

Fungal Genomes that Influence Basic Physiological Processes of Black Grama and Fourwing Saltbush in Arid Southwestern Rangelands

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Abstract: Symbiotic fungi confer multiple benefits such as enhanced photosynthetic rates and drought tolerance in host plants. Shrubs and grasses of southwestern deserts are colonized by symbiotic fungi that cannot be removed by conventional sterilization methods. These fungi were extensively studied in *Bouteloua eriopoda* (Torr.) Torr. and *Atriplex canescens* (Pursh) Nutt. over a wide range of locations and environmental conditions. Fungi were intrinsically integrated with cells, tissues, and regenerated plant cultures. These composite plant-fungus organisms are comprised of more than one fungal species. Fungal association with photosynthetic cells and accumulation of lipids provide evidence for carbon management. Fungal biofilms that coat cells, tissues, roots, and leaves suggests protection of plants from direct exposure to stressed environments. Associations with vascular tissues suggests a role in resource transport. Association with stomata indicates an influence in gas exchange, photosynthesis, and evapo-transpiration. Transfer of fungal endophytes from native plants to non-host plants resulted in substantial modifications in root, shoot morphology and biomass, chlorophyll content, and fruiting. Host plants are modified by fungi at the genetic, cellular, and physiological level and positively enhance ecological fitness.

Introduction

Fungi are unable to synthesize organic carbon, therefore they either assimilate exogenous organic carbon as saprophytes or they form associations with photosynthetic organisms. Lichens provide the simplest symbiotic model where fungi form associations with photosynthetic microorganisms. Evidence supporting similar symbioses between fungi and higher plants is increasingly overwhelming. Fungi reside in healthy tissues of every native plant examined to date and range from destructive pathogens to mutualists that profoundly affect ecological fitness (Arnold and others 2000). Some endophytic fungi occupy the apoplast of leaves, stems, and reproductive organs of many grasses (Clay 1990; Schulz and others 2002). Mycorrhizal fungi colonize roots of most plant species and have significant, well documented ecological roles in the nutrition and survival of host plants in natural ecosystems (Smith and Read 1997). Apparent saprophytic fungi also colonize roots with symptomless endophytic or biotrophic phases in their life cycles that are not obvious to casual observers (Parbery 1996). Most fungal associations are localized, while a few inhabit the entire apoplast and are vertically transmitted by seed (Clay and Schardl 2002). Not surprisingly, fungal endophytes confer multiple benefits to their host, such as enhanced tolerance to disease, drought, herbivory, and heavy metals and enhanced nutrient uptake and photosynthetic efficiency (Arnold 2003; Clay 1990; Ruiz-Lozano 2003; Obledo and others 2003).

Recent attention has been given to a diverse group of ascomycetous fungi designated as dark septate endophytes (DSE). These fungi have been detected microscopically by their inter- and intracellular, darkly pigmented (melanized) septate hyphae and microsclerotia in the root cortex (Barrow and Aaltonen, 2001; Jumpponen 2001). They are found extensively in cold, nutrient-stressed environments where AM fungi do not proliferate (Kohn and Stasovski 1990). A higher incidence of DSE than AM fungi was found in *Carex* sp. in subarctic alpine regions (Haselwandter and Read 1982; Ruotsalainen and others 2002). Non-staining hyaline hyphal extensions from stained or melanized structures were reported by Barrow and Aaltonen (2001), Haselwandter and Read (1982), Newsham (1999), and Yu and others (2001) revealing morphological variability. DSE are the primary fungal associates of dominant native grasses and shrubs in the northern Chihuahuan Desert (Barrow and others 1997).

Barrow and Aaltonen (2001) and Barrow (2003) evaluated *Bouteloua eriopoda* Torr. (BOER) and *Atriplex canescens* (Pursh) Nutt. (ATCA), important forage shrub and grass species of the northern Chihuahuan Desert. Histochemical staining revealed greater colonization of atypical forms of DSE than previously observed using conventional methods. Osuna-Avila and Barrow (2004) regenerated plants from embryonic shoot meristem cells of naturally produced BOER seed and found fungal endophytes to be intrinsically integrated in regenerated plants (Barrow and others 2004).

In this study, naturally colonized roots and leaves of native *Bouteloua eriopoda* Torr. and *Atriplex canescens* (Pursh) Nutt.

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plants are compared with cell cultures, germinating seedlings, and micro-propagated plants of both species using scanning electron microscopy (SEM) and light microscopy with dual-staining methodology. Endophytic fungal endophytes were transferred from BOER and ATCA to non-host plants.

Materials and Methods

Tissue Samples

Root and leaf samples of native and micro-propagated plants and germinating seedlings of BOER and ATCA were analyzed in this study. Roots were sampled every two weeks, from 2001 to the present from native populations of ATCA and BOER on the USDA Agricultural Research Service's Jornada Experimental Range in southern New Mexico. Soil moisture in the chronically dry collection sites are generally less than 3 percent. Significant precipitation events are intense, localized rain showers during the summer monsoons that saturate the soil for only brief periods. Sampling times included a wide range of seasonal, physiological, and climatic conditions. Uniform expression of fungal structures was consistent within the population at each sampling period. Leaves from native plants were sampled over all seasons and included different physiological stages ranging from actively growing to drought induced dormant plants.

