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Plant–fungal interactions in arid and semi-arid ecosystems: Large-scale impacts from microscale processes

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Abstract

The roles of microbes in shaping plant communities have historically been underestimated. Recent improvements in our abilities to detect, identify, and monitor microbial inhabitants of plant tissues are increasing our appreciation of the complex microbial dynamics in arid and semi-arid ecosystems. Microbial endophytes can modify plants at genetic, physiologic, and ecologic levels. These modifications induce profound changes in how plants respond to their environments, with potential consequences in terms of spatial variation in vegetation dynamics. Microscale examination of *Bouteloua eriopoda* (black grama) and *Atriplex canescens* (fourwing saltbush) collected in the northern Chihuahuan Desert revealed diverse fungal communities associated with individual plants at the cellular and subcellular levels. To explore thresholds of plant fitness defined by microbial communities, endophytes from *B. eriopoda* were transferred to *Sporobolus cryptandrus* (sand dropseed), and endophytes from *S. cryptandrus* and *A. canescens* were transferred to *B. eriopoda*. Dramatic, whole-plant differences in morphology and biomass between treated and untreated plants were observed. Treated plants were generally larger, with greater reproductive potential than untreated controls. We hypothesize that these transformed plants will continue to out-perform their native counterparts, propagating changes from the plant–microbe interface to ecologically significant scales.

Keywords: *Atriplex canescens*; *Bouteloua eriopoda*; Endophytic fungi; In vitro propagation; *Sporobolus cryptandrus*

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1. Introduction

Peters and Havstad (2006) identify key elements and spatial hierarchies associated with variability and heterogeneous vegetation patterns in arid and semi-arid ecosystems. Our research examines the role of plant-associated fungi as a source of variability at the finest hierarchial scale (individual plant) described in their paper. In recent decades, the development of improved technologies for examining microscale communities has contributed to increased appreciation for the roles these organisms play in larger scale ecosystems. Using light and electron microscopy, chemical analysis, and molecular markers, it is now possible to detect and quantify the complex microbial communities inhabiting not only soils, but individual plants. For example, Vandenkoornhuyse et al. (2002) used DNA sequence analysis to identify 49 species of fungi from the roots of a single grass plant, *Arrhenatherum elatius*. An even more complex community, consisting of 82 genetically distinct endophytes, has been described in western white pine (Ganley et al., 2004). The majority of these microbes appear to be neither parasites nor simple decomposers (Ganley et al., 2004). Many exist symbiotically within host plants. Clear demonstrations of microbial abilities to enhance plant growth by facilitating nutrient and water uptake (Hildebrandt et al., 2002), increasing biomass production, and modifying expression of chemicals involved in plant defense (Bultman and Bell, 2003; Mucciarelli et al., 2003) all suggest that microscale communities have significant potential to influence vegetative communities.

The complexity of plant–microbe communities creates great difficulty in determining contributions of individual members. We have previously attempted to separate *Bouteloua eriopoda* (black grama) and *Atriplex canescens* (fourwing saltbush) from microbial endophytes in vitro in an effort to examine the contributions of individual fungal endophytes to plant performance. Yet, even under aseptic conditions, fungal endophytes persisted in plant tissue (Barrow et al., 2004).

In this paper, our objectives are to: (1) describe the asymptomatic, systemic fungal communities present in *B. eriopoda* and *A. canescens* following in vitro propagation. Such fungi are likely to interact symbiotically with the plant, since even the extreme sterilization processes used to establish in vitro cultures do not eliminate these associations; (2) compare in vitro (aseptically cultured) and in situ (uncultivated, naturally occurring plants collected from native populations) plant–fungal communities. This control objective was included to verify that fungi observed in vitro were representative of in situ populations rather than incidental associations acquired during the culturing process; (3) describe changes in endophyte community composition that alter plant morphology.

