In Vitro Cell.Dev.Biol.—Plant (2007) 43:65–70
DOI 10.1007/s11627-006-9007-4

PHYSIOLOGY

# Endosymbiotic fungi structurally integrated with leaves reveals a lichenous condition of C4 grasses

**Jerry Barrow · Mary Lucero · Isaac Reyes-Vera · Kris Havstad**

Received: 5 July 2006 / Accepted: 25 October 2006 / Published online: 11 March 2007 / Editor: M. C. Jordan


**Abstract** This paper addresses the assumed autonomy of vascular plants by revealing the presence of an obligate fungus structurally integrated with leaf anatomy of C4 grasses. We examined leaf surfaces of 26 species representing 14 genera of C4 grasses. In all species, we found similarities between leaf surface microhair-like structures and *Uredomycete* teliospores. These bicellular structures produced hyphae and spores, confirming they were fungal, rather than plant tissue. The plant-fungus structural morphology was also observed in *Bouteloua eriopoda* plants regenerated from embryonic meristem cells. The conserved symbiosis between fungi and C4 grasses suggests a lichenous association with evolutionary significance. The structural integration of endosymbiotic fungi with cells and tissues offers novel and unexplored approaches to developing physiological, ecological, and systematic models of C4 grasses.

**Keywords** Plant anatomy · Ecology · Lichen · Regeneration · Symbiosis · Taxonomy

J. Barrow (✉) · M. Lucero · K. Havstad
USDA-ARS, Jornada Experimental Range, MSC, 3JER, New Mexico State University,
P.O. Box 30003, MSC, 3JER, 2995 Knox St.,
Las Cruces, NM 88003, USA
e-mail: jbarrow@nmsu.edu

I. Reyes-Vera
Department of Agronomy and Horticulture, New Mexico State University,
P.O. Box 30003m, MSC, 3JER, 2995 Knox St.,
Las Cruces, NM 88003, USA

## Introduction

Plants are regarded as autonomous organisms and their environmental response was assumed to be based on their individual genetic and physiological processes. However, all plant species are known to host diverse populations of fungi (Berbee, 2001). DNA sequence analysis has revealed the presence of numerous cryptic, plant-associated fungi with unknown functions (Arnold et al., 2000; Vandenkoornhuyse, 2002; Ganley et al., 2004). Presently, symbiosis is considered to be a common and fundamental condition of both plants and animals (Sapp, 2004). Typical symbionts include fungi that contribute multiple benefits to their hosts and profoundly influence enhanced ecological fitness (Clay, 1990; Clay and Schardl, 2002), photosynthetic efficiency (Obledo et al., 2003), and nutrition and growth (Smith and Read, 1997). The classic symbiotic model is found in the lichens, where fungi form symbiotic associations with photosynthetically active algae or cyanobacteria. Lichenous fungi provide shelter, moisture, and nutrients in exchange for photosynthetic carbon (Alexopoulos et al., 1996). Plant–fungal interactions that contribute resistance to stress, herbivory, disease, and temperature extremes are well documented (Clay, 1990; Clay and Schardl, 2002; Redman, 2002; Ruiz-Lozano, 2003). The general array of benefits reported for plants associated with fungal symbionts has led to speculation that fungi are essential for plant survival in stressed environments (Schardl et al., 2004; Lucero et al., 2006).

Fungal endophytes associated with native grasses and shrubs of North American deserts are ubiquitous (Barrow et al., 1997), vertically transmitted and exist as structural components of all major cell and tissue types (Barrow and Aaltonen, 2001; Barrow, 2003). Fungi associated with two native species, *Bouteloua eriopoda* Torr., (black grama grass) and *Atriplex canescens* (Pursh) Nutt., (fourwing saltbush) host plants were

found to persist in *in vitro* regenerated plants (Barrow et al., 2004; Osuna-Avila and Barrow, 2004). Analysis of dual stained leaves of *B. eriopoda* for fungal presence revealed bicellular structures that stained positively for fungal tissue and resembled fruiting bodies of fungi belonging to the *Uredinales*. Taxonomists have regarded these structures as bicellular plant trichomes, and they were used as indicators to distinguish species differences between C4 warm season grasses (*Poacea*) (Ellis, 1971; Johnston and Watson, 1977). Here, we provide light and electron microscopic evidence that these structures, previously thought to be plant cells, are actually fungal cells and that C4 grasses are composite plant fungus organisms.