Tissue Preparation and Staining

Staining and mounting methods were modified by Barrow and Aaltonen (2001) and Barrow (2003) for optimal fungal expression. Tissues were dual stained with trypan blue (TB), which targets fungal chitin, and sudan IV (SIV), which targets lipid bodies found previously to stain lipids attached to fungal structures (Barrow and Aaltonen 2001; Barrow 2003). Samples were analyzed with a Zeiss Axiophot microscope with both conventional and differential interference contrast optics at 1000x. Images were captured with a high resolution digital camera and processed using auto-montage 3-D software by Syncroscopy™ to give a focused image.

Tissue Preparation by Liquid Nitrogen

Roots and leaves from native and propagated plants and germinated seedlings were also placed in liquid nitrogen and dried at 100°C. Tissues were then ground with a mortar and pestle and suspended in a centrifuge vial in 10 ml of 2.5 percent KOH and micro-waved for 1.5 min prior to centrifugation at 3000 rpm until a stable pellet formed. The supernatant was removed by pipette and the pellet was re-suspended in 10 ml dH₂O and centrifuged again. The pellet was re-suspended in 10 ml HCL and micro-waved for 1.5 min. The vial was filled with dH₂O and the cell pellet was harvested again by centrifugation. Next, the cell pellets were suspended in 5ml trypan blue and 5 ml sudan IV and micro-waved for 2 min. The vial was rinsed with dH₂O and centrifuged, and the supernatant was again removed by pipette.

Stained ground material was suspended in a drop of mounting medium on a slide and sealed with a cover slip and microscopically examined. This procedure resulted in tissues fractured in all possible planes permitting analysis of single layers of cells at 1000x magnification. Power settings, time, and sample size must be adjusted to prevent over heating in the microwave.

Scanning Electron Microscopy

Fresh leaves and roots of native and propagated plants were sampled from the field and from micro-propagated plants and placed in moist plastic bags and analyzed within 1h by placing them in a vacuum chamber of a biological scanning electron microscope.

Micro-Propagated BOER Plants

Seeds were manually harvested from native BOER plants; caryopses were separated from florets and surface disinfested and germinated. Embryonic shoots were excised from roots and plated on an auxin supplemented medium to induce callus from meristematic shoot cells. Plants were regenerated via somatic embryogenesis from callus cells after transferring to an auxin-free medium (Osuna and Barrow 2004).

Micro-Propagated ATCA Plants

Seeds of four-wing saltbush, *Atriplex canescens*, were surface disinfested by soaking them in 95 percent ethanol for 1 minute, followed by 2.6 percent sodium hypochlorite (50 percent dilution of commercial liquid bleach) for 7 minutes. Seeds were rinsed three times in sterile distilled water then were placed on hormone-free, modified White's media (White 1934) for germination. Germinated seedlings of *Atriplex canescens* were used to initiate shoot cultures by transferring them to shoot proliferation media consisting of standard Murashige and Skoog (MS) basal salts (Murashige and Skoog 1962) supplemented with 11.42 μ M Indole-3-Acetic Acid (IAA) and 18.58 μ M 6-Furfurylaminopurine (Kinetin). Vitamins were supplemented according to the L2 formulation of Phillips and Collins (1979), 30 g l⁻¹ sucrose, and solidified with 0.8 percent agar. The pH of the medium was adjusted to 5.8 $\pm$ 0.05 prior to autoclaving at 121°C at 125 kPa for 35 minutes. Cultures were grown in 100 x 25 mm polystyrene petri dishes and sealed with Parafilm®. They were subcultured to fresh media every 4 weeks. This protocol represents the standard control media.

Shoots were originally vitrified and were reverted to normal by transferring to shoot proliferation media with the following modifications. Ammonium-free MS (NH₄NO₃ excluded from the original major salts composition) was supplemented with 4.40 g/L of casein hydrolysate to provide an amount of total nitrogen content comparable with the standard MS formulation. Standard MS basal salts served as the control. Vitamin supplements were added according to the L2 formulation by Phillips and Collins (1979) plus 30 g l⁻¹ sucrose. All experimental

media treatments were solidified with 5.0 g/L of Agargel® (Sigma Chemical Co., St Louis MO, USA), a blend of agar and phytagel documented to control vitrification (Pasqualetto and others, 1986). Growth regulator composition consisted of 24.61 μM 6-(γ - γ -Dimethylallylamino) purine (2iP). The pH of the medium was adjusted to 5.8 ± 0.05 prior to autoclaving at 121°C at 125 kPa for 35 minutes and dispensed in polycarbonate Magenta® GA7 vessels (Magenta Corp., Chicago, IL., USA). Culture boxes were closed with either standard Magenta® GA7 vessel covers or vented lids with a 10 mm polypropylene membrane (0.22 μm pore size) Magenta® (Magenta Corp. Chicago, USA). All cultures were incubated at $28 \pm 1^\circ\text{C}$ under continuous fluorescent light (14–18 $\mu\text{mol s}^{-1}$). Normal shoots were removed from culture boxes and subcultured on the reversion medium.

