Examining Endophyte Interactions within Fourwing Saltbush (*Atriplex canescens*)

Mary E. Lucero[1], Jerry R. Barrow[1], Isaac Reyes-Vera[2], and Ruth Sedillo[1]

ABSTRACT

*Cryptic endophyte consortia associated with embryonic tissues of fourwing saltbush (*Atriplex canescens*) are believed to serve mutualistic functions. However, the complexity of these endophyte communities makes cause-and-effect relationships difficult to establish. In fourwing saltbush, cryptic endophytes are thought to enhance drought and salt tolerance, nutrient acquisition, germination, and establishment. Some of these traits can be conferred to alternate host plants by co-culturing seedlings in vitro with micropropagated fourwing saltbush callus. Here we describe an evolving process for; 1) identifying arrays of cryptic, seed borne endophytes associated with fourwing saltbush; 2) evaluating early vigor of grasses and other plants endowed with fourwing saltbush endophytes; 3) selecting plant-endophyte combinations with increased tolerance to abiotic stressors, and 4) monitoring the persistance of transferred endophytes across plant generations in increasingly complex habitats. This process is designed to evaluate specific plant-fungal interactions that influence host tolerance to salinity, but may be broadly adaptable for selecting endophyte-enhanced plant germplasms with a variety of desired traits.*

INTRODUCTION

The utility of fourwing saltbush and other *Atriplex* species for restoration, remediation, and forage combined with global distribution of the genus, halophyte physiology, and complex genetics have helped *Atriplex* to rank among the most widely studied native shrub species (Abeliovich and others 1994; Adair, Andrews and others 1992; Bu, Bai and others 2007; Cibils, Swift and others 1998; Glenn, Olsen and others 1994; Kadereit, Gotzek and others 2005; Karimi and Ungar 1989; Ortíz-Dorda, Martínez-Mora and others 2005; Ostyina, McKell and others 1984; Sanderson and McArthur 2004; Simon, Glenn, Pfister and others 1996; Ueckert and Petersen 1991). Yet many of the interesting traits attributed to *Atriplex* species, including drought and salt tolerance may be heavily influenced by internal fungal and bacterial endophytes (Barrow and Osuna 2002; Barrow, Osuna-Avila and others 2004). Experiments involving co-cultivation of endophyte-laden fourwing saltbush (*Atriplex canescens*) callus with non-host plants suggest cryptic endophytes associated with the plant can be transferred to diverse hosts, where they may increase vigor and establishment potential of the recipient plant (Barrow, Lucero and others 2006; Lucero, Barrow and others 2006; Lucero, Barrow and others 2006; Lucero, Barrow and others 2008). Because the calli used in these studies was derived from embryonic tissue, the putatively transferred endophytes are considered capable of being carried to plant progeny within seeds. Thus, fourwing saltbush (and other *Atriplex* species) may serve as diverse warehouses of valuable endophytes which can be transferred to other species to improve recipient plant tolerance to arid rangeland environments. Unlike the more thoroughly described clavipitaceous endophytes that confer drought tolerance to cool season grasses, fourwing saltbush endophytes are not known to produce toxic alkaloids. Thus, they are deemed less likely than clavipitaceous endophytes to confer negative traits to foreign hosts. Their association with a plant species that thrives in harsh environments suggests significant potential for use in revegetation of arid lands.

A difficulty associated with the study of all microbial communities is that their cryptic, dynamic nature makes cause and effect relationships difficult to establish. Many endophyte species cannot be isolated or cultured independently of host plants (Barrow, Lucero and others 2008). In addition, even under micropropagation, incredible diversity exists within endophyte communities (Lucero, Barrow and others 2008) (Thomas, Swarna and others 2008; Thomas, Swarna and others 2008). Specific markers, such as the novel alkaloids indicative of certain clavipitaceous endophytes, have not been described for most endophyte species known to associate with fourwing saltbush, so detection to date has relied on universal molecular and histochemical methods.