## Materials and Methods

Leaves of 26 species of C4 grasses were collected from native populations in the northern Chihuahuan Desert, an arid region within the Mexican Plateau of the Basin and Range Province of North America. Bicellular microhair-like structures of these samples were examined at several phenological stages using both light and electron microscopy.

*Tissue staining and light microscopy.* Staining methods modified by Barrow and Aaltonen (2001) and Barrow (2003) were used to detect fungal bodies in plant tissues. Slides were mounted and examined with a Zeiss Axiophot microscope with conventional and differential interference contrast optics at 1,000×. Images were captured with a high-resolution digital camera and processed using Auto-Montage 3-D software by Syncroscopy to give a focused image.

*Electron microscopy.* Fresh leaves and roots were collected in moist plastic bags at ambient temperatures. Samples were analyzed within 1 h by placing samples in the vacuum chamber of a Hitachi S3200N scanning electron microscope under variable pressure mode for analysis of fresh biological samples.

## Results and Discussion

Consistent with taxonomic observations, the shapes and sizes of the observed bicellular structures differed between and were consistent within plant species. However, these same structures in all samples exhibited similarities to teliospores produced by the fungal order *Uredinales* (Alexopoulos et al., 1996). They were observed to be connected to hyphae structurally integrated on the leaf surface, and they stained positively for fungal tissue. They were present in both native and in *in vitro* regenerated plants of *B. eripoda*, one of the 26 species examined.

Electron micrographs of leaves from a native population of *B. eriopoda* (Fig. 1) revealed the anatomical plant–fungus composition of the epidermis. Plant cells, stomata, short and long cells, and sharp pointed trichomes developing from short plant cells are readily recognized within the epidermal layer of the bundle sheath. Superimposed on the epidermis is a precisely organized fungal network that is structurally integrated within the epidermal anatomy. This organized pattern differs from randomly distributed lesions of pathogens and is not evident using light microscopy; however, dual stained epidermal cells show fungal presence (not shown).

Elongated hyphae (*ih*) are distinguished from plant cells because they are significantly longer and without septa, grow between and extend past several long (*lc*) or short (*sc*) plant cells. A tightly joined network composed of three individual hyphae (*hn*, hyphal network) completely covers the surface of each long cell. Branching, typical of fungal hyphae rather than plant cells, regularly occurs within the network covering cells adjacent to the stomata. Examination of leaves by both light and scanning electron microscopy revealed that these surface hyphae are connected to all epidermal cells. In *B. eriopoda* plants regenerated from embryonic meristem cells (Barrow et al., 2004), this organized plant–fungus anatomy was also observed developing on embryonic leaves *in vitro* (Fig. 2). Bicellular fungal structures (*bfs*) were attached to fungal hyphae (*h*) that formed parallel to rows of stomata (*st*) similar to those observed in leaves sampled from native

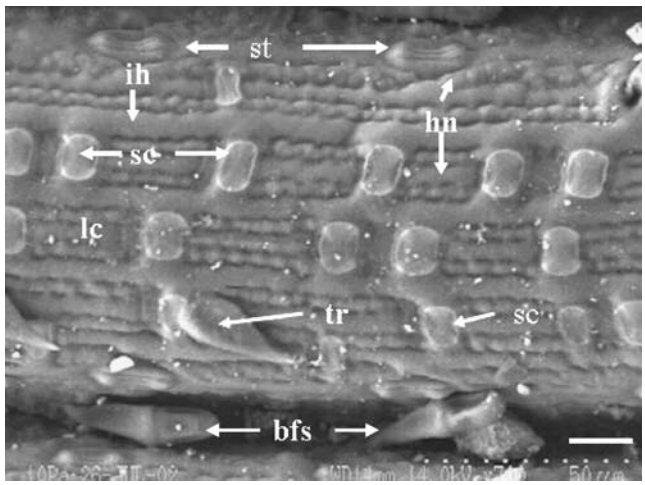


**Figure 1.** A scanning electron micrograph of the abaxial leaf surface of *Bouteloua eriopoda* Torr. sampled from a native population. Plant stomata (*st*), short cells (*sc*), and trichomes (*tr*) are distinguishable in the epidermal layer of the bundle sheath. A precisely organized network of fungal hyphae (*ih*) extends between rows of short (*sc*) and long cells (*lc*), occasionally forming branches. This tightly joined hyphal network (*hn*) completely covers the long cells (*lc*). Bicellular fungal structures (*bfs*) are attached to this fungal network. *Bar*=10 μm.

plants (Fig. 1). In a high magnification scanning electron micrograph (Fig. 3), bicellular fungal structures (*bfs*) with rounded tips, which differ from the sharp, pointed, plant trichomes *(tr*), are attached to hyphae that run parallel to rows of stomata. Bicellular structures (Fig. 4) were strikingly similar to teliospores, characteristic of *Uredomycete* fungi, and were regularly observed with light microscopy in dual stained leaves. They have a transparent hyaline outer wall. The inner terminal and basal cells frequently stained positively with trypan blue specific for fungal tissue (Fig. 4). These cells are also attached to hyphae (*h*), as shown in Fig. 3.

