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Can Phytoremediation-Induced Changes in the Microbiome Improve Saline/Sodic Soil and Plant Health?

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Abstract: Increasing soil salinity and/or sodicity is an expanding problem in the Northern Great Plains (NGP) of North America. This study investigated the impact of phytoremediation on the soil microbiome and if changes, in turn, had positive or negative effects on plant establishment. Amplicon sequencing and gas chromatograph/mass spectrometer analysis compared root metabolites and microbial composition of bulk vs. rhizosphere soils between two soil types (productive and saline/sodic). Beta-diversity analysis indicated that bacterial and fungal communities from both the bulk and rhizosphere soils from each soil type clustered separately, indicating dissimilar microbial composition. Plant species also influenced both root-associated bacterial and fungal communities with separate clustering of operational taxonomic units (OTUs) identified. Canonical correlation analysis (CCA) found a clear association between specific soil characteristics and soil types. Bacterial and fungal OTUs from productive soil were correlated with greater %Ca Sat, %H Sat, and potassium (ppm), especially for OTUs differentially enriched in productive soil. Both bacterial and fungal OTUs from saline/sodic soil are associated with increased Ca (ppm), soil pH, %Na Sat and CEC. Metabolite analysis showed that kochia (*Bassia scoparia*) roots from the saline/sodic soil had a 4.4-fold decrease in pantothenate accumulation ($p = 0.004$). Moreover, two endophytic bacterial isolates, a *Bacillus* spp. and a previously uncultured halophile, isolated from creeping foxtail (*Alopecurus arundinaceus)* grown in saline/sodic soil and used as buckwheat (*Fagopyrum esculentum*) seed inoculants, significantly increased seed germination by >30% and vigor index by 0.2 under osmotic stress (0.2 M NaCl) ($p < 0.05$). This study revealed the importance of soil, root-associated, and endophytic microbiomes. Using native microbes as seed inoculants may help in establishment and growth of species used for phytoremediation of saline/sodic soil.

Keywords: phytoremediation; saline/sodic soil; microbiome; metabolite; seed inoculum

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1. Introduction

Two critical issues that must be addressed in the 21st century are feeding a growing population and reducing greenhouse gas (GHG) emissions. Saline, sodic, and saline/sodic soils are expanding in area, reduce crop yields, and have high N_2O emissions. For example, Fiedler et al. [1,2] showed that N fertilizer derived N_2O was 5 times greater in a North America Northern Great Plains (NGP) saline/sodic soil compared to productive soil at the same location. In addition to high N_2O emissions, saline/sodic soils are often dispersed and barren and have a very high erosion potential. When these areas are seeded with annual crops, yields can be very low [1,2] and results in a negative return on investment. To reduce the financial and environmental risks of these zones, phytoremediation (i.e., growing salt-tolerant plants, preferably perennials, that can thrive in saline/sodic conditions) is a

viable option. However, multiple years often are required to revegetate these zones [3,4]. Techniques to accelerate perennial plant growth without N application in these sensitive soils are needed.

In the NGP, the region's alluvial and glacial soils are underlaid by buried marine sediments. In this area, increased spring rainfall, combined with annual crop replacement of native perennial grasses, are contributing to a rising water table and capillary movement of subsurface salts to the soil surface [5]. Low lying toe and foot slopes are the first topographic positions to exhibit salinity issues. As time progresses and water tables rise, upslope areas can be compromised. Salinity/sodicity problems are already impacting millions of hectares in the NGP and could be responsible for losses up to 50% of farmable land by 2050 [6,7].

Due to the failure of chemical and drainage treatments [8], land managers and producers of NGP have been forced to investigate alternate techniques for stabilizing and mitigating high salinity soils. To restore hydrologic functions, improve plant growth, and stop further soil degradation, phytoremediation has been used worldwide in salt-impacted regions with or without chemical amendments (reviewed in [3]). However, revegetation is anticipated to be difficult in these areas in NGP due to the high salinity and electrical conductivity (EC), nearly constant wetness, and periodic ponding. In addition, the NGP climate conditions are harsh, with extreme low winter temperatures (−30 °C) and a short growing season of ~1300 growing degree days (base 10 °C). Species choices that are salt tolerant, suitable for grazing, and native to the NGP are limited [9].

Plants can form a spectrum of symbiotic to pathogenic relationships with the bacterial and fungal communities surrounding and penetrating their underground tissues, summed as root-associated microbiome. Root-associated microbes can promote plant growth and have the potential to suppress pathogens and remove contaminants [10]. The root-associated microbiome can also improve a plant's response to abiotic stressors through the production of osmolytes (e.g., proline, polyamines, and sugars), antioxidants that are reactive oxygen scavengers, and growth hormones, such as gibberellic acid [11,12]. Individual microbes have been identified as candidates for stress tolerance through growth promotion [13]. Plants grown in saline soil have been reported to select for beneficial root-associated microbiomes that improve plant response to salinity stress [14].

Selective inoculation of root microbiomes with growth-promoting microbes have also been shown to improve crop production [15–17]. Some of the more common microbial inoculation methods to confer stress tolerance include inoculation of the plant or seed via immersion [18], or mixing inoculum directly into the soil [19]. Certain species of bacteria and fungi can produce exopolysaccharides which can bind to soil sodium making it unavailable for plant uptake [20].

Changes in soil microbiomes due to revegetation or changes in plant species are underexplored [21]. This is especially true in challenging soil types where plant growth can be minimal. A relevant NGP on-farm study near Clark, SD determined that perennial grass mixes established well in saline/sodic soils [3]. Samples for this current study were collected from the Clark study site where the saline/sodic areas were initially near-barren and reclamation with perennial species was on-going. Results from the saline/sodic samples were compared to those from annual row crop areas, just up-slope of the saline areas, which had not yet impacted by salinity/sodicity [1]. This study was initiated two summers after sowing perennial grasses in both areas. This unique arrangement allowed for microbiome comparison among different treatments in two distinct but adjacent settings. This study investigated the impact of phytoremediation on the soil microbiome and if microbiome changes in turn had positive or negative effects on plant health. To achieve this, amplicon sequencing was used for microbiome comparison, and gas chromatograph/mass spectrometer was used to analyze differential root metabolites under 'normal' vs. saline/sodic conditions. Furthermore, the study assessed the ability of inoculants derived from root-associated microbes isolated from plants grown under saline/sodic conditions to improve seed germination under osmotic stress.

