PLANT MICROBE INTERACTIONS

Growth of Quailbush in Acidic, Metalliferous Desert Mine Tailings: Effect of *Azospirillum brasilense* Sp6 on Biomass Production and Rhizosphere Community Structure

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Abstract Mine tailing deposits in semiarid and arid environments frequently remain devoid of vegetation due to the toxicity of the substrate and the absence of a diverse soil microbial community capable of supporting seed germination and plant growth. The contribution of the plant growth promoting bacterium (PGPB) *Azospirillum brasilense* Sp6 to the growth of quailbush in compost-amended, moderately acidic, high-metal content mine tailings using an irrigation-based reclamation strategy was examined along with its influence on the rhizosphere bacterial community. Sp6 inoculation resulted in a significant (2.2-fold) increase in plant biomass production. The data suggest that the inoculum successfully colonized the root surface and persisted throughout the 60-day experiment in both the rhizosphere, as demonstrated by excision and sequencing of the appropriate denaturing gradient gel electrophoresis (DGGE) band, and the rhizoplane, as indicated by fluorescent in situ hybridization of root surfaces. Changes in rhizosphere community structure in response to Sp6 inoculation were evaluated after 15, 30, and 60 days using DGGE analysis of 16S rRNA polymerase chain reaction amplicons. A comparison of DGGE profiles using canonical correspondence analysis revealed a significant treatment effect (Sp6-inoculated vs. uninoculated plants vs. unplanted) on bacterial community structure at 15, 30, and 60 days ($p < 0.05$). These data indicate that in an extremely stressed environment such as acid mine tailings, an inoculated plant growth promoting bacterium not only can persist and stimulate plant growth but also can directly or indirectly influence rhizobacterial community development.

Introduction

Metalliferous mine tailings, especially those found in arid or semiarid environments, pose a long-term health hazard for nearby urban populations through exposure to dust originating from the tailings and to tailings distributed into the local environment by water erosion [38, 44]. One strategy to reduce this hazard is phytostabilization—the creation of a vegetative cap that acts to prevent wind and water erosion and to stabilize (precipitate) metals in the root zone while limiting shoot accumulation [36]. The main obstacle to phytostabilization success is that most tailings are a poor substrate for plant growth due to both the abiotic stress posed by some combination of metal toxicity, low pH, high salinity, lack of essential minerals and nutrients, poor soil structure and water retention, and the absence of a suitable microbial community to sustain plant growth. As such, these tailings remain devoid of a vegetative cover for decades or longer [26, 38]. Amendments such as compost, biosolids, and lime, as well as irrigation have been suggested to help plant establishment on tailings [13, 48]. Compost amendments, for example, have been shown to increase the organic carbon and nutrient content, cation exchange capacity (CEC), pH, and neutrophilic heterotrophic bacteria counts of acidic,

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metalliferous mine tailings [37]. Though feasible, such amendments are costly due to the large volumes of tailings at many sites and also the remote location of some sites.

Recent studies have shown that the optimal compost levels required for the establishment of several species of grasses and plants on tailings can be reduced by supplemental amendment with plant growth-promoting bacteria (PGPB) isolated from tailings or areas adjacent to tailings sites [27, 43, 49]. These PGPB have also been found to influence the development of the rhizosphere microbial community [28]. In this study, we explore the application of the extensive research on plant growth promotion by *Azospirillum brasilense* inoculants, to plant growth in acidic, metalliferous mine tailings amended with a suboptimal level of compost (10% wt/wt). *A. brasilense* strains are known for their ability to promote plant growth in agricultural systems [9], desert soils [8], and microalgae in wastewater treatment [20], but have not been evaluated for use in disturbed ecosystems. Previous work has shown that the principle mechanism of plant growth promotion by *A. brasilense* is the production of plant phytohormones, principally indole acetic acid [22], and nitrous oxide [15, 40], which interact to stimulate the growth of lateral roots and root hairs. Recent work has suggested that this stimulation of adventitious root growth by *A. brasilense* not only enhances nutrient uptake but also alleviates the effects of salt stress in different agricultural plant species [3, 16, 21], both of which are common concerns in the phytostabilization of mine tailings in semiarid and arid ecosystems. In addition to plant growth promotion and enhanced resistance to abiotic stress, properties of *Azospirillum* that are relevant to their use in mine tailings include the ability to grow under pH [5, 18] and heavy metal [11] stress conditions.

The objectives of the present study are 1) to determine whether the agricultural inoculant, *A. brasilense* Sp6, an isolate with no previous exposure to mining tailings stress conditions, can support establishment of quailbush in acidic metalliferous mine tailings; 2) to determine how long the Sp6 inoculum survives; and 3) to assess the impact of Sp6 inoculation on the plant rhizosphere bacterial community structure. These objectives were addressed in an irrigation-based factorial greenhouse-scale experiment using compost-amended, acidic (pH 4.5) high-metal content desert tailings from the Klondyke State Superfund site in southern Arizona that have been previously characterized [27]. Previous greenhouse research established the optimal compost rate for the growth of quailbush in these tailings to be 15% [37]. The treatments evaluated here included Sp6-inoculated and uninoculated planted treatments and an unplanted control, all of which were supplemented with a suboptimal compost loading rate of 10%.

Methods

Native Plant

A shrub native to the desert southwest, *Atriplex lentiformis* (Torr.) S. Watson (commonly known as quailbush), was used in this study. Seeds were obtained from Carter Seeds, Vista, CA.

Characterization of Klondyke Tailings and Compost

Tailings were collected and homogenized from the Klondyke mine tailings site located in Aravaipa Valley, Graham County, AZ, where a lead/zinc flotation mill was in operation from 1948 to 1952. Physical and chemical characteristics for the tailings used in this study have been previously described [27]. Briefly, the mine tailings used in this study have a pH of 4.54±0.02, a texture of 51.9% sand, 26.4% silt, and 21.7% clay with major mineral constituents of quartz, orthoclase feldspar, and jarosite and minor constituents including plumbojarosite and goslarite. Chemical properties include a total organic carbon and total nitrogen content of 360±68 and 67±12 mg kg^{-1}, respectively, and electrical conductivity (EC) of 3.0±0.12 dS m^{-1}. The major metals in the tailings are as follows (mg kg^{-1} ± relative SD): As, 91±10%; Cd, 2.4±0.7%; Cu, 653±1%; Fe, $2.66×10^4$±1%; Mn, $2.8×10^3$±1%; Pb, $4.6×10^3$±1%; and Zn, $1.4×10^3$±1%. Culturable neutrophilic heterotrophic bacterial counts (enumerated after 15-day growth on R2A agar) were $2.95±0.97×10^3$ CFU g dry tailings^{-1}. Neutrophilic heterotrophic counts are used as an indicator of tailings toxicity to guide in the determination of compost loading rates and plant selection.

