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Phytoremediation of Secondary Salinity in Greenhouse Soil with *Astragalus sinicus*, *Spinacea oleracea* and *Lolium perenne*

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Abstract: Phytoremediation is an effective and ecological method used to control soil secondary salinization in greenhouses. However, the plant–soil interactions for phytoremediation have not been studied sufficiently. In this study, three crop species (*Astragalus sinicus* (CM), *Spinacea oleracea* (SP) and *Lolium perenne* (RY)) were compared in a greenhouse experiment. The results showed that all three crops increased the soil microbial biomass, the abundance of beneficial microorganisms, available phosphorus and soil pH, and reduced the soil salt content. The crop nutrient accumulation was positively correlated with the relative abundance of bacterial 16S rRNA sequences in the soil. CM and RY respectively increased the relative abundances of *norank_f_Gemmatimonadaceae* and *norank_f_Anaerolineaceae* within the soil bacterial community, while SP increased the relative abundances of *Gibellulopsis* within the fungal community. Correlation analysis revealed that pH and total dissolved salts were the vital factors affecting soil microbial communities in the secondary salinized soil. Our results suggest that phytoremediation could effectively alleviate secondary salinization by regulating the balance of soil microbial community composition and promoting crop nutrient accumulation.

Keywords: phytoremediation; secondary salinization; salt tolerance; microbial diversity; nutrient accumulation

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1. Introduction

Secondary soil salinization, which is mainly caused by intensified anthropogenic agricultural production, has been recognized as an extensive form of land degradation [1–3]. High inputs of agrochemicals, high evaporation, and mineral leaching in the intensified production system significantly intensify secondary soil salinization, as well as high Na^+ accumulation in surface soil, which restricts agricultural productivity worldwide [4,5]. The salinization causes soil compaction and an imbalance in nutrient supply, which directly harms the normal growth of crops. Furthermore, the salinization alters the status of soil microorganisms, thereby indirectly affecting the entire ecological environment, and thus hindering the sustainable development of agricultural production [6].

Phytoremediation can alleviate secondary salinization in facility cultivation soils and reduce the dependence on mineral fertilizers [7]. In previous studies, it has been suggested that soil microbes are susceptible to farming practices, and that selecting an effective crop has positive effects on microbial communities and functions [8]. For example, applying a green manure crop has been shown to significantly change the soil microbial community composition and function [9]. Fungi, bacteria, and actinomycetes have been found to be active in the rhizosphere of Italian ryegrass [10]. The symbioses of these microorganisms accelerated nutrient cycling processes [11]. Therefore, understanding the structure of the soil microbial community and its responses to applications of different types of crops is necessary to elucidate the effects of microbes on secondary salinization.

In this study, it was hypothesized that the saline soil biochemical properties and microbial communities would change consistently with the type of crop species planted. Further, it was postulated that there would be considerable linkage between crop nutrient accumulation, soil salinization indicators and soil biochemical properties during the process of phytoremediation. The objectives of this study were to clarify the impact of different crop species on soil biochemical properties and microbial communities in cultivation facility soil, and to explore the linkage between crop nutrient accumulation and the composition of soil microbial communities.

2. Materials and Methods

2.1. Field Site Setup, Management and Sampling

A greenhouse experiment was performed at the Zhuanghang Comprehensive Experimental Station of the Shanghai Academy of Agricultural Sciences, Fengxian, Shanghai, China (30°53′20.0″ N, 121°23′06.4″ E). The study site was flat and the soil type was calcareous alluvium. Three replicates of four treatments were arranged in a randomized block design using 30 m × 2 m plots constructed in January 2015 (Figure 1). Nylon screen fabric was erected around every plot to avoid runoff effects, and it was buried beneath the soil surface with a height of 40 cm. Four treatments were set up, including the fallow control (CK), Chinese milk vetch (*Astragalus sinicus* L., CM), Spinach (*Spinacia oleracea* L., SP) and Ryegrass (*Lolium perenne* L., RY). CM, SP and RY were selected because they are the major native winter cover crop species that are easily accessible and widely applied to ameliorate soil salinization. Seeds of CM, SP and RY were obtained from Shanghai Nongle Planting Co. Ltd. (Shanghai, China). After 3 years of continuous planting, soil samples were collected on 30 January, 2018. In each separated plot, soil samples from the 0−20 cm surface layer were collected from 8 points to form a mixed composite soil sample, which was then divided into two parts, with one part air dried prior to the determination of basic physicochemical properties, and the other stored at −80 °C prior to the DNA extraction.