2. Materials and Methods

2.1. Study Site

The study area was located at 44°42′11.6388″ N, 97°52′43.8312″ W, and it was on the border of three Köppen climate regimes, Dwa (warm continental climate/humid continental climate), Dwb (temperate continental climate/humid continental climate), and Bsk (cold semiarid climate). The 30-year average annual precipitation (1981 to 2010) was 60.4 cm, and the average annual temperature was 6.2 °C (NOAA 2019).

The experiment was conducted in two soil zones (productive vs. saline/sodic). The soil mapping unit in the productive soil was a Forman-Cresbard loam (fine-loamy, mixed, superactive, frigid Calcic Argiudoll and fine, smectitic, frigid Glossic Natrudoll) having 3% to 6% slope, and the soil mapping unit in the saline/sodic soil was a Cresbard-Cavour loam (fine, smectitic, frigid Calcic Natrudoll) with a 0% to 3% slope. The differences in soil chemical characteristics were described previously [3] where productive and saline/sodic soils had similar soil organic carbon (SOC) levels of about 2.2 mg kg^{-1}. In the top 15-cm of soil, the $EC_{1:1}$ in the productive soil was 0.39 dS/m and in the saline/sodic soil was 3.87 dS/m; the sodium adsorption ratio (SAR) productive and saline/sodic soils were 1.79 and 22, respectively. The $pH_{1:1}$ of the productive soil and saline/sodic soil were similar, averaging about 7.3. However, there were significantly higher Na, Olsen P, K, Mg, sulfate, and chloride levels and lower Ca in the saline/sodic soil [3].

Each soil zone had 4 blocks that contained four randomly located treatments (bare soil (control), corn, and 2 perennial grass treatments called Mix 1 and Mix 2 (mix 1 contained slender wheatgrass [*Elymus trachycaulus*] and beardless wildrye [*Leymus triticoides*], and mix 2 contained slender wheatgrass, creeping meadow foxtail [*Alopecurus arundinaceus*], western wheatgrass [*Agropyron smithii*], and green wheatgrass [*Elymus Hoffmannii*]). In the bare treatment, plants were prevented from growing in 2018. The perennial plants were dormant seeded in the fall of 2017 and corn was seeded in the spring of 2018, 2019, and 2020 [1,2].

2.2. Soil Samples for Microbiome Analysis

Bulk soil samples to a depth of 15-cm were collected in the summer of 2018 and 2019, whereas plant root samples were collected in summer of 2019 and 2020 [1,2]. In 2018, 32 samples were collected from both productive and saline fields pre-planting and in 2019, 4 replicated samples were collected from both productive and saline plots that contained the phytoremediation treatments described above. Samples were placed in coolers containing dry ice immediately after collecting and transferred to −80 °C in the lab until the DNA extraction and isolation of the microorganisms associated with the samples took place.

Representative plant roots from the top 15-cm were collected in 2019 and 2020 from corn (*Zea mays*) from corn plots, volunteer kochia (*Bassia scoparia formerly Kochia scoparia*) from the bare soil control plots, slender wheatgrass (*Elymus trachycaulus*) from Mix 1 plots, and creeping foxtail (*Alopecurus arundinaceus*) from Mix 2 plots, and a halophile across the field regardless of the treatment. In 2019, four plants were taken from each plot, and 2020, six random plants were taken from each plot. After plant excavation, roots were cut off at the base of the stem, shaken to remove excess soil, and placed in storage bags, labeled, and placed on dry ice for transport. Sampling over years was done to ensure consistency or monitor any changes that may have occurred due to differences in environmental conditions.

2.3. Root Sample Preparation for Microbiome Analysis

Root samples were cut into ~1 mm pieces with scissors sterilized by 70% alcohol between samples [22]. A 0.5 g sample was pulverized, and a 0.2 g subsample was placed in a labeled tube containing one metal bead, and a second metal bead was placed on top of the tissue to facilitate bead movement. The tube was placed in a thermo-container containing liquid nitrogen. Tissues were ground into a fine powder using BeadBug Microtube Homog-

enizer (Benchmark Scientific, Stafford, TX, USA). Homogenizing was done in 10-s intervals at least three times to ensure the roots did not thaw until the lysis buffer was added.

2.4. DNA Extraction for Microbiome Analysis

For bulk soil DNA extraction, 0.5 g of each soil was weighed into a tube. The FastDNA Spin Kit for Soil (Cat.NO.116560200, MP Biomedicals, Solon, OH, USA), designed to efficiently lyse all microorganisms, was used following the manufacturers' protocol. Once DNA was extracted, samples were stored at −20 °C until subjected to hexadecyltrimethylammonium bromide (CTAB) purification [23]. To ensure high-quality DNA, the extracts were further purified using the ZYMO DNA Clean and Concentrate Kit (ZYMO Research, Irvine, CA, USA).

After root sample homogenization, 490 µL of water, 20 µL of SDS, and 20 µL of EDTA were added and the tube was vortexed vigorously to ensure thorough mixing. Samples were incubated at 68 °C for 10 min followed by centrifugation at 13,000 rpm for 8 min. The supernatant was transferred to a new microcentrifuge tube, and 30 µL of −20 °C potassium acetate (KOAc) was added. The solution was incubated on ice for 10 min to precipitate protein. This solution was then incubated and centrifuged as described above with the supernatant transferred to a new microcentrifuge tube.

The purified DNA were then sent to the University of Minnesota Genomics Center for sequencing on Illumina MiSeq (Illumina, Inc., San Diego, CA, USA) V3. This analysis is based on 16S (V3–V4) for bacterial community and ITS1 regions for fungal community with paired-end reads of 300 nucleotides long. The returned data were uploaded to NCBI with the following accession numbers, PRJNA521547, PRJNA645437, and PRJNA920718.

