

Strain Selection of AM Fungi for Restoration of Oil Brine Spill Sites

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Abstract

The vast majority of land plants form symbiotic relationships with soil fungi. These fungi are an extension of the plant's root system, granting access to water and nutrients otherwise not available to the plant host. To help understand the role these fungi play in the restoration of oil brine contaminated soils, a greenhouse experiment was established. Soils from naturally regenerating spill sites were collected, and trap cultures were established to produce inoculum. Rye and Bermuda grass were inoculated and then grown in ebb and flow tables with oil brine diluted to 16Dsm/m². Rye grass performed the best under our greenhouse conditions. Bermuda grass had very high mortality across most treatments. Overall, inoculation improved total rye biomass compared to control. Vaminoc G (a commercial AM fungal inoculant) and an inoculant produced using soil samples from Ranger, Texas improved rye biomass significantly compared to control. This data supports the hypotheses that introducing symbiotic soil fungi improves plant performance in contaminated soils. This technique should be considered when developing a restoration plan for remediation of a brine spill site.

1.0 Introduction

More than 90% of the world's terrestrial plant species require mycorrhizal fungi for survival, growth, and reproduction (Trappe 1962). These fungi are an extension of the 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 (see Miller and Jastrow 1992 for a review). These unseen organisms play more than a supportive role in ecosystems; they are directly responsible for nutrient cycling and development of soil structure (Jastrow and Miller 1991). Mycorrhizal fungi are the direct link between the photosynthetic producers in these systems and the rest of the soil food web.

Drilling and production of petroleum result in a large quantity of waste, including brine (Reis 1992; Gevertz et al. 2000). Inappropriate historical disposal and accidental spills have also occurred. Bass (1999) reported 567 accidental brine spills (presumably not limited to oil brine) in Louisiana between 1990 and the first half of 1998 based on data from the LA DEQ. The resulting brine-contaminated sites require restoration. Because no economical method exists to reduce soil salinity, restoration primarily involves revegetation and natural attenuation of salt. Vegetation improves soil surficial chemistry, structure, organic content, water capacity, thermal regime, humidity, and leaching of salt (Evans and Young 1970; Oke 1978; Hopkins et al. 1987, 1991).

Research has been performed on plant species' tolerance to salt and revegetation of saline sites (Szabolcs 1974; Vander Pluym et al. 1981; Marcum and Murdoch 1990; Dagar 1998). A range of species, varieties, and genotypes adapted to saline soils have been identified (e.g. DePew 1998; Seliskar and Gallagher 2000) as well as mechanisms providing salt tolerance (e.g. Mozafar and Goodin 1970; Flowers et al. 1977; Greenway and Munns 1980; Cheeseman 1988; Marcum 1999). Several plant species have been selected and are currently being screened under various salt/oil/brine concentrations in a controlled greenhouse study (Co-PI, PI, current OSRADP funded project). The outcome of this study is discussed below.

A number of vascular plant species identified as salt-tolerant have been independently shown to tolerate petroleum. These species are currently being used in our ongoing greenhouse study: *Cynodon dactylon* (salt: Marcum and Murdoch 1990; oil: Banks et al. 2000); and *Lolium perenne* (salt: Ashraf et al. 1986; oil Banks et al. 2000). Others identified by the Co-PI's literature review include *Distichlis stricta*, *Lotus corniculatus* and *Panicum virgatum*. All of these plant species are considered AM plants.