Normal shoots of three nodes or longer were rooted by culturing on White's media culture (White, 1934) supplemented with 2.46 μM of indolebutyric acid (IBA), 30 g/l of sucrose, and solidified with 2.5 g/L of Phytigel® (Sigma Chemical Co., St Louis MO., USA). Pure phytigel facilitated easy visual evaluation of root initials. The pH was adjusted to 5.5 ± 0.05 prior to autoclaving at 121°C at 125 kPa for 35 min and dispensed in 107 x 107 x 96 mm high LifeGuard® polycarbonate culture boxes closed with vented lids with an opening of 22 mm and a 0.3 μm pore size (Osmotek Ltd, Israel).

Fungal Transfer

Fungi were transferred from BOER and ATCA by placing germinating disinfested tomato seed in contact with callus cultures of both species. Tomato plants with fungi were compared with control plants without fungi under greenhouse conditions.

Ribosomal DNA Analysis

Polymerase chain reactions (PCRs) targeting ribosomal DNA (rDNA) and internal spacer regions (ITS) developed for identification of fungi (White, 1990) were applied to tomato DNA isolated from leaves of control, BOER- and ATCA-inoculated plants. Electrophoresis of PCR products was used to identify products that differed between treatment groups.

Some of the fungal endophytes present in BOER and ATCA inoculated plants have been previously characterized by sequence analysis of cloned PCR products and/or by isolation and morphological identification (Barrow and others 2004; Lucero and others 2004). The ATCA callus used to inoculate tomato was known to contain *Aspegillus ustus*, *Pennicillium olsoni*, and *Bipolaris spicifera*. The BOER callus was also associated with *A. ustus*. In addition, BOER callus contained *Engyodontium album* and an uncharacterized, teliospore-producing fungus (Lucero and others 2004). Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Results and Discussion

Symbiotic fungal presence was verified in plant tissues by light and electron microscopy and DNA sequence analysis. Fungi were found to be intrinsically integrated with all cells tissues and organs of native and aseptically regenerated BOER and ATCA plants. Significant associations with specific cell types and potential functions are presented here. A key observation was the consistent association of fungal tissue, as indicated by positive staining with TB (fig. 1, sf), with all root meristem and daughter cells. The transmission of fungi to daughter cells explains how fungi become associated with all host plant cells. Such an association has significant implications regarding how the host plant may perform at the genetic, physiological, and ecological levels. Internal fungal structures were morphologically variable; many differed from classically recognized structures and escape detection using conventional analytical methods.

Fungi were previously found to be non-destructively associated with xylem and sieve elements of the vascular cylinder, cortical, and epidermis of native BOER and ATCA roots with external hyphal extensions into the soil (Barrow and Aaltonen 2001; Barrow 2003). Such associations suggest the potential enhancement of resource transport in the vascular cylinder by fungal symbionts. Historically, axenically cultured plants have been considered microbe free (Borkowska 2002). However, tissue cultured BOER and ATCA plants were found to have intrinsically associated fungi that were found to be continuous from embryonic shoot meristem cells, to callus and to regenerated BOER plants (Barrow and others 2004). SEM analysis of callus tissue induced from embryonic BOER cells (fig. 2) reveals hyphae (h) enmeshed within the biofilm (bf) that completely encapsulates the axenically cultured callus tissue. This is consistent with observations of Pirttila and others (2002), who also found fungal biofilms on the surface of *Pinus* tissue cultures. Embryos were induced within the callus (Barrow and others 2004). SEM analysis of embryonic roots of developing plantlets (fig. 3) shows a tightly woven hyphal mass (hn) enmeshed within a biofilm (bf). Symbiotic fungal endophytes are shown here to totally encapsulate cells, callus, and roots and prevent their direct exposure to the external environment. Similar fungal layers have been observed to completely cover all root and leaf surfaces of native plants in the field.

Native plants in the northern Chihuahuan are chronically exposed to extreme nutrient and soil water deficits (soil moisture < 3 percent) for long periods, as well as high temperatures, dry atmospheric conditions, and high light intensity. Fungi, as well as other microbes, synthesize exo-polysaccharides that attach to their cell surfaces (Sutherland 1998). Biofilms constitute a protective mode for the microbe (Costerton and others 1999). They have unique characteristics that may be valuable contributions to host plants in an arid ecosystem. They hydrate rapidly on contact with water and assist in the attachment to substrates or to host plants. Biofilms protect against invasions from pathogens and changes in the physico-chemical environment and allow microbes to survive in hostile