The organized fungal integration with germinating seedlings of both native and regenerated *B. eriopoda* plants suggests that these fungi are vertically transmitted and form a heritable symbiosis with the grass host plant, similar to endophytes of cool season grasses (Schardl et al., 2004). Additional evidence of heritability comes from the similarities in structure of endophytes within a particular grass species, the contrast in shape and size of structures from other species, and the utility of these markers as taxonomic indicators for warm season grasses (Ellis, 1971; Johnston and Watson, 1977).

Dual stained leaves collected from native C4 grass populations entering dormancy revealed that some bicellular fungal structures substantially increased in size from approximately 50 to 90 μm during this time. Some (Fig. 5) formed club-like, melanized fungal structures (*mfs*). Occasionally, fungal hyphae (Fig. 6, *h*) extended from the wall of the melanized club-like structures (*mfs*) and formed other external melanized spores (*ms*). The club-like structure differs in shape from the hyaline bicellular fungal structure (*bfs*). Melanization and hyphal extensions are characteristic of fungal, rather than plant tissue, and further confirm that

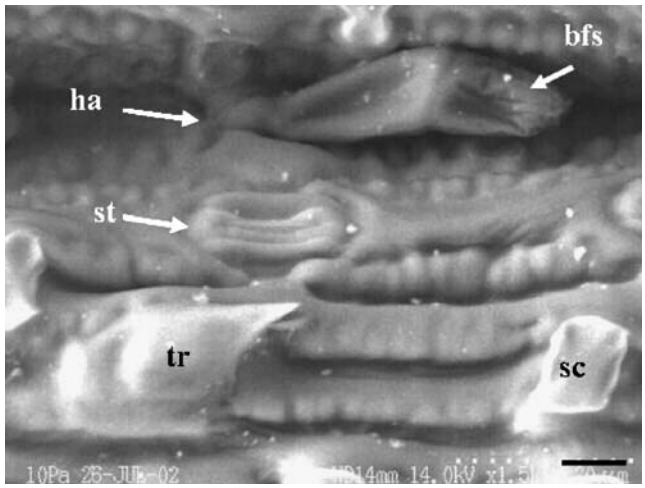


**Figure 3.** A scanning electron micrograph of the abaxial leaf surface of *Bouteloua eriopoda* Torr. sampled from a native population. A bicellular fungal structure (*bfs*) is attached to the hyphae (*ha*). These hyphae lie parallel to rows of stomata (*st*). A pointed, single celled plant trichome (*tr*) is formed from a short cell (*sc*). *Bar*=5 μm.

bicellular microhairs are fungal and not plant cells. Similar club-like structures also developed directly from the wall of the bicellular fungal structure (Fig. 6) and from the same fungal hyphae (*h*) from which the bicellular fungal structures originate (Fig. 5). Single, melanized spores (Fig. 7, *ms*) were also attached to hyphae (*ha*). These subsequently divided to form other melanized, club-like structures containing up to 16 spores (Fig. 8, *mfs*). Trypan blue positively stained the inner wall of the basal cell and the hyaline outer wall of the basal cell indicates fungal tissue.

In C4 grass plants collected in the fall, as they became dormant, all bicellular fungal structures simultaneously decreased in length to approximately 25–30 μm (Fig. 9)

