

ABSTRACT: Both abiotic and biotic factors impact the plant populations of semiarid and arid ecosystems. As we learn more about how microbial populations within these ecological communities impact plant lifecycles, it becomes apparent that preservation and restoration of native plant communities might in part rely on establishing or reestablishing the microbial inhabitants of native plants. Fungi play a crucial role in many ecological processes. Despite this, fungal diversity and function within natural habitats are poorly defined. Within native grasses, fungal endophytes are ubiquitous, suggesting mutualistic or symbiotic relationships that might strengthen the ability of these grasses to survive under adverse conditions. We are interested in the plant-microbe(s) interactions that are present in the native grass *Bouteloua eriopoda*, (black grama), in a rangeland environment, and are using fungal specific oligonucleotide primers and polymerase chain reaction (PCR) to help identify fungal endophytes that closely associate with this grass. Our interest is to characterize the extent of the plant-fungal interaction and to study the persistence of specific fungi across the *B. eriopoda* community.

INTRODUCTION: The romance of the open range during the American westward expansion of the 1800s was extended into southern New Mexico with eye-witness accounts of the beauty of never-ending grasslands, perfect for cattle and sheep production. A major historical component of the rangeland, *Bouteloua eriopoda* (Black grama grass), began its well-documented decline with the introduction of large grazing herds, and is present only as fragmented populations in disturbed areas of rangeland where it was once dominant. This decline also holds true within the Jornada Experimental Range (JER) in southern New Mexico (Fig. 1). Although there appears to be no one single cause, high-intensity grazing and other human-associated disruptions, drought and climate change, and changes in soil composition that are the result of these disturbances, are believed to be factors that greatly contributed to black grama population decreases.

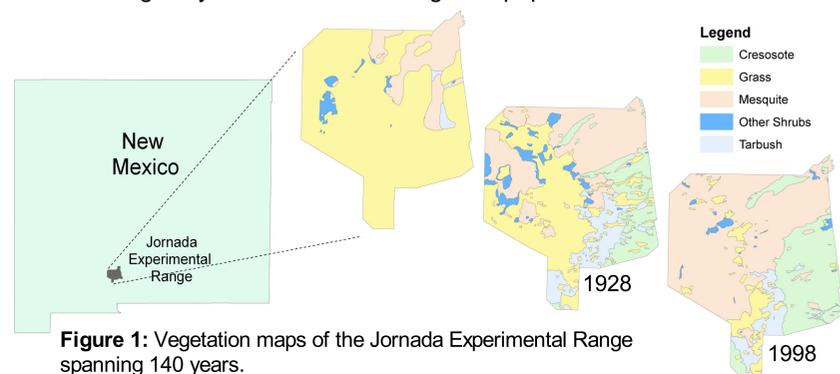


Figure 1: Vegetation maps of the Jornada Experimental Range spanning 140 years.

One factor that might play a significant role in the ability of black grama to overcome environmental stresses is its association with microbial populations within its ecological community. Little is known about the microbial communities found associated with black grama, but there are potentially hundreds of bacteria and fungi that are around, on and even growing within this native grass. In other systems, the potential for microbes to have positive effects on plant growth and establishment is well-documented and even startling. Included are a fungal endophyte that enhances a plant's thermotolerance to endure greater than 50C soil temperatures (Redman, Science: 298, 2002), and an endophytic fungus that increase salt-tolerance and disease resistance in barley (Waller, PNAS: v.102, 2005).

Our focus is to determine if specific fungi are associated with black grama, and whether these fungi have positive effects on the plant, with the potential for their usage in conservation and restoration efforts.

There are many difficulties in studying unknown microbial populations in black grama: sheer number of associated microbes, their relationship to the plant (mutualistic, symbiotic, antagonistic, pathogenic, saprophytic), whether the microbes being characterized are vertically (seed-borne) or horizontally transferred, tissue-specificity of specific microbes, and whether there are biochemical or molecular impacts between the associated organisms are all still virtually unstudied and unknown. There have been attempts to culture and purify fungus linked with black grama, but some of the associated fungi is potentially unculturable.

METHODS AND MATERIALS: We are using polymerase chain reaction (PCR) and oligonucleotide primers for fungal-associated and fungal-specific genes to assess the population of black grama-associated fungi. These primer pairs include those for the internal transcribed spacer (ITS), β -tubulin (β -tub), chitin synthase (CS), and amino adipate reductase (AAR). (Table 1) CS and AAR are fungal-specific genes and the oligonucleotide primers should not recognize plant DNA.

