Field Applications of AM Fungi for Restoration of Oil Brine Spill Sites

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Abstract

Virtually of the world's terrestrial plant species require mycorrhizal fungi for survival, growth, and reproduction. These fungi are an extension of a plant's root system, granting access to water and nutrients otherwise not available to the plant host. These unseen organisms play more than a supportive role in ecosystems; they are directly responsible for nutrient cycling and development of soil structure. This belowground aspect of ecosystems has been overlooked in the development and implementation of most restoration strategies. Restoration of ecosystems will require restoration of the belowground community, particularly where there is little or no organic matter in the topsoil. Mycorrhizal fungal tolerance to contamination varies; however, many researchers have shown strain level differences in performance of these fungi with respect to environmental stressors such as salinity, pH, and heavy metal contaminants in soils. For this study we have hydroseeded several vascular plant species on an oil brine spill site and inoculated them with rhizosphere fungi. We will quantify the survival, growth and fecundity of plants through the growing season. Preliminary results suggest that AM fungi improved the number of seedlings establishing on the spill site.

1.0 Introduction

Drilling and production of petroleum result in a large quantity of waste, including oil brine. Inappropriate disposal and accidental spills of this waste have occurred. The resulting brine-contaminated sites require restoration. Restoration primarily involves revegetation and natural attenuation of salt because no economical method exists to reduce soil salinity. Vegetation improves soil surficial chemistry, structure, organic content, water capacity, thermal regime, humidity, and leaching of salt.

Extensive revegetation of brine-contaminated soil may rely on the presence of mycorrhizal fungi. Mycorrhizal fungi function as an extension of a plant's root system, granting access to water and nutrients otherwise not available to the plant host. This belowground aspect of ecosystems has been overlooked in the development and implementation of most restoration strategies. These unseen organisms play more than a supportive role in ecosystems; they are directly responsible for nutrient cycling and development of soil structure.

Several researchers have shown that arbuscular mycorrhizal (AM) fungi can alleviate some of the plant stresses that will likely be encountered in an oil brine spill, although responses appear to be species/strain specific. Various researchers have shown that AM fungi improve drought tolerance and increase growth of plants under saline conditions. These researchers have also shown that overall water use efficiency, transpiration, carbon dioxide exchange rate, and stomatal conductance were higher in mycorrhizal plants than in non-mycorrhizal controls. Some species/strains of AM fungi collected from saline soils improved growth of plants grown in saline soils where other species strains from non-saline soils did not. This demonstrates a potential for adaptation by the fungi to a saline environment.

A number of vascular plant species identified as salt-tolerant have been shown independently to tolerate petroleum (e.g. *Cynodon dactylon* and *Lolium perenne*) and are currently being used in our ongoing greenhouse studies. Both *C. dactylon* and *L. perenne* are believed to routinely form mycorrhizae with AM fungi in nature. Accordingly these plants with appropriate AM fungi may be used to revegetate brine spill sites.

Our objectives were to quantify the survival and growth of plant species inoculated with selected rhizosphere fungi when grown on an oil brine contaminated spill site. We also tracked the fate of specific fungal strains introduced on an oil brine spill site through a growing season to determine their fate on these contaminated sites. The ultimate goal of this project is to economically restore oil brine spill sites *via* re-establishing self-sustaining plant communities.

2.0 Methods

Soil samples containing rhizosphere fungi were collected from regenerating spill areas. (See Colgan and Vavrek 2003 for full details). Two cultures from a wellhead near Ranger Texas (Ranger 1 and 2), a culture from a seasonally flooded pitcher plant bog (ABFR), and one from a regenerating brine spill site in Smackover AR (Smackover 6) were selected for field trials. Trap cultures were maintained in the Louisiana Tech University greenhouses. We used a simple aeroponic technique based on that developed by Jarstfer and Sylvia (1994). Sheared roots and soil from greenhouse trap cultures was used to inoculate Sudan grass seedlings germinated through polyester batting grown under fluorescent lights. Large ice chests served as watertight 2' x by 3' x 18" deep chambers and large air stones attached to 1/4 inch tubing from the compressed air system continuously misted the roots of inoculated seedlings with a nutrient solution. Inoculated seedlings were planted through polyester plugs in the tops of the chambers, allowing roots and associated fungi to hang down into the nutrient spray. This technique did not work well for our system and did not produce sufficient quantities of inoculum.