Although these methods are helpful for illustrating the presence or absence of endophytes, they do little to distinguish between endophyte species. Thus, efforts to reproducibly detect and monitor transferred endophytes are prone to interpretation errors. For example, denaturing gradient gel electrophoresis (DGGE) techniques reveal DNA banding patterns in treated and untreated plants, supporting the hypothesis that endophytes had transferred from source callus to recipient plants (Lucero, Barrow and others 2006). However, subsequent isolation and sequencing of the co-migrating bands revealed significant differences between bands, with some sequences representing polymorphic plant DNA rather than transferred endophytes (unpublished). Tempered by these and other varied results, we have evolved a complex, cyclical process for examining endophyte interactions influencing fourwing

In: Wambolt, C.L. et al. comps. 2011. Proceedings – Shrublands: wildlands and wildlife habitats; 2008 June 17-19; Bozeman, MT. NREI, volume XVI. S.J. and Jessie E. Quinney Natural Resources Research Library, Logan, Utah, USA.

[1]Mary E. Luceroa, Jerry R. Barrowa, and Ruth Sedilloa, USDA, Agricultural Research Service, Jornada Experimental Range, New Mexico State University

[2]Isaac Reyes-Vera, New Mexico State University.

saltbush biology (figure 1). In this proceedings we will summarize our process, describing various protocols we have developed or evaluated for 1) identifying arrays of cryptic, seed borne endophytes associated with fourwing saltbush 2) evaluating early vigor of grasses and other plants endowed with fourwing saltbush endophytes 3) selecting plant-endophyte combinations with increased tolerance to abiotic stressors, and 4) monitoring the persistance of transferred endophytes across plant generations in increasingly complex habitats. We will describe limitations to these techniques and emphasize the need for improved and complementary methods to monitor and evaluate endophytic microbes in complex habitats.

Identifying Arrays of Cryptic, Seed Borne Endophytes Associated With Fourwing Saltbush

Selection of Source Material

Endophyte communities associated with any plant material are remarkably complex, and may vary by both genotype and habitat (Arnold and Lutzoni 2007; Porras-Alfaro, Herrera and others 2008). Hence, determination of endophytes associated with the species should include representative samples from populations that are both geographically diverse and are associated with varied habitats. We have identified potential sampling populations by utilizing Ecological Site Descriptions (ESD's) to create a spreadsheet database of sites in which *Atriplex* species are listed as part of the vegetative community. Sorting and filtering functions were used within the spreadsheet to identify ecological sites with varied soil and climate features. In this way, fourwing saltbush can be sampled from sites that vary in exposure to environmental stressors such as salinity and drought, and endophytes associated with each population can be evaluated. Comparative phylogenetic analyses and other correlations between endophytes, host plant genotypes, and habitats will be used to reveal endophyte species which are most correlated with host plants exposed to osmotically stressed habitats.