Little work has been done on the role of mycorrhizal fungi in restoration of brine spills. 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. Allen and Boosalis (1983) showed that *Glomus fasciculatus* would improve drought tolerance of winter wheat while *Glomus mosseae* would not. Poss et al. (1985) grew onion and tomato under various salt concentrations with and without fertilizers. They demonstrated that *Glomus fasciculatus* and *G. mosseae* both increased growth of plants under saline conditions, via increased water potential in tomato and increased K nutrition in onion

species. Mechanisms other than mineral nutrition were implicated by Ruiz-lozano et al. (1996). They looked at salt stress in lettuce (*Lactuca sativa*) and found that non-mycorrhizal plants had 35% lower shoot weight than mycorrhizal plants. Overall water-use efficiency, transpiration, carbon dioxide exchange rate, and stomatal conductance were higher in mycorrhizal plants than in non-mycorrhizal controls. At 5g NaCl kg⁻¹, water use efficiency and photosynthetic rates were 100% higher than in non-mycorrhizal plants. The experiment demonstrated that the effect of mycorrhizal fungi on their plant hosts was a system-wide phenomenon that altered the overall metabolism of the plant. Pond et al. (1984) found that some species/strains of *Glomus* collected from saline soils improved growth of tomato grown in saline soils where *Glomus* species/ strains from non-saline soils did not. This demonstrates the potential for adaptation by the fungi to a saline environment.

A number of studies have shown AM fungi to be critical players in redeveloping soil structure after oil spills. Arbuscular mycorrhizae, for example, have been shown to improve survivorship, and above- and belowground mass of *Elaeagnus commutata* and *Shepherdia canadensis* grown on oil sand tailings (Visser et al. 1991). Similar results were found with *Medicago sativa* grown in hydrocarbon-polluted substrate (Cabello, 1999). This same study showed that propagules of AM fungi were reduced in petroleum contaminated soils from Argentina and Germany, and strongly suggested that low propagule numbers were a limiting factor in restoring these sites.

Soils at oil brine spill sites often include metals as well as hydrocarbons and salts (Reis 1992; Gevertz et al. 2000). A number of studies have shown that mycorrhizal fungi help their plant hosts resist metals and other toxic substances. Sharples et al. (2000, 2001) have shown that the mycorrhizal fungus *Hymenoscyphus ericae* is essential to the restoration of mine spoils in the UK. Populations of this fungus have adapted to the high arsenate levels present in these soils and act as "filters," reducing arsenate accumulation in their host plants. This trend has also been shown in fungi found on soils naturally high in heavy metals. Genetic markers for tolerance were documented in the mycorrhizal fungus *Cenocccum geophilum* populations found on serpentine soils (Panaccioni et al. 2001).

The ultimate goal of this project is to economically restore oil brine spill sites by reestablishing self-sustaining plant communities. Specifically, our objectives are to:

- 1) characterize and identify specific strains of vascular plants and AM mycorrhizal and other rhizosphere fungi tolerant to oil brine
- 2) quantify the survival and growth of identified tolerant plant species when grown in saline and oil brine-contaminated soils with potential AM fungal strains

2.0 Methods

Fungi were isolated from soils of several natural habitats and naturally regenerating brine sites. Three soil samples from an oil well near Ranger, Texas; soil samples from a seasonally flooded pitcher plant/long leaf pine stand from south Louisiana; five soil samples from the rhizosphere of regenerating grasses growing on brine and oil contaminated soils from Smackover, AR; and one sample from Jena, LA were collected. The samples were placed in ziploc bags and kept cool until they reached the LA Tech greenhouse. Two types of commercially available AM inoculum were also tested: Vaminoc G from Becker Underwood (Harwood industrial estate, Harwood road, Littleham, West Sussex, UK, BN 177 AU), and AM 120 from Reforestation Technologies, 1324 Dayton Street, Salinas CA 93901.