2. Methods and materials

2.1. Plant material

Shoot and root tissues and seeds from *B. eriopoda* and *A. canescens* were collected from native populations on the USDA-ARS Jornada Experimental Range (JER) near Las Cruces, New Mexico, and *Sporobolus cryptandrus* seeds were purchased from Plants of the Southwest (Santa Fe, NM). Seeds were used to initiate undifferentiated callus cultures, which served as sources of inoculum for unculturable endophytes. Culturable endophytes

were isolated either from field-collected, surface-sterilized roots or from regenerated plants (Barrow et al., 2004; Osuna-Avila and Barrow, 2004).

B. eriopoda and *S. cryptandrus* calli were induced by germinating surface-disinfested seeds under aseptic conditions. Embryonic shoots were excised and cultured on Murashige and Skoog (MS) agar (Murashige and Skoog, 1962) to produce callus. Callus cultures were maintained on MS agar containing 4.52 μM Dicamba (6-dichloro-o-anisic acid) at 25 °C with an 18 h photoperiod (151 μmol $m^{-2} s^{-1}$). The callus was transferred to hormone-free MS agar for multiplication prior to inoculation of foreign host tissue.

Aseptic shoot cultures of *A. canescens* were established as described above, then transferred to a callus initiation media (MS basal salts supplemented with 3.5 μM 4-amino-3,5,6-tricloropicolinicacid (Picloram) and 8.87 μM 6-benzylaminopurine (BAP). Callus was transferred to hormone-free MS agar prior to seedling inoculation. *A. canescens* cultures were incubated at 28 ± 1 °C under continuous fluorescent light (14–18 μmol $m^{-2} s^{-1}$).

2.2. Isolation of endophytic fungi

Field-collected roots were washed in tap water to remove soil, then surface-disinfested by soaking in 70% ethanol for 7 min, followed by 30 min in 3.9% sodium hypochlorite (75% Chlorox™). Sections of disinfested root were cultured on potato dextrose agar (PDA) and incubated in a controlled environment. Fungal hyphae emerging from the nodes or cross-sections of root segments were removed and transferred to PDA. To isolate hyphae from regenerated plants, aseptically cultured tissues were transferred to PDA.

One fungal strain isolated from *B. eriopoda* was identified morphologically as *Aspergillus ustus* by Dr. Marin Klich (USDA-ARS Food and Safety Research Laboratory, New Orleans, LA). A morphologically identical endophyte was also isolated from roots of *A. canescens*. A second species isolated from *B. eriopoda* roots was tentatively identified by Dr. M. Catherine Aime (USDA-ARS Systematic Botany and Mycology Laboratory, Beltsville, MD) as a *Moniliophthora* species, based on sequence analysis of ribosomal DNA. *Penicillium olsonii* and *Bipolaris spicifera*, isolated from *A. canescens* in vitro, and *Engyodontium album*, isolated from in vitro *B. eriopoda*, were identified by sequence analysis of the internal transcribed spacer region (ITS1-5.8S-ITS2 rDNA) of ribosomal DNA. These analyses were performed by the Plant Pathogen Identification Laboratory at North Carolina State University (Raleigh, NC).

2.3. PCR amplification of fungal sequences from B. eriopoda regenerated cell lines

To identify fungi that could not be cultured independently from *B. eriopoda* or *A. canescens*, total DNA was extracted from plant tissues produced in vitro using MoBio Ultraclean™ Plant DNA Kits. DNA was amplified by polymerase chain reaction (PCR) using the primers: Ured1, 5′GCATTCCCAAACAACTCGAC3′ and Ured2, 5′CCTGTTTGAGTGTCATGAAACC3′, AU2 and AU4 (Vandenkoornhuyse et al., 2002), or ITS1 and ITS4 (White et al., 1990) with Taq PCR Master Mix (Qiagen, Inc.) at annealing temperatures of 60, 48, and 53 °C, respectively. The amplified products were cloned into pCR 2.1™ plasmids using an Invitrogen TA Cloning Kit™. Individual clones were sequenced using universal M13 forward and reverse primers and BigDye®

Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in conjunction with an Applied Biosystems 3100 Genetic Analyzer.