2.5. Microbiome Data Analysis

Qiime2 was used as the pipeline for analysis [24]. DADA2 was used to improve the data quality. To improve the output, parameters were selected to optimize the crossover for the forward and backward reads. We determined the expected amplicon size and set the trunc and len values larger than the number. This allowed for the required crossover and provided a high feature count for each sample. Subsequently, a sequence "feature" table was generated and used as a reference throughout the analysis. The term "feature" is used in QIIME2 in place of an operational taxonomic unit (OTU) or a sequence variant to be more inclusive of other potential uses of the pipeline being developed but will be referred to as OTU to reduce confusion. During the denoising process using DADA2, a few samples from both years fell under the preset threshold.

A phylogenetic tree was generated to evaluate OTU composition. Alpha and beta-diversity were analyzed to compare the identified OTU within the samples. Plots were separated based on soil type or plant species. The beta-diversity was determined by Jaccard distance and Unweighted UniFrac distance plots [25].

Taxonomic data analysis allowed for the identification of OTUs present within each sample. The classifier used for 16S analysis was Silva 132, and the classifier used for ITS (Internal Transcribed Spacer) analysis was UNITE version 8.0. Canonical correspondence plots (CCA) were developed for these taxonomic data using XLSTAT version 23.2.1 by Addinsoft.

Analysis of Composition of Microbiomes (ANCOM) identified significantly different OTU within the samples from different soil types at the order (4th), at the family (5th), and at the genus (6th) taxonomic levels. These steps were repeated for each study year, bulk soil and root samples, and 16S- and ITS-amplicon data.

2.6. Plant Materials and Preparation for Metabolite Analysis

Metabolite analyses were conducted on the corn, kochia, creeping foxtail, and slender wheatgrass root samples. For this analysis, a 700-µL aliquot of methanol (−20 °C) was added to finely ground root tissue and vortexed to homogenize. A 5 µL aliquot of ribitol, used as an internal standard for the gas chromatography/mass spectrometry analysis,

was added and the solution was vortexed again [26]. The solution was incubated with mixing for 10 min at 70 °C. The solution was then centrifuged at 11,000 g for 10 min to settle the solid root with the supernatant transferred to a reaction tube. A 375-µL aliquot of chloroform (−20 °C) was added to the supernatant and stored at −20 °C. A 750-µL aliquot of cold (4 °C) water was added to the solution and vortexed for 10 s followed by centrifugation for 15 min at 2200 g to separate the polar/upper phase from the water phase. A 200-µL aliquot of the polar phase was placed into a 1.5 mL microcentrifuge tube and dried using vacuum without heat.

Tubes were removed from the vacuum immediately after drying, and 40 µL of pyridine containing methoxamine (20 mg mL^{-1}) was added to each tube and vortexed for one minute. Controls were made with solutions but contained no root sample. All samples were incubated at 37 °C for 2 h, with vortexing every 10-min to ensure procedural success. Next, a 70-µL aliquot of N-methyl-N-trifluoroacetamide (MSTFA) was added to each tube, followed by a 30 min incubation at 37 °C, with samples vortexed every 5 min. Following this incubation, samples were centrifuged for 10 min at 14,000 rpm. An 80-µL aliquot of the supernatant was placed in a vial for metabolite analysis. The prepared samples were analyzed using a Gas Chromatograph—Mass Spectrometer (GC-MS). Each 1 µL aliquot of the derivatized samples was injected by an Agilent 7693 autosampler into a GC-MS (Agilent 7890A/5975C) with Pulsed Splitless mode at a flow rate of 2.2 mL min^{-1} using hydrogen as a carrier gas. The metabolites were separated by a Zebron ZB-5ms column (30 m × 0.25 mm × 0.25 µm) using the following program: GC was initiated at 70 °C held for 5 min, ramped at 5 °C min^{-1} to reach 310 °C, and then held for 1 min. The scanning mass range of MSD was between 70 and 600 m z^{-1} using the EMV Mode with the Gain Factor 1.00. Data were acquired with Enhanced ChemStation and processed with MSD ChemStation data analysis software (E.02.02.1431). This analysis provided data in the form of many different peaks in the gas chromatogram, and each peak generates a unique mass spectrum used for compound identification. These data were extracted in .AIA format and then uploaded to https://xcmsonline.scripps.edu, accessed on 15 February 2020. The specific metabolites in the root samples were identified using XCMS MassBank spectra libraries. Further quantification of differential metabolites in roots between datasets collected from roots of saline/sodic vs. productive soils were made by pairwise comparisons.

2.7. Sample Culturing and Isolation for Root-Associated Microbial Inoculation of Seeds

Root-associated microbes were cultured from creeping foxtail in saline/sodic soil. A 0.2 g root sample was homogenized using a bead-beating method and plated on LB agar after adding a 1 mL aliquot of sterile water. The LB agar did not have extra salt added.

After about 5 days at room temperature, individual colonies with differing morphologies were selected for isolation. A total of eight isolates were streaked onto new LB agar plates and left at room temperature to grow. The isolated cultures were extracted for genomic DNA [27] which was used as PCR templates for 16S rDNA amplification from 347f and 803R 16S universal primers and were sent to GenScript (Piscataway, NJ, USA) for Sanger sequencing to identify the species.

2.8. Seed Inoculation and NaCl Challenge and Data Analysis

The eight isolated bacterial cultures from the creeping foxtail roots were used as inoculants on buckwheat seeds to determine if they improved germination when exposed to salinity stress at 0.2 M NaCl.

Seed inoculation was conducted using bio-priming that was adapted from Syed-Ab-Rahman et al. [28]. In this method, seeds were surface sterilized with 70% alcohol solution and rinsed with distilled water, repeated three times. Seeds were then placed in centrifuge tubes, submerged in broth containing the isolated bacteria (optical density of 0.5 at 260 nm) [29], shaken at room temperature for one hour and air dried. For control, seeds were shaken in sterile water at room temperature for 1 h. Dried seeds were placed on a sterilized paper towel in a sterile petri dish, and 8 mL of a 0.2 M NaCl solution was

added to each dish. Petri dishes were sealed with Parafilm and set on the benchtop at room temperature to germinate. This procedure was repeated three times. The vigor index is a calculation of the germination percentage multiplied by the average length of the plant root. This method was derived from a method by Gamalero et al. [30]. Statistical analysis was performed using a pair-wise t-test. For samples of three or more, one-way ANOVA was utilized. Whenever a significant effect was observed, Tukey's HSD test was conducted to compare the means of all pairs.