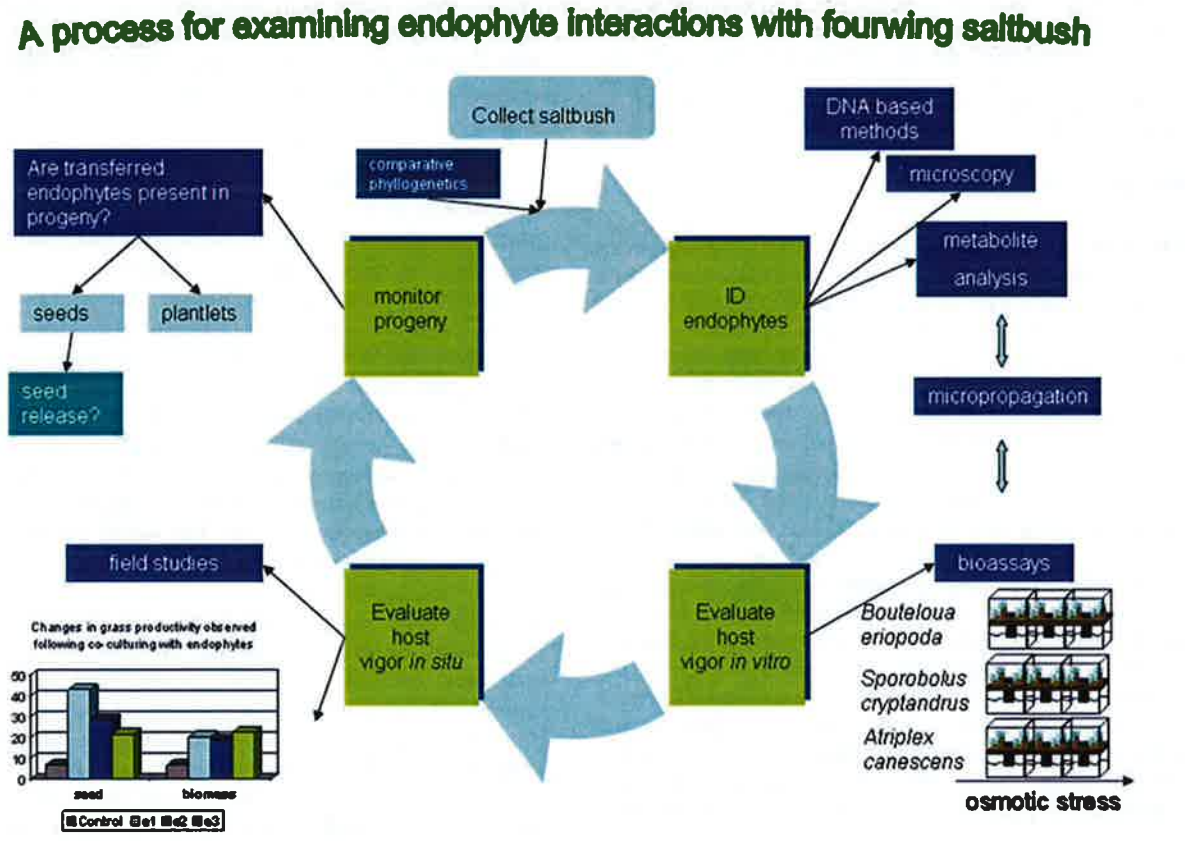

Figure 1—A multi-step process for examining interactions between various host plants and fourwing saltbush endophytes. Saltbush seeds harvested from well-described habitats provide starting material. Endophytes associated with these seeds are identified either as isolates or within aseptically germinated saltbush seedlings. Plant interactions with endophytes are first evaluated in vitro to minimize complexity of associated microbial populations. Shrubs and grasses inoculated with saltbush endophytes are exposed to osmotic stress, and endophytes that increase host stress tolerance are selected for greenhouse or field evaluations (in situ). Endophytes that improve host performance in field settings are examined for persistence in the progeny of inoculated plants.

Isolating Culturable Endophytes
Once source plants have been identified, harvested seeds from plants within each population serve as the source material for endophyte identification. Seeds are excised from the utricle and disinfested by soaking for a minimum of 30 minutes in a 1 percent solution of ZeroTol (BioSafe Systems, LLC.) in sterile water. Disinfested seeds are placed on petri-dishes containing malt extract agar, potato dextrose agar, or nutrient agar and incubated for 30 days. Endophytes detected growing on the agar are isolated, and DNA is extracted, amplified, cloned, and sequenced as previously described (Lucero, Barrow and others 2006). Additional aliquots of DNA are freeze dried for long term preservation so that additional gene regions can be amplified when necessary for more complete identification.

Isolating Unculturable Endophytes
Unculturable endophytes can be separated from the host plant by excising visible structures (usually hyphae) from plant tissues. Hyphae are often seen extruding from root surfaces of germinating seedlings, and are easily confused with root hairs. Sometimes, hyphae will also appear at the nodes between leaves and stems of micropropagated plants. Microdissection would be useful for extracting smaller fungal structures from plant tissues, such as hyphae surrounding stomata.

