal DS fungal tissue were observed in *A. canescens* roots, melanized hyphae, microsclerotia, hyaline hyphae minus lipids, and hyaline hyphae plus lipids. The relative abundances of these structures at different levels of physiological activity are listed in Table 1. Numbers of melanized hyphae (0.37 and 1.24 per mm) and microsclerotia (3.21 and 2.03 per mm of root length) were greatest in roots sampled from dormant plants in the winter at two different dates. Melanized hyphae (0.07 per mm) and microsclerotia (0 per mm) decreased in roots of plants in the spring just before plants initiated new leaves, and hyaline hyphae minus lipids (1.68 per mm) and hyphae plus lipids (2.51 per mm) increased. After new leaves were estab-

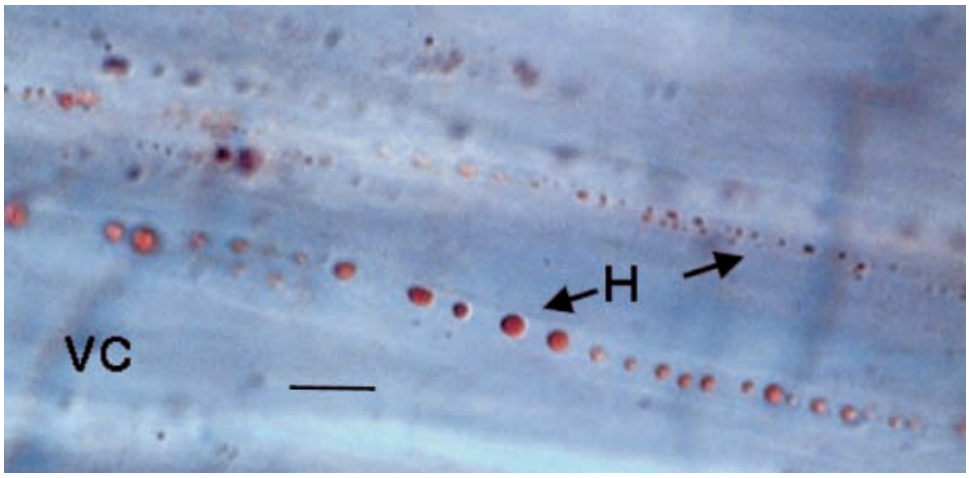

Fig. 6 Hyaline hyphae (*H*) in the vascular cylinder (*VC*). Note that hyphal walls are virtually indistinguishable and only linear-arranged lipid bodies are visible in *A. canescens* (Pursh) Nutt. (*H*)

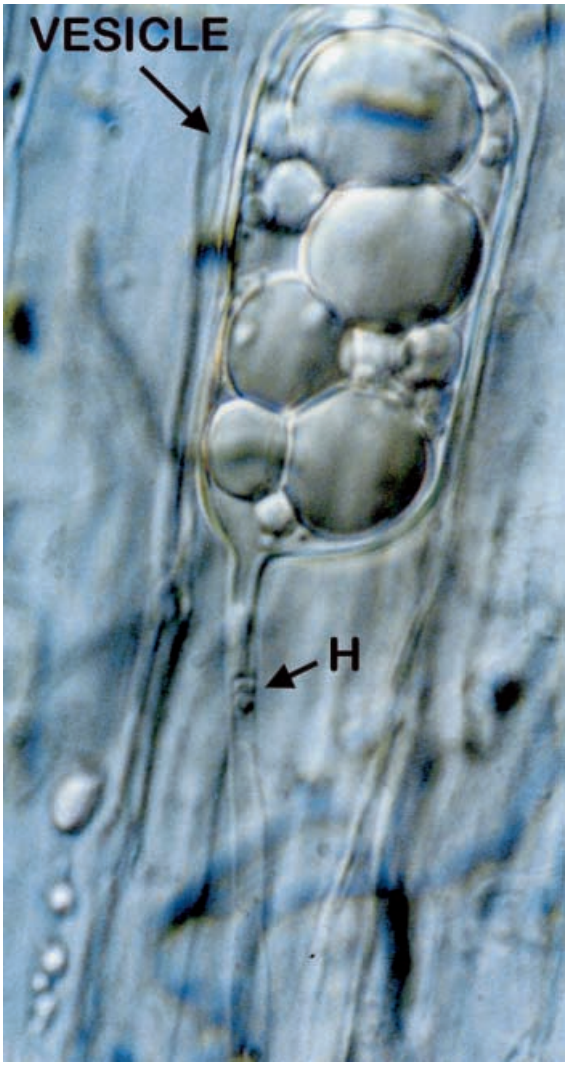

Fig. 7 Hyaline hypha (*H*) and vesicle (*C*) in the cortex of *A. canescens* (Pursh)Nutt. Globular structures failed to stain for lipids with sudan IV after new leaves were formed