2.4. Microscopic examination of plant tissue

Tissues from either field collected or in vitro plant material were stained as described by Barrow and Aaltonen (2004). Tissues were mounted on microscope slides and examined with a Zeiss Axiophot microscope using both conventional and DIC optics at 1000 × magnification. Electron microscopy was performed by Soumitra Ghoshroy (Electron Microscopy Laboratory, New Mexico State University).

2.5. Inoculation of foreign hosts

Callus cultures of *B. eriopoda*, *A. canescens*, or *S. cryptandrus* were incubated with surface-disinfested, germinating seed and cultivated aseptically in MS agar. Seeds from *B. eriopoda* were incubated with callus cultures from *A. canescens* ($n = 30$) or *S. cryptandrus* ($n = 30$), and *S. cryptandrus* seeds were incubated with callus cultures from *B. eriopoda* ($n = 30$). Because *B. eriopoda* seeds incubated with *S. cryptandrus* callus exhibited a high mortality rate, additional seeds ($n = 33$) were incubated in an effort to generate comparable numbers of inoculated plants.

3. Results

Dual staining with Trypan Blue (selective for fungal chitin) and Sudan IV (specific for fungal-associated lipid bodies) revealed the presence of fungi associated with every cell type examined (Fig. 1). Long and short epidermal cells (Fig. 1a), stomatal complexes (Fig. 1b), and mesophyll cells, vascular bundles, and somatic embryos (not shown) all stained positive for fungi. With transmission electron microscopy, vacuolated fungal hyphae were observed between plant cell walls and membranes, even appearing to encircle chloroplasts. Using scanning electron microscopy, fungal networks and biofilms appeared to cover the surface of *B. eriopoda* leaf tissue (Fig. 1c), and teliospores were evident (Fig. 1d). Similar images were obtained from both aseptic and field-collected specimens of *B. eriopoda* and *A. canescens*.

Fungal species isolated and identified from aseptically cultured *A. canescens* include *P. olsonii*, *B. spicifera*, and *A. ustus*. From aseptically cultured *B. eriopoda*, we isolated and identified a second strain of *A. ustus*, *E. album*, and an endophyte tentatively identified as belonging to the genus *Moniliophthora*. In addition, teliospores present on *B. eriopoda* suggest the presence of a fungus similar in morphology to rust-causing pathogens from the genus *Puccinia* (Fig. 1d), and PCR primers targeting *Puccinia* internal spacer regions produced a fragment of the predicted size when used to amplify total DNA from plant cultures. Sequence analysis of this fragment is underway. Finally, a cloned PCR fragment amplified from *B. eriopoda* callus DNA is homologous to an unidentified ascomycete (Genbank accession number AY559361) and similar to several species of *Cercospora*. To summarize, we found evidence of at least six fungal species intrinsically associated with *B. eriopoda*, and at least three species in *A. canescens* callus and regenerated plants.

When *B. eriopoda* was germinated in the presence of callus tissue from *A. canescens*, 90% percent of the inoculated seedlings exhibited greater total biomass (Fig. 2a, right)