Approximately 600ml of collected soil and roots were mixed with equal volumes of steam-sterilized quartz sand into sterilized 15cm greenhouse containers. Sudan Grass (*Sorghum vulgahare var. sudanense*) was seeded into these pots to serve as a trap plant that would be colonized by the AM and other fungi in the sample. Sudan grass is an excellent host for a wide range of AM fungi. The grass grows rapidly and has an extensive root system that is easily colonized by the fungi. These trap cultures were maintained for 12 weeks in the greenhouse to allow AM and other fungi to colonize the root system. The trap cultures were harvested, and the roots, spores, and hyphal fragments were used to inoculate rye grass (*Lolium perenne* L.) and common Bermuda (*Cynodon dactylon* (L.) Pers.). One part trap culture inoculum was mixed with two parts ProMix (peat moss and vermiculite) and placed into cone-tainers. Vaminoc and AM 120 inoculants were mixed at manufacturers' suggested rates with the top 2" of ProMix in the cone-tainers. Seedlings planted in plain ProMix served as non-inoculated controls.

After six weeks, one seedling of each plant species was transplanted into 26 L containers on an ebb and flow system in the Louisiana Tech greenhouse (N= 10). The soil was typical north Louisiana pine forest soil, mixed with one part coarse perlite to five parts soil. At the time of planting, three tablespoons of 13-13-13 Osmocote were mixed into the pots. All ebb and flow chambers were completely randomized for control of environmental heterogeneity within the greenhouse. One week after planting, ebb and flow systems were flooded with oil brine diluted to an electrical conductivity of 16-17ds/m. Ebb and flow systems were then flooded once per week for 20 weeks. Surviving plants were harvested at the end of 20 weeks. Root and shoots were measured to the nearest centimeter, then dried and weighed to the nearest 0.10g.

Plant roots were harvested for analysis of rhizosphere fungi at three points during the study: (1.) trap culture roots and soil were harvested when *Lolium* and *Cynodon* seeds were started, (2.) *Lolium* and *Cynodon* roots were harvested from three randomly selected seedlings when the seedlings were transplanted into ebb and flow tables, and (3.) roots were harvested from three randomly selected *Lolium* and *Cynodon* at the time of final harvest. Selected root sub-samples were cleared in KOH and stained with Trypan Blue (to visually observe fungi in the roots).

To identify the fungi in the trap culture, total DNA was extracted from the roots and closely adherent soil using the MoBio Soil DNA extraction kit (MoBio Labs inc.). PCR amplification of all samples was accomplished using fungal specific primers for the internal

transcribed spacer region using ITS-1f and ITS 4, (Gardes et al. 1991) and the nuclear small ribosomal subunit using primers nu-ssu-817 and nu-ssu-1536 (Borneman and Hartin 2000). All PCR reactions were conducted according to cycling parameters in Borneman and Hartin (2000).

3.0 Results

3.1 AM and Other Rhizosphere Fungi

Characterization and identification of fungi present in the rhizosphere of plants is ongoing. At the time of this report, DNA of all samples has been extracted and purified. Trap culture and rye and Bermuda samples at time of planting have been amplified using both sets of primers. All samples were amplified and showed strong bands on agarose gels of the appropriate sizes for each primer set. Some samples exhibit multiple bands, which strongly indicates multiple fungal groups. Further analyses using denaturing gradient gel electrophoreses and cloning are underway.

Clearing and staining of plant roots confirmed that several types of fungi were present in the rhizosphere of both rye and Bermuda before planting into ebb and flow tables and at time of harvest. Typical AM mycorrhizal structures were seen in several samples. Colonization numbers were largely too low to quantify with the small subsamples of roots cleared and stained. Non-AM fungal types were also seen in the roots. Structures typical of soil and plant associated Ascomycota were present in most samples. Further elucidation of the structure and function of these communities is forthcoming with the molecular data.

3.2 Plant Performance

Rye grass was the best performer in our greenhouse experiment. Overall survival was high across all treatments (158 of 162 seedlings survived 20 weeks). Bermuda grass had much higher mortality (71 of 162 seedlings survived 20 weeks). Total biomass per container (ranging from 81.88g (Vaminoc) to 28.1g (Ranger 4)) was significantly affected by the inoculum source (one way ANOVA $F=5.045$, $P<0.001$) (Fig. 3.1). Overall, nine of 16 inoculation treatments achieved more biomass than controls. However, variance was high, and only Ranger 1 and Vaminoc were significantly different than controls (Dunnet's method, $\alpha=0.05$).