Table 1: Oligonucleotide primer sequences and expected PCR product sizes.

| Targeted Gene | Sequence | Reference | Product Size |
|---------------------------------|--|---|-------------------------------|
| ITS-4 ITS-5 | 5'-TCTCCGCTTATTGATATGC-3' 5'-GGAAGTAAAGTCGTAAACAAGG-3' | PCR Protocols: A guide to Methods and Appls. T.J. White Ed. (1990) | ~530 bp plant and fungus |
| β T3-LM β T10-LM | 5' GAACGTCTACTTCAACGAG 3' 5' TCGGAAGCAGCCATCATGTTCTT 3' | L. Myllys et al., Mycologia 93: 335-343, (2001) | ~750 bp fungus ~1550 bp |
| CS-1 CS-2 | 5'-CTGAAGCTTACNATGYATMATRAGSAT-3' 5'-GTTCTCGAGYTTTATYTCRAARTTYTG-3' | A.R. Bowen et al., Classification of fungal Chitin synthases. PNAS 89: 519-523 (1992) | ~800 bp fungus only |
| AAR-1 AAR-2 | 5'-GGNATHGCNCAYGAYCCNRTNCA-3' 5'-GGYTTTTCNAYTTTNCRRITNGRIT-3' | Kwang-Deuk et al., BMC Evol Biol. 2: 6 (2002) | ~1050 bp fungus only |

DNA was isolated from black grama leaf blades harvested from the JER (Qiagen DNeasy Plant Mini kit). JER harvested tissue was surface sterilized using 95% EtOH (1 min), followed by 15% H₂O₂ (5 min), and washed in sterile diH₂O 3X before use. DNA was also isolated from black grama callus culture and black grama seedlings in tissue culture, grown from surface sterilized seeds.

In addition, surface sterilized black grama leaf blades harvested from the JER were maintained on potato dextrose agar (PDA) to promote culturable fungal growth (Fig. 2) Surface sterilized JER harvested and black grama leaf blades from tissue culture black were also stained with Trypan Blue/Sudan IV and subjected to microscopy for fungal structure visualization (Fig. 3). Finally, DNA was isolated from characterized fungi that were cultured from JER harvested black grama. All isolated DNA was subjected to PCR using the above specified primer pairs (Fig. 4).

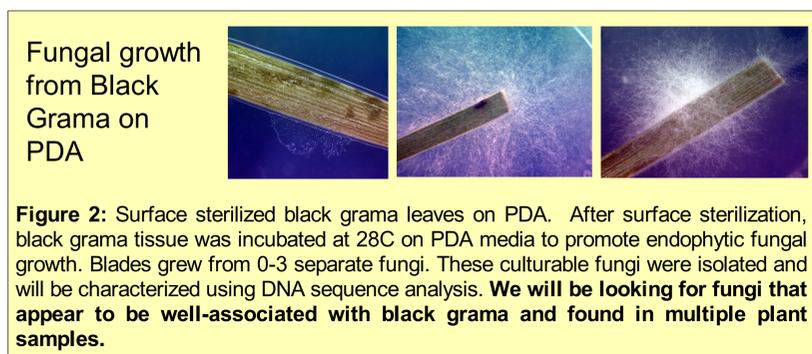


Figure 2: Surface sterilized black grama leaves on PDA. After surface sterilization, black grama tissue was incubated at 28C on PDA media to promote endophytic fungal growth. Blades grew from 0-3 separate fungi. These culturable fungi were isolated and will be characterized using DNA sequence analysis. We will be looking for fungi that appear to be well-associated with black grama and found in multiple plant samples.