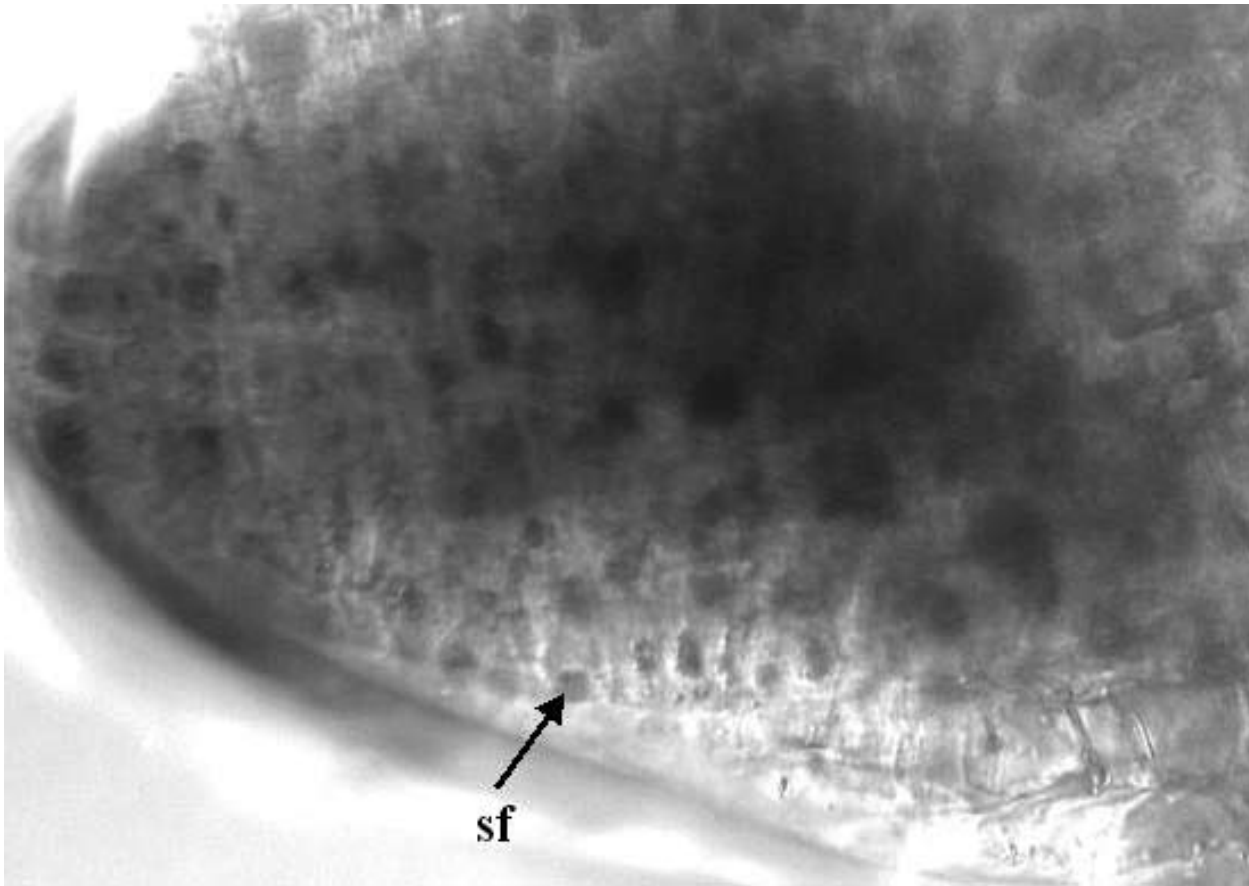


Figure 1. Meristematic cells of a lateral root initial of *Bouteloua eriopoda* seedling. Trypan blue stained fungus associated with all cells (sf).

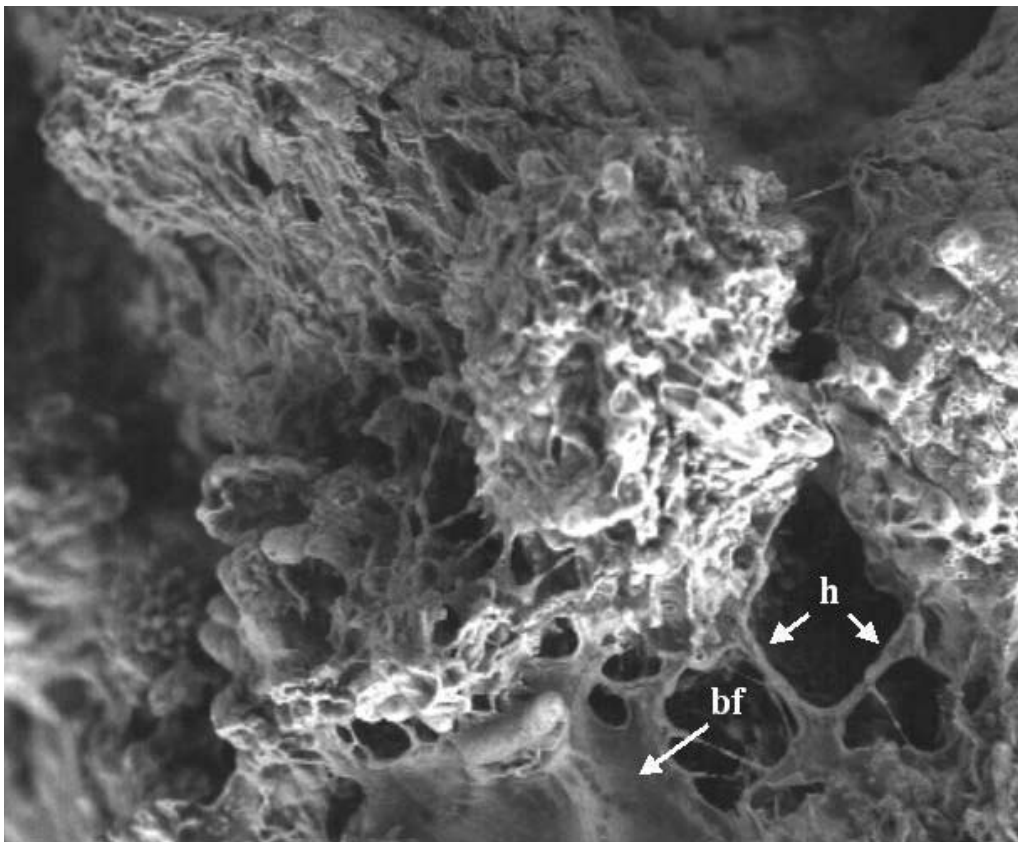


Figure 2. An SEM image of *Bouteloua eriopoda* callus tissue encapsulated with fungal hyphae (h) enmeshed within a biofilm (bf).