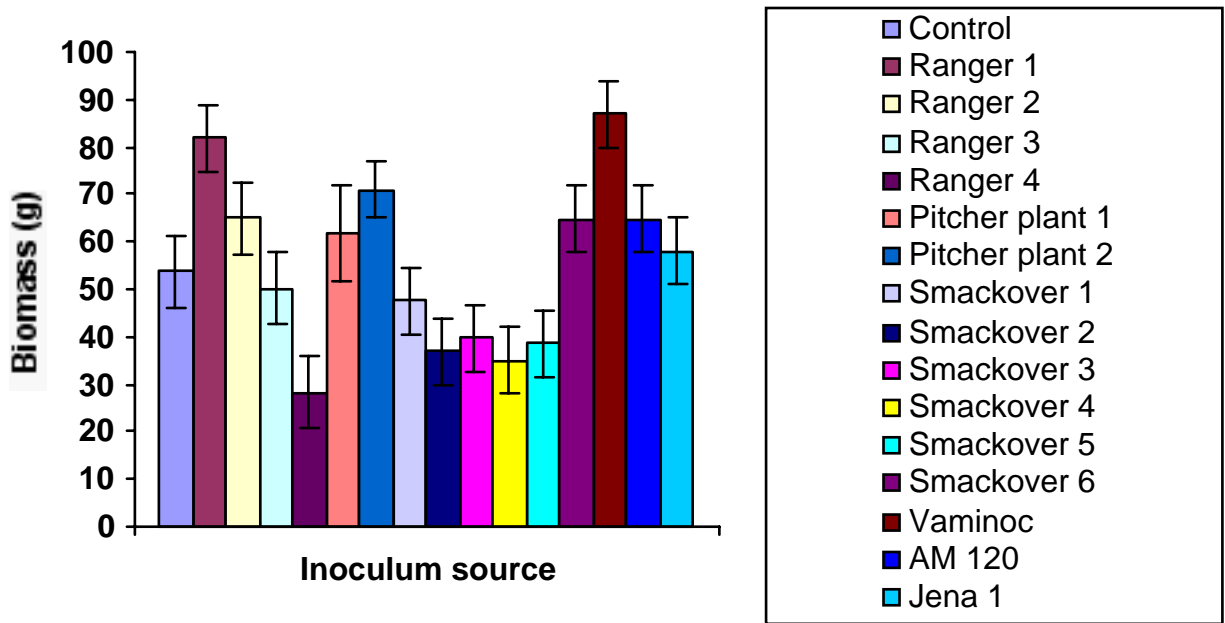


Figure 3.1. Mean total plant biomass per pot produced by treatment. Bars represent \pm SE.

Significant interactions were seen between species and inoculum source (two way ANOVA, Treatment P=0.0105, Species P=<0.001, Treatment*Species P=0.018) (Fig. 3.2).