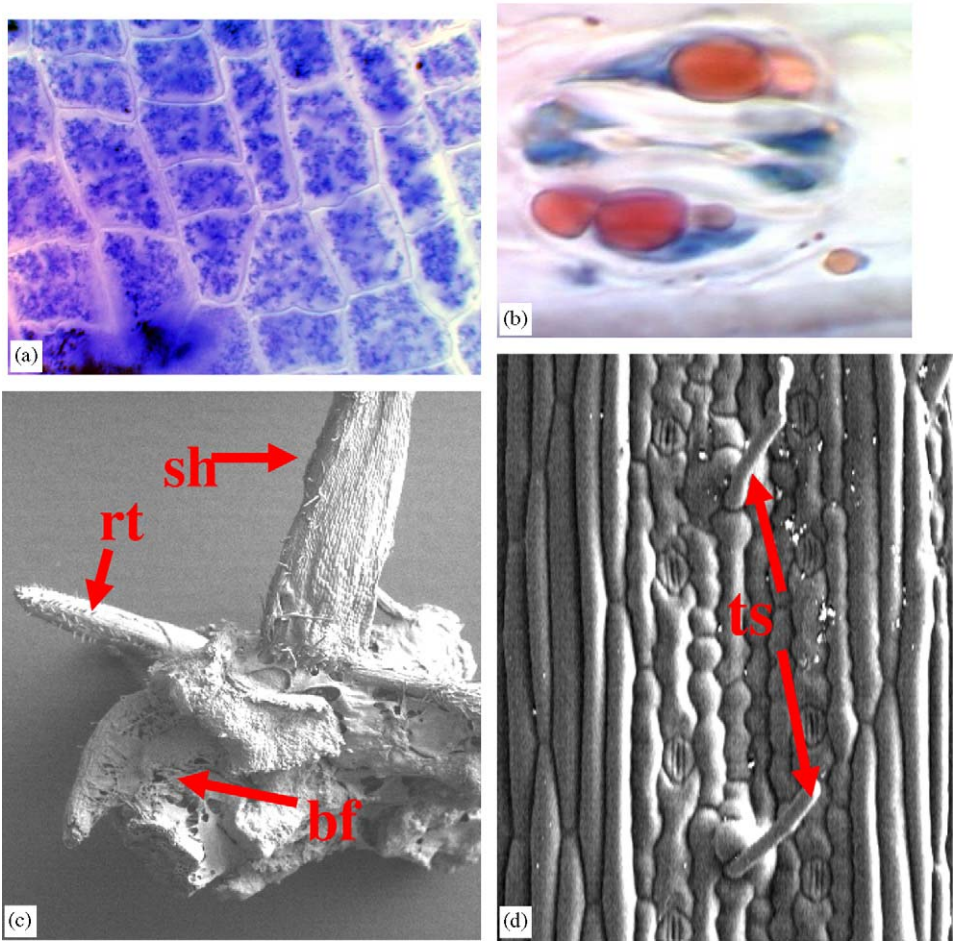

Fig. 1. Fungi associated with major tissues and cell types of aseptically grown *B. eriopoda*. Dual staining reveals fungi scattered throughout surfaces of epidermal tissue (a) and clustered around guard cells of a stomatal complex (b). A biofilm encases emerging roots and shoots of regenerated plants (c), and teleospores characteristic of rust fungi (d) are observed with electron microscopy.

than uninoculated control plants (Fig. 2a, left). Root branching and tiller production were both greater in inoculated *B. eriopoda*, suggesting that fungi induced morphological changes in the host plant. When *S. cryptandrus* was inoculated with *B. eriopoda*, similar results were observed (Fig. 2b). All inoculated plants survived and produced greater shoot and root biomass than uninoculated controls. Results were less straightforward for *B. eriopoda* seedlings inoculated with *S. cryptandrus* callus. For this treatment, only 19% of the seedlings survived (12 of 63 seedlings from two plantings). Eight of these plants were similar in size and morphology to controls; however, four plants produced greater root and shoot biomass than uninoculated controls (Fig. 2a, middle).