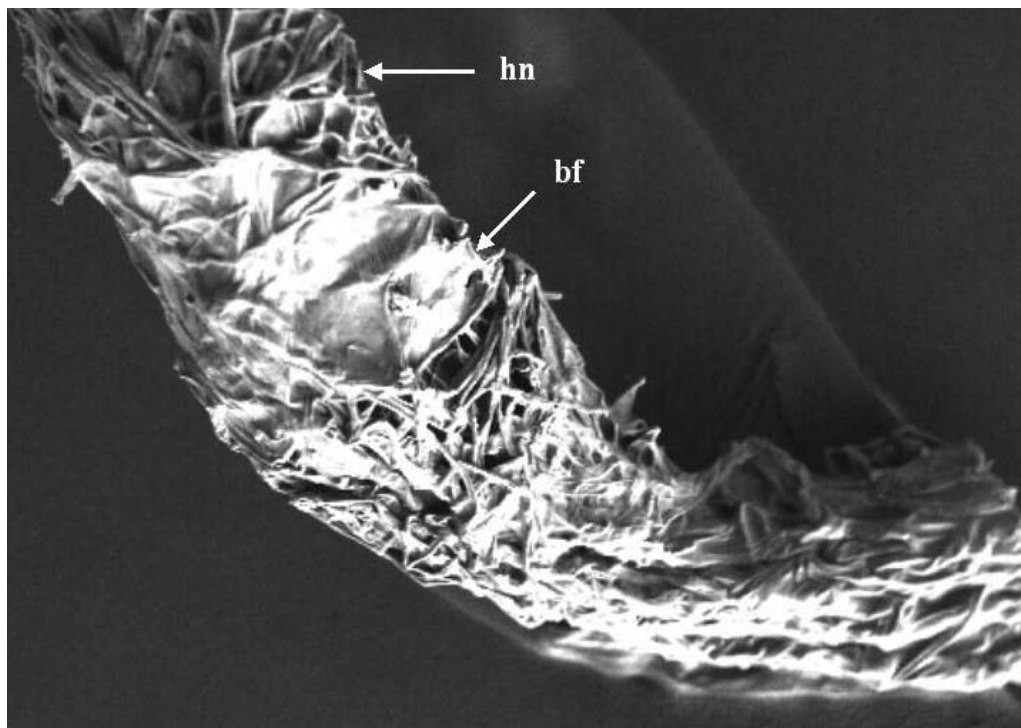


Figure 3. An SEM image of an embryonic root developing from a regenerated *Bouteloua eriopoda* plantlet that is encapsulated with a dense hyphal network (hn) and fungal biofilm (bf).

environments (Costerton and others 1999; Krembs and others 2002; Wotton 2004). Consistent with findings of Deckert and others (2001), we also observed that external hyphae on root and leaf surfaces that were embedded within a mucilaginous (polysaccharide) matrix. The fungus with a protective biofilm, makes fungi an attractive cohort for native plants that enables them to function under extreme stress. This barrier would be expected to confer multiple benefits to host plants, protecting plant surfaces from dry soil and atmospheric conditions, temperature extremes, intense light, salinity, absorption of heavy metals, or infection by pathogens.

Also significant was the association of symbiotic fungi with photosynthetic cells and the stomatal complex. Fungal associations with all photosynthetic cells of native and regenerated plants of BOER and ATCA were observed. Figure 4 shows stained fungal structures (sf) associated with photosynthetic bundle sheath cells (bs) and mesophyll cells (mp) of BOER. Note that the fungi assume the shape of the respective cells. The interpretation of extensive observations is that fungal tissue adheres to the outer plasmalemma of most cells and is transmitted to new cells at division. Positively stained fungal structures (fig. 5, sf) were also observed associated with bundle sheath cells of native and regenerated ATCA plants. The xylem vessels (st) also stained positively for fungal tissue. Fungi bind with and assume the shape of the lignified xylem vessels. Fungal association with photosynthetic cells suggests a potential site where these biotrophic endophytes may access carbon from the host plant. The quantity of exo-polysaccharides produced by these fungi for protective biofilms suggests considerable carbon expenditure for protection of the hybrid plant-fungus organism. Associations with xylem vessels suggest a role of active water transport by fungi and enhancement of drought tolerance.

Fungal association with cells of the stomatal complex with both native and regenerated plants further confirmed their distribution to all cells and tissues and their existence as a composite plant-fungus symbiota. Figure 6 shows stained fungal tissue (stf) associated with guard cells of stomata (st) in a regenerated ATCA plant. Similar fungal staining was consistently observed in cells of the stomatal complex of native and regenerated BOER plants (fig. 7). The staining pattern in BOER, was a consistent staining of the terminal ends of guard cells (gc), while in ATCA, staining of the entire guard cells was observed (fig. 6). Staining was also regularly observed in the subsidiary cells (sc) of the stomatal complex. Colonization of stomata of these native plants suggests that these endophytes influence stomatal regulation and evapotranspiration. Minimizing water loss in arid ecosystems would enhance drought tolerance. A suggested fungal role may be to maximize gas exchange and photosynthetic efficiency, while reducing water loss by transpiration.