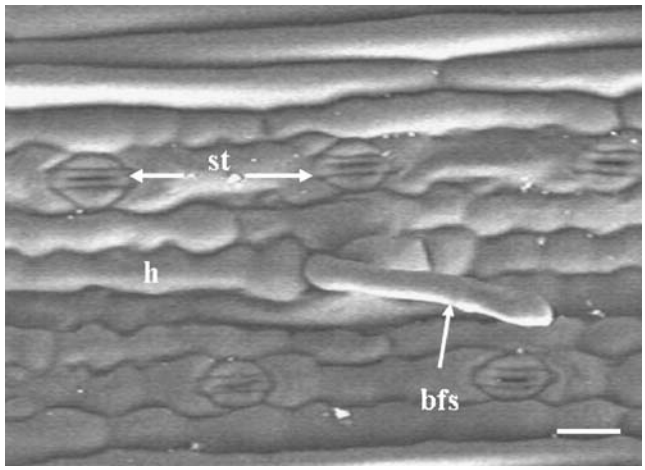


**Figure 2.** A scanning electron micrograph of an embryonic shoot of *Bouteloua eriopoda* Torr. plantlet regenerated from embryonic meristem cells initiated *in vitro* (Barrow et al. 2004). *Rows* of stomata (*st*) are illustrated. A bicellular fungal structure (*bfs*) forms from an integrated fungal hyphae (*h*). *Bar*=10 μm.

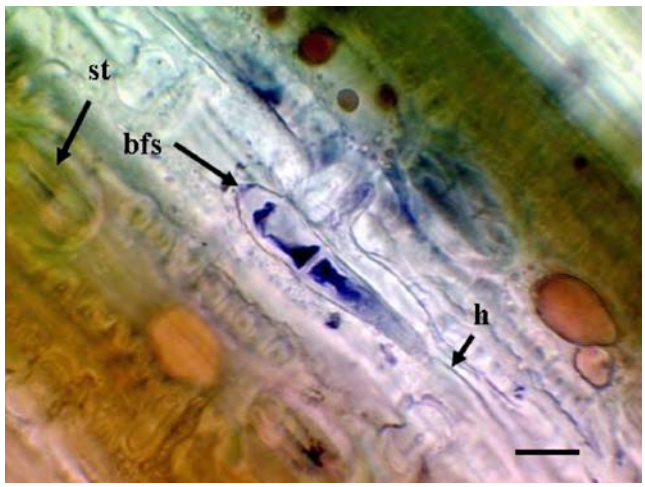


**Figure 4.** A light micrograph of a dual stained *Bouteloua gracilis* (H. B.K.) Lag. leaf sampled from a native population. A bicellular fungal structure (*bfs*) stains positively with trypan blue specific for fungal chitin. It is attached to a fungal hyphae (*h*) that lies parallel to *rows* of stomata (*st*). *Bar*=10 μm.

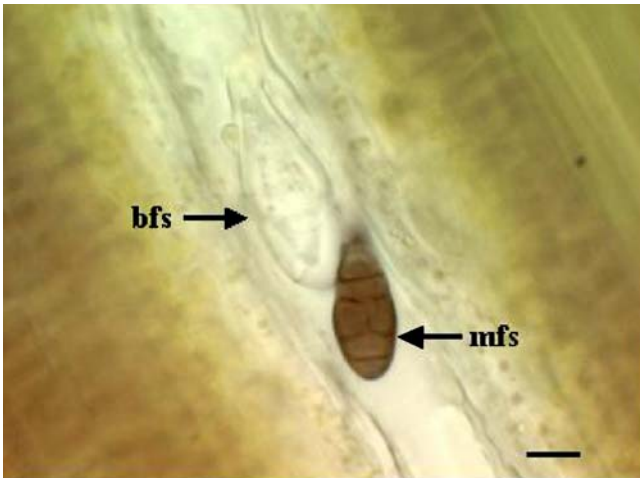


**Figure 5.** A light micrograph of a dual stained *Bouteloua curtipendula* (Michx.) Torr. leaf sampled from a native population. A hyaline (*transparent*) bicellular fungal structure (*bfs*) is illustrated. A club-like melanized fungal structure (*mfs*) filled with compartmented fungal cells is attached to the same fungal hyphae as the bicellular fungal structure (attachment not visible from the photographed view). *Bar*=10 μm.