Optimizing Endophyte Detection
Fungal endophytes associated with fourwing saltbush can be detected using light microscopy with selective staining (Barrow and Aaltonen 2001; Barrow and Aaltonen 2003). However, this technique does not distinguish species, nor does it differentiate systemic fungi from those located only in a particular tissue, such as the roots. The addition of micropropagation to minimize interference from external microbes has provided an invaluable control, ensuring that only those microbes retained in embryonic tissues are detected (Barrow, Osuna-Avila and others 2004). Initiation of healthy micropropagated lines from embryos reduces the microbial community to highly endophytic species with potential for vertical transmission (Reyes-Vera 2008; Reyes-Vera, Potenza and others accepted).

Such cryptic endophyte communities are notoriously difficult to identify. Molecular techniques based on amplification and sequencing of conserved microbial gene sequences can provide a good starting point, but without specific primers, it is unlikely that all species present within a plant host will be detected (Lucero, Barrow and others 2008). Meanwhile, even within controlled, micropropagated lines, endophyte communities are complex and dynamic, such that species successfully detected at a given point in time may disappear from subsequent harvests. For this reason, newly established lines are considered to be more representative of natural populations, and combined detection and analysis techniques are most likely to provide informative results.

We continue to use PCR based methods in combination with micropropagation and microscopy to provide information about endophytes associated with fourwing saltbush. We are also exploring biochemical profiles of plants and isolated endophytes in the hopes of identifying chemical indicators of individual endophyte species.

Evaluating Early Vigor of Grasses and Other Plants Endowed With Fourwing Saltbush Endophytes

Micropropagation for Controlled Plant-Endophyte Interaction Bioassays
In addition to aiding identification of uncultured microbes, micropropagation has been valuable for exploring the physiological responses of plants to endophytes. Initial screening is accomplished by inoculating micropropagated plants with isolated endophytes, or with endophyte-laden callus (Lucero, Barrow and others 2006). These experiments have provided evidence that saltbush endophytes may improve establishment and reproductive potential of native grasses and of crop plants (Barrow and Lucero 2005; Barrow, Lucero and others 2006; Lucero, Barrow and others 2008). However, as noted above, detection and monitoring of mixed endophtye populations, even within micropropagated systems, remains problematic (Lucero, Barrow and others 2006; Lucero, Barrow and others 2008).

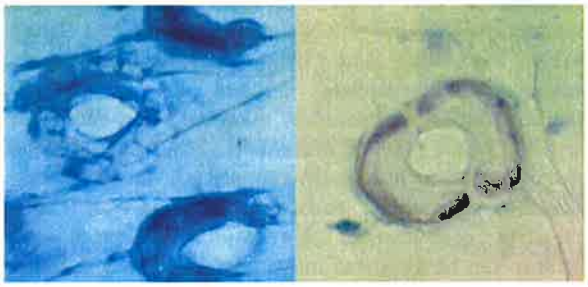

Figure 2—Leaf stoma of micropropagated *A. griffithsii* shoot cultures. The left image (untreated) contains blue staining fungal hyphae associated with guard cells of the stomatal complex. Treatment with the contact fungicide 26 GT® (Bayer Environmental Science) reduced endophyte colonization of stomata detectable with trypan blue (right image). 26 GT® contains the active ingredient iprodione (3-(*3, 5-dichlorophenyl)-N-(1-methylethyl)-2, 4-dioxo-1-imidazolidinecarboxamide*)), which inhibits the germination of fungal spores and mycelium.

Recently, treatment of micropropagated *A. griffithsii* shoot cultures with the contact fungicide 26 GT® (Bayer Environmental Science) has reduced endophyte colonization of stomata detectable with trypan blue (figure 2). 26 GT® contains the active ingredient iprodione (*3-(3, 5-dichlorophenyl)-N-(1-methylethyl)-2, 4-dioxo-1-imidazo lidinecarboxamide*)), which inhibits the germination of fungal spores and mycelium. It is not yet clear whether

repeated doses will eliminate all trypan blue staining structures associated plant tissues, or how such treatments will affect culture viability. However, this is a positive step towards the development of an endophyte-reduced line, which would provide an invaluable control for examining plant interactions with various endophytes.