The compost was a mixed dairy-green waste material produced by the University of Arizona Agricultural Center, Tucson, AZ. The total carbon and nitrogen contents of the compost are 135.5±7% g kg^{-1} (91% organic carbon) and 2.7±16% g kg^{-1}, respectively. The major elements present in the compost include the following (g kg^{-1} ± relative SD): Ca, 40.4±0.6%; Fe, 16.5±2%; K, 10.4±11%; Al, 7.5±4%; Mg, 5.2±3%; Na, 1.5±5%; and Mn, 0.24±7%. Tailings compost mixtures were homogenized immediately prior to planting.

Plant Growth Promoting Bacterium

The PGPB used in this study, *A. brasilense* Sp6 (Katholieke Universiteit Leuven, Belgium), has been shown to produce indole acetic acid and stimulate the number and length of lateral roots as well as the distribution of root hairs [4]. This strain is also a known growth promoter of wheat [4] and microalgae [19]. To prepare Sp6 for inoculation, the bacterium was cultivated on tryptone-yeast extract-glucose

(TYG) medium supplemented with microelements [8]. Incubation was for 24 h at 30°C, with gyratory shaking at 120 rpm as described previously. Following incubation, cultures were formulated into alginate microbeads [8]. The alginate microbeads were mixed with the quailbush seeds to achieve an inoculation level of 1.2×10^6 CFU seed^{-1}. This inoculation level is based on inoculation optimization experiments using *A. brasilense* and alginate microbeads as inoculant carriers for growth of tomatoes and wheat in desert soils [8].

Experimental Design and Greenhouse Conditions

An initial pilot greenhouse study demonstrated that Sp6 inoculation was beneficial for the growth of quailbush (data not shown) in compost-amended Klondyke mine tailings under irrigated conditions. Therefore, a second greenhouse study was performed to evaluate the effect of Sp6 inoculation of quailbush grown in tailings amended with a suboptimal level of compost and further to examine the impact of Sp6 on rhizosphere community structure during plant growth. The second study was a completely randomized factorial experiment with two planted treatments (Sp6-inoculated and uninoculated) and an unplanted control. All treatments were conducted in irrigated tailings amended with 10% compost (wt/wt), a level previously shown to support slightly suboptimal growth of uninoculated quailbush plants in Klondyke tailings [37]. Each treatment/control comprised five replicate black plastic pots (15× 10 cm, diameter).

The tailings/compost mixture was prewetted to field capacity 48 h prior to initiation of the experiment (sowing). For the Sp6-inoculated treatment, 20 seeds inoculated with Sp6 alginate microbeads were planted in each pot. For the uninoculated control, 20 seeds without Sp6 inoculation were planted in each pot. The unplanted control comprised pots containing compost amended tailings that did not receive either seeds or Sp6 inoculation. Seeds were sown at a depth of approximately 0.5 cm using sterile tweezers and, following germination, were thinned to 10 seedlings per pot. An additional plant was removed after 10, 15, 30, and 60 days, to leave a total of 6 plants per pot at the end of the experiment. Plants removed at 15, 30, and 60 days were used to collect rhizosphere samples for subsequent rhizosphere community structure and fluorescent in situ hybridization (FISH) analyses.

Following planting, pots were placed in a greenhouse located at the University of Arizona's Controlled Environment Agriculture Center (Tucson, AZ) for 60 days. Each pot was irrigated three times daily via drip irrigation, distributing a total of 1.5 cm pot^{-1} d^{-1}. The greenhouse was maintained at a temperature that ranged from 20°C (night) to 30°C (day). Fluorescent supplemental lighting

($200\ \mu m\ m^{-2}\ s^{-1}$) was used to extend the daily photoperiod to 13 h d^{-1} as necessary.

After 60 days, all remaining plants were harvested and rhizosphere samples were collected for subsequent rhizosphere community structure and FISH analyses. Root and shoot tissues were separated and rinsed gently under running water to remove all remaining tailings and compost particles. Plant growth parameters were evaluated including shoot and root length, and leaf number. Plant dry mass was determined by placing root and shoot tissues into separate aluminum foil packets. Tissues were dried for 48 h in a 60°C oven and weighed [7].

Denaturing Gradient Gel Electrophoresis Analysis of Rhizosphere Soils

Bacterial community profiles associated with plant rhizospheres were evaluated by polymerase chain reaction (PCR)–denaturing gradient gel electrophoresis (DGGE) analysis. DGGE analysis was used to track the survival of the Sp6 inoculum in rhizosphere soils and to characterize the community structure of the dominant bacterial populations targeted by the selected primer set [41]. At 15, 30, and 60 days, rhizosphere soil samples were collected from plants removed from each treatment (Sp6-inoculated, uninoculated), and bulk soil was collected from the unplanted control. For each treatment, three plants were selected (randomly from three of the five replicate pots). After removing bulk soil from the plant root by shaking, the rhizosphere soil sample was collected by scraping the remaining soil from the root surface with a sterile spatula. Each soil sample was placed into a sterile 1.5-mL snap-cap tube and stored at −80°C until analyzed. DNA was extracted from a 0.4-g soil sample using the Fast DNA SPIN for soil kit (MP Biomedicals, Solon OH), following the manufacturer's protocol. Humic acids were removed by rinsing the Binding matrix-DNA complex with saturated 5.5 M guanidine thiocyanate (Flucka Sigma-Aldrich GmbH, Buchs, Switzerland) until the supernatant lost its brown tint [45].

Extracted DNA was quantified with a TBS 380 Fluorometer (Turner Biosystems, Sunnyvalley, CA), using the protocol for Quant-iT PicoGreen dsDNA (Molecular Probes, Eugene, OR). Approximately 100 pg μL^{-1} DNA template was used for each PCR reaction.

The V7/V8 variable region of the 16S rRNA gene was amplified following a modified protocol originally described by Colores et al. [14] using the universal bacterial primers 1070f and 1406r-GC [24]. Briefly, each 25 μL reaction contained 2.5 μL of 10× buffer with 15 mM MgCl$_2$ (Qiagen Sciences, Germantown, MD), 200 μM dNTP, 0.4 μM of each primer, 5% dimethyl sulfoxide (Sigma, St. Louis, MO), 0.4 g L^{-1} unacetylated bovine serum albumin (Sigma), 0.5 U HotStarTaq DNA polymerase (Qiagen Sciences), and 100 pg

of community DNA extract. The amplification protocol used 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. PCR products were visualized on a 2% agarose gel (GenePure LE ISC BioExpress, Kaysville, UT) using an Alpha Imager (Alpha Innotech, San Leandro, CA).

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 7% acrylamide and a 45%–70% urea-formamide denaturing gradient (100%=7 M urea and 40% formamide). Each gel included an external reference ladder containing three bands, the second being the *A. brasilense* Sp6 band included to aid in visualizing *A. brasilense* Sp6 on the gels. The reference bands at the top and bottom of the ladder were selected to represent high and low GC content markers to aid in scoring the gel and have no specific association with mine tailing bacterial populations. Gels were run at 60°C and 50 V for 16.5 h, then stained in 2× SYBR Green (Molecular Probes) for 20 min and imaged. Microbial community banding profiles on DGGE gels were analyzed using the Quantity One software package (Bio-Rad Laboratories, Inc., Hercules, CA).