3. Results

3.1. Microbiome Analysis of Bulk Soil and Root Samples

The alpha-diversity was assessed using the Faith's Phylogenetic Diversity (PD) measure [31]. This measurement calculates the sum of all branch lengths within a phylogenetic tree [32]. Bulk soil data shows that the bacterial community's alpha diversity based on Faiths PD measure was similar between the soil types (good vs. saline) in both 2018 and 2019 (Figure 1A,B). The fungal diversity in bulk soil decreased in high-salt soil compared to productive soil in 2018 and 2019 (Figure 1C,D). Productive soil exhibited no difference compared to high-salt soil in bacterial diversity in 2018 but showed greater fungal biodiversity in 2018 and 2019.

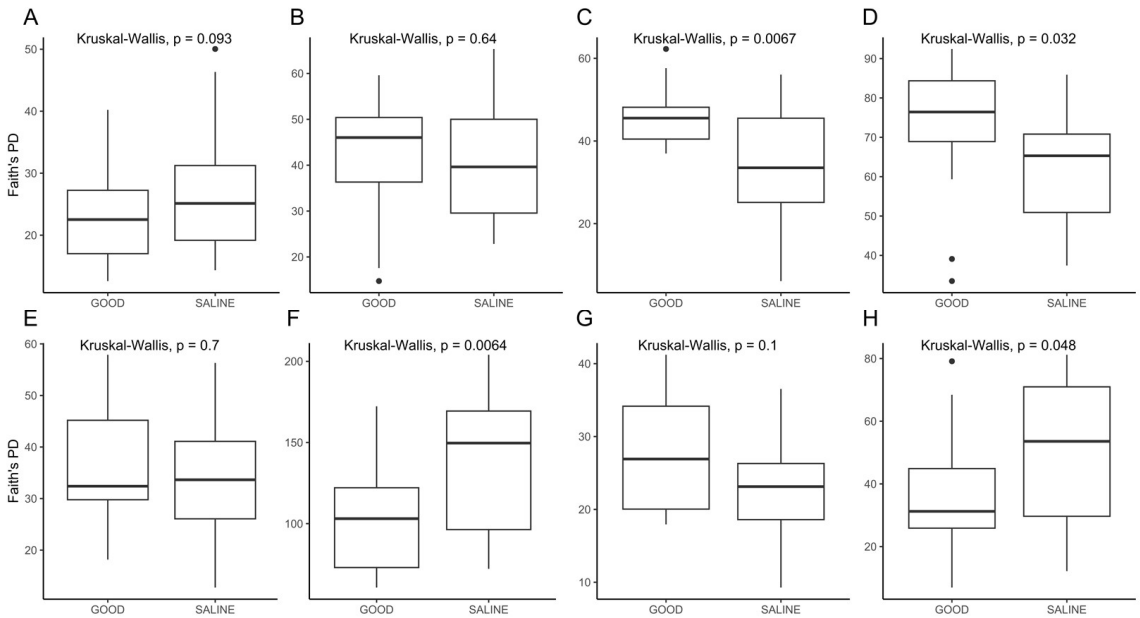

Figure 1. Alpha diversity of bulk and root microbiome samples taken from productive (GOOD) or high-salt (SALINE) fields collected in 2018–2020 calculated by Faith's phylogenetic diversity and calculated by Kruskal-Wallis. (**A**,**B**) Bulk soil bacterial diversity in 2018 and 2019. (**C**,**D**) Bulk soil fungal diversity in 2018 and 2019. (**E**,**F**) Root bacterial diversity in 2019 and 2020. (**G**,**H**); Root fungal diversity in 2019 and 2020.

The beta-diversity analysis shown as Jaccard plots of bulk soil samples collected in 2018/2019 provided additional information for understanding the shared microbial composition within the soil types. The Jaccard plots produced for the bacterial community analysis of bulk soil (Figure 2A,B) and the fungal community (Figure 2C,D) indicated large differences between the saline/sodic and productive soils and showed few shared OTUs between soil types. The fact that the bacterial and fungal communities between soil types with OTUs clustering separately verified the two zones (productive vs. saline/sodic) were delineated by electrical conductivity ($EC_{1:1}$) and sodium adsorption ratio as previously published studies have reported [1].

