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Effect of arbuscular mycorrhizal fungi on plant biomass and the rhizosphere microbial community structure of mesquite grown in acidic lead/zinc mine tailings

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ABSTRACT

Mine tailings in arid and semi-arid environments are barren of vegetation and subject to eolian dispersion and water erosion. Revegetation is a cost-effective strategy to reduce erosion processes and has wide public acceptance. A major cost of revegetation is the addition of amendments, such as compost, to allow plant establishment. In this paper we explore whether arbuscular mycorrhizal fungi (AMF) can help support plant growth in tailings at a reduced compost concentration. A greenhouse experiment was performed to determine the effects of three AMF inocula on biomass, shoot accumulation of heavy metals, and changes in the rhizosphere microbial community structure of the native plant *Prosopis juliflora* (mesquite). Plants were grown in an acidic lead/zinc mine tailings amended with 10% (w/w) compost amendment, which is slightly sub-optimal for plant growth in these tailings. After two months, AMF-inoculated plants showed increased dry biomass and root length ($p<0.05$) and effective AMF colonization compared to controls grown in uninoculated compost-amended tailings. Mesquite shoot tissue lead and zinc concentrations did not exceed domestic animal toxicity limits regardless of whether AMF inoculation was used. The rhizosphere microbial community structure was assessed using denaturing gradient gel electrophoresis (DGGE) profiles of the small subunit RNA gene for bacteria and fungi. Canonical correspondence analysis (CCA) of DGGE profiles showed that the rhizosphere fungal community structure at the end of the experiment was significantly different from the community structure in the tailings, compost, and AMF inocula prior to planting. Further, CCA showed that AMF inoculation significantly influenced the development of both the fungal and bacterial rhizosphere community structures after two months. The changes observed in the rhizosphere microbial community structure may be either a direct effect of the AMF inocula, caused by changes in plant physiology induced by AMF, or a combination of both mechanisms.

1. Introduction

Natural plant colonization of mine tailings is affected not only by metal toxicity but also by the physical–chemical and microbial properties of the mine tailings (Mendez and Maier, 2008). Successful establishment of a vegetation cover on mine tailings requires selection of plants that are tolerant to heavy metals and adapted to the environmental conditions of the site. This research is focused on desert mine tailings and in particular on the ability of arbuscular mycorrhizal fungal (AMF) inocula to aid in establishment of mesquite (*Prosopis juliflora*) in acidic mine metalliferous tailings. The AMF were evaluated both as plant growth promoting microorganisms and as a contributor to the re-establishment of microbial communities in the mine tailings. Criteria used to evaluate the AMF tested included plant biomass production, metal uptake by plant shoots, and the impact of the AMF on both bacterial and fungal community structure in the tailings.

Mesquite was selected for this study because it has shown potential for growth in metal-contaminated soils from urban industrial sites (Senthilkumar et al., 2005) and tolerance to Pb, As and Cr under hydroponic conditions and on agar media (Aldrich et al., 2004; Arias et al., 2010). This native desert plant is a salt- and drought-tolerant deciduous, large crowned and deep rooted bush or tree with the ability to establish symbiosis with nitrogen-fixing bacteria and to form AMF associations. Mesquite has been widely valued in the desert regions of northwest Mexico and the United States southwest for its use as fuel wood and in the manufacture of hardwood products and also for use in habitat restoration for eco-tourism (Stanton et al., 2001).

AMF have previously been examined for establishment of vegetation on polluted sites such as heavy metal-contaminated soils or mine tailings including taconite iron ore tailings and copper smelter factory fly ash (Dueck et al., 1986; Khan, 2005; Noyd et al., 1996). AMF are known for their ability to protect plants against heavy metal toxicity by mediating the interaction between metals and plant roots. For example, AMF can bind heavy metals in their cell wall, compartmentalize them in the vacuole or chelate them into the cytoplasm restricting the influx of heavy metals into the plant (Leyval et al., 1997). Plants colonized by AMF also have greater ability to absorb

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nutrients like P, N, K, Ca, Mg, and water which results in better survival under stressed conditions (Auge and Stodola, 1990). AMF have also been shown to interact with different groups of soil bacteria and modify the rhizosphere microbial community. For example, Albertsen et al. (2006) showed that both bacterial and saprotrophic fungal biomass increased in the presence of *Glomus intraradices* in a root-free sand environment. Wamberg et al. (2003) examined the effect of AMF inoculation on community structure in the pea rhizosphere using denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA amplicons from community DNA extracts. Results showed that while DGGE profiles were quite similar between AMF-inoculated and uninoculated treatments, there were four to five specific bright bands in uninoculated treatments that were not present in *G. intraradices* inoculated treatments. These types of changes have yet to be studied in the extreme conditions characteristic of mine tailings.

There is currently only one published study on AMF–metal–mesquite interactions. In this hydroponic study Arias et al. (2010) studied chromium accumulation into mesquite stems and leaves. Chromium was accumulated at greater than 2-fold levels in stems and leaves of mesquite plants inoculated with *Glomus deserticola* (5000 and 2600 mg kg^{-1}, respectively) than in uninoculated plants (2300 mg kg^{-1} and 990 mg kg^{-1} respectively).

The desert mine tailings used in this study were obtained from the Klondyke mine tailings site, an Arizona State Superfund site located in the southeast corner of Arizona (Grandlic et al., 2008). The Klondyke tailings are acidic and contain high levels of heavy metals including lead and zinc (4620 and 1400 mg kg^{-1}, respectively). Plant establishment in these tailings has been shown to require 15% w/w compost to achieve growth similar to that in a soil collected adjacent to the tailings (Mendez et al., 2007). We used these tailings to test the hypothesis that inoculation with AMF would enhance biomass production and influence the development of the rhizosphere community structure found in plants grown in the tailings at a sub-optimal level (10%) of compost amendment. The specific goals of this study were: 1) to evaluate the effects of three different AMF on the establishment and growth of mesquite (*P. juliflora*), a representative species of the Sonoran-Arizona desert ecosystem, in mine tailings, 2) to characterize metal uptake into mesquite shoot tissue in the presence and absence of AMF, and 3) to determine the impact of the AMF on both bacterial and fungal community structure in the tailings following plant establishment.