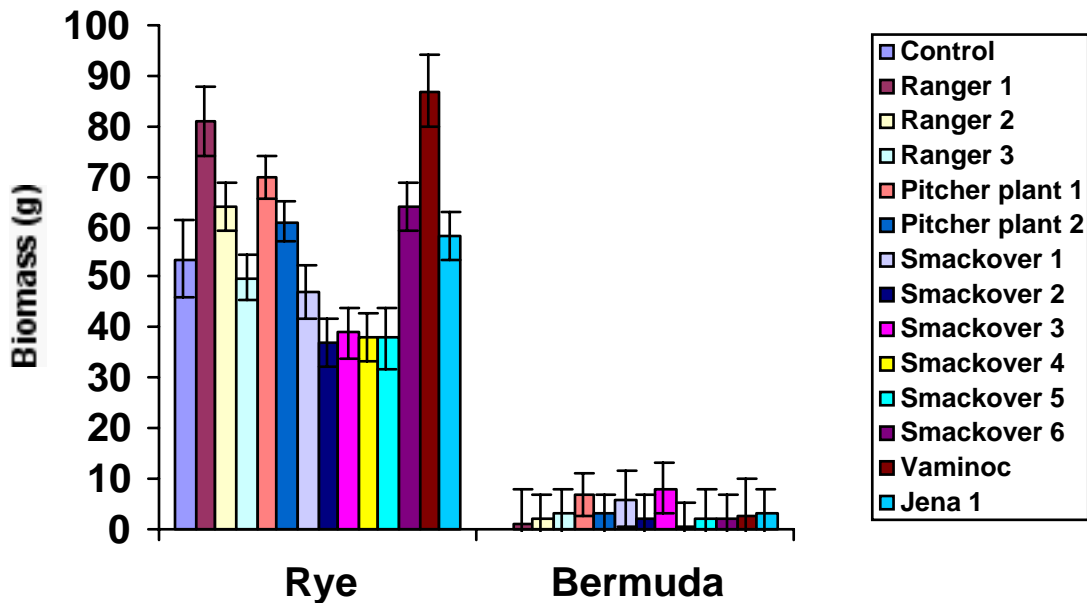


Figure 3.2. Least squares mean for total biomass by species. Treatments with no survival of Bermuda grass were excluded. Bars represent \pm SE.

For rye, shoot height and biomass showed similar trends, but root length did not. Mean shoot height and biomass was greatest in the Vaminoc inoculated seedlings and were

significantly different than control (height, ANOVA $P < 0.001$; biomass ANOVA $P = 0.001$ Dunnet's method, $\alpha = 0.05$). Rye shoot height was lowest in the Smackover 1 inoculated soil (Fig. 3.3, 3.4). Shoot height ranged from 74cm (Vaminoc) to 50.6cm (Smackover 1). Shoot biomass ranged from 60.9g (Vaminoc) to 23.8g in Ranger 4 inoculum. Control seedlings had the lowest mean root length. Root length ranged from 41.2cm with Smackover 5 inoculum to 26.3cm in controls. Rye seedlings inoculated with Smackover 5 inoculum had significantly greater root length than control (Dunnet's method, $\alpha = 0.05$) (Fig. 3.5). Root biomass showed a similar trend to shoot biomass (ANOVA $P < 0.001$). Root biomass ranged from 6.2g (Smackover 2) to 32.5 (Ranger 4). Only Ranger 4 was significantly different from control (Dunnet's method, $\alpha = 0.05$) (Fig 3.6).