Meanwhile, micropropagated systems with mixed endophyte consortia remain useful, if not ideal, for examining endophyte influences on host plant physiology. Both host plant genetics and abiotic environments are tightly controlled, and microbial variability is minimized. Hence, the influence of various endophytes on plant performance can be evaluated by inoculating host and non-host plants with either isolated endophytes or uncultured endophyte consortia retained in saltbush callus.

Selecting Plant-Endophyte Combinations With Increased Tolerance to Abiotic Stressors

Salt Tolerance Bioassays

The ability of fourwing saltbush to tolerate arid environments is due in part to its halophytic traits. Hence, saltbush endophytes likely to influence host plant tolerance to salt may be identified by screening plant-endophyte combinations for increased tolerance to high concentrations of mineral salts. We perform salt tolerance bioassays in culture tubes containing Murashigie and Skoog's medium supplemented with 0 – 3 percent Instant Ocean®, a commercially prepared mixed salt (Murashige and Skoog 1962). Inoculated and uninoculated seedlings or micropropagated plantlets cultured at varied salt concentrations are compared by measuring root and shoot areas and whole plant biomass. Differences can often be detected within as little as two weeks growth, facilitating evaluation of multiple endophyte combinations. Plant-endophyte combinations that exhibit increased salt tolerance can be evaluated microscopically for changes in endophyte distribution patterns, bladder cell formation, stomatal density, and other variables important for osmoregulation.

Both saltbush and native grass hosts have been utilized as endophyte recipients in these bioassays. Advantages to using grasses as recipients in bioassays include rapid germination, small plant size, ease of micropropagation, and freedom from intrinsic fourwing saltbush endophytes. Grasses are also useful for evaluating the potential of saltbush endophytes in alternate hosts, where the ability to confer salt tolerance could be valuable not only for native plant restoration, but also for agricultural production or biofuel development.

Alternate Bioassays

Although our efforts are currently focused on assays to screen for endophytes that improve host plant productivity against varied salinity gradients. Since many of the same biochemical pathways regulate other abiotic stressors, such as drought and heat stress responses, it is likely that selected plants will also exhibit tolerance to other arid land conditions. Hence, assays to specifically select for heat, drought, or cold tolerance are not being implemented at this time.

Instead, selected plants are monitored for differences in endophyte populations, and endophytes that appear to confer salt tolerance are monitored for persistence in plants as they are hardened and transferred from in-vitro to greenhouse propagation.

Monitoring the Persistence of Transferred Endophytes Across Plant Generations In Increasingly Complex Habitats

Co-cultivation of fourwing saltbush callus with native grasses has produced grass plants that exhibit high productivity in field trials (Lucero, Barrow and others 2008). However, questions regarding the nature of transferred endophytes and the degree to which they persist in novel hosts remain. Efforts to detect transferred endophytes with DNA analysis have provided variable results, suggesting transferred endophytes have either diminished over time, or have escaped detection in the presence of other microbes. Clearly, more robust monitoring methods are needed in order to evaluate the persistence of transferred microbes. Yet even methods that prove robust in laboratory trials may be prone to error in more complex greenhouse and field environments (Lueders and Friedrich 2003; Acinas, Sarma-Rupavtarm and others 2005). Therefore, validation of candidate markers for endophyte monitoring mandates availability of genetically similar control- and endophyte-enhanced plant materials cultivated in increasingly complex habitats.

To provide a series of varied, complex environments in which to evaluate the long term performance of grasses inoculated with endophytes from fourwing saltbush we are preparing a series of common garden plantings. Two black grama genotypes will be inoculated with saltbush endophytes as previously described (Lucero, Barrow and others 2008). DNA harvested from treated and untreated plants will be screened to evaluate changes in associated microbial communities. Replicate control and inoculated plants will be transplanted and maintained on three disturbed sites in southern New Mexico. Annual monitoring of transplants using a grid point-intercept method will be used to evaluate the establishment and productivity of treated and untreated plants. Seasonal changes in endophyte populations will be monitored using a combination of DNA based methods and microscopy. If planned biochemical analysis of saltbush endophtye communities reveals chemical indicators, such as alkaloids, that can be used to monitor endophyte presence, these will be included in subsequent monitoring.

