Influence of Mycorrhizal Source and Seeding Methods on Native Grass Species Grown in Soils from a Disturbed Site

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Abstract—Mycorrhizal fungi are crucial elements in native plant communities and restoring these fungi to disturbed sites is known to improve revegetation success. We tested the seedball method of plant dispersal for restoration of plants and mycorrhizal fungi to disturbed ecosystems. We tested the seedball method with a native mycorrhizal fungi inoculum, and a commercial inoculum. We found that the native culture and commercial inoculum were not viable sources of mycorrhizae.

The role of mycorrhizal fungi in ecological restoration has been a topic of great interest to scientists for more than two decades. Mycorrhizal fungi are known to aid plants in acquiring water and nutrients, most notably phosphorus, in exchange for carbohydrates and sugars provided by the plants through photosynthesis. This relationship is thought to benefit not only individual plants, but entire plant communities (Allen and others 1995; Francis & Read 1994; Francis & Read 1995). However, human activities which cause severe soil disturbance may result in the reduction or complete loss of mycorrhizal propagules from the soil (Allen & Allen 1980; Allen and others 1987; Reeves and others 1979). Several ecologists, including Allen and others (1987) and Reeves and others (1979), believe that successful plant community restoration may ultimately depend upon the re-establishment of this mycorrhizal relationship.

Unfortunately, few methods have been developed for re-introducing mycorrhizal fungi to disturbed soils. The method most typically employed involves inoculating nursery plants with the fungi prior to field planting. While this method has been shown highly successful in forest/shrub land restoration, planting nursery raised plants is expensive, labor intensive and impractical when attempting restoration of grass and forb dominated plant communities (i.e. tundra and grassland). Practical and affordable methods for restoring mycorrhizal plants to disturbed grassland and tundra environments are greatly lacking. In fact, we know of no published research investigating such methods. However, a method developed by an organic farmer in Japan may provide an excellent tool for restoring herbaceous mycorrhizal plants to disturbed landscapes.

For over fifty years, Masanobu Fukuoka, a Japanese scientist and farmer, has been practicing a method of sustainable organic farming which centers around the planting of seedballs (Bones 1996). Seedballs are simply a mixture of clay, soil humus, and plant seed rolled into small balls the size of deer droppings. Fukuoka combines seed from over 100 species of vegetables and fruits into these balls and scatters them by hand throughout his field. The clay provides a barrier against seed herbivory, re-dispersal of seeds by wind, and exposure to harsh environmental conditions. The soil humus provides an immediate source of mineral nutrients and soil micro-organisms. The purpose of including seed from a high number of plant species is to provide a soil seed bank from which any number of seeds may germinate in a given year depending upon current climatic conditions. Fukuoka's crop productivity has consistently rivaled or surpassed those of neighboring farmers employing more labor intensive and costly practices that utilize more traditional farming methods (fertilizer inputs, tilling, flood irrigation, etc.).

While seedballs have primarily been used for growing food crops, the concept is intriguing for use in native plant community restoration. By substituting native grass and forb seeds for agricultural crop seeds, land managers restoring disturbed areas with seedballs may benefit from increased ground cover and native plant diversity compared to seeding using traditional broadcasting methods. Seedballs may also be an effective means of restoring a much needed seed bank to severely disturbed soils. Furthermore, by incorporating mycorrhizal fungi into the seedball mixture, emerging seedlings will benefit from the increased water and nutrient uptake in disturbed soils supplied by the mycorrhizal fungi. These methods for restoring mycorrhizal grasses and forbs, however, have never been experimentally tested.

It is important to note that limited options for re-establishing mycorrhizal grasses and forbs to disturbed ecosystems is not the only problem facing restoration ecologists. A difference of opinion has recently surfaced over the best methods for developing mycorrhizal inoculum for restoration plants. The few companies known to us who engage in the production of mycorrhizal inoculum, utilize a method which involves promoting reproduction of arbuscular mycorrhizal (AM) fungi collected from a single

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source. One to a few fungal species are carefully selected and progeny from this "mother" culture are used to inoculate plants for different restoration sites. Inoculum developed from AM collected in the Pacific Northwest, therefore, may be used to inoculate plants for a restoration site in New Mexico. The fungal species bred for these bulk cultures are generally selected because they are aggressive root colonizers, are relatively easy to grow and are present in soils world-wide.