2. Materials and methods

2.1. Tailings and compost

The Klondyke mine tailings were previously characterized (Grandlic et al., 2008). Briefly the tailings have a pH of 4.54 ± 0.02, total organic carbon and total nitrogen of 0.36 ± 0.068 and 0.067 ± 0.012 g kg^{-1}, respectively, and electrical conductivity of 3.0 ± 0.12 dS m^{-1}. The tailings texture is 51.9% sand, 26.4% silt and 21.7% clay. Neutrophilic heterotrophic counts in the tailings range from 10 to 75 CFU g^{-1} dry tailings, while iron- and sulfur-oxidizer counts range from 10^5 to 10^6 MPN g^{-1} dry tailings. In comparison, a soil collected immediately adjacent to the tailings had a heterotrophic count of 8×10^4 CFU g^{-1} dry tailings and undetectable iron- and sulf-oxidizer counts. Total and bioavailable metals in the tailings are shown in Table 1. The compost used in this study is a mixture of dairy manure and green waste and was obtained from the University of Arizona Controlled Environment Agricultural Center, Tucson AZ. The compost is characterized by a total carbon, organic carbon, and total nitrogen in the compost of 135.5 ± 10.02 g kg^{-1}, 123.3 g kg^{-1} and 2.7 ± 0.44 g kg^{-1} respectively.

2.2. AMF inocula

Three AMF inocula were used in this study including two from a commercial source: *G. intraradices* and a mix of *G. intraradices* and *G. deserticola* (desert inoculum) (Reforestation Technologies International, Salinas CA, USA) and a native inoculum (described later). Inocula were added to the tailings as mixtures of sand and mycorrhizal propagules (spores, mycelia and colonized root segments). For the commercial inocula, 2400 AMF propagules were mixed with 20 g sand for each pot (0.8 propagules gram dry mine $tailings^{-1}$). The native inoculum (2400 propagules) was placed into 40 g sand for each pot (0.8 propagules gram dry mine $tailings^{-1}$).

2.2.1. Isolation and identification of the native inoculum

The native inoculum was generated from a soil sample obtained from underneath a mesquite tree in Tucson, Arizona. A primary inoculum was created by mixing the sample with sand ≤ 1 mm particle size (1:3 w/w) using sorghum as a host plant. The primary inoculum was subcultured once into sand after three months. Spores were isolated by wet sieving and decanting (Gerdemann and Nicholson, 1963) and examination with a dissection microscope yielded three unique morphotypes. Spores of each morphotype were disinfected in 2% NaOCl for 10 min, soaked in gentamycin (100 mg L^{-1}) three times for 10 min, rinsed four times with sterile distilled water, and then crushed in 0.25 M NaOH. DNA was extracted as described by Redecker et al. (1997) and the supernatant was collected and extracted with an equal volume of 1:1 phenol to chloroform/isoamyl alcohol (24:1) followed by an equal volume of the aqueous phase with chloroform/isoamyl alcohol. The aqueous phase sample was stored at −20 °C.

Nested-PCR was used to amplify the DNA. First round primers were NS1(f) and ITS4(r) (Rodriguez-Echeverria and Freitas, 2006). Each 20 μL reaction contained 2 μL of 10× buffer containing 15 mM

Table 1
Effect of AMF on accumulation of metal(loid)s and phosphorus in mesquite shoot tissues after 60 days of growth in Klondyke mine tailings. Values are means ± SD ($n = 5$). Values with different letters are significantly different at $p < 0.05$ (one-way ANOVA, Tukey's test) for each row.

	Total[a]	Plant available[b]	DATL[c]	Accumulation of metals in the shoot (mg kg^{-1})				
Metal	mg kg^{-1}			Control uninoculated	Desert inoculum	*Glomus intraradices*	Native inoculum	Average AF[d]
As	91	0.03	≤30	0.16 ± 0.05a	0.15 ± 0.02a	0.14 ± 0.07a	0.14 ± 0.02a	0.001
Cd	2.4	1.17	≤10	0.13 ± 0.04a	0.18 ± 0.06a	0.17 ± 0.07a	0.20 ± 0.06a	0.07
Cr	36	0.04	≤5	0.11 ± 0.04a	0.05 ± 0.04ab	0.02 ± 0.02b	0.01 ± 0.01b	0.001
Cu	653	96.89	≤40	11.4 ± 4.07a	11.7 ± 3.82a	11.8 ± 1.63a	21.5 ± 2.99b	0.02
Mn	2811	171.99	≤2000	115 ± 55.4a	152 ± 45.1a	117 ± 55.3a	116 ± 8.41a	0.04
Pb	4620	149.34	≤100	3.83 ± 1.80a	3.89 ± 0.83a	2.88 ± 1.11a	3.48 ± 1.71a	0.0008
Zn	1400	442.80	≤500	46.1 ± 21.0a	63.3 ± 17.6a	73.4 ± 18.5a	111 ± 50.8a	0.05
P	131	0.21	–	2003 ± 842.8a	1775 ± 166.5a	1973 ± 222.3a	4024 ± 1065b	19

[a] Total metal(loid) concentration in the Klondyke tailings before planting (Grandlic et al., 2008).
[b] Plant available metal concentrations in Klondyke tailings before planting. Determined by diethylenetriaminepentaacetic acid (DTPA) extraction (Hayes et al., 2009).
[c] DATL = domestic animal toxicity limit. Values listed are maximum tolerable levels for cattle (National Research Council, 2005).
[d] AF = accumulation factor which is the total element concentration in the shoot tissue/total element concentration in the mine tailings (Brooks, 1998). The AF presented is the average of the four treatments.

$MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer, 1 U of the HotStarTaq DNA polymerase (QIAgen Sciences, Maryland), and 0.8 ng of DNA was used as template. The amplification protocol used was: 95 °C for 15 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 40 s, and 68 °C for 2 min + 5 s per cycle, followed by a 68 °C extension for 7 min. PCR products were visualized on a 2% agarose gel (GenePure LE ISC BioExpress, Kaysville, UT) using an Alpha Imager (Alpha Innotech, San Leandro, CA). PCR products were diluted 500 times (if a band was visible on the gel) or 350 times (if a band was not visible on the gel) with sterile deionized water and then used in the second round of PCR. Second round primers were NS31(f) and AM1(r) (Rodriguez-Echeverria and Freitas, 2006). Each 25 μL reaction used the same conditions as described earlier with the following amplification protocol: 95 °C for 15 min, 35 cycles of 92 °C for 30 s, 61 °C for 60 s, and 68 °C for 50 s + 1 s per cycle, followed by a 68 °C extension for 5 min.

Final PCR amplicons were purified using the Qiaquick PCR purification kit, (Qiagen Sciences, Maryland USA) and sequenced by University of Arizona Research Laboratories Genomic Analysis and Technology Core using an ABI3730xl DNA analyzer (Applied Biosystems, Foster City, CA). The sequences were compared to sequences in the National Center for Biotechnology Information (NCBI) database using BLAST analysis.

2.3. Greenhouse experiment

A greenhouse experiment was performed to determine the effect of AMF inoculation on the growth of mesquite in Klondyke tailings. All tailings received a 10% (w/w) compost amendment which is less than the optimal amount for plant growth in these tailings. Here we define optimal amount of compost as that which produces a comparable amount of biomass in tailings to the biomass produced in a control treatment of plants grown in soil collected adjacent to the tailings. A previous study by Mendez et al. (2007) showed that 15% w/w compost is optimal for these tailings and that there was an average of a 78% increase in biomass production between the 10% and 15% compost treatments. Four treatments were evaluated: an uninoculated mesquite control; mesquite + commercial *G. intraradices*; mesquite + commercial desert inoculum; and mesquite + native isolate inoculum. The experiment was conducted in a greenhouse (natural light, temperature day/night 32 °C/24 °C) located at the University of Arizona Controlled Environment Agriculture Center (Tucson, AZ).

Black plastic pots with drainage (17 cm top d × 18 cm height × 13.5 cm bottom d) were 3/4 filled with the tailings–compost mixture. A thin layer of the appropriate AMF inoculum was added and then the pots were filled to the top with tailings–compost. Ten mesquite seeds (*P. juliflora*, Desert Nursery, Phoenix, AZ) were sown in each pot at a depth of approximately 0.5 cm. The experimental design was completely randomized with 5 replicate pots per treatment. Pots were watered with tap water using drip irrigation every other day (80 mL pot^{-1}). Selected total elements of interest in the tap water (μg L^{-1}: As, 4.36; Cd, not detected; Cu, 1.0; Cr, 0.01; Mn, 1.50; Zn, 3.0; Pb, not detected) were analyzed by ICP-MS by the University of Arizona Laboratory for Emerging Contaminants. Germination occurred after approximately 3 days and after 10 days, the pots were thinned to one seedling.

2.4. Sample collection and plant analysis

Plants were harvested at 60 days. Each plant was carefully removed from the pot and the shoots separated from the roots. Mine tailings and organic matter attached to the roots were shaken off vigorously to represent rhizosphere samples which were collected in sterile 1.5 mL snap cap tubes and stored at −20 °C for microbial analysis. Shoots were placed into a pre-weighed paper bag. Roots were washed with tap water to remove mine tailings and organic matter and then placed into pre-weighed foil packets. Shoot and root tissues were dried for 72 h at 70 °C, and weighed to determine dry biomass.

To determine shoot metal concentrations, samples were ground with a Wiley Mill for one min, passed through a 30 mesh (0.595 mm) screen, microwave digested using EPA Method 3051 for total elements (As, Cd, Cu, Cr, Mn, Zn, and Pb), and analyzed by ICP-MS by the University of Arizona Laboratory for Emerging Contaminants. Prior to analysis, the Wiley Mill was evaluated for potential metal contamination of the samples. Twelve randomly selected plant biomass samples were cut to pass a 40 mesh sieve using stainless steel scissors to evaluate potential for metal contamination. Identical samples, processed with and without grinding, were analyzed for metal concentration. There were no significant differences in metal concentrations between the two methods. A standard reference material, pine needles 1575a was obtained from the National Institute of Standards and Technology (Gaithersburg, MD) to evaluate metal recoveries which were: As, 103%; Cd, 94%; Cu, 115%; Cr, 97%; Mn, 103%; Pb, 96%; Zn, 83%.

2.5. AMF root colonization

The percentage of root colonization by AMF was obtained from a random subsample of approximately 1.0 g of fresh roots. Root segments were cut into 1.0 cm pieces, cleaned in 10% KOH for 10 min at 90 °C, rinsed in water, acidified with 10% HCl and stained with 0.1% trypan blue for 10 min at 90 °C. Percentage root colonization was determined as described by Phillips and Hayman (1970) placing 100 segments of stained roots on microscope slides and recording the number of segments with any infection with the use of a compound microscope.