lished and before flowering, neither vacuoles nor vesicles stained positively for lipids (Fig. 7). In roots sampled at three different dates from relatively inactive plants in dry soil during the summer, melanized hyphae (0.78 and 1.88 per mm) increased, and microsclerotia were less frequent (0.07 and 0.18 per mm) than in the winter. Hyaline hyphae plus lipids increased (6.18 per mm) dramatically in response to precipitation events, with a corresponding decrease of melanized hyphae, microsclerotia, and hyaline hyphae minus lipids. In the fall, as mean temperatures decreased and seeds matured, mean numbers of hyaline hyphae plus lipids were again increased (6.89 per mm), while melanized hyphae, microsclerotia, and hyaline hyphae minus lipids were near zero.

Discussion

Examination of *A. canescens* roots stained with sudan IV with DIC microscopy revealed extensive internal colonization by hyaline hyphae that was not evident using conventional fungus staining methods. Consistent with the observations of Haselwandter and Read (1982) and Newsham (1999), these hyphae were found to be continuous with melanized hyphae. This method indicates the dynamic and polymorphic nature of DS fungal endophytes and is an improved method for studying their internal interfaces and potential function.

Stained, melanized, and hyaline fungal tissues differ structurally and their variable response to staining can be attributed to the content of chitin and melanin in their wall structure. The characteristic dark color of DS fungi (Jumpponen and Trappe 1998) results from the natural pigment, melanin, and staining by trypan blue and other fungus-specific stains is evidence for chitin. Chitin and melanin are natural components of most fungal walls and directly affect their morphology. These components increase rigidity and reduce permeability of the wall and are thought to protect fungi as they invade harsh environments (Cousin 1996; Henson et al. 1999; Money et

al. 1998). Microsclerotia are most frequent in dormant plants and are considered to be vegetative propagules with thick melanized walls which protect them during stress until conditions are favorable for germination. External hyphae likewise have thick melanized walls which allow them to function in dry soil. Internal non-staining hyaline hyphae, presumed to be protected by the internal environment of the root, lack chitin and melanin. Their prevalence in physiologically active roots and their reduced diameter and visibility suggests thinner and often microscopically invisible walls. Thin hyaline walls are presumed to be more permeable with increased potential for resource exchange with the host and are considered to be active phases of DS fungi. A similar pattern of wall thickness and chitin content has been documented for arbuscular mycorrhizal fungi, in which spores and extraradicle hyphae have thick composite walls with high chitin content. A progressive reduction of wall thickness is found in internal hyphae and coils and is thinnest in the finest arbuscular branches (Smith and Read 1997). The staining of all internal arbuscular mycorrhizal fungal structures indicates that they differ structurally and in chitin composition from non-staining hyaline DS fungi.

The simultaneous colonization of *A. canescens* roots by DS and aseptate fungi (Barrow et al. 1997) allows for some interesting comparisons. Aseptate colonization occurred occasionally at relatively more mesic high-elevation sites and rarely at more xeric sites. During this study, the southwestern USA was experiencing an extended drought and roots were exclusively colonized by DS fungi. The extensive colonization of dominant native plants by DS fungi, particularly those considered to be mycorrhizal, suggests that DS fungi are better adapted to plants in arid ecosystems than aseptate fungi.

DS fungi also differ in the manner in which they interface with plant roots. Arbuscular mycorrhizae interface with cortical cells as thin-walled arbuscules, while DS fungi interface not only with the cortex, but grow into sieve elements (primary plant tissue for carbon transport) as very small diameter, thin-walled hyphae. These hyphae persist in a microscopically invisible form. As they accumulate lipids during conditions in which the host is physiologically active, their lipid-containing vacuoles become visible. At times, these vacuoles were observed in hyphae with either hyaline or melanized walls. There is an increase of visible hyaline hyphae containing lipids in both the vascular cylinder and the cortex when roots are physiologically active. As physiological activity decreases, the visibility of these structures decreases, with a corresponding decrease in vacuole size and lipid content. The accumulation of lipids in fungal vacuoles within sieve elements suggests a potential site for carbon exchange between the host and fungus.

Native plants adapted to arid ecosystems are severely challenged by extreme nutrient and water stress. Presumably, these plants have developed unique mechanisms and symbiotic associations that alleviate stress and enhance nutrition and survival. The abundant and consistent colonization of dominant shrubs and grasses in arid southwestern USA rangelands by DS fungi suggests that they have a significant ecological function. The method reported here reveals substantial, not previously recognized, internal colonization by DS fungi and provides an additional tool for their study. The extraction of substantial quantities of carbon from healthy physiologically active *A. canescens* roots raises important questions as to how these fungi affect the survival of native plants. The implementation of innovative microscopy, histochemicals, labeling, and molecular methods will be necessary to answer these and other pertinent questions relating to these plant-fungal relationships.

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