Research Signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Emerging Concepts in Plant Health Management, 2004: 61-67 ISBN: 81-7736-227-5
Editors: Robert T. Lartey and Anthony J. Caesar

5 A staining method for systemic endophytic fungi in plants

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Abstract

Native desert plants are extensively colonized by dark septate fungal endophytes (DSE). These are characterized by stained or pigmented hyphae and microsclerotia growing inter- and intra-cellularly within the root cortex. A dual staining method was developed using trypan blue that targets fungal chitin and sudan IV that targets internal fungal lipid bodies. Roots and leaves of dominant grasses and shrubs of arid southwestern rangeland were dual stained to determine the nature and extent of colonization by DSE fungi. Fungal structures ranged from lipid bearing protoplasts, hyaline, stained and melanized hyphae and microsclerotia. They inhabit the

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apoplastic spaces of roots and shoots. Predominant fungal structures are protoplasts in physiologically active tissues that are identified with stained associated lipid bodies. This method shows colonization by DSE fungi is more extensive than was previously thought.

Introduction

Of the estimated one million fungal species, one hundred thousand are associated with higher organisms. Many unknown fungal species occupy every living and non-living niche on the earth [1]. Pathogens injure or destroy plant tissues and saprophytes decompose dead or dying tissue, while many fungi reside within living plant tissue. Pathogenic and mycorrhizal fungal colonization is generally localized in plant tissues such as roots, stems or leaves [2]. Some endophytic fungi reside in the intercellular spaces of plant tissues without adverse effects on the welfare of the plant [3].

Fungi are distinguished microscopically from plant tissue because of specific wall components chitin and melanin. Chitin, a primary polysaccharide, directly affects structural stability and permeability of the fungal wall [4]. Melanins are darkly pigmented phenolic or indolic polymers that provide structural strength and protect organisms against environmental stress. They affect hyphopodial turgor, permeability, and wall rigidity [5]. Melanin production is in response to age or exposure to environmental stress from toxic metals, desiccation, hyperosmotic conditions, temperature extremes, antagonistic microbes, limited nutrients, pH shock and UV or ionizing radiation [6].

Conventional methodology for analyzing mycorrhizal colonization of roots has been developed by Bevage [7], Brundrett et al. [8], Kormanik et al. [9] and Phillips and Hayman [10]. These methods involve the clearing of natural root pigments and the use of fungus specific stains that target chitin. Commonly used stains are trypan blue, chlorazol black [8] and acid fushin [11]. Staining of internal fungal structures is highly variable. For example, arbuscules are transient and staining intensity can be affected by plant growth and soil conditions [11, 12]. Hyaline walls lack chitin and do not stain [13].

Native grasses and shrubs in arid rangelands are more extensively colonized by dark septate fungal endophytes [14] than by traditional mycorrhizal fungi. These endophytes are characterized by melanized hyphae and microsclerotia [15] and are readily visible using conventional mycorrhizal staining methods. Hyphae and microsclerotia of DSE fungi are structurally variable and may either stain or be pigmented depending upon environmental conditions.

Barrow and Aaltonen [13] observed high levels of morphological variability of DSE fungi in plant roots. They modified conventional fungus staining methodology where roots and shoots of native plants were stained

with a fungus specific stain, trypan blue and sudan IV. Sudan IV specifically stains lipids and was used as a histochemical to stain internal fungal lipids [16-18]. Roots were examined at a much higher magnification (1000X) using differential interference contrast microscopy than is used in conventional methods. They found that active DSE structures lacked distinguishable walls, in physiologically active roots. In more dormant tissues, structures with hyaline, stained or pigmented walls were more prevalent. Staining with sudan IV revealed copious quantities of lipid bodies of varied shapes and sizes and were mainly associated with fungal and not plant cells. It was determined that conventional methodology reveals only a small fraction of the total colonization and that DSE colonization is systemic and essentially occupies the intercellular spaces of the entire plant. The massive quantity of fungal lipids observed within fungal tissue indicates their potential ecological role of manageing carbon within the host.

Tissue preparation, clearing and staining

Staining methods developed for analysis of mycorrhizal fungi by Bevege [7], Brundrett et al. [8], Kormanik et al. [9], and Phillips and Hayman [10] were modified for optimal visualization of DSE fungi in native grass roots [13, 19]. The method reported here was effective in analyzing roots of fourwing saltbush, *Atriplex canescens* (Pursh) Nutt. and black grama, *Bouteloua eropida* (Torr.) Torr., important shrub and grass species in the northern Chihuahuan Desert. It has also been used effectively on other native grasses in the region. We would expect modifications would be necessary in staining other plant species. For example root clearing is not necessary in all plants.