CONCULSION

Although endophytes appear universally associated with higher plants, the difficulties associated with detection and monitoring have largely limited studies of endophyte influences on plant communities to those endophyte species that can be monitored with specific indicators, including unique alkaloids or known molecular markers (Hahn, Nickel and others 1999) (Bacon, Porter and others 1977; Barrow, Lucero and others 2008). Nonetheless these limited studies have clearly demonstrated profound effects endophytes can have on larger ecosystems (Clay and Schardl 2002; Bultman and Bell 2003; Rudgers, Koslow and others 2004; Clay, Holah and others 2005).

A variety of dark septate and amorphic fungal endophytes have been observed in apparently mutualistic association with fourwing saltbush (Barrow, Havstad and others 1997; Barrow and Aaltonen 2001; Barrow and Aaltonen 2001; Barrow and Osuna 2002; Barrow 2003; Barrow, Osuna-Avila and others 2004). These endophytes are worthy of in depth study because their beneficial association with a hardy, arid land forage shrub and their potential to confer desirable traits to alternate hosts may provide a variety of useful applications for restoration and production agriculture (Lucero, Barrow and others 2006; Lucero, Barrow and others 2008). The complexity of the saltbush endophyte community mandates implementation of a complex, multifaceted system for identification and characterization of those endophytes which contribute to plant hardiness.

Our combined approach of comparing endophytes from diverse, well defined saltbush habitats to identify plant- and habitat-specific correlations; developing biomarkers for monitoring specific endophytes; evaluating endophyte interactions with host and non-host plants in highly controlled, in-vitro bioassays; and examining endophyte persistence in habitats of varied complexity is expected to reveal important plant-endophyte interactions that can be utilized to improve establishment and productivity of a variety of plant species in arid lands.

ACKNOWLEDGEMENTS

This work was supported by funds appropriated to the USDA Agricultural Research Service Jornada Experimental Range and an interagency agreement with the Bureau of Land Management Las Cruces Field Office. We thank Jaime Midez for assistance with in vitro and greenhouse bioassays, Jason Dunwell for maintenance of micropropagated plant lines.

REFERENCES

Acinas, S.G., Sarma-Rupavtarm, R., Klepac-Ceraj, V., Polz, M.F. 2005. PCR-induced sequence artifacts and bias: Insights from comparison of two 16S rRNA clone libraries constructed from the same sample. Applied and Environmental Microbiology. 71(12):8966-8969.

Adair, L.S., Andrews, D.L., Cairney, J., Funkhouser, E.A., Newton, R.J, Aldon, E.F. 1992. Characterizing gene responses to drought stress in fourwing saltbush *Atriplex canescens* (Pursh.) Nutt. Journal of Range Management. 45:454-461.

Arnold, A.E., Lutzoni, F. 2007. Diversity and host range of foliar fungal endophytes: Are tropical leaves biodiversity hotspots? Ecology. 88(3): 541-549.

Bacon, C.W., Porter, J.K., Robbins, J.D., Luttrell, E.S. 1977. *Epichloe typhina* from toxic tall fescue grasses. Applied and Environmental Microbiology 34(5):576-581.

Barrow, J., Aaltonen, R. 2003. A staining method for systemic endophytic fungi in plants. Emerging Concepts in Plant Health Management. R.T. Lartey and Caesar A.J. Kerala, India, Research Signpost: 61-67.

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

Barrow, J.R. 2003. Atypical morphology of dark septate fungal root endophytes of Bouteloua in arid southwestern USA rangelands. Mycorrhiza 13(5):239.

Barrow, J.R, Aaltonen, R.E. 2001. Evaluation of the internal colonization of *Atriplex canescens* (Pursh) nutt. roots by dark septate fungi and the influence of host physiological activity. Mycorrhiza 11(4):199-205.