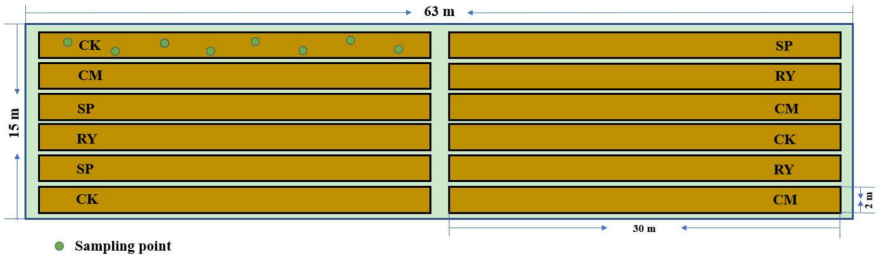

Figure 1. The schematic for field site setup, management and sampling. CK, control; CM, *Astragalus sinicus*; SP, *Spinacia oleracea*; RY, *Lolium perenne*.

2.2. Crop Yield and Nutrient Accumulation

The experimental crops were planted by sowing the equivalent of 75 kg ha^{-1} of seed per plot in early October, and the crops were grown and harvested until the end of January annually. Equal amounts of irrigation water were supplied to each plot and no fertilization was used during the period of phytoremediation. The former crop for all treatments was pakchoi (*Brassica chinensis* L.), and urea (N 46%); compound fertilizer (17-17-17) and potassium sulphate (K_2O 52%) were applied as the N, P and K fertilizer, with application rates of N 120 kg ha^{-1}, P_2O_5 45 kg ha^{-1} and K_2O 90 kg ha^{-1}. Crops were harvested at the same time as the soil samples were taken. The selected uniformly growing plants were taken to the laboratory immediately and were dried to a constant weight in preparation for nutrient determination. The total nitrogen (N), phosphorus (P) and potassium (K) contents of the dry matter were quantified using the Kjeldahl nitrogen determination method, the vanadium-molybdenum-yellow photometric method, and the flame photometry approach, respectively [12].

2.3. Determination of Soil Physicochemical Properties

Soil chemical properties, including the pH, total N, soil organic matter (SOM), alkali-hydrolyzable nitrogen (AMN), available P (AP), available K (AK), and total dissolved salts (TDS), were tested according to the methods of Bao (2000). The soil pH was tested using a soil-to-water ratio of 1:2.5. The soil total N was determined via the Kjeldahl method and SOM was determined via the potassium dichromate oxidation method. The soil AMN content was measured using the alkaline hydrolysis diffusion method. The AP and AK were measured using the molybdenum blue colorimetric method and the flame photometry method, respectively. The TDS in the soil were determined using the gravimetric method. The soil microbial biomass C (MBC) and N (MBN) were measured using the chloroform fumigation method [13].

2.4. Soil DNA Extraction and Microbial Community Analysis

Bacterial and fungal DNA were extracted as three replicates from each soil sample using a FastDNA Spin Kit for Soil and were stored at −80 °C. The bacterial V3–V4 region of the 16S rRNA gene was amplified using the primers 338F and 806R [6]. The internal transcribed spacer (ITS) region of the fungal rRNA gene was amplified using ITS1F and ITS2R [14]. All PCR reactions were performed according to the methods described by Cai et al. [15]. Pyrosequencing was carried out by Majorbio Bio-Pharm Biotechnology Co., Ltd., Shanghai, China, using the Illumina Miseq PE250 platform. After high-throughput sequencing and optimization, 566,160 bacterial 16S rRNA sequences with 235,957,809 bp were obtained from the four treatments (N = 12), and the average sequence length was 416.8 bp. Meanwhile, 727,233 fungal ITS sequences with 173,088,882 bp were obtained, and the average sequence length was 238.0 bp. Sequence data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the accession number SRP273207.

2.5. Real-Time Quantitative Polymerase Chain Reaction (qPCR)

qPCR was used to examine the effects of bioremediation on soil microbial abundance. Standard reactions were performed for all samples in triplicate with an ABI7500 Real-time PCR System (Applied Biosystems INC, Foster City, CA, USA) using the SYBR green qPCR method. The standard curves and amplification curves are shown in Figures S1–S4. The qPCR mixture (20 μL) contained 10 μL of Maxima SYBR green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 0.8 μL of each primer, 1.0 μL of template DNA and 7.4 μL of dd H_2O. The amplification conditions of 16S rRNA comprised pre-denaturation for 5 min at 95 °C, followed by 30 amplification cycles of denaturation at 95 °C for 30 s, annealing for 30 s at 55 °C and extension at 72 °C for 1 min. The amplification conditions of ITS were nearly the same despite the difference of annealing temperature at 62 °C. The gene abundances of each reaction were calculated based on the constructed standard curves and then converted to copies per gram of soil, assuming 100% DNA extraction efficiency.