To produce sufficient quantities for field application, we divided the trap culture root balls into four equal parts and added fresh sterile sand to new 3.75L pots. Each pot was over seeded with 75 to 100 Sudan grass seeds and maintained in the greenhouse. This process was repeated several times to propagate fungi in the roots. Twenty-five to 30g of sheared roots and adherent soil served as inoculum for field plots. Vaminoc G^{tm}, a commercially available inoculum, was obtained from Becker-Underwood and applied at double the recommended rate based on recommendations from Becker-Underwood representative (Larry Butler pers. com.).

Two sites near Jena LA were selected with assistance from Justiss Oil Company. Both sites have a history of repeated oil and oil brine discharges. Field sites were tilled with a small disk cultivator towed behind a 4 wheeler to a depth of 2 to 3 inches to break crust. Lime was applied at a rate of 420lb./acre based on previous soil analysis (Vavrek and Colgan 2003).

Figure 2.1. *One of two field sites selected for our study, near Jena LA. Notice the almost complete lack of vegetation in foreground.*

Twenty-four 29.25 m^2 circular plots were delineated; 12 on each spill site. Plots were separated by at least one meter to control cross-plot contamination. The circular plot shape reduced the perimeter to area ratio and simplified spray application. One full hydroseed tank mixture was sufficient for two plots. The hydroseeding mixture was applied with a Turbo Turf HS-50 hydroseeding system (Turbo Technologies, Inc.) in two layers. Mulch, inoculum, and tackifier were applied in the first layer, and the hydroseeder tank pump and hose were cleaned with a 10% bleach solution between each treatment. Seed, fertilizer, mulch, and tackifier were sprayed in the top layer of all plots. The basic spray mixture contained 190 L of water, seed of five species (Table 1), tackifier (Turbo Tack, Turbo Technologies, Inc.; 17.7 g per 190 L), and cellulose mulch. The cellulose mulch was made from recycled newsprint (paper mulch, Applegate Environmental) and was added at 3.7 kg per 190 L of water as recommended by the manufacturer. Since the site was relatively remote, an electric generator and submersible pump were used to fill the hydroseeder from a small creek. The first application of the hydroseed mix began in late February; Site Two was seeded in early March 2004. A second application to Site One was required and was performed in early April. To control erosion, hay was applied on top of the mix to a depth of 1 to 2 cm. No additional water was provided to the plots.

Table 2.1. Plant species used in a hydroseed mix applied to an oil brine spill site in central Louisiana. Application rates are standard for surfaces lacking vegetation.

Species	Common name	Application rate
Festuca arundinacea Schreb.	Tall fescue	3.60 kg ha^{-1}
Panicum virgatum L.	Switchgrass	1.26 kg ha^{-1}
Cynodon dactylon (L.) Pers.	Common bermuda	0.72 kg ha^{-1}
Lolium perenne L.	Ryegrass	4.32 kg ha^{-1}
Trifolium repens L.	White clover	0.72 kg ha^{-1}

Plant species were selected based on their tolerance to oil and salt exposure, and their presence in Louisiana. The species mix included a warm and cool season grass species, which expands the period of productivity for the regenerating vegetation. Planting a mix of species also improves vegetative structure and persistence.

DNA of rhizosphere fungi was isolated from root zones of trap culture plants as inoculum was being prepared, and from plants growing on the site 10 weeks after hydroseeding. Fungal DNA was amplified using protocols from Colgan and Vavrek (2003). A combination of PCR, cloning, and sequencing of amplicons to confirm the identity of fungi in the rhizosphere was used to track the fate of the fungi introduced via hydroseeding. Percent cover was measured at 10 weeks post hydroseeding using a 1M square point intercept table. Aboveground biomass was estimated via three harvesting and three random 0.14M square areas within each plot.