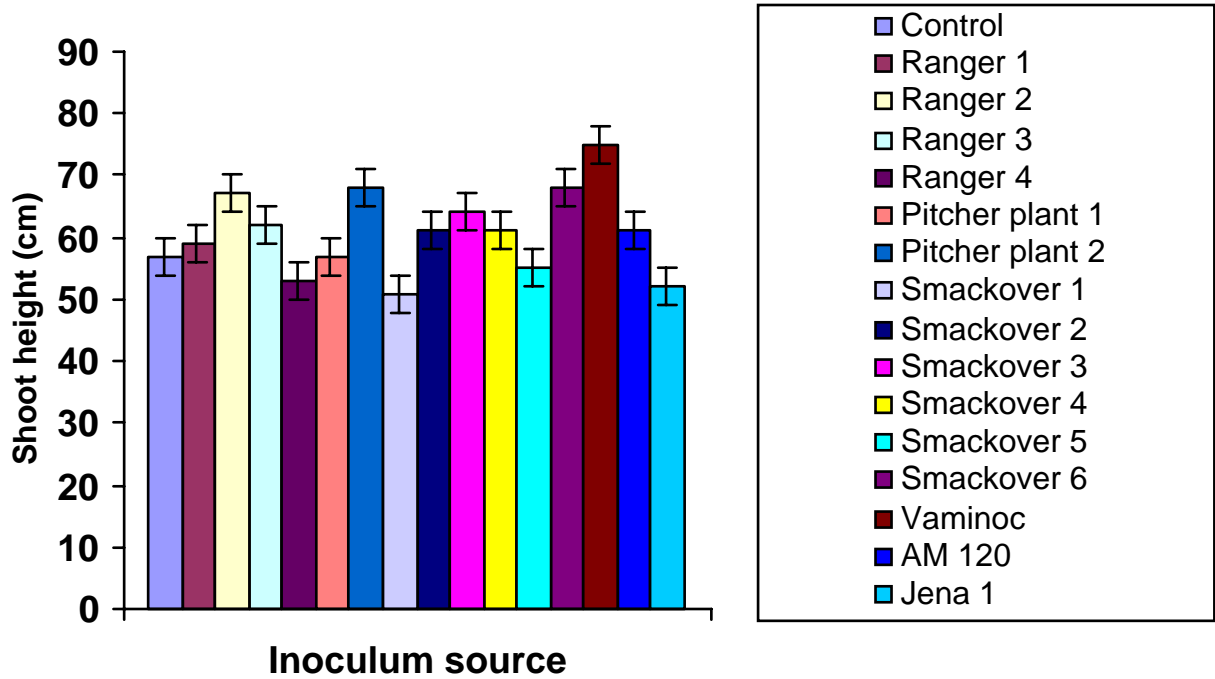


Figure 3.3. Mean shoot height for rye after 20 weeks by inoculum source. Bars represent \pm SE.

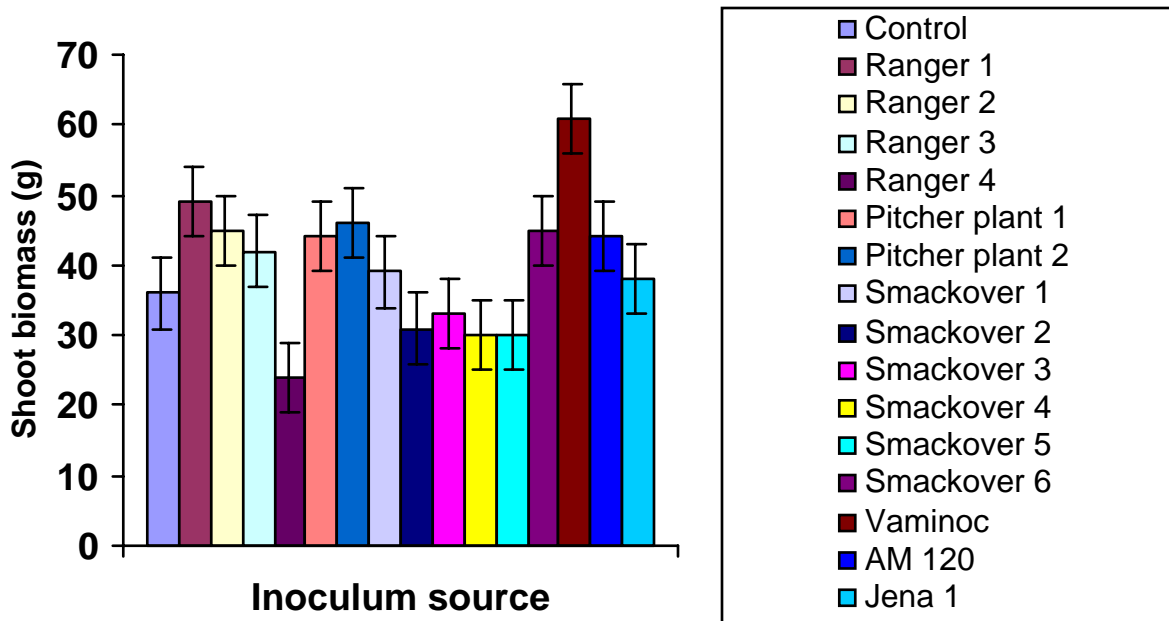


Figure 3.4. Mean shoot biomass for rye after 20 weeks by inoculum source. Bars represent \pm SE.