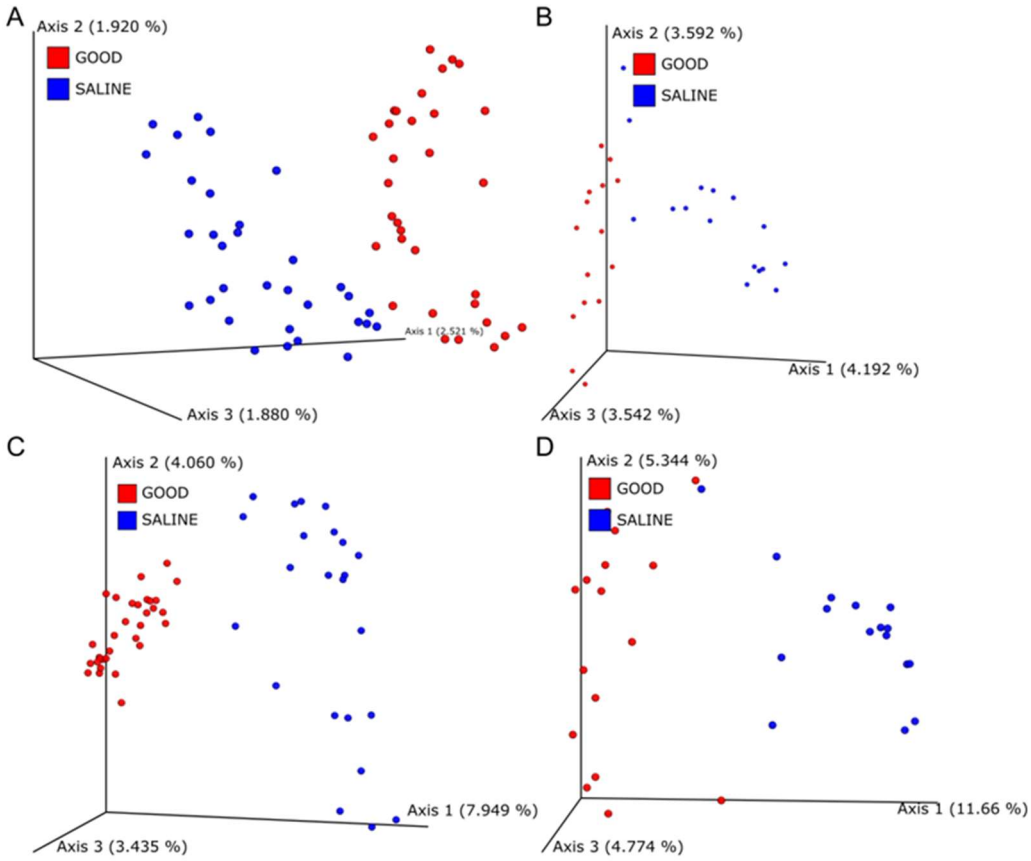

Figure 2. Jaccard Plot showing the microbial community beta diversity between bulk soil samples collected from productive area (red) and saline/sodic area (blue). (**A**) year 2018, bacterial community; (**B**) year 2019, bacterial community; (**C**) year 2018, fungal community; (**D**) year 2019, fungal community. The red dots clustered separately from the blue dots demonstrating that the two types of soil harbor very dissimilar composition for both bacterial and fungal communities in both years.

In both years, the microbiome composition of both soils contained more bacteria than fungi. There were approximately 4.5 times more OTUs identified in bacterial community from root samples in 2020 (55,000 OTU) than in 2019 (12,000 OTU). For the root fungal community analysis, 3000 OTU were identified in 2019 samples, and 5200 OTU in the 2020 samples. This nearly 2-fold increase suggests that the saline/sodic soil was becoming more suitable for microorganisms. This increase is associated with a decrease in the saline/sodic soil $EC_{1:1}$ from 8.42 in 2018 to 6.7 dS/m in 2020. In the productive soil, $EC_{1:1}$ decreased from 0.38 to 0.26 dS/m from 2018 to 2020.

The bacterial community analysis of the 2018 bulk soil identified slightly less than 10,500 total OTUs. Similarly, the 2019 bulk soil analysis identified just under 11,000 OTUs. These values suggest that the bacterial community present in the bulk soil samples was similar across the two years. However, different results were observed for the fungal community that increased from approximately 4800 identified OTUs in 2018 to approximately 7500 OTUs in 2019. This increase suggests that the soil was becoming more habitable for fungal organisms. Using the OTU abundance and the soil testing results, canonical correlation analysis (CCA) plots were generated for the bacterial community and fungal community taxa identified in the bulk soil for 2018 (Figure 3A,B) and showed that specific OTUs are associated with specific soil indicators for the two soil zones. Additionally, Table S1 lists the significantly different relative abundant OTU identified from the ANCOM analysis between bulk soil samples gathered from saline/sodic plots.

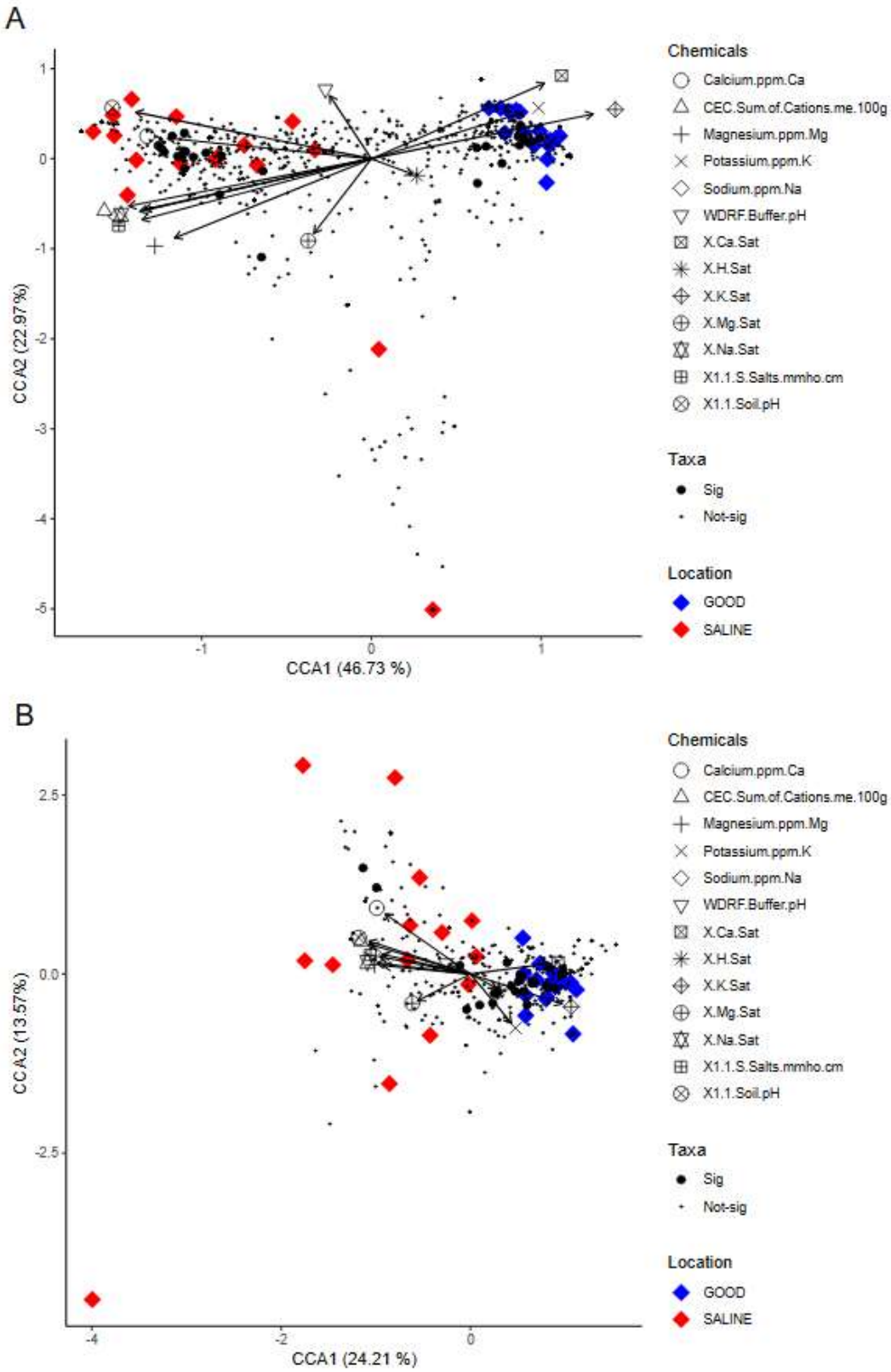

Figure 3. CCA plot showing taxa with more association with specific soil characteristics and soil types. (**A**) 2018 bacterial community; (**B**) 2018 fungal community. For example, bacterial and fungal OTUs from productive soil are correlated with greater %Ca Sat, %H Sat, and %K Sat, especially the ones that are differentially enriched in productive soil.