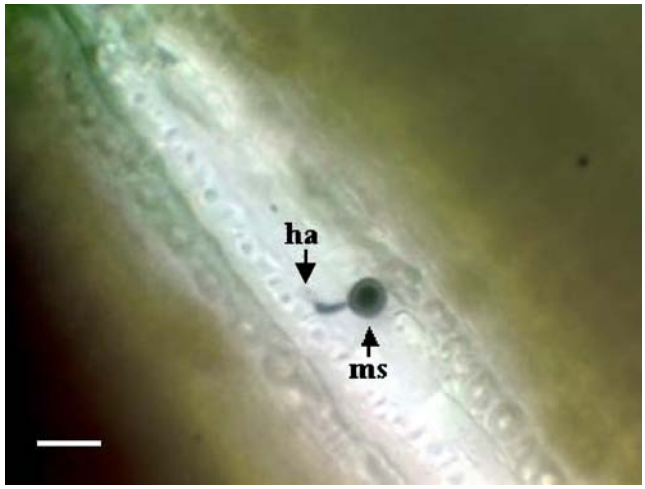


**Figure 7.** A light micrograph of a dual stained *Bouteloua curtipendula* (Michx.) Torr. leaf sampled from a native population. A melanized spore (*ms*) forming from a hyphae (*ha*) attached to the fungal network is shown. *Bar*=10 μm.

and assumed a more spherical shape (Fig. 10). Trypan blue stained sectors (*sth*) were frequently observed on the terminal surface of the bicellular fungal structures. Hyphae extended from these stained sectors (Fig. 11, *hex*). The bicellular fungal structures are interpreted as having an outer hyaline wall and an inner wall that formed on the terminal and basal cells. Hyphae (Fig. 12*h*) that extend from these structures (attachment not shown) likewise have an outer hyaline wall and a stained inner wall. As native plants became fully dormant, mucilage formed on the leaf surfaces, making it impossible to microscopically view any further development of the bicellular structures.

The sexual and life cycle phases, typical of the *Uredomycetes*, were not observed in the C4 grass-associated fungi. The bicellular microhair structures are structurally similar to rust teliospores produced by *Uredomycetes* (order *Uredinales*), yet their differences are important. Rust fungi produce teliospores on telia in randomly distributed pathogenic lesions on the leaves of susceptible host plants. In contrast, the bicellular fungal structures on C4 grasses form a precisely organized protective covering on the leaf surface (Figs. 1, 5, and 9), suggesting a symbiotic relationship. Rust

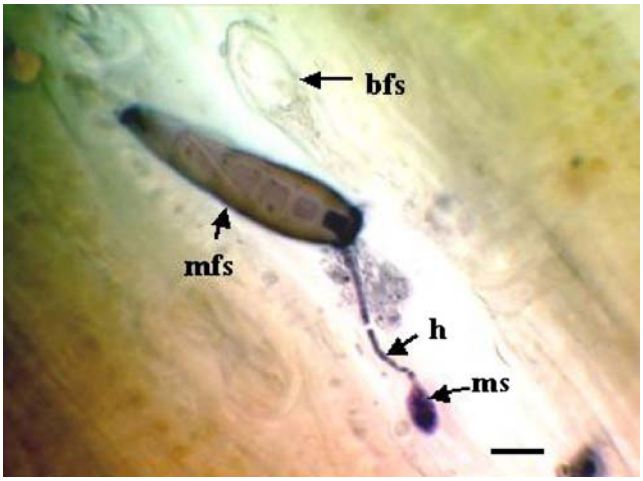


**Figure 6.** A light micrograph of a dual stained *Bouteloua curtipendula* (Michx.) Torr. leaf sampled from a native population. A hyaline bicellular fungal structure (*bfs*) is attached to a fungal hyphae. A melanized fungal structure (*mfs*) shows a trypan blue stained fungal hyphae (*h*) extending from a fungal compartment in the melanized structure. Note a hyaline (*transparent*) outer wall and a stained inner wall that forms a melanized fungal spore (*ms*) on its terminal end. *Bar*=10 μm.

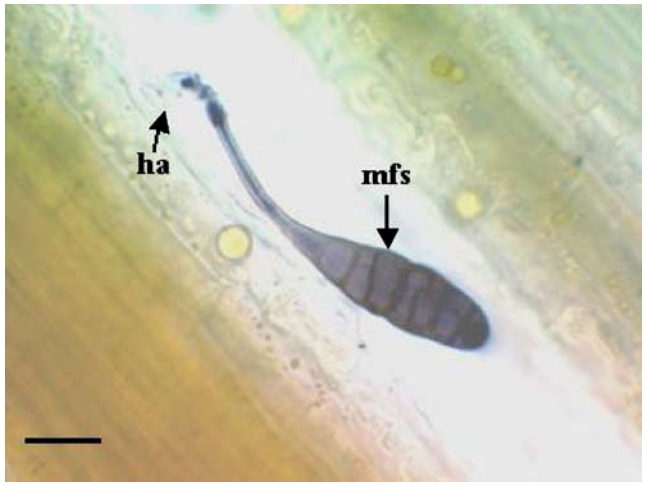


**Figure 8.** A light micrograph of a dual stained *Bouteloua curtipendula* (Michx.) Torr. leaf sampled from a native population. A multicompartmented club like melanized fungal structure (*mfs*) attached to a fungal hyphae (*ha*) shows development of melanized spores. *Note* the basal portion with the hyaline outer wall and the trypan blue stained inner wall. *Bar*=10 μm.

