

Symbiotic Fungi that Influence Vigor, Biomass and Reproductive Potential of Native Bunch Grasses for Remediation of Degraded Semiarid Rangelands

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Abstract—A steady decline of perennial bunch grasses in arid rangelands has resulted in losses of productivity and germplasm. Remediation is costly and rarely successful. Cryptic symbiotic fungi, structurally integrated with cells and organs of native plants cannot be separated from host plant tissue. However, they were successfully transferred from cell cultures of native plants to other native grasses and tomato. Fungi in recipient plants were heritable and substantially enhanced vigor, biomass and reproductive potential. The transfer of novel fungal symbionts to other native or crop plants may offer an alternative method of improving plant performance without modifying native plant germplasm.

Introduction

Native perennial bunch grasses have steadily declined in arid southwestern USA rangelands in the past century as a result of complex biotic and abiotic stress. Attempts to re-establish them have been costly and rarely successful. On the other hand, invasive woody shrubs and grasses that produce seed and establish seedlings, particularly during years of below normal precipitation, have effectively invaded these ecosystems. Disturbed native rangelands have suffered substantial losses in productivity, soil and native plant germplasms.

Mycorrhizal fungi and fungal endophytes of cool season grasses have demonstrated roles in regulating ecosystem structure, growth and tolerance to biotic and abiotic stress (Smith and Read 1997; Clay and Schardl 2002). Recent studies suggest that symbiotic microbes are important components that have regulated the sustainability of native ecosystems. *Bouteloua eriopoda* Torr. (black grama) and

Atriplex canescens (Pursh) Nutt. (fourwings saltbush), a native grass and shrub of the northern Chihuahuan Desert, were extensively colonized by non-destructive endophytic fungi (Barrow and others 1997; Barrow and Aaltonen 2001; Barrow 2003). Specific fungal staining revealed a novel group of fungi that differed morphologically from traditionally recognized hyphae, spores, and fruiting bodies in all leaf and root cells (Barrow and Aaltonen 2001; Barrow 2003). Fungal interfaces with physiologically important cells, such as photosynthetic, vascular, and stomata suggests that these fungi may significantly regulate physiological processes to insure ecological success in this water and nutrient stressed desert ecosystem.

Traditional experimental protocol to test the efficacy of a plant associated microbe requires comparing plants with and without the microbe. Plant associated microbes could not be removed from germinating seedlings using standard disinfestation methods. Therefore we attempted to regenerate microbe free *B. eriopoda* plants from embryonic meristem cells of disinfested germinating seedlings (Osuna and Barrow 2004). Unexpectedly, regenerated plants were intrinsically integrated with different fungal symbionts (Barrow and others 2004; Lucero and others 2006). Light and electron microscope studies of cells and tissues of all phases of the regeneration process revealed a continuous fungal presence from precursor embryonic meristem cells to regenerated plants. After thousands of observations of native plants over years and seasonal variation and following their transfer from single cells to regenerated plants, key endosymbionts were determined to be structural components of all *B. eriopoda* cells, tissues, and organs suggesting co-evolutionary development. These symptomless endophytes have escaped detection because they are not always expressed as hyphae or commonly recognized fungal structures. Scanning electron microscopy revealed a fungal enmeshed biofilm that protectively encapsulated axenically grown callus tissue. Pirttila and others (2002) reported similar fungal biofilms in callus cultures derived from *Pinus* bud cultures. This structural integration of fungi with cells and tissues prevented their removal by traditional sterilization or separation methods. To test their influence in plant performance, fungi were transferred from cell cultures of native plants to roots of germinating seedlings of non-host native grasses and crop plants. Here we report a general response of fungi integrated with cell cultures of native desert plants when transferred to other native grasses and tomato that resulted in a phenomenal enhancement of vigor, biomass and reproductive potential.

In: Kitchen, Stanley G.; Pendleton, Rosemary L.; Monaco, Thomas A.; Vernon, Jason, comps. 2008. Proceedings—Shrublands under fire: disturbance and recovery in a changing world; 2006 June 6–8; Cedar City, UT. Proc. RMRS-P-52. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station.