Bulk inoculum containing one to a few ubiquitous fungal species may not be the best approach to inoculating restoration plants. One argument is based upon the fact that different mycorrhizal fungi may be active on the same host plant at different times in the year (Allen and others 1995; Sanders & Fitter 1992; Siguenza and others 1996). Inoculum containing only one or two species, therefore, may provide little or no functional benefit to its host during certain times of the growing season or under variable environmental conditions. Another argument is that even though a given AM fungi species is ubiquitous, different genotypes probably exist which influence plant function differently depending on the biome from where it was collected. For example, Allen and others (1995) found that similar morphotypes of AM fungi collected from different sites confer different physiological benefits to the same plant species. These arguments have led us to ask the question: Will plants used in ecological restoration efforts derive greater benefit (increased growth and survival) from local AM fungi inoculum compared to non-local, commercial inoculum containing one or a few AM fungi species?

We addressed this question in a controlled greenhouse experiment while simultaneously investigating the seedball seeding method for seed germination and inoculation. We believe these methods, once perfected, have great promise for large scale application.

Methods

Greenhouse

Soils used for the greenhouse experiment were collected from several locations within a 200 acre Saltcedar (*Tamarix chinensis*) / Russian olive (*Elaeagnus angustifolia*) stand on Santa Ana Pueblo in Bernalillo, NM. The soil was collected from 27 random sites from an area of known low salinity. We sieved the soil through 2 mm sieves to remove organic debris. We sterilized the soil by microwaving it for 150 seconds per kilogram of soil. The soil reached mean temperatures of 97 °C (s.d. 10.7). One untreated, and one sterilized representative sample were sent to the SWAT Laboratory at New Mexico State University, Agronomy and Horticulture Department and analyzed for macro and micro nutrients, organic matter, pH, texture, and electro-conductivity (table 1).

The soils were placed in flat cedar boxes (15" x 20" x 12") in the Rocky Mountain Experiment Station greenhouse and seeded with three locally native grassland species according to the following treatments: 1) seedballs with cultured native AM fungi inoculum; 2) seedballs with commercial AM fungi inoculum; 3) seedballs with sterilized inoculum (control); and 4) broadcast seeding. We planted each box on July 30, 1997. The species of grass utilized were *Sporobolus crytandrus, Hilaria jamesii*, and *Bouteloua gracilis*. Each treatment was replicated five times, yielding twenty boxes (samples) total.

We made seedballs by combining specific quantities of clay, soil humus, plant seed and water, then rolling them into small balls the size of elk droppings. These quantities follow methods outlined by Harris (1996). In addition, mycorrhizal fungi from two sources were incorporated into the seedball mix. Seedballs in treatment 1 had native AM fungi inoculum cultured in Santa Ana greenhouse and seedballs in treatment 2 had commercial AM fungi inoculum purchased from a bulk producer and distributor. Methods for culturing indigenous AM fungi followed Menge (1984) and Morton (1996).

Bioassays

Plant contents from each sample were harvested when the majority of plants approached senescence on October 19, 1997. Upon harvest, individual plants were separated and species composites were formed. We collected 0.3 g of root segments from these composites to determine percent mycorrhizal root infection. In addition, oven dry weight measures of root biomass and shoot biomass were measured for each composite sample. Finally, portions of the dried shoots from each composite sample were ground and sent to the SWAT laboratory for tissue phosphorus and nitrogen analysis.

Table 1—Analyses of untreated, and sterilized soil samples for pH, electro-conductivity, nutrient content, and texture.