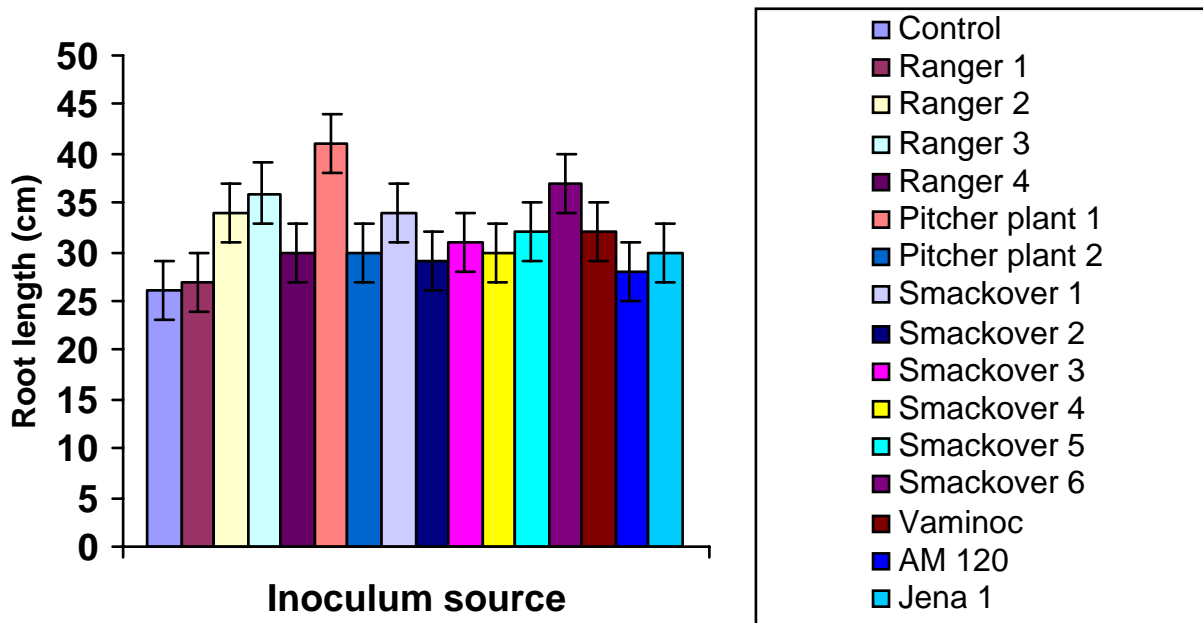


Figure 3.5. Mean root length for rye after 20 weeks by inoculum source. Bars represent \pm SE.