Root and leaf samples processed for this method were harvested from native populations of *B. eropida* located on the USDA Agricultural Research Service=s Jornada Experimental Range, located in the Northern Chihuahuan Desert in southern New Mexico. Healthy feeder roots of uniform maturity and appearance were washed in tap water to remove soil. Roots approximately 0.25 mm in diameter were randomly selected and cleared by placing in an autoclave in 2.5% KOH. Temperature was increased to121°C over 5 min, maintained for 3 min and after 8 min, samples were removed from the autoclave. Roots were rinsed in tap water, bleached in 10% alkaline H_2O_2 for 10 to 45 minutes to remove pigmentation, and placed in 1% HCL for 3 min. Decolorized roots were rinsed for three minutes in dH_2O before staining with either trypan blue (TB), Sudan IV (SIV) or they were dual stained with TB and SIV.

For staining with TB, roots were placed in prepared TB stain (0.5g trypan blue in 500ml glycerol, 450ml dH_2O and 50ml HCL), autoclaved at 121°C for 3 min and stored in acidic glycerol (500 ml glycerol, 450 ml H_2O

and 50 ml HCL). For SIV staining, roots were placed in prepared SIV (3.0g Sudan IV in 740ml of 95% ETOH plus 240ml dH_2O and filtered) and were autoclaved at 121°C for 3 min and stored in acidic glycerol. For dual staining, roots were stained first in TB and autoclaved as above and destained 24 h in the acidic glycerol. Roots were then transferred to the SIV stain and autoclaved as above. Dual stained roots were destained in dH_2O for three minutes and roots were stored in acidic glycerol until mounting. Both green and dry leaves were similarly processed.

Ten to 12, two cm root or leaf segments were placed on a microscope slide in several drops of permanent mounting medium. Leaf tissues were macerated to obtain single layers of epidermal cells. A cover slip was placed over the root sections and pressed firmly to facilitate analysis at high magnification. Analysis was done with a Zeiss Axiophot microscope using both conventional and DIC optics at 1000x. Grass leaves were similarly prepared and stained.

Colonization of roots and leaves of *B. eropida* by DSE fungi

These staining methods revealed several unique features of DSE fungi that are not observed using conventional fungus staining methodology [13, 19]. Colonization was systemic and formed an intimately integrated interface in the intercellular spaces of most if not all sieve elements, cortical, epidermal, meristematic cells of the roots and the vascular, parenchyma epidermal and stomatal cells of the leaves. Internal DSE fungal morphology was highly variable and often were atypical from commonly recognized fungal structures.

Some of this variability is illustrated. Figure 1 shows melanized hyphae and microsclerotia (arrow ms) characteristic of DSE fungi. Lipid filled fungal protoplasts (arrow ph) were also observed in the root cortex of *B. eropida*. Fungal structures without visible hyaline, stained or pigmented walls are not detected using conventional methods. For the most part, fungal structures are present, but not observed in the sieve elements, except by stained associated lipid bodies. Figure 2 shows dual stained lipid bodies attached to trypan blue stained fungal tissue (arrow ds) and intercellular lipid filled fungal protoplast (ih) within the sieve elements in roots of plants entering dormancy. Lipid bodies (lb) attached to stained fungal tissue (sf) were observed in cortical cells as activity decreased (Figure 3). Figure 4 shows a cluster of meristematic cells in a lateral root with stained fungal tissue (sf) and lipid filled fungal protoplasts (lp) in the apoplastic spaces.

Figure 5 shows stained fungal filaments (arrows) within transparent, non-staining hyphae. These hyphae form a loose network embedded within a mucilaginous gel on the root surface. Figure 6 shows lipid bodies (lb) attached to

stained fungal tissue (sf) within photosynthetic bundle sheath cells of the leaf similar to those observed in cortical cells (Figure 3). In Figure 7, stained fungal tissue was associated, at times, with terminal ends of the guard cells (gc) of the stomata. Lipid bodies within hyaline hyphae and fungal protoplasts (fpp) were also present (Figure 7). Figure 8 shows stained fungal tissue associated with both guard cells (gc) and the subsidiary cells (sc) of the stomatal complex. These illustrations indicate the systemic pattern of DSE colonization.

Characterization of nature and extent of DSE fungi colonization of roots and leaves by dual staining