Identification of *A. brasilense* Sp6 in DGGE Profiles

Putative Sp6 bands from each sampling day were excised from DGGE profiles with a sterile razor blade, placed in sterile 1.5-mL snap-cap tubes with 100 μL elution buffer (0.5 M ammonium acetate, 1 mM EDTA, pH 8.0) [2], incubated at 50°C for 30 min and then centrifuged at 15,000×*g* for 2 min. The supernatant was diluted 1:50 in sterile nanopure water, and 1 μL was used as template DNA for PCR using the primers and reaction conditions described above. PCR products were compared with the original profiles by DGGE analysis (45%–70% gradient) to confirm band purity and identity. DGGE-PCR cycles were repeated until pure bands were obtained for sequence analysis. The PCR product was then purified with a QIAquick PCR Purification kit (Qiagen Sciences) prior to submitting the samples to the University of Arizona Research Laboratories Genomic Analysis and Technology Core (Tucson, AZ) for sequencing in both directions with an Applied Biosystems 3730X1 DNA Analyzer (Applied Biosystems, Foster City, CA) using the primers 1070f and 1406r. Excised band sequences were compared with that of the original inoculum, which was sequenced at the time of the experiment.

FISH Analysis

FISH analysis was performed with two oligonucleotides probes: EUB338 [1] specific for the domain *Bacteria* and an *A. brasilense*-specific probe (KO 205—Probe Base accession number: pB01271) [25] targeting 1,012–1,030 bp of the 16S rRNA gene. This probe was checked for specificity using both the Ribosomal Database Project (RDP—http://www.microbial-ecology.net/probebase) and Greengenes (http://greengenes.ibl.gov). Oligonucleotides probes were labeled at the 5′ end with the fluorescent dyes Cy3 (*Azospirillum* specific probe) and Cy5 (universal probe; IDT, Coraville, IA). Specificity of the KO 205 was checked at 15% stringency using pure cultures of *A. brasilense* Sp6 and A. brasilense Cd (positive controls) and *Bacillus pumilus* (negative control) as described by Daims et al. [17].

Root samples were harvested at 15, 30, and 60 days from the same plants used for the DGGE analysis of rhizobacterial communities. Roots were carefully separated from the adhering soil, washed with 1× phosphate-buffered saline (PBS; 200 mM NaH_2PO_4, 200 mM Na_2HPO_4), and fixed with 4% paraformaldehyde (Acros, Morris Plains, NJ) for 2 h at −4°C. After fixation, roots were washed with 1× PBS and stored in a mix of 1× PBS/96% ethanol (1:1 vol/vol) at −20°C until hybridization. FISH analysis was performed as described by Iverson and Maier [32]. Briefly, slices of the fixed root were thawed, placed on a gelatin (0.1% wt/vol, 0.01% wt/vol cromium potassium sulfate)-coated microscope slide, fixed to the slide by adding 1 drop of warm low melt agarose solution (0.2% wt/vol, agarose LMP; Promega, Madison, WI), and dried at 37°C for 45 min. Samples were dehydrated by successive 50%, 80%, and 96% ethanol washes (3 min each) and finally air dried. Hybridization was performed at 15% stringency at 46°C for 2 h. The final concentration of the probe was 3 ng μL^{-1}. Samples were washed at 48°C for 15 min with 50 mL of prewarmed washing buffer. The slides were rinsed with ice-cold deionized water and air dried. Slides were stored at −20°C in the dark until visualized.

For visualization, the slides were mounted in AF1 antifading reagent (Citifluor–Electron Microscopy Sciences, Hattfield, PA). Epifluorescent microscopy was performed for *Azospirillum* Sp6 pure cultures hybridized with the specific KO 205 probe using an Axioplan 2 Imagin epifluorescence microscope (Zeiss, Thornwood NY). Cells were observed under oil immersion using a 100× objective. Root samples were examined using confocal laser microscopy on a Zeiss LMS 510 Meta confocal scanning laser microscope (Zeiss). *A. brasilense* and members of the domain *Bacteria* were visualized simultaneously using two helium neon lasers for excitation of the Cy3 dye at 543 nm and the Cy5 dye at 633 nm. The pinhole was set for both channels to 1.2 Airy units. Samples were observed with a 63× water immersion objective (Zeiss). To distinguish visualization of *A. brasilense* Sp6 and other bacteria, fluorescence from Cy3 and Cy5-labeled oligonucleotides probe was assigned green and red colors, respectively. Thus, *A. brasilense* cells appear yellow in the image, and other bacteria appear

red. Combining and analysis of images were performed with the ZEISS software package LSM 510 ver. 4.2 (Oberkochen, Germany). Images were processed with Adobe Photoshop ver. 8.0 (Adobe Systems Incorporated, Mountain View, CA).

Statistical Analysis

Plant growth parameter data were analyzed using Student's *t*-test at the significance level of $p \leq 0.05$. Data in percentages were converted to arcsin before analysis. All statistics were performed using Statistica software (V. 6; Statsoft, Tulsa, OK).

Multivariate analysis of rhizobacterial community DGGE banding patterns evaluated with the Quantity One software was conducted using canonical correspondence analysis (CCA), a form of correspondence analysis widely used in community ecology [34, 42]. CCA finds axes of variation in banding patterns that are maximally related to explanatory variables or treatments (e.g., inoculum vs. no inoculum). CCA eigenvalues represent the strength of the relationship between DGGE profile bands and the treatment and are tested against the null model of no relationship using a permutation test [47]. CCA also allows simultaneous visualization of different treatments, samples, and DGGE bands in few dimensions using a triplot. Treatments in CCA triplots are typically represented by centroids; i.e., points that represent the weighted mean of a treatment multivariate data set along all axes. According to the centroid rule, the proximity of a population (e.g., a DGGE band) to a centroid or sample point is directly related to the occurrence of that population in that centroid (treatment) or sample (replicate). PAST, version 1.90, was used for CCA [29].

Results

Effect of Inoculation on Quailbush Growth

Inoculation with *A. brasilense* Sp6 significantly improved several quailbush growth parameters including root length, and root and shoot biomass production in this 60-day experiment (Table 1). Both root and shoot biomass were increased 2.2-fold in Sp6 inoculated plants, whereas root length was increased 1.5-fold. Plant growth parameters that were not significantly improved include shoot length and number of leaves, both of which are shoot tissue-related parameters. The overall structure of the Sp6 inoculated plants was more robust than that of the uninoculated plants. Stems were thicker, the leaves were larger, and there were more lateral branches, all of which is reflected in the significantly higher shoot biomass parameter recorded for this treatment. The Sp6 inoculum also improved the germination rate (measured after 5 days), which increased from 24% for uninoculated seeds to 72% in inoculated seeds (data not shown).