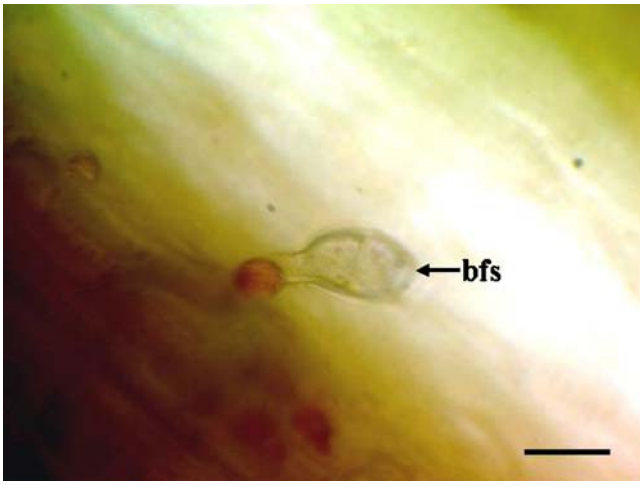


**Figure 9.** A light micrograph of a dual stained *Bouteloua curtipendula* (Michx.) Torr. leaf sampled from a native population as plants entered early dormancy, illustrating a shortened bicellular fungal structure (*bfs*). *Bar*=10 μm.

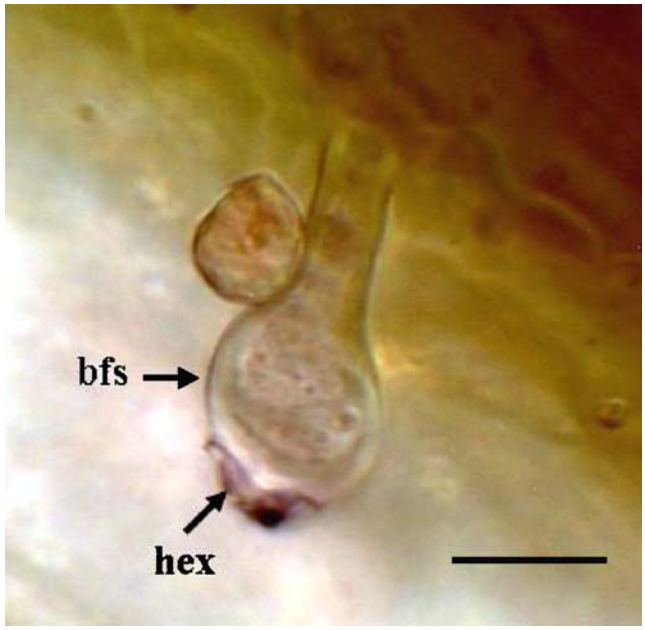


**Figure 11.** A light micrograph of a dual stained *Bouteloua curtipendula* (Michx.) Torr. leaf sampled from a native population as plants entered dormancy. The bicellular fungal structure (*bfs*) has a thickened wall. A hyphal extension (*hex*) with a hyaline outer wall and a trypan blue stained inner wall is emerging from the surface of the hyaline wall of the terminal cell. *Bar*=10 μm.

fungi comprise approximately 7,000 species, all of which are pathogens (Alexopoulos et al., 1996). These teliospore-producing fungi are structural symbionts with C4 grasses and are suspected to be a novel unreported class of *Uredomycetes*.

We propose that C4 grasses exist as symbiotic plant–fungal communities similar to lichens. The functional–anatomical roles served by the grass-associated fungi remain largely unexplored, but we hypothesize that, as in lichens, the fungi have considerable influence regulating basic plant physiological responses to biotic and abiotic stress. They may also influence photosynthetic pathways and heavy metal tolerance as described in other plant systems (Clay, 1990; Obledo et al., 2003; Ruiz-Lozano, 2003; Schardl et al., 2004). Similar structures identified as bladder cells on *A. canescens* are perceived to enhance salt tolerance and are also believed to be fungal components of the leaves (Barrow et al., 2004).