Alpha diversity calculated by Faith's phylogenetic diversity was used to determine the phylogenetic diversity of root microbiomes collected from plants in productive and saline/sodic plots in 2019 and 2020. In 2019, bacterial diversity in plant root samples showed no difference between the plants collected from good and saline soil but showed a greater bacterial diversity in saline soils in 2020 (Figure 1E,F). Root microbiomes in 2019 and 2020 showed a similar trend in fungal diversity in root samples with no differences in 2019 but an increase in the diversity in 2020 (Figure 1G,H).

The beta-diversity analysis using 3-dimensional scatter plots with metadata indicates that the saline/sodic and productive soils had different plant root microbiome compositions. Jaccard distance plots also were used to determine a similarity/difference among samples in productive/saline plots [33]. In 2019 and 2020 samples clustering shows separation of both bacterial (Figure 4A,B) and fungal community compositions (Figure 4D,E). Unweighted UniFrac plots take into consideration the OTU of collected plant roots [34] (Figure 4C,F). In 2019, no clear clustering of the root microbiome was found based on plant species, but clear clustering can be seen in 2020 when the perennial grasses had greater coverages. The point color indicates the plant species of the sample, and spheres and stars represent the productive and saline/sodic soils, respectively. In both years, the microbiome clusters separately according to the plant species from the productive plots. These data demonstrate that root microbiomes shifted by plant species representing the phytoremediation treatment.

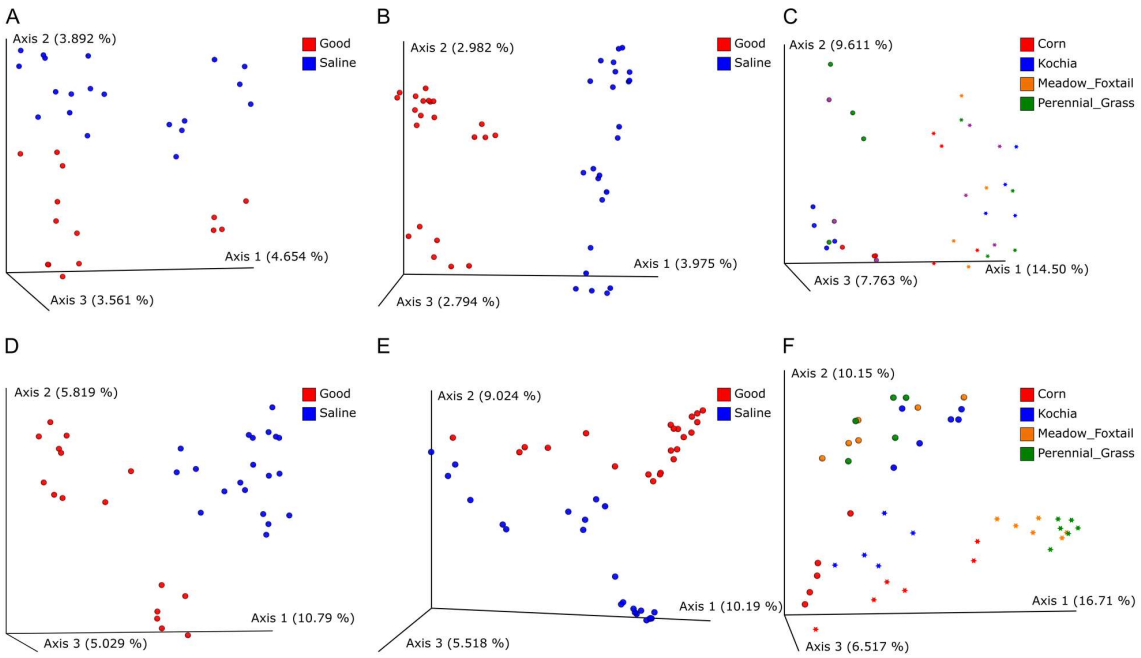

Figure 4. Beta-diversity and unweighted UniFrac models were determined for bacterial and fungal OTUs in root samples taken from productive (good) and high-salt (saline/sodic) fields in 2019 and 2020. High dissimilarities in bacterial and fungal OTUs were found between good and saline fields. (**A**,**B**) Bacterial community's beta diversity of good and saline samples shows clustering of OTUs by two soil zones in both 2019 and 2020 respectively. (**C**) Unweighted UniFrac plot of root samples' bacterial community colored by OTUs detected from productive (spheres) and high-salt (stars) fields shows clustering by plant species in 2020. (**D**,**E**) Fungal community's beta diversity of good and saline samples shows clustering by two soil zones in both 2019 and 2020 respectively. (**F**) Unweighted UniFrac plot of root samples' fungal community colored by OTU detected from productive and high-salt fields shows clustering by plant species in 2020.

Table S2 lists the significantly different relative abundant OTU identified from the ANCOM analysis between root samples gathered from saline/sodic soil and root samples gathered from the productive plots. Under saline stress, the increase and decrease of specific bacterial community in root microbiome was observed. Increases were observed for

Pelagicoccus, Helomonas, Azoarcus, Diplosphaera, Planktosalinus, Flavobacteriaceae, Algoriphagus, Methylophaga, Truepera, Cyclobacterium and Xanthomonas. Decreases were observed for Acidibacter, Lacunishphaera, Lechevalieria, Pyrinomonadaceae, Bradyrhizobium, Micromonosporaceae, Chthoniobacter, Actinocorallia, Ohtaekwangia and others. The increases and decreases in specific bacterial OTUs are mainly a result of selection by high salinity/sodicity. However, it is unknown whether these microbiome changes can impact plant establishment. Some of the species may be helpful for the phytoremediation process, which led us to look at the root metabolite and isolation of endophytes as seed inoculum.