Barrow, J.R., Havstad, K.M., Hubstenberger, J., McCaslin, B.D., Barrow, J.R. 1997. Seed-borne fungal endophytes on fourwing saltbush, *Atriplex canescens*. Arid Soil Research and Rehabilitation 11(4):307.

Barrow, J.R., Lucero, M. 2005. Transfer and incorporation of heritable symbiotic fungi into non-host plants. Online: http://patents.com/us-7901927.html. Patent Application S.N. 11/500,702.

Barrow, J.R., Lucero, M.E., and Reyes-Vera, I. 2006. Symbiotic fungi that influence vigor, biomass and reproductive potential of native bunch grasses for remediation of degraded semiarid rangelands. Shrublands Under Fire: Disturbance and Recovery in a Changing World. Fourteenth Wildland Shrub Symposium, Cedar City, Utah, USDA, Rocky Mountain Research Station. Proceedings RMRS-P-52.

Barrow, J.R., Lucero, M.E., Reyes-Vera, I., Kavstad, K.M. 2008. Do symbiotic microbes have a role in plant evolution, performance and response to stress? Communicative & Integrative Biology 1(1):1-5.

Barrow, J.R., Osuna-Avila, P., and Reyes-Vera, I. 2004. Fungal endophytes intrinsically associated with micropropagated plants regenerated from native *Bouteloua eriopoda Torr.* and *Atriplex canescens* In Vitro-Cellular & Developmental Biology-Plant 40(6):608-612.

Barrow, J.R., Osuna, P. 2002. Phosphorus solubilization and uptake by dark septate fungi in fourwing saltbush, *Atriplex canescens* (Pursh) Nutt. Journal of Arid Environments 51(3):449-459.

Bu, Q., Bai, X., Wang, B. 2007. Accumulation and distribution of salt in leaves of *Atriplex triangularis* under salt stress. Chinese Journal of Applied and Environmental Biology 13(2):192.

Bultman, T., Bel, G. 2003. Interaction between fungal endophytes and environmental stressors influences plant resistance to insects. Oikos 103(1):182-190.

Cibils, A.F., Swift, D.M., McArthur, E.D. 1998. Plant Herbivore interactions in *Atriplex*: current state of knowledge. Rocky Mountain Research Station, Ogden, Utah, USDA Forest Service. RMRS-GTR-14.

Clay, K., Holah, J., Rudgers, J.A. 2005. Herbivores cause a rapid increase in hereditary symbiosis and alter plant community composition. Proceedings of the National Academy of Sciences of the United States of America 102(35):12465-12470.

Clay, K., Schardl, C. 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. American Naturalist 160 (4 SUPPL.):S99 – S127.

Glenn, E., Pfister, R., Brown, J.J., Thompson, T.L, O'Leary, J. 1996. Na and K accumulation and salt tolerance of *Atriplex canescens* (Chenopodiaceae) genotypes. American Journal of Botany 83(8):997.

Glenn, E.P., Olsen, M., Frye, R., Moore, D, Miyamoto, S. 1994. How much sodium accumulation is necessary for salt tolerance in subspecies of halophyte *Atriplex canescens*? Plant, Cell and Environment 17(6):711.

Hahn, D., Nickel, A., Dawson, J. 1999. Assessing *Frankia* populations in plants and soil using molecular methods. FEMS Microbiology Ecology 29(3):215-227.

Kadereit, G., Gotzek, D., Jacobs, S., Freitag, H. 2005. Origin and age of Australian Chenopodiaceae. Organisms Diversity and Evolution 5(1):59.

Karimi, S.H., Ungar, I.A., 1989. Development of epidermal salt hairs in *Atriplex triangularis* Willd. in response to salinity, light intensity, and aeration. Botanical Gazette 150(1):68-71.

Lucero, M., Barrow, J., Osuna-Avila, P., Reyes-Vera, I., Duke, S. 2008. Enhancing native grass productivity by co-cultivating with endophyte-laden calli. Rangeland Ecology and Management 61:124–130.