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Materials and Methods

Seed Sources

The following plant materials were used in this experiment. Black grama, *Bouteloua eriopoda* Torr. (BOER) and sand dropseed, *Sporobolus cryptandrus* (Torr.) Gray (SPCR) were obtained from Plants of the Southwest, Santa Fe, NM. Seed of fourwing saltbush, *Atriplex canescens* (Pursh) Nutt., (ATCA), alkali sacaton, *Sporobolus airoides* Torr. (SPAI) and creosote bush, *Larrea tridentata* (DC.) Coville (LATR) were collected from native populations on the USDA-ARS Jornada Experimental Range in south-central New Mexico. Tomato seed, *Lycopersicon esculentum* Mill. var. Bradley was purchased at a local farm and ranch supply.

Callus Initiation

Callus was initiated from BOER, SPCR, SPAI, ATCA and LATR, all dominant grasses and shrubs of the northern Chihuahuan Desert (Osuna and Barrow 2004; Barrow and others 2004). Seeds of each species were surface disinfested by soaking for 10 min in 50 percent ethanol, followed by 25 min in 50 percent bleach. Seeds were rinsed three times in sterile distilled water, and allowed to germinate on Murashigie and Skoogs medium (MS) (Murashigie and Skoog 1962). Shoot apices were dissected from seedlings (15 d old) and transferred to fresh plates of MS supplemented with 1 mg/l 2,4-D for callus induction. The calluses were maintained with monthly subcultures to fresh MS medium with the same growth regulator concentration. Fungal presence was detected in all cultures initiated from all native plant sources by either microscopy or by isolation. Endophytes isolated from some callus lines are described in Lucero and others (2006).

Fungal Transfer to Non-Host Plants

Systemic fungal endophytes structurally integrated with cell, tissues and organs of native plants could not be separated from host plants. Therefore, to determine their function, they were transferred to non-host BOER and SPCR and tomato. Seeds of each non-host species were surface disinfested as described above and were germinated on MS media and incubated at 26 °C in a growth chamber receiving light (151 μ mol m⁻²s⁻²) over a 12-hour/day photoperiod. Seedlings with emerging radicles were aseptically transferred to callus cultures of each donor species, with the radicle in direct contact with the callus for 20 days. Fungi from BOER, SPAI, LATR and ATCA were transferred to tomato. Fungi from ATCA, BOER, and SPAI were transferred to SPCR and reciprocal fungal transfers were made from callus tissue of ATCA, SPCR and SPAI to non-host BOER seedlings.

Seedling Establishment

After 20 days in contact with fungal integrated callus, healthy grass seedlings of non-host recipient plants were transplanted to 2-inch peat pots containing a mixture of 50 percent commercial potting soil and 50 percent soil collected from the field site located on the Jornada Experimental

Range. Tomato seedlings were transplanted to commercial potting soil. Plants were grown for 20 days in an environmental chamber described above, then transferred to a heated greenhouse for 45 days. During this time, seedlings were irrigated with tap water as needed.

Plant Culture

Tomato plants were transferred to 30 x 38 cm pots with commercial potting soil and grown to maturity in the greenhouse. Native grasses were transplanted to a field site on the Jornada Experimental Range. The field site (32 x 50 ft) was fenced to exclude large animals and rodents, and tilled to a depth of 6 inches. Drip irrigation lines were installed, 18 inches apart, with emitters spaced 18 inches apart. A bag of steer manure (1.415 m³) ("Grow King," Western Organics) was uniformly incorporated into the soil, and Roundup™ herbicide was applied according to manufacturer's instructions 2 weeks prior to planting to reduce weeds. Seedling flats were moved to the field site and maintained in peat pots for 1 week for acclimation prior to transplanting. Seedlings were planted by hand on 18-inch centers at each water emitter. BOER and SPCR plants were planted in a randomized block design with 16 plants per replicate and three replicates per endophyte treatment. Edge effects were reduced by placing two additional rows of grasses around the entire plot. Water was applied once a week for 45 min (approximately 1 liter/plant). Weeds were removed manually with a scuffle hoe or by hand as required throughout the growth season.