3.2. Root Metabolite Analysis

Running a pairwise comparison between saline/sodic and productive soil samples allowed for the side-by-side viewing of plant metabolites found in each soil location. One of the few identified metabolites was pantothenate from the kochia root. Pantothenate is the precursor to the Coenzyme A biosynthetic pathway [35]. Levels of pantothenate have been reported to decrease in plants exposed to stress [36]. In this study, lower concentrations of pantothenate were found in the kochia roots from saline/sodic plots than those from the productive soil ($p = 0.02$) (Figure 5A,B), suggesting depletion of pantothenate due to salt stress, which can be used as a biomarker for stress tolerance.

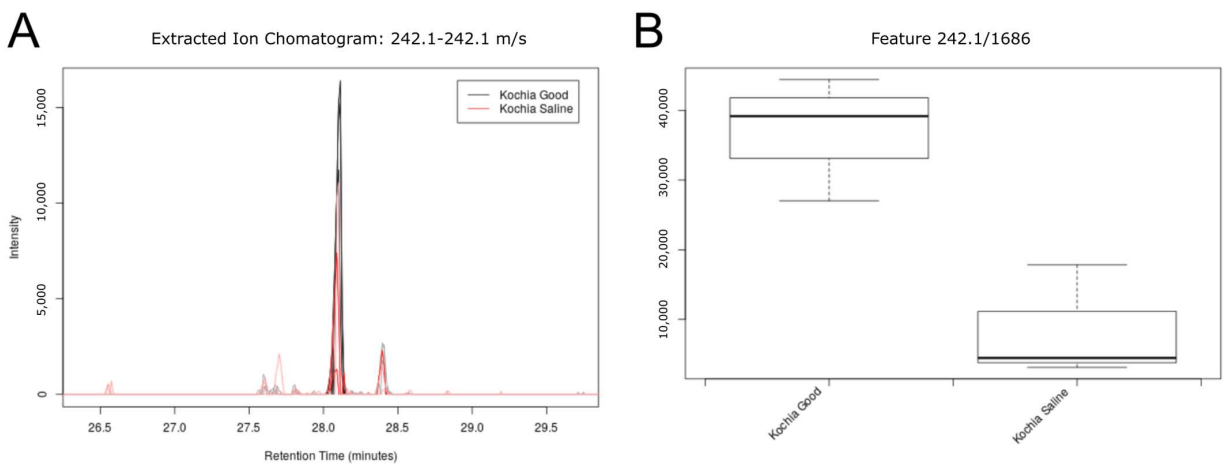

Figure 5. The comparison between the amount of pantothenate as a metabolite present in samples of kochia roots gathered from saline/sodic vs. productive areas. (**A**) Box plot showing the different abundance. (**B**) Extracted ion chromatogram comparing the intensity of peaks at the specific retention time associated with the pantothenate metabolite.

3.3. Endophyte Inoculation

The results of the Sanger sequencing identified eight bacterial isolates that could be classified to the genera but not species level. Results indicated that three of the bacterial isolates have not been previously cultured or identified. SMF23 was determined to be an unidentified species within the genus of *Bacillus*. The nucleotide sequence associated with SMF27 is associated with no known bacterium.

Out of the 8 bacterial isolates tested summarized in Table 1, two (SMF 23 and SMF 27) increased buckwheat seed germination and root growth when grown in 0.2 M of NaCl (Figure 6A). Germination was similar in these two bacterial isolates; however, there was a difference ($p = 0.07$) in vigor between the isolates (Figure 6B).

Table 1. Potential species identity based on Sanger sequencing results of bacterial isolates from saline/sodic creeping foxtail (*Alopecurus arundinaceus*) root.

Sample Name	Suggested Species
SMF22	- *Bacillus* sp. (in: Bacteria) [firmicutes] - *Bacillus cereus* [firmicutes] - *Bacillus thuringiensis* [firmicutes]
SMF23	- *Bacillus* sp. (in: Bacteria) [firmicutes] - *Bacillus pacificus* [firmicutes] - *Bacillus mycoides* [firmicutes]
SMF24	- *Pseudomonas putida* [g-proteobacteria] - *Pseudomonas hunanensis* [g-proteobacteria] - *Pseudomonas fluorescens* [g-proteobacteria]
SMF25	- *Bacilli* bacterium So2Pw_1330 [firmicutes] - *Bacillus cereus* [firmicutes] - *Bacillus thuringiensis* [firmicutes]
SMF26	- uncultured bacterium [bacteria] - *Comamonadaceae* bacterium Ri2Ps_5065 [b-proteobacteria] - *Comamonas koreensis* [b-proteobacteria] - *Delftia* sp. H214 [b-proteobacteria]
SMF27	- uncultured bacterium [bacteria] - uncultured *Chloroflexi* bacterium [GNS bacteria] - uncultured *Anaerolinea* sp. [GNS bacteria]
SMF28	- uncultured bacterium [bacteria] - *Stenotrophomonas* sp. DAIF1 [g-proteobacteria] - *Stenotrophomonas maltophilia* [g-proteobacteria]
SMF29	- *Bacillus* sp. (in: Bacteria) [firmicutes] - *Bacillus cereus* [firmicutes] - *Bacillus thuringiensis* [firmicutes] - *Bacillus subtilis* [firmicutes]