Test parameter	Untreated	Sterilized
pH	7.75	7.48
Electro-conductivity	2.20 mmhos/cm	2.55 mmhos/cm
Magnesium	2.40 meq/L	3.25 meq/L
Calcium	6.31 meq/L	8.44 meq/L
Sodium	12.05 meq/L	13.10 meq/L
Sodium Absorbtion ratio	5.77	5.42
Calculated Exchangeable NA pct-ESP	6.8	6.3
Organic material - pct	1.29	1.61
NO3-N 1:5 (soil:water) extract	1.1 ppm	1.1 ppm
Phosphorus (NaHCO3 extracted	17.6 ppm	8.1 ppm
K 1:5 (soil:water extract)	64 ppm	63 ppm
Texture of soil by feel	sand	sand

Table 2—Means and standard errors of percent AM fungi colonization, root biomass, shoot biomass, tissue N, tissue P for each of the treatments; control (sterilized inoculum), cultured native inoculum (native), commercial inoculum (commercial), and broadcast seeding. F-probability statistic shown for each variable. Letters reflect significant groupings by one way ANOVA analysis.

Treatment	Per. colonized	Root biomass	Shoot biomass
Control	0.40 (s.e. 0.30)	40.89 (s.e. 5.20)	35.8 (s.e. 3.6)
Native	0.99 (s.e. 0.38)	46.65 (s.e. 5.01)	43.9 (s.e. 4.2)
Commercial	4.51 (s.e. 2.14)	54.14 (s.e. 5.06)	38.4 (s.e. 2.7)
Broadcast	1.50 (s.e. 0.58)	56.89 (s.e. 2.62)	36.5 (s.e. 1.6)
F-prob	0.055	0.097	0.30

Treatment	Tissue N	Tissue P
Control	0.61 (s.e. 0.03) **C**	0.08 (s.e. 0.01)
Native	1.04 (s.e. 0.05) **A**	0.09 (s.e. 0.01)
Commercial	0.82 (s.e. 0.08) **B**	0.09 (s.e. 0.01)
Broadcast	0.58 (s.e. 0.04) **C**	0.09 (s.e. 0.01)
F-prob	0.0000	0.86

The 0.3 g root segments used for percent root infection were immediately placed in tissue cassettes and immersed in 50 percent ethanol solution. We cleared the roots prior to staining by alternating them between a 10 percent potassium hydroxide solution and a 1 percent hydrogen peroxide solution for approximately 50 hours or until each sample was observed to be cleared. The samples soaked in each solution for at least 1 hour and up to 10 hours before changing to the other solution. After clearing, we stained the roots in a 5 percent trypan blue solution in lactoglycerin. Fungal colonization of the roots was evaluated using the gridline intersection method described in Brundrett and others (1994).

Samples of the commercial inoculum, cultured inoculum, and microwaved soil were sent to Joe Morton's lab at West Virginia University in Morgantown, WV for an infectivity assay. The assay determined the presence, quantity and identity of mycorrhizal spores by utilizing a mean infection percentage assay (Morton, 1996).

Results

Treatment Effects

A one-way ANOVA test was performed for analysis of treatment effects on each of the following variables; percent of root length colonized by arbuscular-mycorrhizal (AM) fungi, root biomass, shoot biomass, tissue nitrogen, and tissue phosphorus. The treatment effects were analyzed across all species (table 2).

The commercial inoculum treatment resulted in relatively high percent AM colonization, but there were no significant treatment effects. The values obtained for percent AM colonization were so low as to be negligible in all treatments.

No significant treatment effects were recorded for root or shoot biomass.

There were significant treatment effects for the plant tissue nitrogen. The highest accumulation occurred in the native inoculum treatment followed by the commercial inoculum treatment. The control treatment and the broadcast treatment were not significantly different. The tissue phosphorus values did not differ significantly between the treatments.

Species Effects

A T-Test was used to test for species effect since the *H. jamesii* plants were inadvertently removed from the boxes during the experiment. Plant tissue phosphorus was significantly greater in the *S. crytandrus* than in the *B. gracilis*, but there were no significant species effects for plant tissue nitrogen (table 3). Other analyses were not performed due to missing data. All tests were conducted on Statistical Software for the Social Sciences (SPSS 5.0).

Infectivity

The infectivity assays for the cultured inoculum, the commercial inoculum and the microwaved soil all had the same result: there were no viable spores in any of the samples.

Discussion

In this experiment we attempted to test two new techniques for restoring mycorrhizal plants to disturbed areas, the seedball method of dispersal, and utilizing either native

Table 3—Means and standard errors for tissue N and tissue P for two of the grass species, *Bouteloua gracilis* and *Sporobolus crytandrus*. F- probability statistic is shown for each variable. Letters reflect significant groupings by T-Test.