Results and Discussion

Callus tissues derived from native plants, examined by selective staining and light microscopy and also scanning electron microscopy, revealed that all callus tissues were encapsulated with a protective fungal biofilm. Immediately upon contact with seedling germinants, fungal hyphae was observed with a stereo microscope growing from the callus cultures to the seedling radical, followed by swelling of the root tissue and induction of lateral rootlets. Within a week, substantial increases in rooting and shooting were observed in both tomato and seedling grasses.

Phenomenal responses were observed in tomato. Figure 1a compares tomato control plants (the top row) with plants with fungi transferred from SPAI, BOER, LATR and ATCA. In each of these cases plants receiving fungi were more vigorous and ranged from 3- (BOER) to 8- (LATR) fold increases in root and shoot biomass. Substantial increases in root and shoot branching were also observed. Plants receiving fungi had more and larger stems and leaves with greater chlorophyll and tissue phosphorous in the leaves. It can be seen from the photos that there is good uniformity within each transfer, yet there are differences between fungal treatments. This suggests that each native plant species transfers a different fungal symbiont that differentially influences the performance of the recipient host.

Figure 1b shows an initial response of fungal transfers from ATCA and SPAI to SPCR, where greater root and shoot biomass was observed within 3 weeks of fungal transfer. This response was generally observed in both grasses, but was greatest in the SPCR recipient plants. Plants in the