Persistence of Sp6 in the Rhizosphere

A reference ladder containing the Sp6 band was used in the DGGE analysis to track the presumptive persistence of the inoculant in rhizosphere samples of plants harvested randomly from three of the five replicate pots at 15, 30, and 60 days (Fig. 1). To investigate the presence of Sp6, a putative band located in the appropriate position was excised from one of the triplicate DGGE profiles from each time point and sequenced in both directions. Results reveal that a sequence with 100% identity to the Sp6 inoculum was recovered from each time point, suggesting that the inoculated PGPB survived in the plant rhizosphere for 60 days. The likelihood that the excised bands represented the inoculant and not a related indigenous *Azospirillum* strain was evaluated by comparing the nearly full-length Sp6 sequence to the 80 most closely related (96%–99% similar) sequences in the GenBank database. This analysis revealed that DGGE (targeting the V7/V8 variable region) can differentiate between *Azospirillum* strains at the species level and in many cases at the strain level. Strains with ≥99% nearly full-length16s rDNA sequence identity to the Sp6 inoculum could not be differentiated using the DGGE analysis alone, but 60% of the strains with 98%–99% sequence identity, 92% of those with 97%–98% sequence identity, and 100% of those with 96%–97% identity to the Sp6 full-length sequence could be differentiated by the DGGE analysis used in this study.

Table 1 Effect of *A. brasilense* Sp6 inoculation on quailbush growth parameters in acidic, high-metal content mine tailings

Treatment	No. of leaves[a]	Length (cm)		Root/shoot length ratio	Dry mass (g)	
		Root[a]	Shoot[a]		Root[a]	Shoot[a]
Uninoculated	7.0±0.6a	3.5±0.2a	6.4±0.7a	0.54	3.70±0.53a	23.48±2.15a
Inoculated	5.9±0.3a	5.4±0.3b	7.2±0.3a	0.75	8.64±1.40b	51.23±3.05b

[a] Plant growth parameter values are presented as the average and the standard error of five replicates. For each growth parameter, means with different letters are significantly different at *p*<0.05
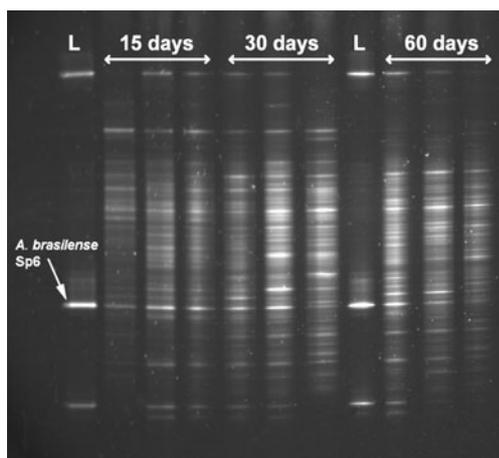
Figure 1 A DGGE gel showing triplicate bacterial community rhizosphere profiles from Sp6 inoculated tailings after 15, 30, and 60 days. A ladder (lanes marked L) composed of high and low GC-content environmental isolates bracketing the Sp6 strain, which is indicated on the gel

There is a possibility that other *A. brasilense* strains were introduced into the tailings with the compost amendment, but the GenBank data analysis indicates that only a small percentage of *A. brasilense* strains with <98% overall sequence similarity to Sp6 would have DGGE bands with the 100% sequence similarity to the Sp6 inoculum detected in this study. We believe that this 16S rRNA gene analysis strongly suggests that the excised bands represent the original inoculum, thus supporting the likelihood that the inoculant persisted in the rhizosphere for the 60 days. Though Sp6 appears to have persisted in the rhizosphere throughout the study, a decrease in the DGGE band brightness (Fig. 1) by 60 days indicates that the Sp6 population density declined relative to the overall rhizo-bacterial community.

## Rhizoplane Colonization of Quailbush Plants by *A. brasilense* Sp6

FISH analysis was performed to document the colonization by Sp6 of the rhizoplane and to examine root colonization in general. Root samples were prepared by carefully removing the adhering soil, washing with PBS, then rinsing multiple times during the FISH preparation procedure, a protocol that removes all rhizosphere soil, thus limiting the analysis to the rhizoplane populations. FISH was performed using two probes, a universal probe (arrows indicating red colonies) and a Sp6 specific probe (arrows indicating yellow colonies). After 15 days (Fig. 2a), root surfaces of uninoculated plants exhibited limited colonization, whereas those of Sp6-inoculated treatments showed extensive colonization of root tips and at the sites of emerging lateral roots (Fig. 2b–d). Most bacteria on the root surfaces of inoculated plants hybridized with the Sp6-specific probe

(yellow) indicating strong colonization at this early time point by Sp6, whereas no Sp6-probe hybridization was observed on the uninoculated root surfaces (Fig. 2a). As is typical for *Azospirillum*, large aggregates of Sp6 were observed on some of the inoculated roots examined (e.g., Fig. 2b). As with the DGGE analysis, FISH results showed a dominant presence of Sp6 in the rhizoplane communties at 15 days, but by 60 days, the proportion of Sp6 in relation to other populations was no longer dominant (Fig. 2e, f).

Effect of Inoculation on the Rhizosphere Bacterial Community Structure

The rhizosphere bacterial community was monitored on plant samples harvested randomly from three of the five replicate pots for each treatment. In addition, bulk soil samples were taken simultaneously from the unplanted control to monitor compost-induced community changes. The community was analyzed at three separate time points: 15, 30, and 60 days. CCA analysis of DGGE profiles of the Sp6-inoculated treatments (Fig. 3a) revealed a significant effect of sample time on rhizosphere bacterial community structure ($p=0.003$), suggesting a temporal shift in the community structure during the study. We further examined the relationship between the Sp6-inoculated and uninoculated rhizosphere communities and the unplanted control at each of the time points. The results reveal a significant treatment effect at all three time points, ($p = 0.002$ at 15 days, $p = 0.002$ at 30 days, and $p = 0.004$ at 60 days; Fig. 3b–d). The first axis accounted for 71%, 83%, and 76% of the variation for the 15-, 30-, and 60-day time points, respectively. At all three time points, each of the treatment replicates clustered together, and the separation of the Sp6-inoculated rhizosphere community from the other soil communities was explained primarily by the first axis, whereas the variation between the uninoculated treatment and the unplanted control was explained by the second axis. Thus, the rhizosphere communities of the uninoculated quailbush plants were more similar to the unplanted bulk soil than to the Sp6-inoculated rhizosphere community. Taken together, these results demonstrate that the Sp6 inoculum significantly influenced the rhizosphere community structure at all time points measured and that the community structure changed significantly as plants established and grew in the tailings.