2.6. Data Analysis

The effects of the different crop treatments on the physicochemical soil properties and crop biomass values were tested using one-way ANOVA in SPSS 17.0 (SPSS Inc., Chicago, IL, USA). QIIME (1.7.0) software was used to calculate the alpha and beta diversities of the soil bacterial and fungal communities. The OTUs were used to characterize the alpha diversity. The Chao1, ACE, Shannon and Simpson indices were calculated. Principal coordinates analysis (PCoA) of the unweighted UniFrac distances between the samples was used to characterize the similarities (beta diversity) in the bacterial and fungal communities among the treatments [16]. The vegan data package in R was used for redundancy analysis (RDA), which was used to identify factors that affected microbial community structure.

3. Results

3.1. Response of Soil Biochemical Properties to Green Manure Crops

The application of green manure in the form of the three different crops improved the biochemical properties associated with soil fertility (Table 1). Compared to CK, all cultivation treatments displayed lower TDS contents, but higher soil pH, MBC and MBN contents ($p < 0.05$). The AP and AK contents were also significantly higher than in the control, irrespective of the type of crop applied ($p < 0.05$). The AMN and MBN contents were significantly higher in the CM treatment than in the other treatments ($p < 0.05$). The AP and AK contents were significantly higher in RY than in the other treatments ($p < 0.05$).

Table 1. Biochemical properties of the soil in each bioremediation treatment.

Treatments	pH	TDS (g kg^{-1})	SOC (g kg^{-1})	AMN (mg kg^{-1})	AP (mg kg^{-1})	AK (mg kg^{-1})	MBC (mg kg^{-1})	MBN (mg kg^{-1})
CK	4.98 c	3.25 a	11.70 b	120.58 b	45.56 c	205.20 a	108.45 c	38.70 c
CM	5.29 b	2.81 b	12.58 ab	139.33 a	90.47 b	336.92 c	192.94 ab	62.60 a
SP	5.33 b	2.74 b	11.79 b	104.06 c	92.44 b	282.98 b	188.36 b	52.56 b
RY	5.52 a	2.52 b	13.09 a	95.89 c	113.60 a	426.08 d	194.84 a	54.92 b

Note: TDS, total dissolved salts; SOC, soil organic carbon; AMN, alkali-hydrolyzable nitrogen; AP, available phosphorus; AK, available potassium; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen. CK, control; CM, *Astragalus sinicus*; SP, *Spinacia oleracea*; RY, *Lolium perenne*. Different letters in the same column indicate a significant difference between treatments at the 0.05 level ($n = 3$).

3.2. The Yield and Nutrient Uptake and Accumulation of the Different Crop Species

Crop yield significantly differed both in terms of shoot biomass and root biomass (Figure 2). RY had a significantly higher yield than SP and CM ($p < 0.01$), with the whole fresh biomass of RY reaching 83.8 kg ha^{-1}. As shown in Figure 3, the three crops displayed the highest cumulative uptake for K, followed by N, and then P. The cumulative K uptake by RY was significantly greater than that exhibited by SP and CM ($p < 0.01$). It was also observed that the root-to-shoot ratios of dry weight for SP and CM were significantly higher than the ratio of RY (Table S1). Further, significant differences were observed in the nutrient contents of the three crops (Figure S5). The N content of RP was significantly lower than that of CM and SP. The P content was higher in SP than in CM and RY. The average K content of the three crops was in the following order: SP > RY > CM.