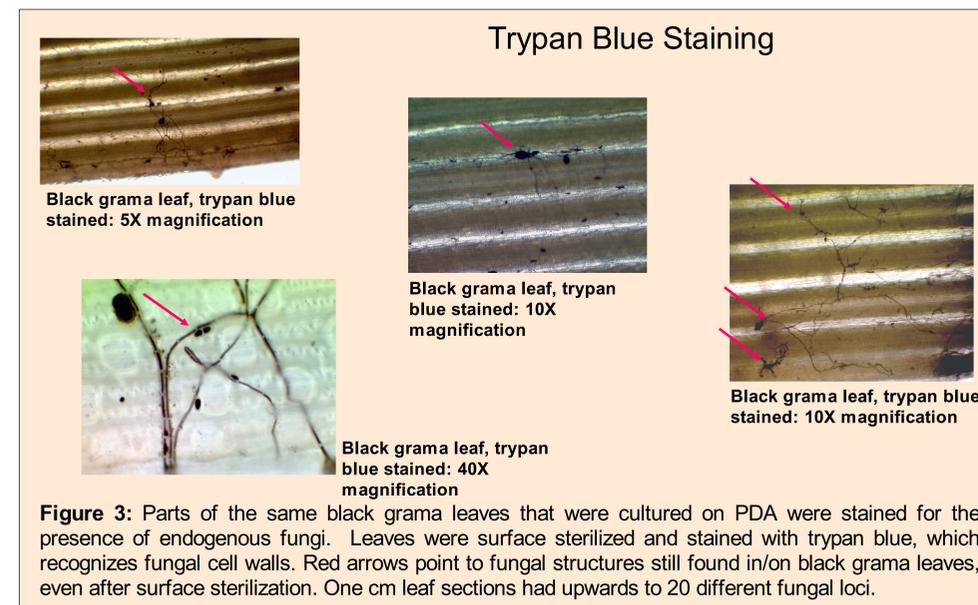


Figure 3: Parts of the same black grama leaves that were cultured on PDA were stained for the presence of endogenous fungi. Leaves were surface sterilized and stained with trypan blue, which recognizes fungal cell walls. Red arrows point to fungal structures still found in/on black grama leaves, even after surface sterilization. One cm leaf sections had upwards to 20 different fungal loci.

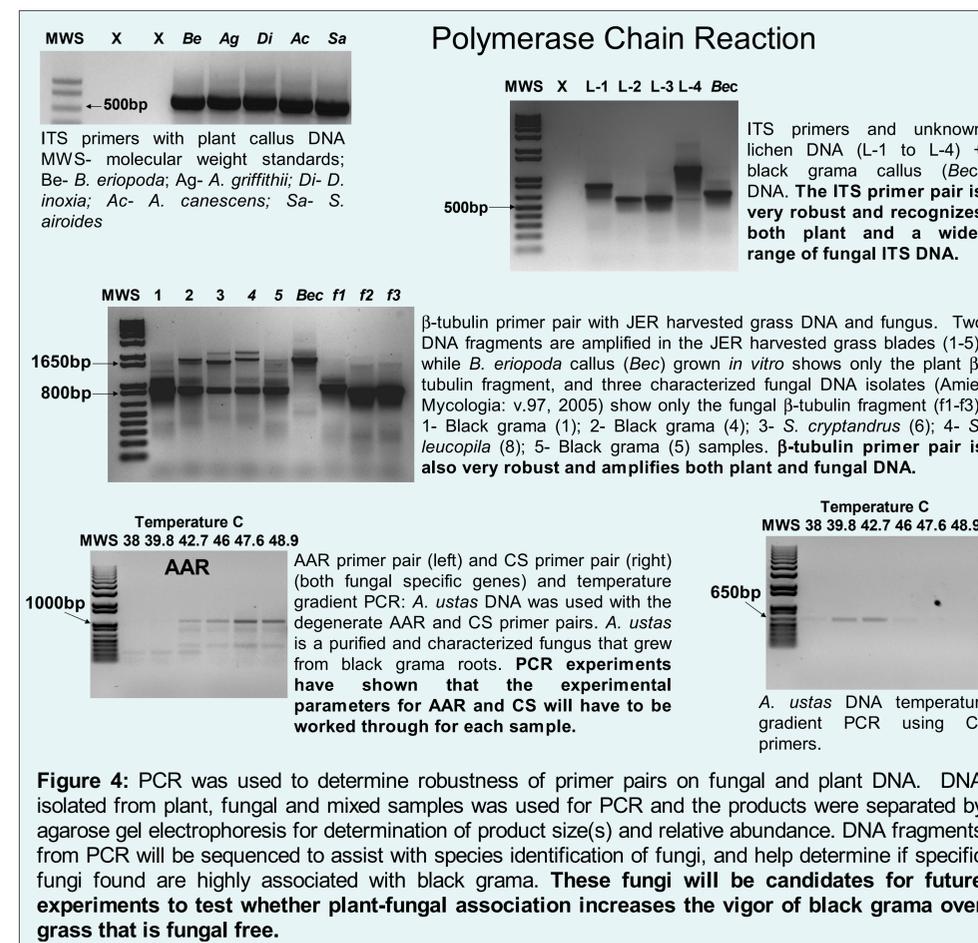


Figure 4: PCR was used to determine robustness of primer pairs on fungal and plant DNA. DNA isolated from plant, fungal and mixed samples was used for PCR and the products were separated by agarose gel electrophoresis for determination of product size(s) and relative abundance. DNA fragments from PCR will be sequenced to assist with species identification of fungi, and help determine if specific fungi found are highly associated with black grama. These fungi will be candidates for future experiments to test whether plant-fungal association increases the vigor of black grama over grass that is fungal free.

Conclusions: Our experiments show that black grama leaves carry multiple fungi that probably consists of many types or species. The high number of fungi on black grama leaves is probably due to horizontal transfer, and NOT specific association of fungal species with black grama. Although further characterization experiments will be completed on the culturable fungi found in black grama leaves, we will begin to focus on other tissues (roots and seed) for black grama fungi that may play a role in plant health and be useful in restoration efforts.