CCA allows visualization of the distribution of specific bacterial populations (e.g., bands on the DGGE gel) among the treatments tested. If one makes the assumption that each DGGE profile band is associated with a unique population or combination of populations within a community, then the CCA triplot provides information about the relative proportion of populations uniquely associated with specific treatments as compared with populations shared more equally
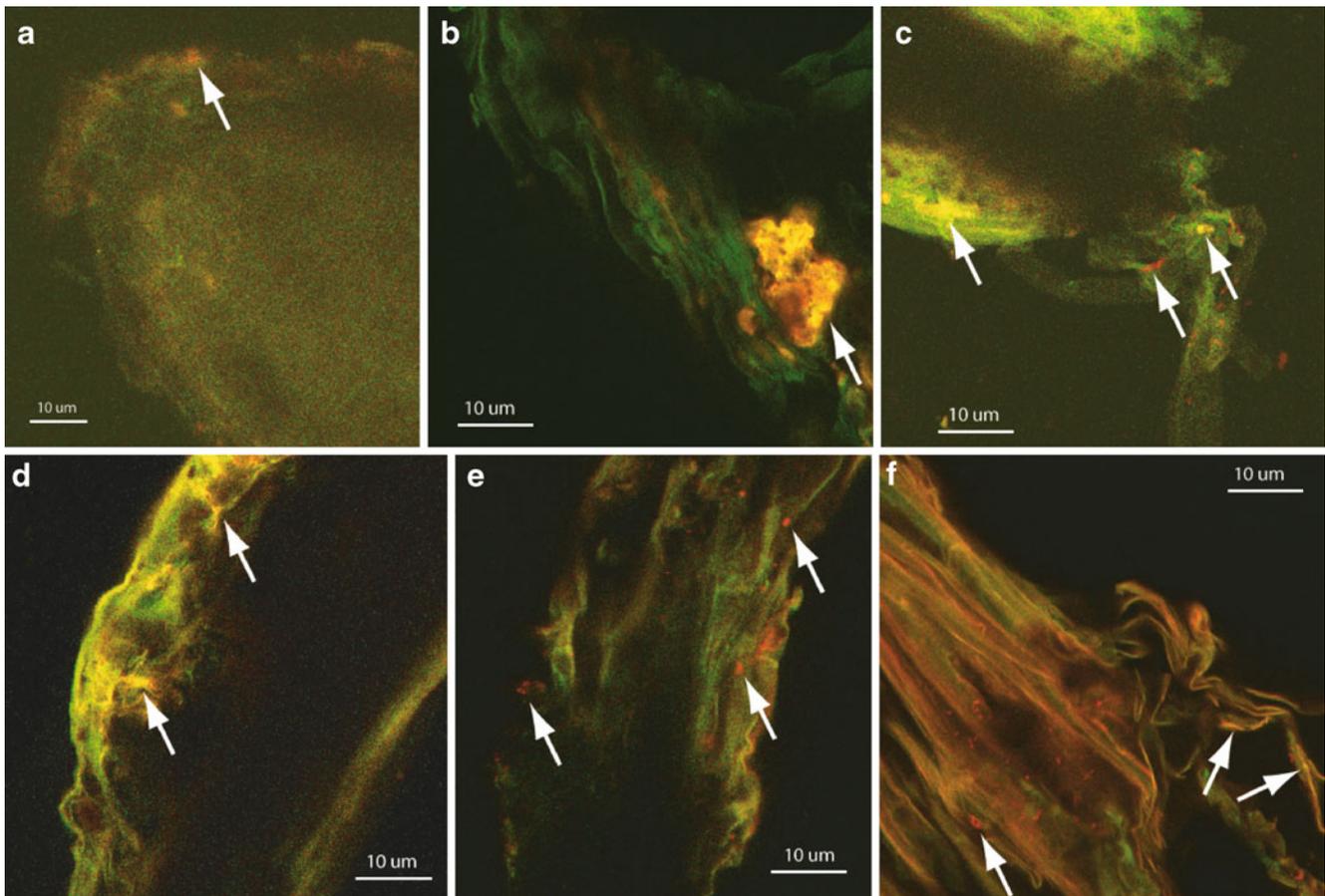
Figure 2 FISH analysis of root colonization of quailbush by *A. brasilense* Sp6 using two probes: KO 205, which is specific for Sp6 (*arrows indicating yellow color*), and the universal probe, EUB338 (*arrows indicating red color*). The *white arrows* in each panel point out bacteria or bacterial colonies. **a** An inoculated plant root tip after 15 days showing little colonization (arrow indicates red colonies); **b-d**. All arrows in **b** and **d** and the left and right arrows in **c** indicate yellow colonies. The center arrow in **c** indicates a red colony. **e, f**: Sixty-day-old root tips from plants inoculated with Sp6 showing that colonization by 60 days is dominated by bacteria other than Sp6 (*arrows indicate red colonies*)

between treatments. Figure 4 shows the CCA triplots for 15, 30, and 60 days results from Fig. 3. In this figure, each band beginning at the top of the gel was assigned a number (in numerical order) for each possible vertical location among the lanes analyzed. Analysis of the bands uniquely associated with only one of the three treatments (Fig. 4, numbers in text boxes) suggests that the number of specific populations associated with the planted treatments increased over time, whereas the number of bands uniquely associated with the unplanted control remained fairly constant (15 days, 3 bands; 30 days, 4 bands; and 60 days, 2 bands). At 15 days, two unique bands (3% of all bands) were associated with each of the planted treatments, Sp6 inoculated and uninoculated (Fig. 4a). By 30 days, this number increased to four bands (9%) for the Sp6 inoculated and 5 bands (11%) for the uninoculated planted treatments (Fig. 4b). Finally, by 60 days, seven (13%) and 9 (16%) bands were uniquely associated with the Sp6 and uninoculated treatments, respectively (Fig. 4c). This pattern indicates that over time,

both of the planted treatments developed an increasing number of bacterial populations uniquely associated with the rhizosphere of that specific treatment, a development that did not occur in the unplanted bulk soil. It is interesting to note that the magnitude of the phenology effect was similar for both of the planted treatments, but the populations uniquely associated with each treatment were distinct, and the uninoculated rhizosphere community was more similar to the unplanted bulk community than to the Sp6-inoculated rhizosphere community