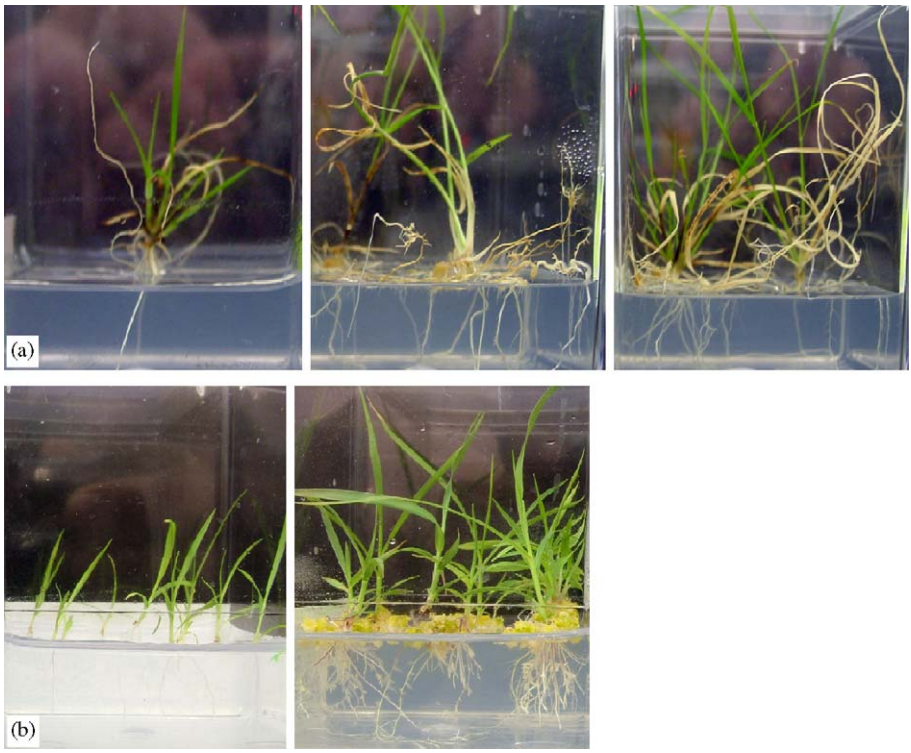

Fig. 2. (a) Differences in root and shoot biomass between germinating seedlings of *B. eriopoda* (left) and *B. eriopoda* inoculated with callus from *S. cryptandrus* (middle) or *A. canescens* (right). (b) Shows germinating seedlings of *S. cryptandrus* (left) and *S. cryptandrus* inoculated with callus from *B. eriopoda* (right).

4. Discussion

The intricacy of fungal networks observed within individual cells and tissues of *B. eriopoda* and *A. canescens* in vitro suggests that physiological processes within these plants are influenced by surrounding fungi. Demonstrations of the unique roles of each member of this plant–fungus community have been hindered by difficulty in isolating obligate fungal species that cannot grow apart from host plants. Equally challenging has been the isolation of endophyte-free plant cells, since endophytes are present even at single cell and embryonic stages. The induction of fruiting bodies, crucial for morphological identification of fungi, does not always occur within plant tissues, especially under the arid conditions found in natural settings. It is notable that all fungal species described herein were isolated from plants grown in vitro, either from callus tissue or regenerated plants. Thus, even if fungal endophytes serve tissue-specific functions within a developed plant, the species identified in this study were all associated with undifferentiated plant cells (callus). Equally important, these species have also been detected in field samples collected from a number of sites on the JER, assuring that endophytic associations observed are natural components of the plant species examined.

The teliospores illustrated in Fig. 1d are characteristic of rusts. Historically, *B. eriopoda* has been described as a host plant for *Puccinia cacabata*, the causative agent for Southwest

Cotton Rust. *Puccinia* species are generally obligate, and cannot be cultured independently of the host. To determine whether the observed teliospores were produced by a *Puccinia* species, PCR primers were designed by aligning internal spacer sequences of rDNA from *Puccinia* sequences in Genbank, *B. eriopoda*, *A. ustus*, *Moniliophthora* spp., and *E. album*, using the ClustalW algorithm (Thompson et al., 1994). Regions of the alignment that were conserved only among *Puccinia* were selected for forward and reverse PCR primers. The BLAST Search for short, nearly exact matches (McGinnis and Madden, 2004), used to test primer specificity, invariably returned *Puccina* or other *Uredomycetes* as matches, and the primers amplified a product of the predicted size when used to amplify DNA from *B. eriopoda*. However, the amplified sequence exhibited weak homogeneity when aligned with *Uredomycetes* in Genbank. Failure to align with previously described species suggests that either the teliospore-producing fungus belongs to a novel species or the amplified PCR product was from a different endophyte present in *B. eriopoda*. Analysis of longer segments and FISH (Fluorescence In situ Hybridization) techniques using *Puccinia*-specific probes are currently underway to completely characterize the teliospore-producing species.