Species	Tissue N	Tissue P
Bouteloua gracilis	0.73 (s.e. 0.06)	0.08 (s.e. 0.00) **B**
Sporobolus crytandrus	0.74 (s.e. 0.052)	0.10 (s.e. 0.00) **A**
F-prob	0.91	0.002

or commercial AM fungi inoculum. Both of these techniques have great potential, and further work may address the complications we encountered in this experiment.

Seedball Method

We made seedballs with a commercially available red pottery clay substrate. None of the seedballs dissolved completely during watering in the greenhouse though the boxes were thoroughly soaked daily. We attribute this to adding too much clay to the seedball mixture. The plants that grew in the seedball treatments sprouted directly out of the seedballs but only the seeds on the periphery of the seedballs germinated. Although there was no significant difference in biomass between treatments, we observed that grasses from the seedball treatments were substantially larger in size than the broadcast treatments. The observed size difference between plants in seedball and broadcast treatments is attributed to fewer numbers of individuals in the seedball treatments and greater competition for nutrients in the crowded broadcast treatment samples.

AM Fungi Inoculum

The question of using an AM fungi inoculum in the seedball is complicated. Although we found significant treatment effects in the tissue nitrogen analysis, the infectivity assay results showed that our inoculums, both cultured and commercial, had no viable spores.

AM fungi propagates by two methods, spores and hyphae. The spores are the sexually produced propagules. Hyphae are the "body" of the fungi and penetrate the root cortex cells of the host plant and extend out into the rhizosphere. Often hyphae will extend from one host plant's roots to the roots of another neighboring plant. By this "vegetative" means, the hyphae of a single fungi are spread throughout a community.

We attempted to infect our experimental grasses with AM fungi spores harvested from the native culture we generated in the Santa Ana Greenhouse, and from AM fungi spores we ordered from a commercial supplier. Both of these sources were later found to be unreliable for viable spores. We checked the roots of the native culture host plants for percent of root length colonized by AM fungi prior to the experiment and found them to be highly mycorrhizal (mean 45.7 percent). So why were there no viable spores in the native inoculum? It has been hypothesized that sporulation will not occur for an individual AM fungi until root colonization reaches values of 30- 40 percent of root length (Morton 1998, personal comm.). Southwestern AM-fungi has high levels of diversity, and although the root length colonized in the native culture host plants appeared to be high enough to have sporulation, there were no spores. This phenomenon may be explained by the presence of more than one species of AM fungi colonizing the roots although it is not possible to identify AM species without spores. Multiple species occupying the same host plant has been observed in other studies (Allen and others 1995; Sanders & Fitter 1992; Siguenza and others 1996). In our culture host plants, one species of AM fungi may be colonizing 20 percent of the root length, another may be colonizing 10 percent, while a third species may occupy the remaining 15 percent. It is nearly impossible to distinguish between species of AM fungi in this type of assessment. So what we thought was extensive colonization by one species of AM fungi may have been 2 to 3 different species colonizing the same plant. Given this hypothesis, AM fungi in the Southwest would rely more on hyphal propagation than sporulation due to the high diversity of AM fungi available for colonization. Future attempts to inoculate restoration plants using seedballs may require relying upon AM hyphae by mixing segments of the roots of the native culture host plants into the seedballs, rather than relying upon spore production. The hyphae within these root segments, therefore, will then colonize the roots of target plants "vegetatively."

We returned the commercial inoculum for a refund. Repeated analyses of various commercial inoculums have shown similar results. Commercial sources of mycorrhizal inoculum should be tested for viable propagules prior to experimental or practical application.

The differences we observed in our experimental treatments between tissue phosphorus were probably not due to mycorrhizal effects, evidenced by the extremely low values of root colonization. They may be attributed to competitive effects related to the clumped dispersal of the plants emerging from the seedballs.

We still believe that seedballs can be excellent method of seed dispersal for restoration projects. Incorporating root segments of the native culture host plants containing hyphae into the seedballs may accomplish both goals of restoring not only the seeds of native plants, but the native AM fungi that have been removed from a disturbed area.

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