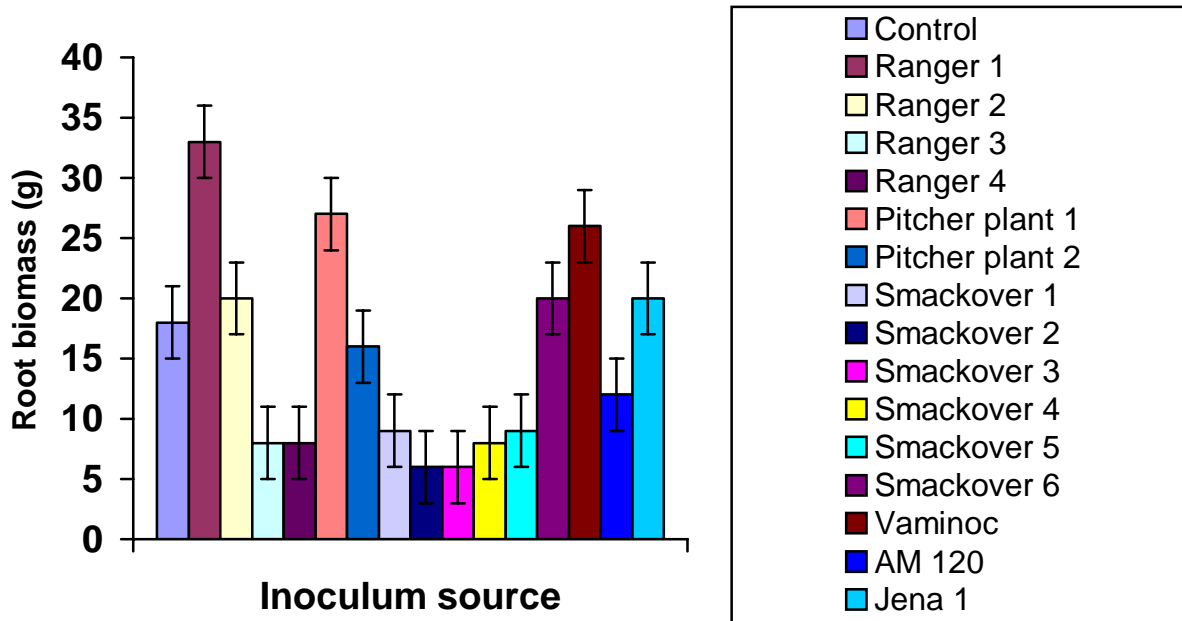


Figure 3.6. Mean root biomass for rye after 20 weeks by inoculum source. Bars represent \pm SE.

4.0 Discussion

Oil brine spill sites require a number of remedial treatments to restore ecosystem attributes. Return of function is dependent upon soil structure, presence of bacteria, fungi, and plants, as well as a reduction in contaminants. To achieve these characteristics, spill sites generally require fertilizer to accelerate degradation of residual hydrocarbons. Soil structure is improved by amending soils with organic matter and calcium. Lastly, sowing seeds or outplanting nursery stock is used to reestablish vegetation. Symbiotic fungi may assist in plant recruitment and performance on these disturbed sites, and our data support the idea that for rye at least, inoculation with fungi did enhance biomass production.

High mortality of Bermuda grass limited our ability to make inferences about the role it may play in restoration of a brine spill. The fact that there was a significant interaction suggests that the inoculum source needs to be matched with plant species to best enhance restoration. The three inoculum sources that provided best growth of Bermuda were some of the poorer performers for rye. Further analyses of the molecular data should help resolve this challenge. The AM fungi are not generally considered to be specific in their association with plant hosts. The Smackover inoculum sources that showed the best performance for Bermuda actually reduced rye performance. Only the Pitcher plant 1 inoculum provided a modest increase in performance for both species. Bermuda performed well in last season's research (Vavrek et al. 2001). The particular seed source and cooler than normal conditions in the greenhouse may have contributed to the poor performance. However, specific toxicity or carryover of Bermuda grass pathogens may have also occurred from arboretum soil or trap cultures. However, the latter is unlikely since one of the treatments with total Bermuda mortality was the control. Improved inoculum production techniques, such as aeroponic production, should reduce the chance of pathogen carryover. Our best interpretation of the data is that seed viability was low, and greenhouse temperatures were too low for the first several weeks of the study for Bermuda to

grow well, regardless of the inoculum source. These environmental factors need to be considered when choosing plants (or timing of application) to revegetate oil brine spill sites.

Both commercial inoculum sources reported here contain a mix of AM species. The manufacturers of Vaminoc tout the optimization of their product for turf grasses, and our data supports this. Overall, Vaminoc G provided excellent benefits for rye and no measurable decrease for Bermuda. Field trials of the best performing inoculum types are planned for next year. At this early date, we cannot say for certain whether these plant/inoculum combinations will show the same results under field conditions. We are confident, however, that a mixture of plant species and fungal species/strains will prove beneficial under real world conditions.

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