Unexpected, was the intrinsic association of endophytic fungi with cell cultures, calluses, and axenically cultured plants, previously expected to be microbe free (Barrow and Osuna-Avila 2004; Borkowska 2002). Also of interest, was that these fungi did not grow on the carbon-mineral rich culture medium as long as the plant tissues were healthy. However, after the eventual death of plants, *A. ustus*, *Crinipellis* and *Engyodontium album* were isolated and grew well on the culture medium. This suggested an obligate affinity of these species to healthy living tissue compared to their saprophytic potential on artificial medium after plant health is impaired.

Attempts to completely separate BOER and ATCA cell cultures from fungi have not been successful. Therefore, in order to observe the effects that BOER- and ATCA-associated fungi might have on their prospective host plant, callus

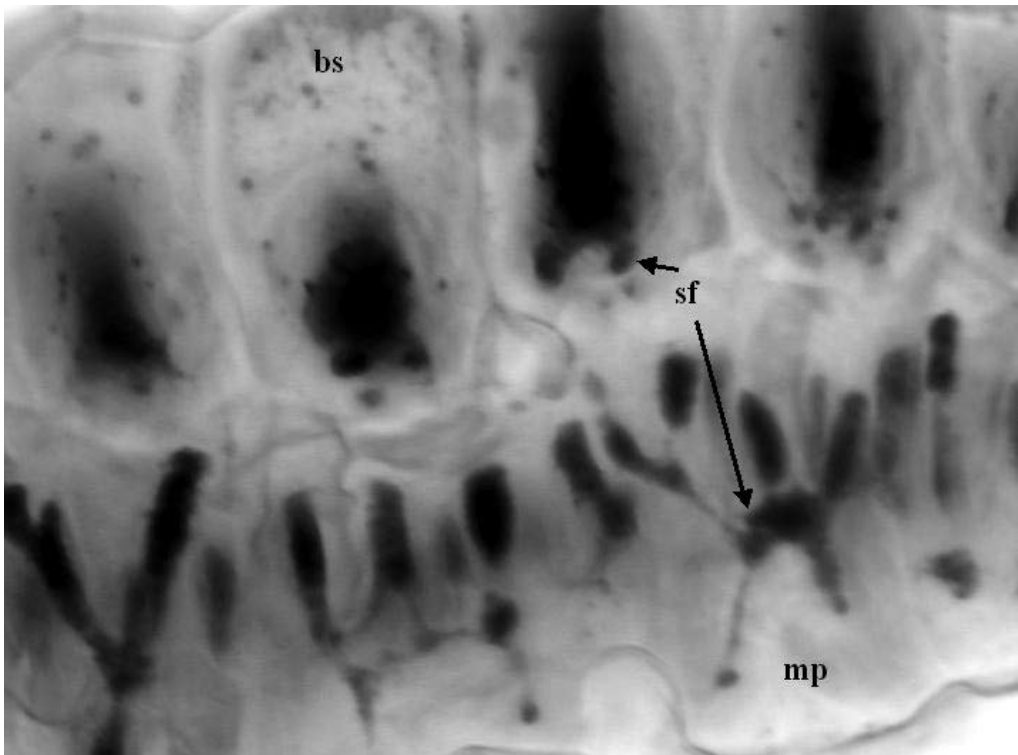


Figure 4. Trypan blue stained fungal tissue (sf) within photosynthetic mesophyll cells (mp) and bundle sheath cells (bs) of native *Bouteloua eriopoda*.