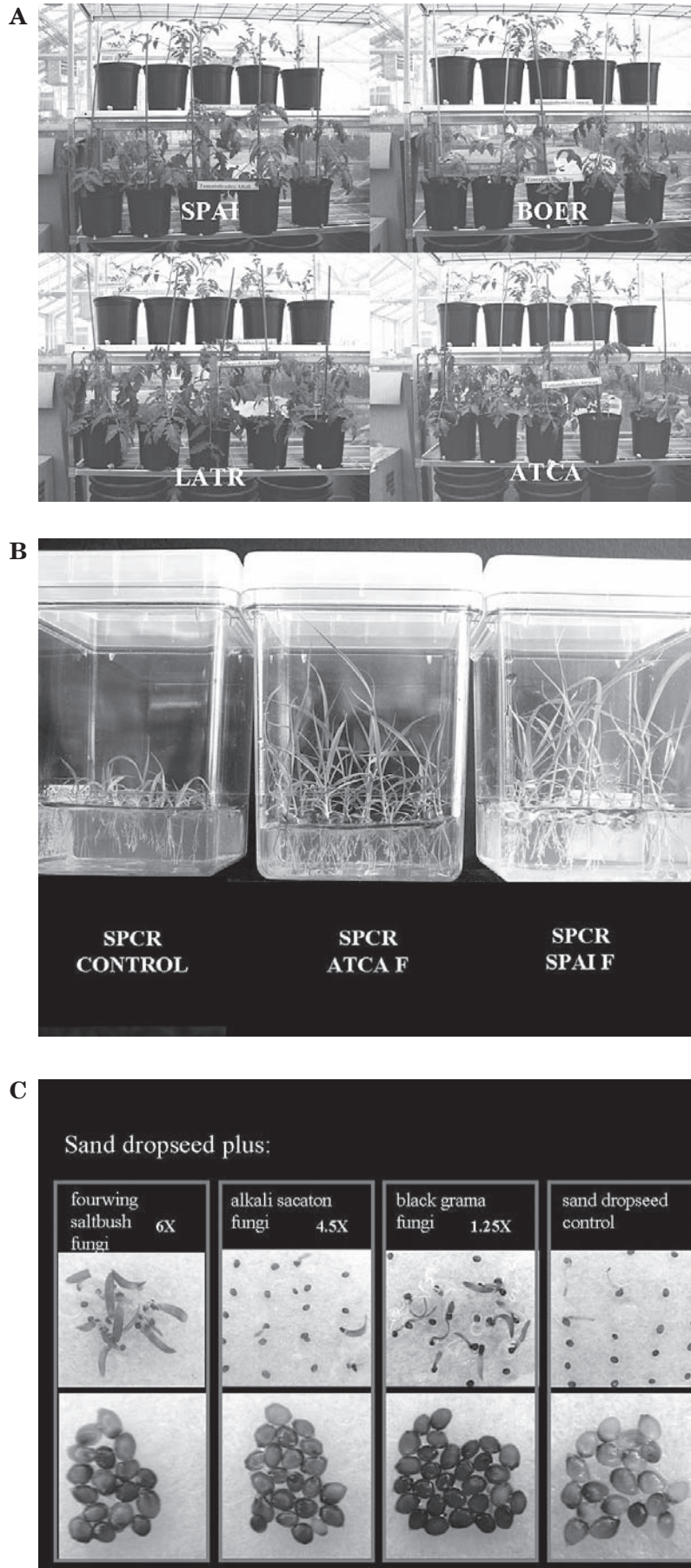


Figure 1—(A) Tomato plants var. Bradley with fungi transferred from sand dropseed (SPAI), black grama (BOER), creosote bush (LATR) and fourwing saltbush (ATCA) on bottom row compared to control plants without fungi in top row of each photo. All plants with transferred fungi are larger than control plants. (B) Sand dropseed plants with fungi transferred from fourwing saltbush (ATCA) and alkali sacaton (SPAI). Substantially greater root and shoot biomass is observed in each transfer. (C) Comparison of seed harvested from sand dropseed plants with fungi transferred from black grama (1.25 times more seed); alkali sacaton (4.5 times more seed) and fourwing saltbush (6 times more seed). In each transfer, emerging shoots were larger than control germinants in the far right photo.

field receiving symbiotic fungi expressed greater vigor, crown diameters, and seed production, and less mortality than control plants receiving no fungi. The experimental procedure and statistical analysis of these traits are being prepared for publication elsewhere.

One significant example, reported here, is seed production of SPCR receiving fungi from ATCA, SPAI and BOER compared to the controls (fig. 1c). Seed production from plants with fungi from ATCA was 6 times greater, fungi from SPAI was 4.5 times greater and fungi from BOER was 1.25 times greater than control plants. Germinants from each fungal transfer were more vigorous, with larger and greener shoots than control germinants.

The multiple benefits of these indigenous fungal symbionts confer similar benefits as are reported for mycorrhizal fungi (Smith and Read 1997) and endophytes of cool season grasses (Clay and Schardl 2002), and contribute multiple benefits to their host plants. These include enhanced nutrition and water economy, and tolerance to abiotic stress, pathogens and herbivores. Redman and others (2002) demonstrated phenomenal increases in heat tolerance. Waller and others (2005) showed that barley inoculated with an endophyte, *Piriformospora indica*, discovered in plants of the India Thar desert, exhibited enhanced productivity and tolerance to salt and pathogens.

Multiple benefits conferred by symbiotic microbes in agriculture are well documented, but their incorporation into mainstream agriculture has generally met with difficulties. The transfer of structurally integrated symbiotic fungi indigenous to native grasses and shrubs generally, but not uniformly, induced substantial and multiple benefits to recipient native grasses. These included enhanced vigor, biomass, seed production, and seedling vigor in the second generation. Indigenous fungi apparently have a major role in regulating performance of native host plants. Increased vigor, biomass, and reproductive potential conferred by these novel fungi provide attractive and alternative approaches to improving native grasses for the remediation of degraded arid rangelands.

Our findings indicate that once transferred to other non-host species, such as other native grasses or tomato, they become vertically transmitted or heritable units in their new hosts. Thus the benefits are transferred to succeeding generations. The conferral of multiple benefits described in this paper far exceeds any expectations resulting from gene transfers by conventional breeding or recombinant DNA. Fungal transfer via cell cultures is simple and the results are immediate in the first generation, compared to high inputs of capital, time, and technological skill required for breeding or recombinant DNA. Another advantage is that time tested plant and fungal materials adapted to

arid rangelands are recombined, resulting in synergistic plant performance. Plant genomes are not modified, for example BOER or SPAI remain genetically unchanged, but are enhanced by the integration of unique symbiotic fungi. This method may allow the preservation of these valuable, yet declining germplasms. Cell cultures of nine different native grasses and shrubs (Reyes-Vera, unpublished data) have been generated and each has transferable symbiotic fungi. Hundreds of native plant species adapted to these arid rangelands represent a vast untapped reservoir of genetic variability for improvement of both native and crop plants. This research has only scratched the surface of the potential of symbiotic microbes. More research is required to understand their value in improving native and crop plants in dynamic stressed ecosystems.

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