A two way fixed effects analysis of variance was preformed to test for differences in cover between inoculum sources and site. The normality of the data was assessed with normal quartile plots and transformed as necessary.

3.0 Results and Discussion

Brine spill sites are exceptionally harsh and heterogeneous. On Site One, all or part of the study area was inundated by floodwaters in late March, and several plots were damaged by equipment movement. These events made it necessary to reinstall the study area in April 2004.

The resulting vegetative cover data are highly variable, ranging from zero to almost 30% cover 10 weeks after hydroseeding; aboveground biomass data was equally variable (see Figs. 3.1, 3.2). The high variability made it impossible to determine differences between treatments. However, site significantly influenced biomass ($p<0.05$).

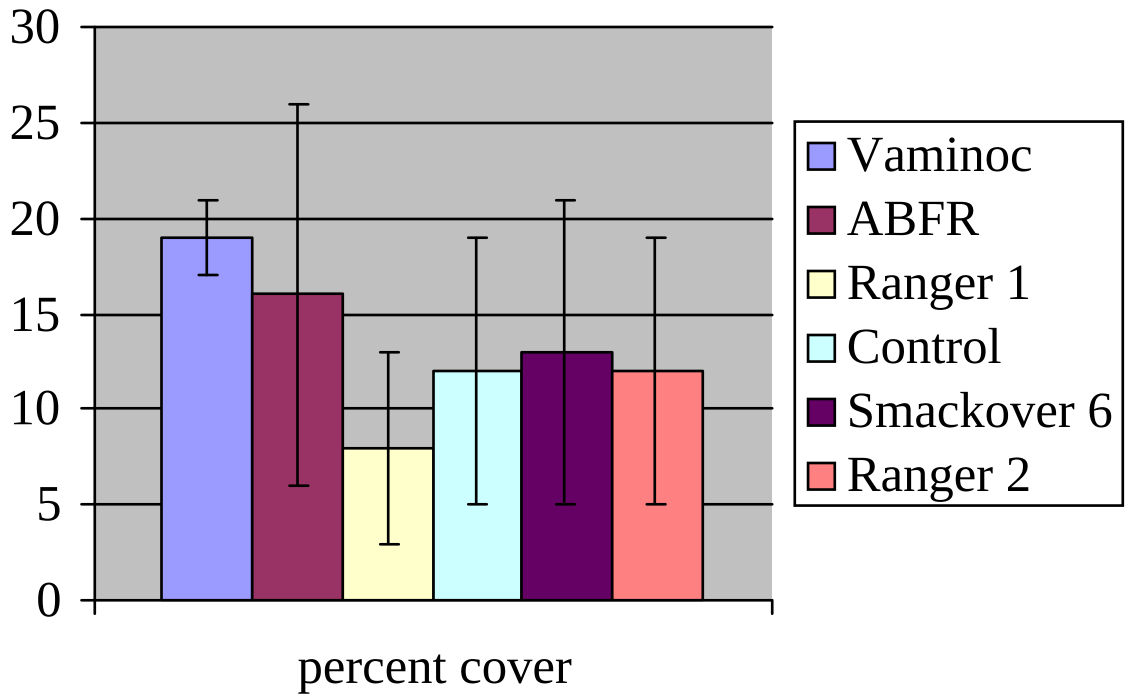

Figure 3.1 *Percent cover per square meter; bars represent the standard error of the mean. One control plot determined to be an outlie rvia Mahalonobis distances was excluded from the analysis.*