The probability that eukaryotic genes isolated from C4 grass tissues could be derived from fungal cells is high, and control measures designed to identify non-plant genes

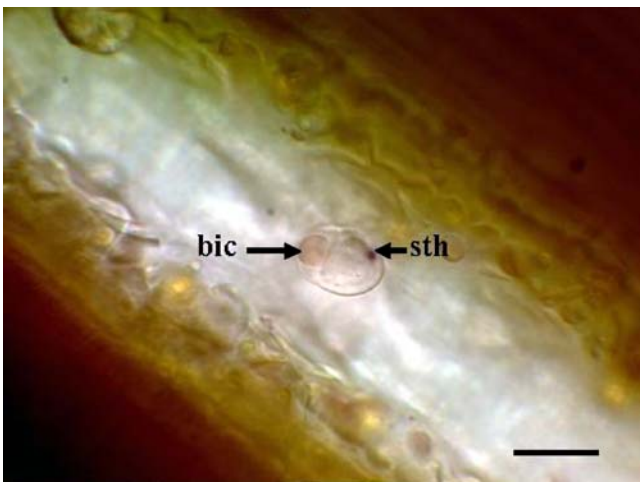


**Figure 10.** A light micrograph of a dual stained *Bouteloua curtipendula* (Michx.) Torr. leaf sampled from a nearly dormant native population. A *red*, sudan IV stained lipid body is visible in the basal cell of a bicellular fungal structure (*bic*). The attachment to the hyphal network is not visible. A typan blue stained hyphal bud (*sth*) is visible on the hyaline wall of the terminal end. *Bar*=10 μm.

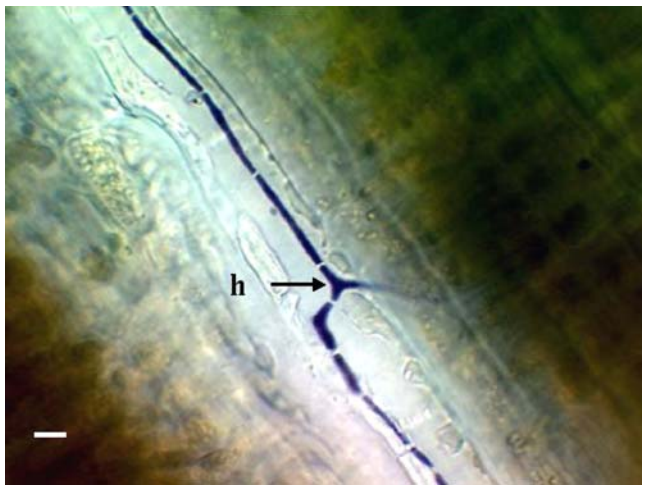


**Figure 12.** A light micrograph of a dual stained *Bouteloua curtipendula* (Michx.) Torr. leaf sampled from a native population as plants entered dormancy. Branched hyphae (*h*) with a hyaline outer wall and a Trypan blue stained inner wall typical of hyphae extending from bicellular fungal structures. Attachment not shown. *Bar*=5 μm.

derived from plant tissues are inadequate. Such controls rely on sequence and codon preference comparisons to previously described and typically culturable microbes. The genetics and taxonomy of cryptic fungal symbionts are largely unknown. Although knowing that all plants harbor symbiotic microbes and even suggested that they behave as lichens (Atsatt, 1988), this concept was slow to penetrate mainstream plant biology. Nevertheless, increasingly sophisticated methods for detection and characterization of plant symbionts will reveal additional novel plant–fungal interactions. Consistent with the paradigm that symbiosis is a universal condition of plants (Sapp, 2004), we promote the concept that vascular plants harbor populations of symbiotic microbes and have evolved as high order lichens.

We conclude that bicellular microhair-like structures of warm season grasses are fungal cells in a highly conserved, symbiotic association with C4 grasses. We propose that the endosymbiotic fungi were structural components of grasses from their evolutionary origin. Consistent with observations of Ganely et al. (2004), we propose that these are obligately associated with plant cells and tissues and are likely related to, but differ from, free living pathogens and saprophytes. This lichenous nature contradicts preconceived notions that these graminoids are autonomous plants. This novel concept transforms current models of C4 grass physiology and ecology by offering unlimited mechanisms through which fungal interactions may impact host plant growth, development, evolution, and gene transfer. Failure to recognize the presence of endophytic microbes throughout host plant tissues limits the ability of plant scientists to accurately describe and manipulate host anatomy, taxonomy, and physiology.