Lucero, M., Barrow, J.R., Sedillo, R., Osuna-Avila, P., Reyes-Vera, I. 2006. Genetic characterization of uncultured fungal endophytes from *Bouteloua eriopoda* and *Atriplex canescens*. Shrublands Under Fire: Disturbance and Recovery in a Changing World. Fourteenth Wildland Shrub Symposium, Cedar City, Utah, Rocky Mountain Research Station RMRS-P-52.

Lucero, M.E., Barrow, J.R., Osuna-Avila, P., Reyes-Vera, I. 2008. A cryptic microbial community persists within micropropagated *Bouteloua eriopoda* (Torr.) Torr. cultures. Plant Science 174:570-575.

Lucero, M.E., Barrow, J.R., Osuna-Avila, P., Reyes-Vera, I. 2006. Plant-fungal interactions in arid and semiarid ecosystems: large scale impacts from microscale processes. Journal of Arid Environments 65(2):276-284.

Lueders, T., Friedrich, M. 2003. Evaluation of PCR amplification bias by terminal restriction fragment length polymorphism analysis of small-subunit rRNA and mcrA genes by using defined template mixtures of methanogenic pure cultures and soil DNA extracts. Applied and Environmental Microbiology 69(1):320-326.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497.

NRCS, Natural Resource Conservation Service. (12 November 2003). Ecological Site Information System, USDA.

Ortiz-Dorda, J., Martínez-Mora, C., Correal, E., Simón, B., Cenis, J.L. 2005. Genetic structure of *Atriplex halimus* populations in the Mediterranean Basin. Annals of Botany 95(5): 827.

Ostyina, R.M., McKell, C.M., Malecheck, J.M., Van Epps, G.A. 1984. Potential of Atriplex and other chenopod shrubs for increasing range productivity and fall and winter grazing use. General Technical Report – USDA, Forest Service (INT-172):215.

Porras-Alfaro, A., Herrera, J., Sinsabaugh, R.L., Odenbach, K.J., Lowrey, T., Natvig, D.O. 2008. A novel root fungal consortium associated with a dominant desert grass. Applied and Environmental Microbiology: AEM.02769-07.

Reyes-Vera, I. 2008. Dissertation. Plant and Environmental Sciences. Las Cruces, New Mexico State University.

Reyes-Vera, I., Potenza, C., Barrow, J. 2008. Hyperhydricity reversal and clonal propagation of four-wing saltbush (*Atriplex canescens*, cultivated in vitro. Australian Journal of Botany. 56:358-362.

Rudgers, J.A., Koslow, J.M., Clay, K. 2004. Endophytic fungi alter relationships between diversity and ecosystem properties. Ecology Letters 7(1):42-51.

Sanderson, S.C., McArthur, E.D. 2004. Fourwing saltbush (*Atriplex canescens*) seed transfer zones. USDA, Forest Service, Rocky Mountain Research Station. General Techinical Report RMRS-GTR-125.

Simon, R.D., Abeliovich, A., Belkin, S. 1994. A novel terrestrial halophilic environment: The phylloplane of *Atriplex halimus*, a salt-excreting plant. FEMS Microbiology Ecology 14(2):99-109.

Thomas, P., Swarna, G., Patil, P., Rawal, R. 2008. Ubiquitous presence of normally non-culturable endophytic bacteria in field shoot-tips of banana and their gradual activation to quiescent cultivable form in tissue cultures. Plant Cell, Tissue and Organ Culture 93(1):39-54.

Thomas, P., Swarna, G., Roy, P., Patil, P. 2008. Identification of culturable and originally non-culturable endophytic bacteria isolated from shoot tip cultures of banana cv. Grand Naine. Plant Cell, Tissue and Organ Culture 93(1):55-63.

Ueckert, D.N., Petersen, J.L. 1991. Selecting *Atriplex canescens* for greater tolerance to competition. Journal of Range Management 44(3):220.