2.6. Microbial community structure analysis

2.6.1. DNA extraction and small subunit RNA gene PCR

Community DNA was extracted from (i) a 0.5 g rhizosphere sample collected during plant harvest and (ii) from each AMF inoculum using the Fast DNA SPIN for soil kit (MP Biomedicals, Solon OH, USA) with some modifications to the manufacturer's protocol to enhance the DNA yield. Both vortexing and centrifugation of the Lysing Matrix tube was increased to 15 min, and the binding matrix–DNA complex was rinsed multiple times with saturated 5.5 M guanidine thiocyanate (Fluka Sigma-Aldrich GmbH, Buchs, Switzerland) until the supernatant lost its brown tint (Rosario et al., 2007). In addition, the spin filters containing the binding matrix were air-dried under a laminar flow hood overnight prior to DNA elution, and the water used to elute the DNA was preheated to 60 °C. The extracted DNA was quantified as described previously and added at a concentration of 200 pg μL^{-1} for each PCR reaction. Analysis of the rhizosphere bacterial and fungal communities was performed on three of the five replicates in each treatment (chosen at random). In addition, analyses of the three AMF inoculum fungal communities were performed.

For bacteria, the V7/V8 variable region of the 16S rRNA gene was amplified following a modified protocol described by Colores et al. (2000) using primers 1070(f) and 1406(r) with a 40-bp GC clamp (Ferris et al., 1996). Each 25 μL reaction contained 2.5 μL of 10× buffer containing 15 mM $MgCl_2$ (QIAgen Sciences, Maryland), 0.2 mM dNTP, 0.4 μM of each primer, 5% dimethyl sulfoxide (Sigma, St. Louis, MO), 0.4 μg μL^{-1} unacetylated albumin bovine serum (Sigma, St. Louis, MO), 0.625 U HotStarTaqDNA polymerase (QIAgen Sciences, Maryland) and 200 pg of template DNA. The amplification protocol was 95 °C for 15 min followed by 30 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 30 s followed by a 72 °C extension for 7 min.

For fungi, approximately 350 bp of the 18S rRNA gene was amplified with the primer pair NS1(f) and GC Fung(r) with a 40 base GC clamp (May et al., 2001). Each 50 μL reaction contained 5 μL of 10× buffer containing 15 mM $MgCl_2$ (QIAgen Sciences, Maryland), 0.2 mM dNTP,

0.8 μM each primer, 1.25 U HotStarTaqDNA polymerase, 0.4 μg μL^{-1} unacetylated albumin bovine serum (Sigma, St. Louis, MO), and 200 pg of DNA template. The amplification protocol used was 95 °C for 15 min followed by 40 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 3 min, followed by a 72 °C extension for 10 min. All bacterial and fungal amplification products were visualized as described earlier.

2.6.2. Denaturing gradient gel electrophoresis (DGGE) analysis

Amplicons were separated on DGGE gels (a uniform concentration of PCR product from each sample was used based on quantification from an agarose gel) containing a 45 to 65% or 20 to 45% urea-formamide denaturing gradient for bacteria and fungi, respectively. Gels were run at a constant voltage of 50 V for 17 h at 60 °C, then stained in 2× SYBR Green (Molecular Probes, Eugene, OR) for 20 min and imaged. Microbial community banding profiles on DGGE gels were analyzed using Quantity One 4.5.2 (Bio-Rad Laboratories, Hercules, CA) to generate presence–absence species matrices for bacteria and fungi for further analysis.

2.7. Statistical analysis

Plant biomass, mycorrhizal colonization percentage and heavy metal concentration data were analyzed using SAS version 9.1 (SAS Institute Inc., Cary, NC). Significant differences were detected by employing a one-way analysis of variance (ANOVA) ($p<0.05$). Significant differences between means were determined by Tukey's test ($p<0.05$).

Presence–absence matrices of DGGE bacterial and fungal profiles were analyzed by canonical correspondence analysis (CCA), using the CANOCO 4.5 software to determine the extent to which the different treatments (inoculation vs. no inoculation) could explain patterns of similarity in rhizosphere microbial community composition (ter Braak and Smilauer, 2002). CCA is a direct gradient ordination method, better described as a constrained variation of correspondence analysis (CA), which is widely used in community ecology (Palmer, 1993). The result from a CCA is that the axes of the final ordination are a linear combination of the environmental variables and the species data. So, in our case the CCA will find axes of variation in DNA fingerprinting patterns (response variables) that are maximally related to the treatment types (explanatory variables). Our analysis focused the scaling on inter-species distances and was performed using untransformed presence–absence species data and the treatments as nominal environmental variables. Biplots of the treatment replicates and the environmental variable centroids arranged along the first two CCA ordination axes were generated with the CanoDraw 4.14 software by constraining the axes to be linear combinations of environmental variables scores (ter Braak and Smilauer, 2002). From the analysis, CCA eigenvalues for each canonical axis were generated to represent the importance of each axis in explaining the relationship between DGGE fingerprinting profile patterns and the treatment from which they were obtained. The statistical significance of the CCA analysis was tested using a Monte Carlo permutation test (1000 unrestricted random permutations; $p<0.05$) (ter Braak and Wiertz, 1994) of residuals from a reduced model against the null hypothesis that microbial community composition was unrelated to the treatments as nominal environmental variables.