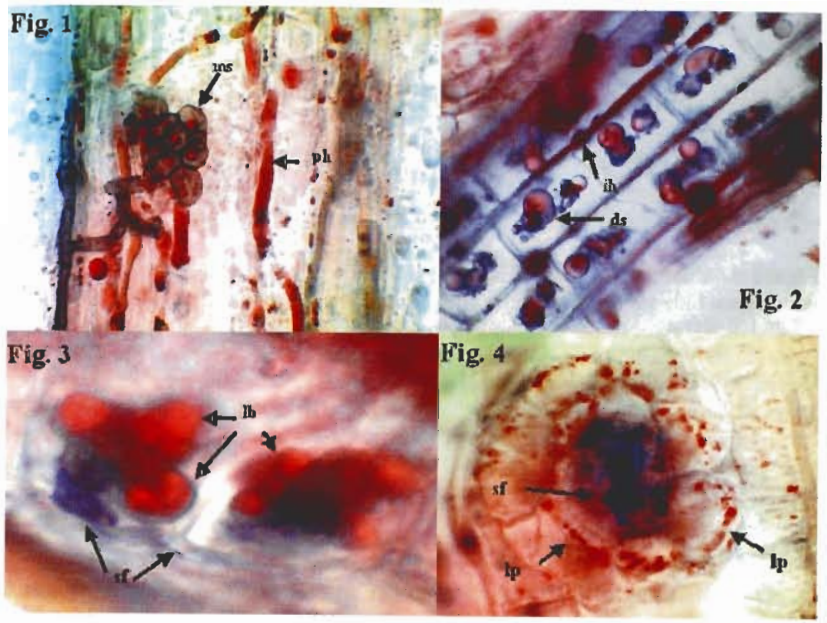

Figure 1. Melanized (pigmented) hyphae and microsclerotia (ms) and sudan IV stained lipid bearing fungal protoplasts on the surface of dual stained *Bouteloua eriopoda* roots. **Figure 2.** Intracellular (SIV stained) lipid bodies attached to trypan blue stained fungal tissue (ds) and intercellular lipid bearing fungal protoplasts associated with sieve elements of the root vascular cylinder of *Bouteloua eriopoda*. **Figure 3.** Intracellular (SIV stained) lipid bodies (lb) attached to trypan blue stained fungal tissue (sf) in cortical root cells of *Bouteloua eriopoda*. **Figure 4.** Cluster of root meritem cells of *Bouteloua eriopoda*, showing trypan blue stained fungal tissue (sf) and intercellular SIV stained lipid bearing protoplasts (lp) in the apoplastic spaces.

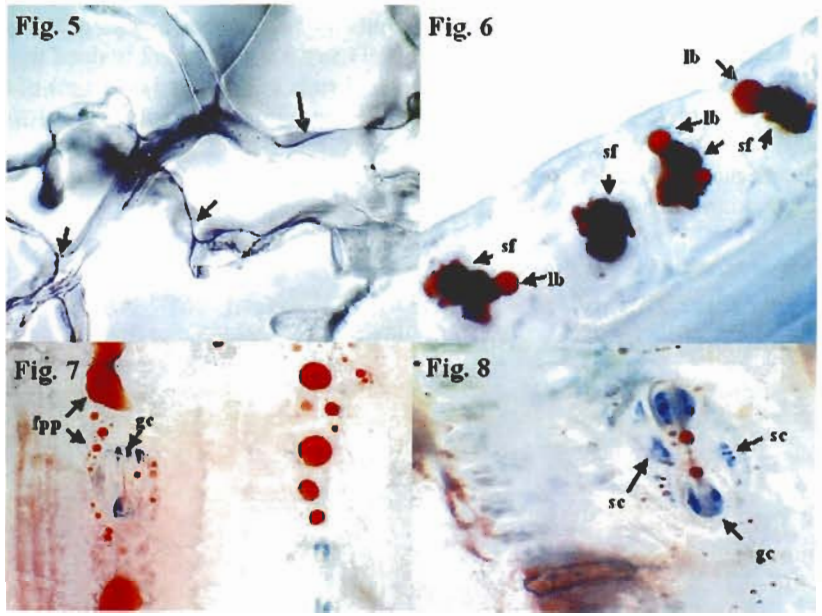

Figure 5. Mucilaginous fungal network on the surface of *Bouteloua eriopoda* roots with trypan blue stained filaments (arrows) within mucilaginous hyphae. **Figure 6.** SIV stained lipid bodies (lb) attached to trypan blue stained fungal tissue (sf) formed intracellularly within photosynthetic bundle sheath cells of *Bouteloua eriopoda* leaves. **Figure 7.** SIV stained fungal protoplasts (fpp) adjacent to the stomata. Terminal ends of guard cells (gc) positively stained with trypan blue of developing *Bouteloua eriopoda* leaves. **Figure 8.** Trypan blue stained fungal tissue associated with terminal ends of guard cell (gc) and subsidiary cells (sc) of the stomatal complex of *Bouteloua eriopoda* leaves.

Summary

Dual staining with trypan blue and sudan IV and analysis at 1000X with differential interference microscopy reveals active fungal structures that escape detection using conventional fungus staining methods. Fungal structures in physiologically active roots are identified primarily by associated lipid bodies and stained or pigmented structures are rare. Stained and pigmented structures, typical of DSE fungi are increasingly evident in more dormant roots. The quantity and size of associated lipid bodies and colonization of photosynthetic cells suggests an ecological role of carbon management. For a better understanding of the full nature and extent of DSE colonization of native plants, new and innovative microscopic and histochemical methods should also

be employed such as confocal laser scanning microscopy, epi-fluorescence and electron microscopy have improved the definition of internal structures of both endo- and ectomycorrhizal colonization compared to conventional methods [11, 20, 21].

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