## References


Alexopoulos, C. J.; Mims, C. W.; Blackwell, M. Introd. Mycol. (John Wiley & Sons, New York. Chapter 20, 26. [Fourth Edition]; 1996.

Arnold, A., Maynard, Z.; Gilbert, G. S.; Coley, P. D.; Kursar, T. A. Are tropical fungal endophytes hyperdiverse? Ecol. Lett. 3:267–274; 2000.

Atsatt, P. R. Are vascular plants “inside-out lichens”? Ecol. 64:17–23; 1988.

Barrow, J. R. Atypical morphology of dark septate fungal root endophytes of *Bouteloua* in southwestern USA rangelands. Mycorrhiza 13:239–247; 2003.

Barrow, J. R.; Aaltonen, R. E. A method of evaluating internal colonization of *Atriplex canescens* (Pursh) Nutt. roots by dark septate fungi and how they are influenced by host physiological activity. Mycorrhiza 11:199–205; 2001.

Barrow, J. R.; Havstad, K. M,; McCaslin, B. D. Fungal root endophytes in fourwing saltbush, *Atriplex canescens*, on arid rangelands of southwestern USA. Arid Soil Res. Rehabil. 11:177–185; 1997.

Barrow, J. R.; Osuna-Avila, P.; Reyes-Vera, I. Fungal endophytes intrinsically associated with micropropagated plants regenerated from native *Bouteloua eriopoda* Torr. and *Atriplex canescens* (Pursh) Nutt. In Vitro Cell Dev. Biol. Plant 40:608–612; 2004.

Berbee, M. L. The phylogeny of plant and animal pathogens in the Ascomycota. Physiol. Mol. Plant Path. 59:165–187; 2001.

Clay, K. Fungal endophytes of grasses. Ann. Rev. Ecolog. Syst. 21:275–297; 1990.

Clay, K.; Schardl, C. Evolutionary origins of ecological consequences of endophyte symbiosis with grasses. Am. Nat. 160:s99–s127; 2002.

Ellis, R. P. A procedure for standardizing comparative leaf anatomy in the *Poaceae*. II. The epidermis as seen in surface view. Bothalia 12:641–671; 1971.

Ganley, R. J.; Brunsfeld, S. J., Newcombe G. A community of unknown, endophytic fungi in western white pine. Proc. Natl. Acad. Sci. USA 101 (27):10107–10112; 2004.

Johnston, C. R.; Watson, L. Microhairs: a universal characteristic of non-festucoid genera? Phytomorphology. 26:297–301; 1977.

Lucero, M. E.; Barrow, J. R.; Osuna, P.; Reyes I. Plant–fungal interactions in arid and semi-arid ecosystems: large-scale impacts from microscale processes. J. Arid Environ. 65:608–612; 2006.

Obledo, E. N.; Barragan-Barragan, L. B.; Gutierrez-Gonzalez, P.; Ramirez-Hernandez, B. C.; Rameriz, J. J.; Rodriguez-Garay B. Increased photosynthetic efficiency generated by fungal symbiosis in *Agave victoria-reginae*. Plant Cell Tissue Organ Cult. 74:237–241; 2003.

Osuna-Avila P.; Barrow, J. R. Regeneration of black grama (*Bouteloua eriopoda* Torr.) via somatic embryogenesis. In Vitro Cell Dev. Biol. Plant 40: (3):303–310; 2004.

Redman, R. S.; Sheehan, K. B.; Stout, R. G.; Rodriguez, R. J.; Henson, J. M. Thermotolerance generated by plant/fungal symbiosis. Sci. 298:1581; 2002.

Ruiz-Lozano, J. M. Arbuscular mycorrhizal symbiosis and alleviation of osmotic stress. New perspectives for molecular studies. Mycorrhizae 13:309–317; 2003.

Sapp, J. The dynamics of symbiosis: an historical overview. Can. J. Bot. 82:1046–1056; 2004.

Schardl, C. L.; Leuchtmann, A.; Spiering, M. J. Symbioses of grasses with seedborne fungal endophytes. Annu. Rev. Plant Biol. 55:315–340; 2004.

Smith, S. E.; Read, D. J. *Mycorrhizal Symbiosis*. Ed 2, Academic Press, San Diego, London 59–60; 1997.

Vandenkoornhuyse, P., Bauldauf, S. L., Leyval, C., Straczek, J., Young, J. P. W. Extensive fungal diversity in plant roots. Sci. 295:2051; 2002.