3. Results and discussion

3.1. Identification of the native inoculum

The native inoculum was composed of three morphotypes and sequence similarity analysis confirmed that each of the three morphotypes was unique and belonged to the *Glomus* genus. Morphotype 1 (accession number HM357114) was characterized by small spores of 32 to 80 μm in diameter and was observed sporulating inside of dead roots. The closest match, with 99% similarity, was to uncultured *Glomus* clones TY159_A06; 28_10.B-NT; M0607; M0102 (accession numbers: FJ913026.1; AM412099.1; AB365813.1; AB365803.1, respectively). Morphotype 2 (accession number HM357115) was composed of sporocarps ranging from 260 to 310 μm in diameter. This morphotype was most similar (99%) to uncultured *Glomus* clones GPR-A11; GP-A11; TY432_G04; ael9 (accession numbers: GU183714.1; GU183701.1; FJ913048.1; FM875888.1, respectively). Morphotype 3 (accession number HM357116) corresponded to spores ranging from 150 to 320 μm in diameter surrounded by a tight peridium observed singly or in aggregates of two or four. The closest match (100% similarity) was to five uncultured *Glomus* clones OC6_36C3Y; OC6_22C3Y; 4D.7; 4D.3; 1bf14 (with the respective accession numbers: EF177643.1; EF177633.1; DQ164834.1; DQ164832.1; AY243568.1) and *Glomus mosseae* strain BEG69 (accession number: U96141.1).

3.2. Effect of AMF inocula on plant biomass and AMF colonization of roots

Control uninoculated mesquite plants produced 7.4 ± 1.7 g dry biomass per plant during the two month growth period. AMF-inoculated plants had a 44 to 76% increase in dry biomass ($p<0.05$) compared to the uninoculated control producing from 10.7 ± 1.9 to 13.1 ± 0.7 g dry biomass per plant depending on the inoculum type (Fig. 1). Recall that the compost level used in this experiment, 10%, was sub-optimal and that Mendez et al. (2007) reported a 78% difference in plant biomass produced between 10% and 15% compost treatments (with no AMF inoculum). The results of this study suggested that the AMF-inoculated treatments containing 10% compost have potential for higher levels of biomass production that are comparable to that reported from increasing the compost amendment from 10 to 15%. Thus, AMF inoculation effectively reduces the amount of compost required to achieve a given level of biomass production in mine tailings.

In examining the three AMF inocula, the *G. intraradices* inoculated treatment had the highest biomass production although it was not significantly different from the other two inoculated treatments. Each of the treatments had similar shoot to root ratios. Root length increased from 61.4 ± 12.6 cm in the uninoculated control to between 78.2 ± 2.5 and 90.4 ± 6.1 cm (a 27 to 47% increase) in the AMF inoculated treatments (Fig. 2; $p<0.05$). The commercial desert inoculum was the best performing AMF in terms of root length

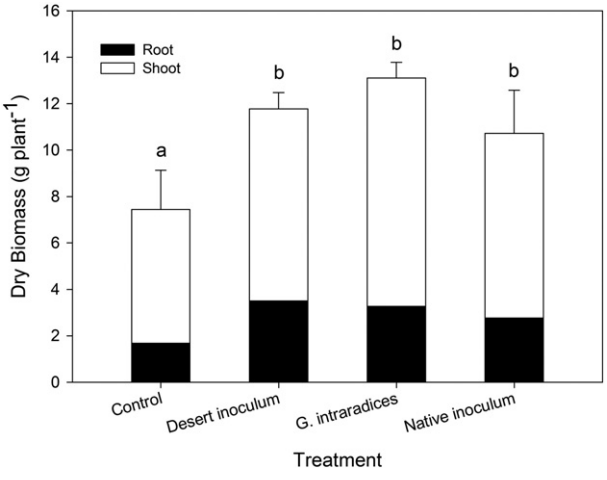

Fig. 1. Shoot and root dry biomass of mesquite plants grown for 60 days in Klondyke mine tailings. Treatments include inoculation with AMF (either *G. intraradices*, desert inoculum, or native inoculum) or uninoculated (control). Bars represent the mean of each treatment, and error bars indicate standard deviation for the total biomass ($n=5$). Means with different letters are significantly different at $p<0.05$ (one-way ANOVA, Tukey's test).

although again it was not significantly different from the other two AMF treatments.

There was no apparent root colonization by AMF in the uninoculated control plants as indicated by the lack of fungal structures in all 100 root segments examined, suggesting that there are no indigenous AMF in either the tailings or the compost that was used. In contrast, root colonization in the AMF-inoculated treatments ranged from 38 to 77% indicating effective colonization occurred during the two month growth period (Fig. 3). Microscopic examination of the roots showed that the mycorrhizal infection was well established and showed the formation of typical arbuscles and vesicles structures. Arbuscles are important structures for nutrient exchange (Smith and Gianinazzi-Pearson, 1988) and were observed on 12 to 47% of the roots examined indicating a functional interaction between AMF and plant. Vesicles are storage organs that may also function as propagules (Brundrett et al., 1996) and were observed on 7 to 18% of the roots.

The native inoculum, which was isolated from a mesquite tree in a climatic location similar to that of the Klondyke mine tailings, showed higher root colonization and higher formation of arbuscles than the two commercial inocula ($p<0.05$). Interestingly, even though the native inoculum was most efficient at colonization of the mesquite roots, it did not stimulate significantly better biomass production than the two commercial inocula. Previous work has shown that while different AMF may colonize a specific host species to a similar extent, the symbiotic efficiency as measured by growth response, may vary substantially (Feddermann et al., 2010; Smith et al., 2004). For example, an AMF species with relatively low root colonization but with a large soil mycelium can support their host with similar efficiency to species with a high intra-radical colonization (Feddermann et al., 2010; Smith et al., 2004).