Discussion

Effect of *A. brasilense* Sp6 on Plant Biomass Production

Commerical *Azospirillum* inoculants are currently used to enhance wheat and maize production in Argentina and Mexico [12]; however, the potential application of this
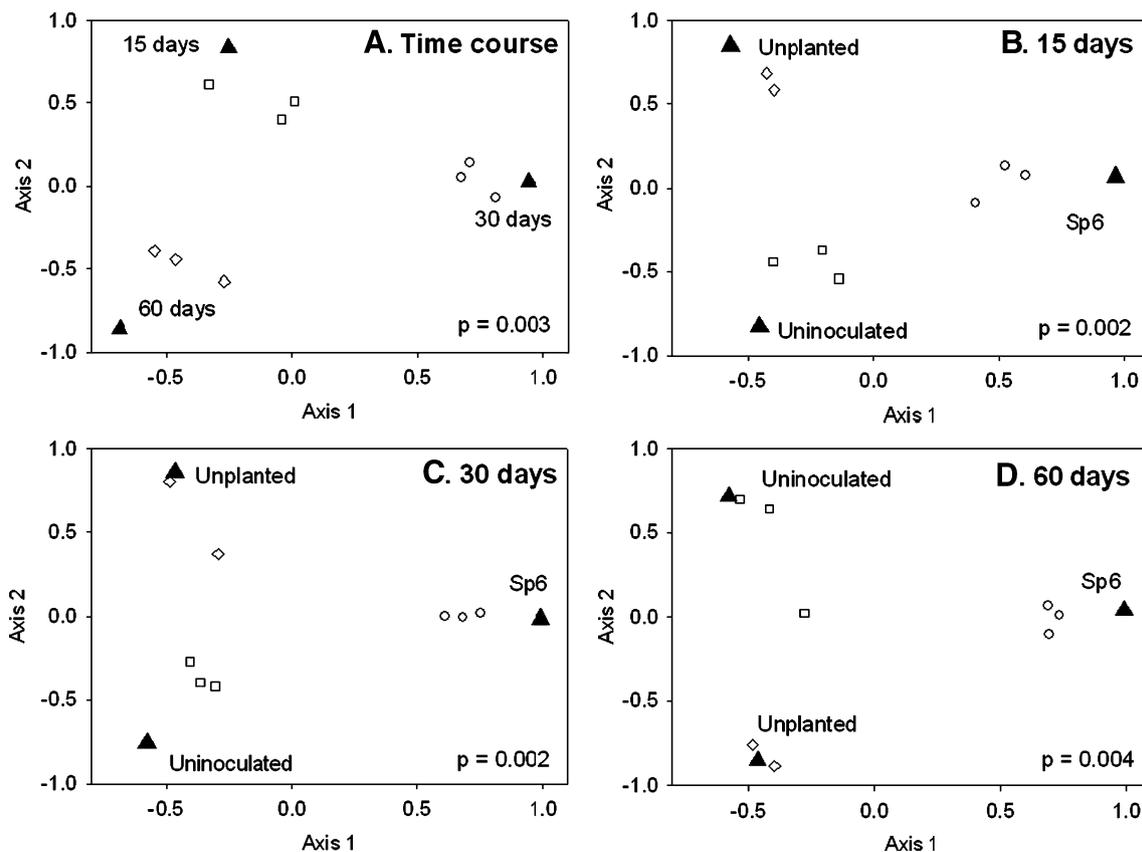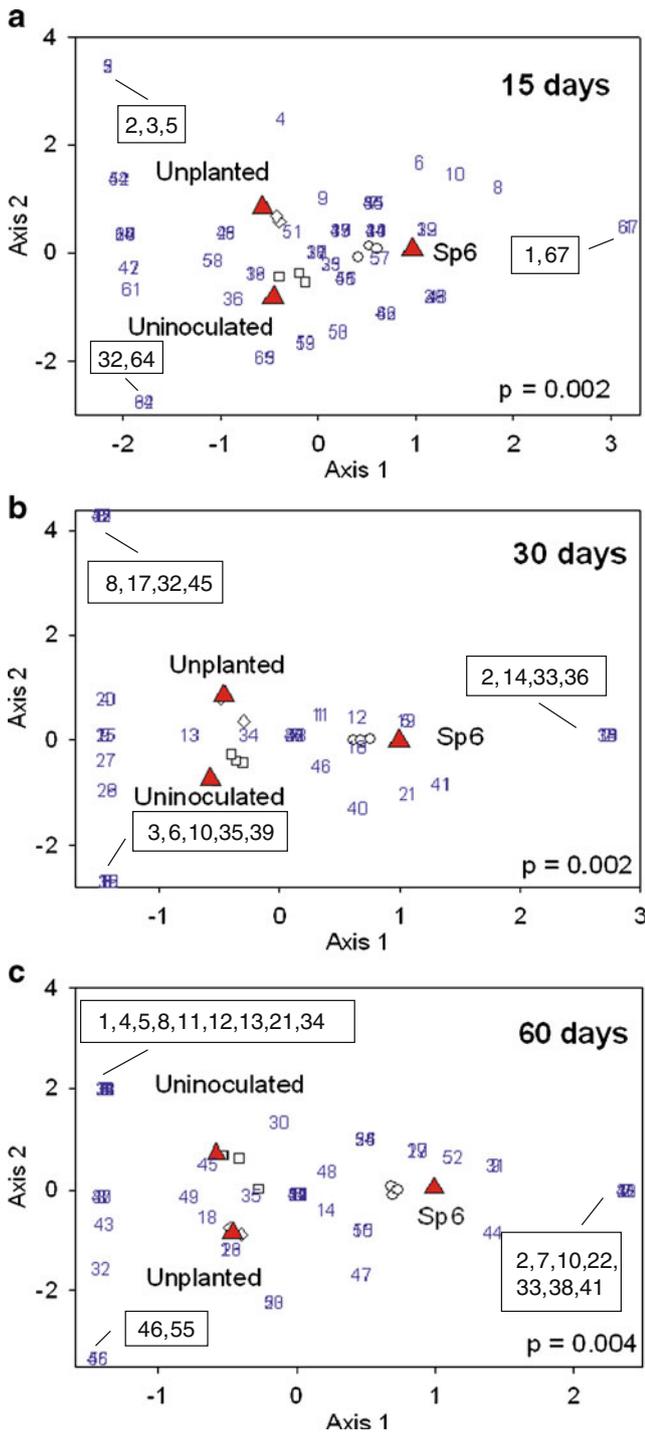
Figure 3 DGGE-CCA analysis of the rhizosphere bacterial community of quailbush grown in an acidic, high-metal content tailings. The filled triangles (▲) represent the CCA centroid for each treatment, while the open symbols represent the individual replicates for that treatment. Following CCA for each analysis, a permutation test was performed (1,000 permutations) to evaluate the influence of time (**a**) or treatment (**b–d**) on the bacterial community structure. A *p*-value <0.05 confirms a significant treatment effect. **a** A time course comparison of the Sp6-inoculated treatments at 15 (□), 30 (○), and 60 days (◊). Axis 1 and axis 2 account for 62% and 38% of the variance, respectively. **b** A comparison of the Sp6-inoculated (○), uninoculated (□), and unplanted control (◊) treatments at 15 days. Axis 1 and axis 2 account for 71% and 29% of the variance, respectively. **c** A comparison of the Sp6-inoculated (○), uninoculated (□), and unplanted control (◊) treatments at 30 days. Axis 1 and axis 2 account for 83% and 17% of the variance, respectively. **d** A comparison of the Sp6-inoculated (○), uninoculated (□), and unplanted control (◊) treatments at 60 days. Axis 1 and axis 2 account for 76% and 24% of the variance, respectively

biotechnology for the phytostabilization of disturbed ecosystems has received less attention. One approach to discovering PGPB that may be useful in the phytostabilization of disturbed ecosystems with high levels of abiotic stress such as mine tailings is the isolation of potential PGPB from mine tailings or from native plants growing adjacent to tailings sites. In this study, we propose the investigation of PGPB known to be effective in agricultural settings. The former process requires the isolation and purification of multiple potential strains followed by laboratory-scale screening for PGPB traits and finally greenhouse screening studies to identify successful candidates. While this approach has been used successfully to target strains suited to the arid ecosystem and capable of survival in the stressed tailings environment, it is labor intensive [27, 28].