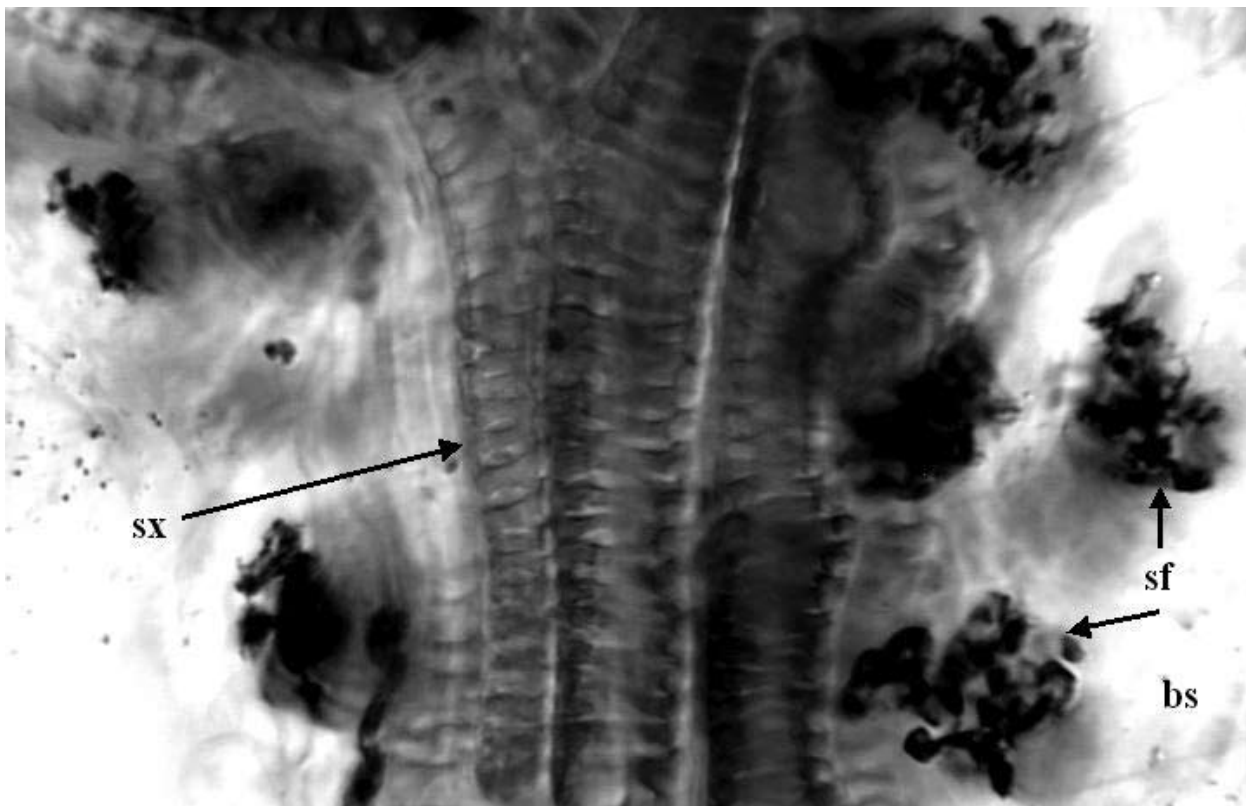


Figure 5. Trypan blue stained fungal tissue (sf) in photosynthetic bundle sheath (bs) cells in leaves of regenerated *Atriplex canescens* leaves. Lignified xylem vessels (sx) also stain positively for fungal tissue.

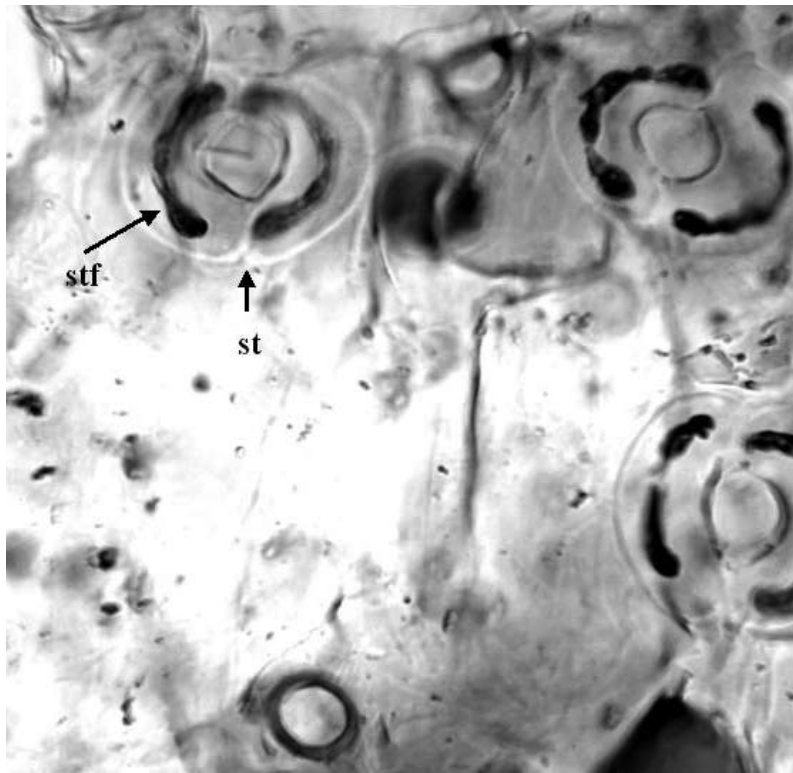


Figure 6. Trypan blue stained fungal tissue (stf) within guard cells of the stomata (st) in leaves of regenerated *Atriplex canescens* plantlets.