Our observation of fungal networks from both monocots and dicots in vitro combined with observations of similar fungal structures in tissues collected from more than 35 species (not shown) indicate these relationships are highly conserved. It is generally assumed that highly conserved features serve crucial functions. We speculate that biofilms enveloping external plant surfaces shield plants from the harsh environments encountered in arid rangelands. Fungi observed in stomatal complexes may have a direct role in regulating evapo-transpiration, while hyphae observed in colonized roots function like mycorrhizae by increasing the surface area available for uptake of nutrients (Barrow and Osuna, 2002).

The identification of five fungal endophytes associated with a single grass species in aseptic cell and tissue cultures has profound implications for plant ecologists, especially in light of the dramatic changes in plant morphology observed when endophyte communities are changed. Of equal interest is the discovery of *A. ustus* behaving as an endophyte in both a grass and a shrub. This fungus assists with phosphorus uptake in *A. canescens* (Barrow and Osuna, 2002), and can propagate vegetatively or sexually in soil. If the same genotype can inhabit multiple plant species, this microbe may provide an unexplored source of connectivity between plant species in a single community. Clearly, analysis of microbial communities associated with vegetation would improve our understanding of the existing variability among plant communities.

Such analysis was once considered an insurmountable task, but increasingly available DNA sequence information coupled with modern high-throughput techniques in molecular biology have made the task of monitoring changes in microbial communities feasible. A microarray containing well-defined ribosomal marker sequences collected from key plant and soil types could provide valuable information regarding site conditions pre- and post-disturbance. As data accumulate relating site conditions to microbial communities, changes in species composition may be useful for predicting changes in plant communities.

Perhaps the most significant aspect of the results described herein concerns the startling changes in plant morphology and biomass observed following inoculation of species with endophyte-containing callus. The enhanced growth of *S. cryptandrus* and *B. eriopoda* following inoculation with endophyte-containing callus suggests endophyte manipulation may provide a powerful tool for developing plant cultivars capable of thriving in harsh environments. This observation parallels the findings of Redman et al. (2002) that

tomatoes survived constant temperatures of 50 °C following inoculation with thermotolerant fungi. With the diversity of endophytes present in a single plant species, not all combinations of plants and fungi would be expected to produce positive results. Hence, the 80% germination failure of *B. eriopoda* seedlings in the presence of *S. cryptandrus* callus was not surprising. It is plausible that one or more of the endophytes present in *S. cryptandrus* is incompatible with *B. eriopoda*. Perhaps this incompatibility also explains, at least in part, the tendency of *S. cryptandrus* to successfully invade *B. eriopoda* grasslands. It is unclear why a small percentage of the surviving plants exhibited enhanced growth following inoculation. Genetic analysis of the *S. cryptandrus* callus has not been performed; therefore, it is unknown how many endophyte species are associated with this callus. Possibly, different endophytes are responsible for the variability in performance among individuals. This hypothesis will be tested in upcoming studies.

5. Conclusions and global implications

Clearly, more research is needed to define changes in plant morphology that can be achieved by modifying plant endophyte communities in arid and semi-arid ecosystems. Equally important will be the identification of key endophytes that can enhance growth of desirable plant species and environmental conditions under which growth enhancements can be achieved. However, existing data demonstrate that fungi have remarkable abilities to interact at plant/interspace scales (Peters and Havstad, 2006). These fine-scale interactions may propagate to whole plant changes, thus modifying the competitive ability of plants. Such modifications would be expected to impact vegetation dynamics at broader scales. The possibilities implied by results obtained to date mandate continued examination of plant–fungal interactions as critical components of plant ecology not only in arid lands worldwide but in all natural and agricultural plant systems.

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