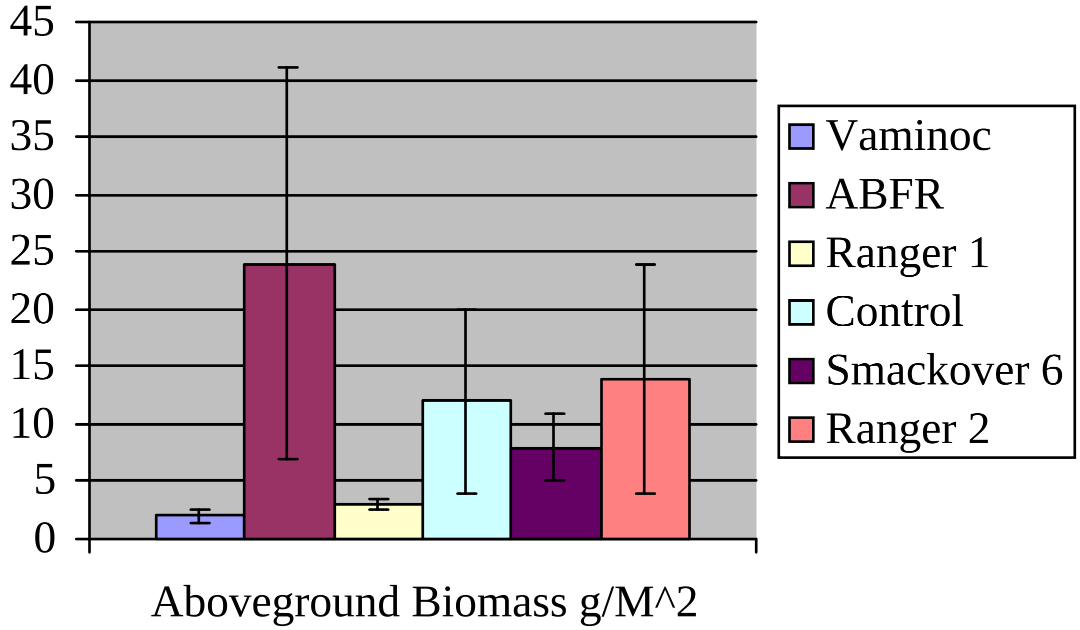

Figure 3.2 *Dry weight biomass in gram per square meter by treatment type; bars represent the standard error of the mean. One control plot determined to be an outlier via Mahalonobis distances was excluded from the analysis.*

Thus far we have characterized the fungal communities found in the rhizosphere of the trap cultures using phylogenetic analysis techniques and BLAST searches of the NCBI database. These techniques have allowed us to identify several fungi in the rhizosphere that would be beneficial to remediation of an oil or oil brine spill. The overwhelming majority of fungi are members of the Ascomycota (Fig 3.3). One of the better performing inoculum sources, (designated ABFR in this study) from greenhouse study (Colgan and Vavrek 2003), included AM mycorrhizal fungi that matched closest to members of the genus *Auclospora*. To our knowledge, this genus is not currently being used in commercial inoculum blends and deserves further study. Our field trial with this inoculum source produced fewer but larger plants, suggesting that low density or unequal distribution of inoculum on the plot may have occurred. Ongoing studies are underway to confirm or refute this hypothesis. One of the ascomycetes found was matched closely with the genus *Monascus*. This genus of fungi has been shown to degrade hydrocarbons and is responsible for clogging fuel filters in jet aircraft (Alexopolos et al. 1996). The role of this fungus in the rhizosphere of plants growing on oil and oil brine sites also deserves further study.