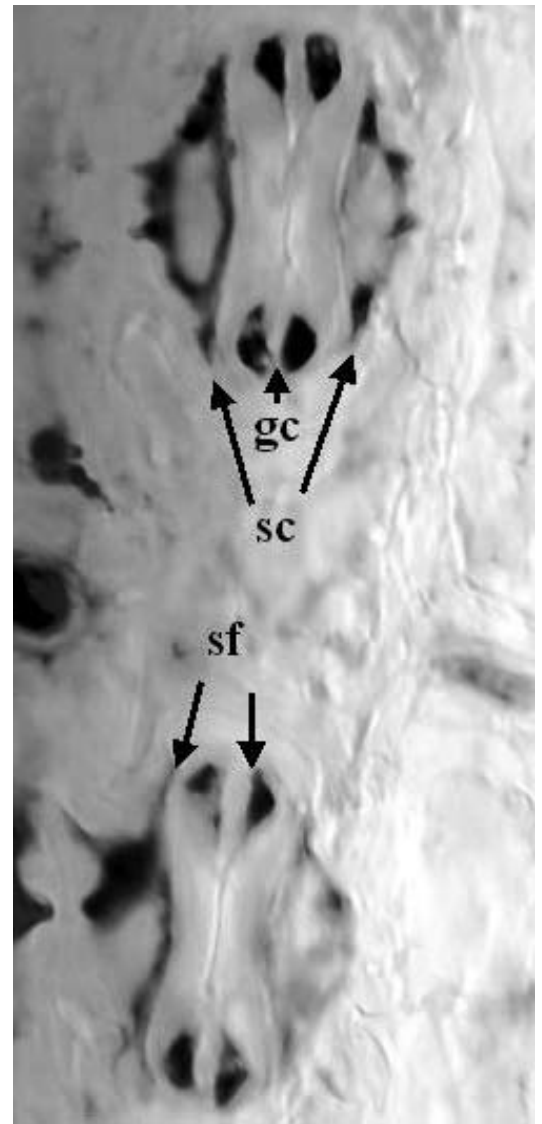


Figure 7. Trypan blue stained fungal tissue (sf) associated with terminal ends of guard cells (gc) and subsidiary cells (sc) of the stomatal complex of native *Bouteloua eriopoda* leaves.

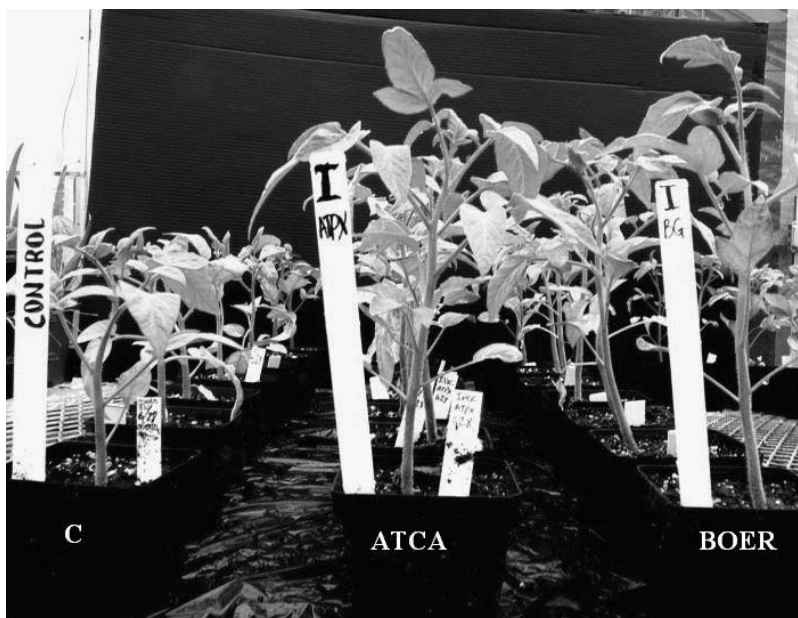


Figure 8. Control tomato plants, var. Bradley (without fungi). Fungi transferred to plants from *Atriplex canescens* (ATCA) callus. Fungi transferred to plants from *Bouteloua eriopoda* (BOER) callus.



Figure 9. PCR products obtained from amplification of rDNA from control tomato plants, var. Bradley (without fungi), and from tomato plants (var. Bradley) inoculated with *Atriplex canescens* (ATCA) callus or *Bouteloua eriopoda* (BOER) callus. Left to right: Lane 1-Molecular Weight Marker, Lane 2- Control tomato, Lane 3- Tomato inoculated with BOER callus, Lane 4- Tomato inoculated with ATCA callus.

from cell cultures was used to inoculate tomato seedling. Evidence for their transfer was a three- to five- fold increase in root and shoot biomass. Figure 8 compares standard tomato variety, Bradley, control plants (c) to plants with fungi transferred from ATCA callus, and to plants with fungi transferred from BOER callus. Transferred fungi immediately take up residence in the apoplastic spaces of the non-host tomato. Substantial increase in shoot biomass is observed with earlier and larger fruit production. Fungi increased root biomass from three to five times. Differences in responses were observed, suggesting that different fungi may have been transferred with ATCA and BOER callus.

When the primers NS1 and NS4 were used with defined protocols (White 1990), a band was seen in both BOER and ATCA treated samples that was absent from the control (fig. 9). Curiously, this band has not been seen in PCR products that have been amplified from endophytes such as the putative rust, the *Crinipellis* and the *Aspergillus ustus* (Lucero and others, submitted), which have previously been characterized in ATCA and BOER cultures. This suggests yet another fungal endophyte resides in these cell cultures. If the different effects observed in the BOER- and ATCA-inoculated treatment groups are indeed due to different fungi, then sequence analysis should produce a different sequence for each of the bands shown in figure 9. The intrinsic integration of symbiotic fungi with all cells and tissues suggests that fungi modify plant behavior at genetic, cellular, physiological, and ecological levels and that fungal genes contribute multiple benefits to the host plant. It is unlikely that plants bearing such large fungal populations would continue to thrive under any environment unless the fungi contributed benefits to the host plant in exchange for substantial quantities of organic carbon they consume.

The simplicity with which fungi can be transferred to new hosts, combined with the benefits conferred on plants by fungi, raises interesting possibilities for native and crop plant improvement. Continued research in the identification and characterization of fungal endophytes promises to yield a complex assortment of species that may serve as tools for plant improvement. Clearly, microbial diversity in our arid rangelands has been a largely overlooked and underutilized resource.

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