3.3. Heavy metal uptake into mesquite shoot tissue

Metal (Pb, Zn, Mn, As, Cr, Cd, and Cu) accumulation in shoot tissues after 60 days of growth in Klondyke tailings was similar for the inoculated and uninoculated treatments with two exceptions (Table 1). Chromium was accumulated at higher levels in the uninoculated control than in the inoculated treatments. Specifically, Cr was present in 5 to 10-fold lower levels in two of the three inoculated treatments (*G. intraradices* and native inoculum) ($p<0.05$). The desert inoculum also reduced Cr accumulation 2-fold but this was not significantly different from the uninoculated control. The second exception was for Cu where the native inoculum accumulated 2-fold more Cu than the other inoculated treatments and the uninoculated control ($p<0.05$).

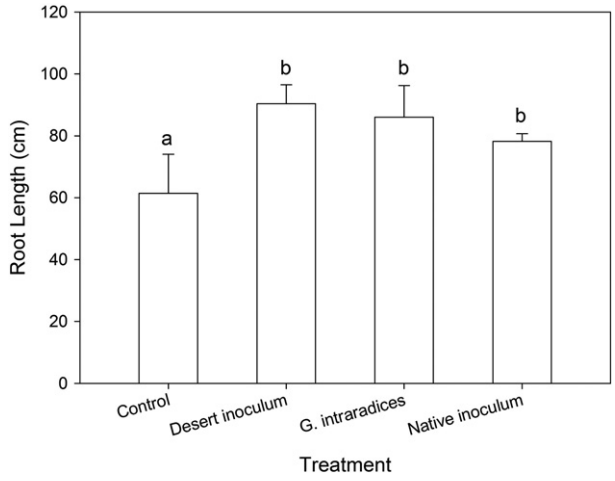

Fig. 2. Root length of mesquite plants grown for 60 days in Klondyke mine tailings. Treatments include inoculation with AMF (either *G. intraradices*, desert inoculum, or native inoculum) or uninoculated (control). Bars represent the mean of each treatment, and error bars indicate the standard deviation ($n=5$). Means with different letters are significantly different at $p<0.05$ (one-way ANOVA, Tukey's test).

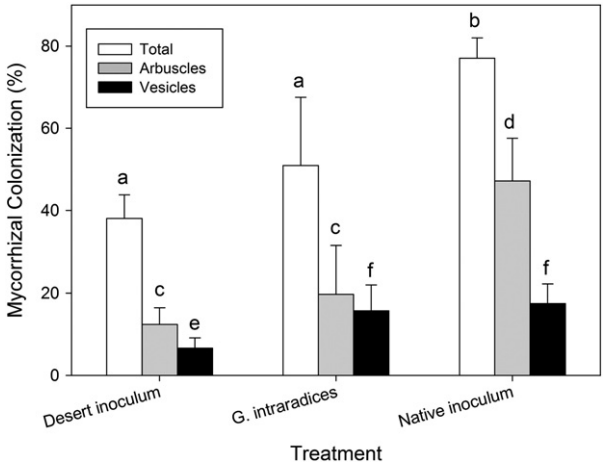

Fig. 3. Percent mycorrhizal colonization of mesquite roots grown for 60 days in Klondyke mine tailings. Treatments include inoculation with either *G. intraradices*, desert inoculum, or native inoculum. Bars represent the mean of each treatment, and error bars indicate standard deviation ($n=5$). Means with different letters are significantly different at $p<0.05$ (one-way ANOVA, Tukey's test).

Metal accumulation in mesquite shoot tissues did not exceed domestic animal toxicity limits (DATL) in either the inoculated or uninoculated treatments thus, shoot metal concentrations are not a health concern (Table 1). Demonstrating that metal uptake was low, is the accumulation factor which was $\ll 1$ for all metals across all treatments (Table 1). The accumulation factor is the amount of metals in the shoot tissue divided by the amount of metals in the tailings (Brooks, 1998). In general, accumulation factors that are <1 indicate plants that do not shoot accumulate metals and are more suitable for phytostabilization while accumulation factors >1 indicate plants that are more suited to phytoextraction of metals. Taken together these results suggest that 1) mesquite is a good candidate for phytostabilization because of its low shoot accumulation of metals, and 2) the AMF tested did not increase metal accumulation from tailings into mesquite shoot tissues compared to the uninoculated control with one exception. This was the native inoculum for which Cu accumulation in shoot tissue was doubled in comparison to other treatments, but still lower than the DATL.

In contrast to this study, previous reports have suggested that mesquite may accumulate metals in the shoots. For example, in a hydroponic system spiked with Pb, the accumulation factor for Pb in stem tissue ranged up to 75 (depending on the amount of spiked Pb) although in the same system the accumulation factor for leaf tissue was much lower (~1) (Aldrich et al., 2004). Senthilkumar et al. (2005) reported a mesquite shoot accumulation factor of 3.5 to 6.6 for Cu and 43 to 55 for Cd in five metal-contaminated soils collected in the vicinity of metal-waste producing industries in Coimbatore, India. Thus, metal uptake by mesquite may be growth medium dependent and require understanding of the impact of the physico-chemical characteristics of the growth medium (e.g., hydroponics vs. soils vs. tailings) including pH, cation exchange capacity and salt content, metal bioavailability, organic matter and microbial activity on metal speciation (Kabata-Pendias and Pendias, 2001).

The literature also shows variable effects of AMF on metal uptake by plants in general with some reports showing that AMF result in increased accumulation of metals (Killham and Firestone, 1983; Weissenhorn and Leyval, 1995) and others reporting reduced accumulation (Gildon and Tinker, 1983; Weissenhorn et al., 1995).