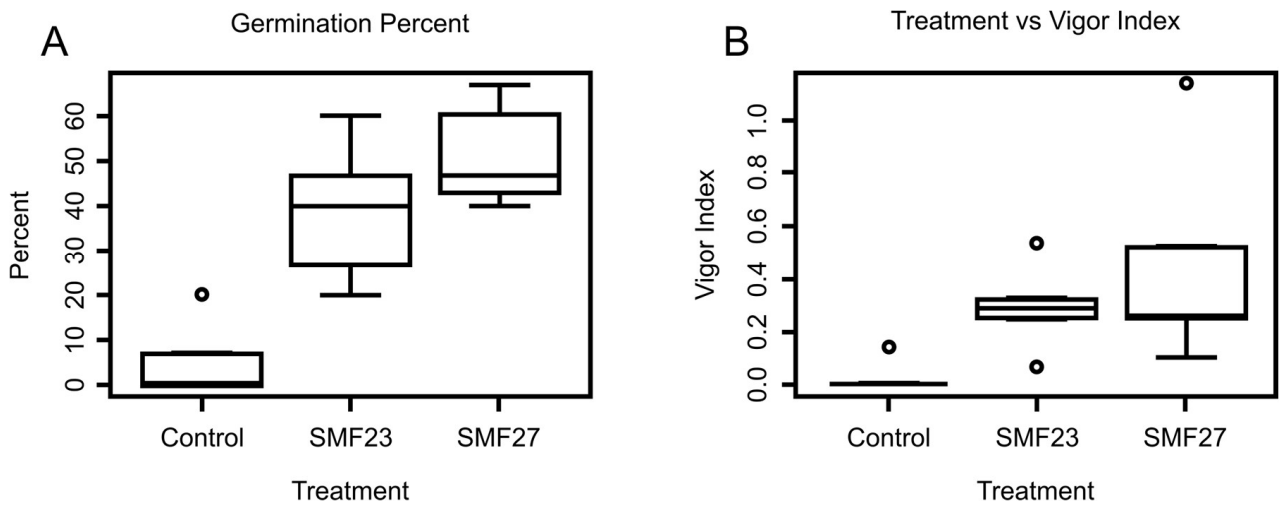

Figure 6. (**A**) Boxplot comparing germination percent of seeds treated with inoculants vs. control; (**B**) Boxplot comparing vigor indexes of treatments.

4. Discussion

To determine the effects of saline/sodic soil on microbial composition and on plant metabolite accumulation, we used amplicon sequencing for microbiome comparison and characterized metabolites associated with plant roots, gathered from soil environments (saline/sodic vs. productive). Using a gas chromatograph/mass spectrometer, the production of non-targeted metabolites was compared between root samples from saline/sodic and productive soil types. The differences in microbiome and metabolites may explain the impact of plants/microbiome interactions that increased plant tolerance to salinity. We also tested whether plant growth can be improved by isolated endophytes and root-associated microbes to improve salt stress tolerance. The positive effect of these cultured microorganisms can be tested using the microorganisms as inoculants. Notably that soil organic matter (SOM) are similar in two soils [3], so the observed differences in microbiome are not associated with SOM.

In this experiment, approximately 100,000 OTU were identified that could promote plant growth. Faith's PD showed that the 2019 root samples collected from the two soils had similar alpha diversity levels for the bacterial and fungal communities. However, the 2020 root samples from saline/sodic soil had higher alpha diversity for both bacterial and fungal communities than productive soil samples. This information is important when considering the success and stress tolerance of plants growing in saline/sodic conditions. The biodiversity of microorganisms within plant roots is crucial to its growth and success, especially when growing in stressful conditions [37]. The increasing biodiversity within plant roots could be a key factor in the success of the plant establishment in saline/sodic soil and needs to be confirmed by monitoring it for a longer period of time.

The alpha diversity of bulk soil provided different information than the root microbiome data. In both 2018 and 2019, the bulk soil bacterial communities were similar in alpha level diversity between soil types. However, the fungal community analysis had different results with the productive soil having greater biodiversity than the saline/sodic soil. The results raise a question: is biodiversity in bulk soil important? If a high amount of biodiversity in bulk soil was required for a plant's successful growth in saline/sodic soil, then these results would contradict that requirement. It is likely that bulk soil diversity has less effect on the successful growth of plants in saline/sodic soil, and the biodiversity of the microbial composition of endophytes and the rhizosphere have a much larger effect on plant's stress tolerance and growth in saline/sodic soil.

Beta-diversity is a measure of the differences between two or more biological communities and samples were analyzed using a variety of tests. The Jaccard index, a commonly used metric for assessing beta-diversity of the microbiome, measures the proportion of shared taxa between two samples. Examining Figure 2A–D indicates that there is a separation of clusters from different soil types. Interestingly, the beta diversity among plant species in different soil types was similar, so the soil type appears to be the overriding driver for microbial composition, even though plant species and environmental gradients have been reported to be the drivers of composition shifts [38,39]. Interestingly, corn and kochia root samples were most similar within each soil type, clustering away from creeping foxtail and slender wheatgrass-associated microbial communities. These results suggest that a benefit of phytoremediation using multiple plant species is to support different root-associated microbiomes, which may improve overall revegetation success.

ANCOM analysis detected differentially abundant taxa between samples. Based on the results, we can further hypothesize that the OTUs enriched for root samples from saline/sodic soil (Table S2) may have plant growth promoting capabilities and increase salinity stress tolerance in plants. Although the exact mechanisms behind these benefits are unknown, future studies may identify how these saline/sodic-associated taxa benefit and improve overall soil health.

The results of the inoculation experiment are promising for future uses of isolated bacterial and fungal endophytes and root-associated microbes as inoculants to improve stress tolerance in plants. The high concentration of NaCl disrupted root emergence

from the uninoculated seeds, which would be deleterious to future plant growth and development. However, both SMF23 and SMF27 increased the germination of buckwheat in the NaCl solution. The effectiveness of the treatment is very promising for the future use of these isolates to inoculate other plant species to determine if early salt stress tolerance is improved. Based on the results of the Sanger sequencing, SMF23 is likely a species of *Bacillus*, which have been shown to increase plant growth and reduce salt stress in various plant species [20,40,41].

This study revealed the importance of soil, root-associated, and endophytic microbiomes. The knowledge gained may enable broader application of phytoremediation on saline/sodic soil. The next steps would be to understand the specific mechanism(s) for improved germination, determine if these inoculants improve seed germination of other species in saline/sodic soil, and determine if isolate application to bulk soil would be effective in aiding plant establishment and growth. However, more research is required to address the research questions raised by this study in order to fully utilize microbial contributions to restore vegetation and ecosystem services to these degraded soils.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14010029/s1, Table S1. ANCOM abundance results of bulk soil collected from saline vs. productive plots in 2018 and 2019 show differing abundances of OTUs between the soil types. Table S2. ANCOM abundance results of root samples collected from saline vs. productive plots in 2019 and 2020 show differing abundances of OTUs between the soil types.

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