The latter approach, taken in this study, focuses on available and proven PGPB strains that have been shown to be rhizosphere competent in agricultural settings and have been extensively characterized [6]. We investigate whether a commercial *A. brasilense* strain known to promote growth of agricultural crops in relatively undisturbed soils can successfully colonize a native plant of the arid southwest in an abiotically stressed mine tailings ecosystem. This approach allows phytostabilization efforts to take advantage of the extensive research available documenting the effectiveness and modes of action of this commercially available root colonizing bacteria. *A. brasilense* was selected for this trial due to the documented plant growth promoting effects of this PGPB for a broad range of agricultural crops resulting from stimulation of root development including increased root branching and a higher density of root hairs [9, 35]. This phytostimulatory mode of action provides increased surface area for nutrient absorption, a quality vital to the survival of plants in highly impacted, low-nutrient, mine tailings environments. In

Figure 4 DGGE-CCA analysis of the rhizosphere bacterial community of quailbush grown in an acidic, high-metal content tailings showing the relationship of the bands on the DGGE profiles with the treatments tested. The filled triangles (▲) represent the CCA centroid for each treatment, and the open symbols represent the replicates for the unplanted control (◇), the uninoculated (□), and Sp6- inoculated (○) treatments. Following CCA for each analysis, a permutation test was performed (1,000 permutations) to test the influence of treatment on bacterial community structure. A *p*-value <0.05 confirms a significant treatment effect. The *small numbers (blue)* represent all the possible band locations on the DGGE gel where the highest possible vertical location was labeled starting with the number 1. Note that numbers cannot be compared among different gels; i.e., they do not represent the same band in each gel. **a** A comparison of the Sp6-inoculated, uninoculated, and unplanted control treatments at 15 days. A total of 67 unique band positions were identified on this gel. **b** A comparison of the Sp6-inoculated, uninoculated, and unplanted control treatments at 30 days. A total of 47 unique band positions were identified on this gel. **c** A comparison of the Sp6-inoculated, uninoculated, and unplanted control treatments at 60 days. A total of 55 unique band positions were identified on this gel

from barren desert areas with rich desert soils collected beneath the canopy of mesquite trees [8].

In this study *A. brasilense* Sp6 successfully promoted the growth of quailbush in acidic, metalliferous mine tailings amended with a suboptimal level of compost (10% wt/wt). Previous work established that a 15% compost amendment is needed to support growth of quailbush in the Klondyke tailings comparable with that observed in an offsite control soil [37]. Thus, the results suggest that *Azospirillum* may have potential for environmental application in acidic, metal-contaminated soils. Sp6-inoculated plants demonstrated an increase in seed germination rate from 24% to 72%, as well as significant increases in root length and biomass. The Sp6 inoculum stimulated not only root growth but also general plant health as indicated by the 2.2-fold increase in shoot biomass. Previous research has established that the alginate matrix used to encapsulate the PGPB does not promote enhanced seed germination or plant growth of tomato or wheat in uncontaminated soils [8] or quailbush in Klondyke mine tailings [28]. In fact, the quailbush survival rate, defined as the number of seeds that germinated and survived for the duration of the experiment, was only 7% for the seeds inoculated with PGPB-free alginate microbeads as compared with 21% for the alginate-free uninoculated seeds. In addition, the total biomass was consistently lower for plants inoculated with PGPB-free alginate microbeads than for alginate-free plants, though the difference was not statistically significant [28]. Thus, the alginate matix alone does not enhance plant growth in general and may in fact be detrimental to the growth and biomass production of quailbush. These results confirm that the growth-promoting effects observed in response to inoculation with alginate-encapsualted Sp6 are due to the Sp6 inoculum and not the alginate matrix.

addition, increased root mass is desirable for the physical stabilization of mine tailings against wind and water erosion. Finally, *Azospirillum* strains have also been shown to enhance salt tolerance in a range of plants including maize, wheat, and lettuce [21], a condition of concern for plants in arid mine tailings. In addition, *A. brasilense* Cd was used successfully to promote growth of tomato and wheat plants in a 1:1 mixture of poor arid soils obtained

The growth-promoting effects presented here support results reported by Saad et al. [46] showing that *Azospirillum* aided in growth of sweet potato in a nutrient-poor, sandy material from a tin mining site. The physicochemical characteristics of the tin tailings were not described other than to indicate that they were low in nitrogen and phosphorus and were >90% sand. Results showed that similar sweet potato growth parameters were achieved with inoculated plants grown at one third the nitrogen application as uninoculated controls at full nitrogen application.

Survival of *A. brasilense* Sp6 in Mine Tailings

The results suggest that Sp6 was able to successfully colonize the roots of quailbush and survive in the rhizosphere of these compost-amended tailings for the duration of the study. At 15 days, significant colonization of the root tips and the emerging lateral roots by Sp6 was confirmed using FISH-specific probes for *A. brasilense*. After 60 days, Sp6 cells were still detected on the root surface, but their numbers were significantly reduced relative to the total population. The persistence of Sp6 in rhizosphere soils at 15, 30, and 60 days following inoculation was supported by the presence of a DGGE band in the appropriate position. Identity with the Sp6 inoculum was confirmed by excision and sequencing of the band. Despite this isolation of a16S rRNA gene fragment from the inoculated plant rhizosphere with 100% similarity to the Sp6 inoculum, the possibility cannot be ignored that this DNA could represent an indigenous Sp6-type strain that was stimulated by the rhizosphere of inoculated plants. However, this scenario is unlikely because like the rhizoplane colonizers, rhizosphere numbers of *Azospirillum* relative to the total population declined rather than increased over time (by 60 days, the brightness of the DGGE band was diminished). Both FISH and DGGE are indicators of relative population density; thus, the observed decrease in Sp6 population density reflects a decrease relative to the total bacterial population of the rhizoplane and rhizosphere. A similar pattern was observed in a maize field trial where the concentration of the *Azospirillum* inoculum on the root surface was sustained at 7 and 35 days after sowing, but had declined by 65 days [10].

Ecological Impact of *A. brasilense* Sp6 on the Rhizosphere

A significant phenology effect was observed in the rhizosphere of Sp6-inoculated plants over the time course of the experiment. Further analysis revealed that an increasing number of specific populations were uniquely associated with the Sp6-inoculated rhizosphere as time progressed. Of particular interest is that while the same pattern was observed for the uninoculated plants, the specific populations associated with each treatment were distinct and the overall community structure of the uninoculated plants was more similar to the unplanted control than to the Sp6-inoculated treatment. In addition, the number of divergent populations associated with each of the planted treatments continued to increase over time despite the fact that the relative dominance of the Sp6 population on both the rhizoplane and in the rhizosphere of the inoculated plants declined by 60 days.