3.4. Phosphorus uptake into mesquite shoot tissue

Phosphorus (P) is essential for energy metabolism in plants. AMF have been well-studied for their ability to increase P uptake by plants (Harrison, 1998). The amount of P required by most plants is

0.2% on a total plant dry mass basis (Taiz and Zeiger, 2006). Results from this study showed an equivalent P content of approximately 0.2% plant dry mass for the uninoculated control treatment and two of the AMF-inoculated treatments (desert inoculum and *G. intraradices*). Thus, increased P availability over the uninoculated control treatment does not explain the 44 to 76% increase in plant biomass for these two AMF-inoculated treatments. In contrast, the native inoculum treatment showed a doubling of P uptake to approximately 0.4% plant dry mass (Table 1, $p<0.5$). Despite this, the native inoculum treatment did not result in higher production of plant biomass than the other inoculated treatments suggesting that either P is not limiting or that the different AMF inocula have different mechanisms involved in stimulating production of mesquite biomass.

3.5. Bacterial and fungal community structure

Triplicate rhizosphere samples from each treatment were analyzed using PCR-DGGE to generate community profiles for both bacteria and fungi. DGGE community profiles were analyzed using CCA to test the hypothesis that an AMF inoculum will influence both the bacterial and fungal community structure found in the rhizosphere after a 60 day growth period. For bacteria, the triplicate samples in the control and the three AMF inoculated treatments generally clustered together (Fig. 4). A permutation test was performed to test the null hypothesis that community composition is not related to treatment with the four treatments tested (uninoculated and three inoculated with different AMF). Results showed that there was a significant difference among the four treatments ($p=0.002$, Fig. 4). For the bacterial community CCA, the cumulative species–treatment relationship for axis 1 (17.2%) and axis 2 (13.1%) was 30.3%. This result suggested that an AMF inoculum impacts the bacterial community structure that develops during the first 60 days of growth for mesquite. Other studies have shown that addition of plant growth promoting bacteria significantly impact rhizosphere bacterial community development in plants grown in tailings including quailbush (*Atriplex lentiformis*) and buffalo grass (*Buchloe dactyloides*) (de-Bashan et al., 2010; Grandlic et al., 2009).

A similar analysis was done for the fungal community showing that the fungal community structure was also significantly different among the treatments tested ($p=0.001$) (Fig. 5). For the fungal community CCA, the cumulative species–treatment relationship for axis 1 (15%) and axis 2 (13.6%) was 28.6%. These data showed that in addition to affecting the bacterial community, AMF inoculation affects the fungal community in mesquite rhizosphere after 60 days of growth.

An additional DGGE analysis was then performed to compare the profiles of the individual inocula with the rhizosphere sample profiles (Fig. 6). In this case, two replicate rhizosphere samples from each of the four treatments (uninoculated and the three AMF-inoculated) were compared with each individual AMF inoculum. Interestingly, the individual AMF inocula all had very complex profiles exhibiting 40 (desert), 34 (native), and 39 (*Glomus*) bands respectively. It is unclear why the inocula have such complex profiles. It is likely there are non-AMF fungi present and it is also possible that there are several closely-related AMF strains in each inoculum or that each AMF strain contributes multiple bands to the profile. In terms of band number, the inoculum profiles are similar to the rhizosphere sample profiles which contain from 21 to 43 bands across all treatments including the uninoculated control. Due to the complexity of the inoculum profiles we were not able to determine which of the bands in the inoculum profiles were the actual AMF and by extension whether the AMF bands were in the rhizosphere sample profiles. However, a CCA analysis comparing the three inoculum profiles (as one group) with the rhizosphere samples (as four separate groups) showed a significant difference among the groups tested ($p=0.001$).

These results suggested that for mine tailings, AMF inoculation influences the development of unique bacterial and fungal rhizosphere communities that may aid in more effective establishment and growth of mesquite. The PCR DGGE results reported support previous studies showing that both the bacterial and fungal community composition in the plant rhizosphere and even the activity (as measured by enzymatic assays) of those communities can be affected by AMF colonization of

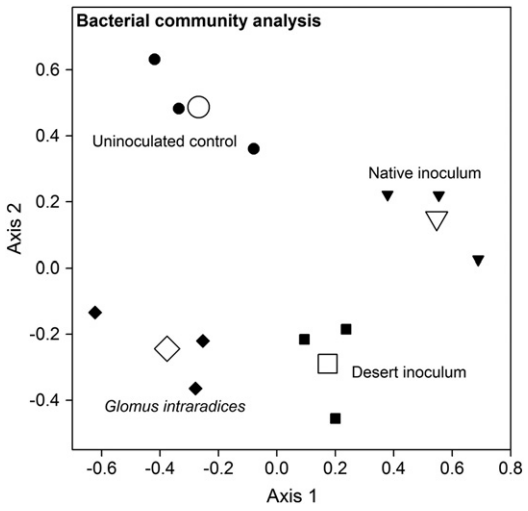

Fig. 4. Canonical correspondence analysis (CCA) of triplicate DGGE bacterial community profiles from mesquite rhizosphere samples after 60 days of growth in mine tailings. Solid symbols represent replicates from the four different treatments: uninoculated control (●), native inoculum (▼), desert inoculum (■), and *Glomus intraradices* inoculum (♦). The open symbols correspond to centroids for each treatment. The distance between each replicate in the ordination diagrams approximates the dissimilarity of their species composition measured by their Chi-square distance. The optimal CCA models represented by the diagrams were produced with scaling of scores focused on inter-species distances. The first two canonical axes are shown and the value given on each axis label represents the percentage of the total variance explained by that axis. An unrestricted Monte-Carlo permutation test was performed (1000 permutations) to determine the statistical significance of the relationship between the treatment variables ($p=0.002$).