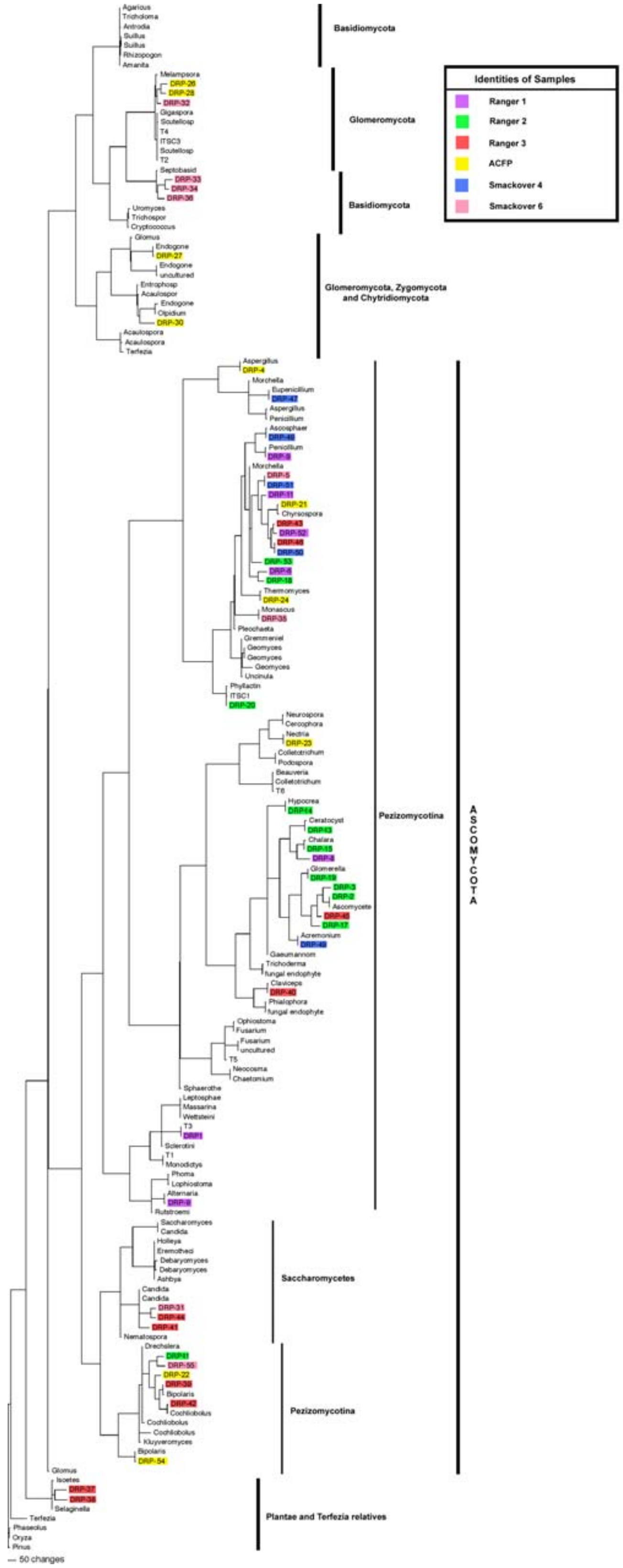

Figure 3.3 *A phylogram of fungi found in the rhizosphere of Sudan grass grown in soils from naturally regenerating oil and oil brine spill sites.*

4.0 Conclusions

Accidental oil brine spills are widespread in Louisiana. Historically these spill sites have been difficult to restore because of the multiple plant stresses associated with the spills as well as the degraded soil environment. Greenhouse studies have suggested that augmenting soils with symbiotic fungi improves plant performance. Our current data from field application of seed and fungal inoculum are equivocal.

Our study plots inoculated with the ABFR inoculum had fewer but bigger plants. The commercially available Vaminoc G^{TM} may improve the number of seedlings establishing. Several reasons may explain the differences between the greenhouse and field studies. Inoculum density and distribution may be adversely affected by hydroseeding. A finer grained inoculum than we could produce may improve homogeneity in the hydroseed mix. Environmental heterogeneity also makes detection of differences difficult. Severely disturbed sites such as ours may also require additional amendments (calcium, gypsum, organic matter, etc.) before they can support plant establishment.

We will continue to monitor these plots and re-evaluate the productivity at the end of the growing season. We will also continue to track the fate of introduced fungi in the field using molecular tools. Maintenance of trap cultures will continue, and we will attempt to collaborate with other experts to improve inoculum production. Additionally the use of inoculated plug stock, hydrosprigging, and fiber roll technology deserves further consideration to help elucidate the role of these fungi in contaminated soil.

Since fungi are ubiquitous, amending soils with AM fungal inoculum to enhance revegetation and soil structure is applicable to all habitats within Louisiana. In addition, hydroseeding provides a rapid, non-invasive, cost-effective technique to sow seeds and add spores, mulch, fertilizer and water. Therefore, by addressing some of the applied and fundamental questions of biotic interaction in these sites and providing limiting resources, our results can lead to a novel, effective methodology to restore oil brine spill sites.

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