Several studies have examined the ecological impact of *Azospirillum* inocula on rhizobacterial structure in agricultural soils. Two recent greenhouse studies examined the impact of *A. brasilense* inoculation on the corn plant rhizosphere. The inoculum had little to no effect on the general bacterial community structure or on the structure of specific bacterial divisions (as determined by 16S rRNA PCR-DGGE or automated ribosomal intergenic spacer analysis) [30, 31, 35]. In contrast, one greenhouse study evaluating inoculation of tomato with *A. brasilense* Sp245 [23] and a second report on maize inoculation with *Azospirillum lipoferum* CRT1 in a field study [10] found shifts in the composition of the indigenous rhizobacterial communities. Felici et al. [23] found a slight increase in the number of bands and the variability of the DGGE profiles of rhizosphere samples taken 45 days after sowing from tomato plants inoculated with either *A. brasilense* Sp245 or *Bacillus subtilis* strain 101 when compared with the indigenous profiles from uninoculated plant rhizospheres. The observed increase in diversity was attributed to the influence of the inoculants on either root exudation or the release of molecular signals. Using automated ribosomal intergenic spacer analysis fingerprints, Baudoin et al. [10] documented a transient, but statistically significant shift in the structure of the rhizobacterial communities at 7 and 35 d following sowing of *Azospirillum lipoferum* CRT1 inoculated maize, but the effect was no longer apparent at 65 d. The loss of the inoculum-based impact on the rhizosphere community structure by 65 d was accompanied by a concurrent decrease in the number of *Azospirillum* inocula present on the plant roots as stated above.

The results present here are similar to those of Baudoin et al. [10], with the exception that the apparent decline in relative inoculum concentration on the rhizoplane and in the rhizosphere at 60 days was not associated with a loss of the ecological impact on the rhizobacterial community structure. This difference could be attributed to that fact that the Baudoin study used a different *Azospirillum* strain and was conducted in the field rather than the greenhouse. Alternatively, the Sp6-induced increase in root biomass observed for the inoculated plants and the potential influence on root exudation patterns could lead to a more sustained influence on the compromised rhizosphere bacterial communities of the nutrient poor mine tailings than on

the communities of a comparatively more favorable agricultural soil. Previous work has indicated that the stimulation of root growth by *A. brasilense* indirectly affects the ecology of the rhizosphere [9, 31]. The increased root biomass provides increased surfaces for rhizobacterial colonization as well as a significant source of organic carbon. Herschkovitz et al. [31] suggest that a secondary effect of the more developed root system may be a higher rate of C, N, and P turnover per unit soil volume, a factor that would be significant in the nutrient poor mine tailings ecosystem. *A. brasilense* has been shown to stimulate not only root development but also overall plant root exudation [31, 33]. The potentially enhanced nutrient status of the Sp6-inoculated rhizosphere could explain the observation that the community structure of the uninoculated rhizosphere was more similar to the compost-amended bulk soils than to the inoculated rhizobacterial community. Taken together, these results indicate that the Sp6 inoculation had a unique effect on the development of the rhizobacterial community and suggest that this effect may be an indirect response to the influence of the Sp6 inoculum on plant root growth and/or plant exudates.

These results are similar to our recent report using a different plant (*Buchloe dactyloides*, buffalo grass) and a different inoculum (a mix of two *Arthrobacter* spp. with different PGPB characteristics) [28]. In this report, the structure of the rhizosphere bacterial communities following 75 days of growth was also found to be influenced by the specific PGPB inoculum. In the present study, we expanded our analysis to include a temporal analysis of the rhizosphere effect accompanied by an investigation of the persistence of the inoculum over time. The significant differences in rhizosphere community structure were found to be not only PGPB dependent but also age dependent. Significant differences between PGPB-inoculated and uninoculated plants were already present 15 days following planting and continued to develop throughout the course of the experiment. This pattern is unique when compared with previous studies evaluating the effect of *Azospirillum* inoculation of plants grown in agricultural soils [10, 23]. In addition, we confirmed that the Sp6 PGPB used in this study colonized both the root surface and the rhizosphere and was detected in both locations for 60 days. Interestingly, we found that while the relative dominance of the inoculum population decreased over time, the observed rhizosphere effect persisted. We suggest that this pattern can be attributed to the fact that the Sp6 inoculum influences the rhizosphere community structure indirectly by influencing plant root development and exudations. Finally, we have shown that a well-characterized commercial inoculant known to enhance root development and the production of root exudates can be successfully exploited to enhance plant establishment in metal-contaminated mine tailings. Future work will characterize the changes in microbial diversity indicated by the shifts in microbial community structure observed following inoculation with PGPB.

As indicated in the previously described studies, a primary concern with PGPB inoculation in agricultural systems is the disruption of rhizosphere microbial community structure [10, 23, 31, 35]. Unlike the stable ecosystems evaluated in these reports, mine tailings represent degraded ecosystems with severely compromised bacterial communities lacking the rich diversity found in established soil communities [39]. We have found that neutrophilic heterotrophic plate counts are a good indicator of plant growth potential in acidic mine tailings where tailings with low counts have less potential for successful plant establishment [37]. A greenhouse study evaluating growth of uninoculated quailbush in Klondyke tailings found that plant establishment stimulated a significant increase in neutrophilic heterotrophs accompanied by a decrease in sulfur- and iron-oxidizing populations [37]. Prior to compost amendment, the number of neutrophilic heterotrophs in these tailings was very low (~3000 CFU g dry tailings^{-1}) reflecting the acidic pH, low amounts of organic C, N, and P present, and the high toxic metal concentrations. Mendez et al. [39] compared the bacterial diversity of two different samples of these Klondyke mine tailings with an offsite control soil and found that both the Chao 1 richness estimate and the Shannon diversity index decreased with pH for two tailings samples of pH 2.7 and 5.7 when compared with the offsite control soil of pH 7.7. The phylogenetic diversity followed a similar pattern where the tailings diversity was represented by 4 and 7 phyla for the pH 2.7 and 5.7 samples, respectively, both of which were dominated by bacteria belonging to the *Firmicutes* and γ-*Proteobacteria* divisions. In contrast, the offsite soil, which is a natural habitat for quailbush, was characterized by 11 phyla dominated by α-*Proteobacteria* and *Gemmatimonadetes*.

In summary, the goal of phytoremediation is not only to establish a vegetative cap to stabilize metal contaminants in the root zone but also to begin the successional transformation of the tailings from a toxic environment incapable of sustaining plant growth to a substrate supporting a diversity of native plants. This transition would ideally include the development of a more diverse indigenous microbial community capable of supporting plant growth and nutrition. The results presented here demonstrate that inoculation with *A. brasilense* Sp6 not only enhances the plant growth supported by compost amendment but also creates a sustained ecological impact on the rhizosphere microbial community that is distinct from the impact created by the uninoculated plants characterized by less robust plant and root growth. Future work will determine whether the ecological impact observed in the rhizosphere of inoculated plants can create a sustained halo effect capable of enriching the diversity of the bulk soil microbial community.

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