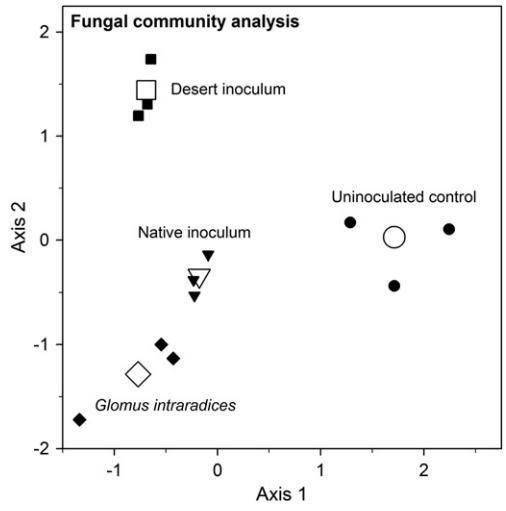

Fig. 5. Canonical correspondence analysis (CCA) of triplicate DGGE fungal community profiles from mesquite rhizosphere samples after 60 days of growth in mine tailings. Solid symbols represent replicates from the four different treatments: uninoculated control (●), native inoculum (▼), desert inoculum (■), and *Glomus intraradices* inoculum (♦). The open symbols correspond to centroids for each treatment. The distance between each replicate in the ordination diagrams approximates the dissimilarity of their species composition measured by their Chi-square distance. The optimal CCA models represented by the diagrams were produced with scaling of scores focused on inter-species distances. The first two canonical axes are shown and the value given on each axis label represents the percentage of the total variance explained by that axis. An unrestricted Monte-Carlo permutation test was performed (1000 permutations) to determine the statistical significance of the relationship between the treatment variables ($p=0.001$).

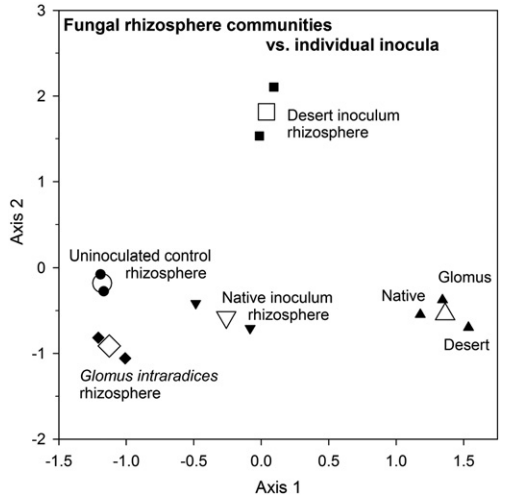

Fig. 6. Canonical correspondence analysis (CCA) of duplicate DGGE fungal community profiles from mesquite rhizosphere samples after 60 days of growth in mine tailings and of each mycorrhizal inoculum culture. Solid symbols represent replicates from the four different treatments: uninoculated control rhizosphere (●), native inoculum rhizosphere (▼), desert inoculum rhizosphere (■), *Glomus intraradices* inoculum rhizosphere (♦), and the three mycorrhizal inocula (▲). The open symbols correspond to the centroids for each treatment and for the three mycorrhizal inocula. The distance between each replicate in the ordination diagrams approximates the dissimilarity of their species composition measured by their Chi-square distance. The optimal CCA models represented by the diagrams were produced with scaling of scores focused on inter-species distances. The first two canonical axes are shown and the value given on each axis label represents the percentage of the total variance explained by that axis. An unrestricted Monte-Carlo permutation test was performed (1000 permutations) to determine the statistical significance of the relationship between the treatment variables ($p = 0.001$).

roots (Linderman, 1988; Marschner et al., 2001; Wamberg et al., 2003). The changes we have observed in the mesquite rhizosphere microbial community structure may be either a direct or indirect effect of the AMF inoculum used. One type of direct interaction between the AMF and bacterial populations in the rhizosphere is the stimulation or suppression of one or more susceptible populations (Vazquez et al., 2000). For example, AMF-bacterial interactions can play a significant role in the prevention of plant infection by pathogens. Filion et al. (1999) observed that exudates of the extraradical mycelium of *G. intraradices* significantly reduced the conidial germination of the plant pathogen *Fusarium oxysporum* in an *in vitro* culture of transformed carrot roots. In contrast, *Pseudomonas chlororaphis*, which has positive effects on plant growth, was strongly stimulated in the same system. Similarly, Elsen et al. (2008) demonstrated that *G. intraradices* induced systemic resistance in banana plants against the parasitic nematodes *Radopholus similis* and *Pratylenchus coffeae*.

AMF are well-known to excrete substances that influence the immediate environment. Exudates including amino acids and whole proteins can have a direct selective effect on the microbial community in the rhizosphere (Marschner and Baumann, 2003). AMF can also induce changes in plant physiology, for instance in root exudation (Graham et al., 1981) and carbohydrate metabolism of the plant (Buwalda and Goh, 1982), which can indirectly affect the microbial community. Either way, the impact of AMF on plant growth and rhizosphere microbial populations, as a consequence of microbial groups supported by a particular AMF inoculum, may be decisive for the successful establishment of plants under limiting soil conditions such as mine tailings (Medina et al., 2003).

4. Conclusions

AMF inoculation of mesquite seeds with either commercial or native inocula helped support plant growth in an acidic metalliferous mine tailings resulting in a 44 to 76% increase in plant biomass production, depending on the inoculum used, after 60 days. Analysis of shoot tissue at the end of the experiment showed that heavy metal accumulation did not exceed DATL in either uninoculated or AMF-inoculated plants. A second finding is that the AMF inocula tested had a significant effect on both the bacterial and fungal microbial community structure in mesquite rhizosphere samples at the end of the experiment. Taken together these results suggested that commercially available AMF inocula can support revegetation of mine tailings and that identification of key microbial populations that are correlated with improved biomass production will help in understanding the role of the microbial community in supporting plant growth in mine tailings. The next important step for this type of research is to determine whether these greenhouse results can be successfully translated to the field.

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