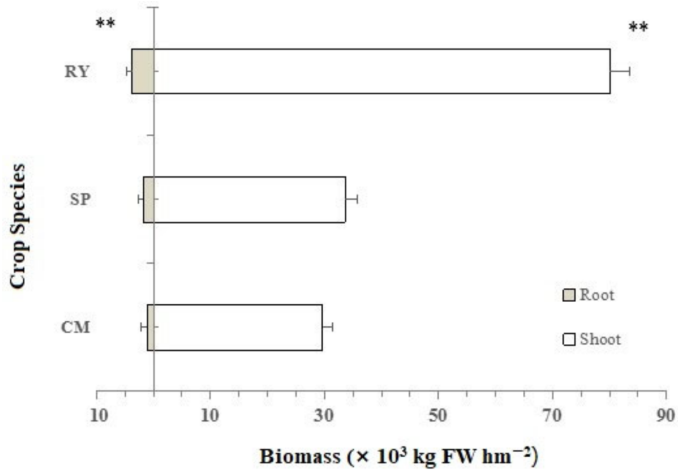

Figure 2. Shoot and root biomass (fresh weight [FW]) of *Lolium perenne* (RY), *Spinacia oleracea* (SP) and *Astragalus sinicus* (CM). Vertical bars represent the standard error of the mean. ** denotes statistically significant differences between crop varieties ($p < 0.01$, Duncan's test).

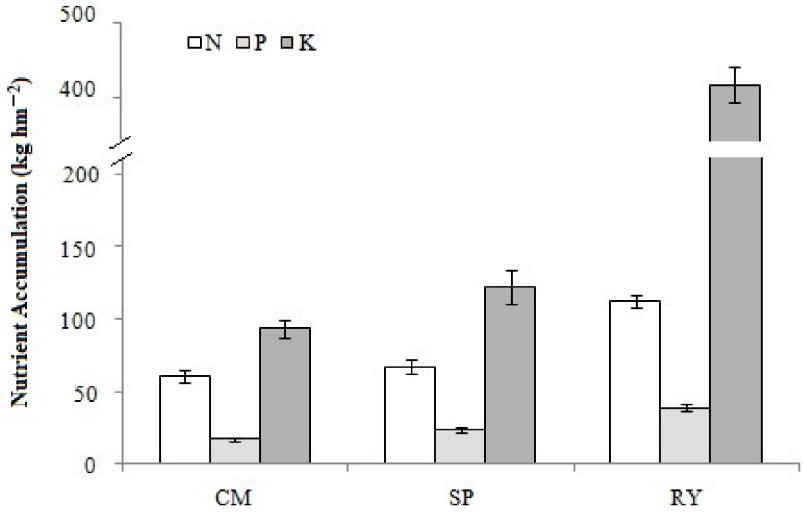

Figure 3. Nutrient accumulation of the three different crop species, *Astragalus sinicus* (CM), *Spinacia oleracea* (SP) and *Lolium perenne* (RY). Vertical bars represent the standard error of the mean.

3.3. Abundance and Diversity of Doil Microbial Communities

Compared to the CK samples, significantly more 16S rRNA sequences but fewer ITS copies ($p < 0.05$) were found in the CM and SP samples (Table 2). However, RY had no significant effects on the number of ITS sequences in the soil compared with CK. Quantitative PCR (qPCR) data also indicated that the bacteria-to-fungi (B/F) ratio declined in the following order: SP > CM > RY > CK.

Table 2. Bacterial (16S rRNA) and fungal (ITS) gene copy numbers in soil samples.

Treatments	16S Gene Copy Numbers (Copies × 10^{10})	ITS Gene Copy Numbers (Copies × 10^{8})	B/F (Bacteria/Fungi 10^{3})
CK	2.50 d	1.44 a	0.17 c
CM	4.21 b	0.82 b	0.51 b
SP	2.83 c	0.26 c	1.11 a
RY	6.69 a	1.47 a	0.46 b

Note: CK, control; CM, *Astragalus sinicus*; SP, *Spinacia oleracea*; RY, *Lolium perenne*. Different letters in the same column indicate a significant difference between treatments at the 0.05 level ($n = 3$).

The α-diversity analysis showed that the bacterial and fungal community richness (Chao1 and ACE) and diversity (Shannon and Simpson) indices varied markedly among the treatments (Table S2). Crop application increased the bacterial and fungal richness indices ($p < 0.05$) and the bacterial Shannon indices ($p < 0.05$) when compared to the control.

The crop treatments were related to an increase in the relative abundance of Proteobacteria and Bacteroidetes, and a decrease in the relative abundance of Actinobacteria for soil bacteria at the phylum level (Figure 4). The fungal community at the phylum level was comparable among all soil samples except for SP samples, which had a high abundance of Basidiomycota and Unclassified_k_Fungi (Figure 5). With respect to the bacterial community at the genus level, CM increased the relative abundances of *norank_f_Gemmatimonadaceae*. With respect to the fungal community, CM and RY both increased the relative abundances of *Chaetomium* and *Humicola*. A higher relative abundance of *Gibellulopsis* was observed in the SP samples.

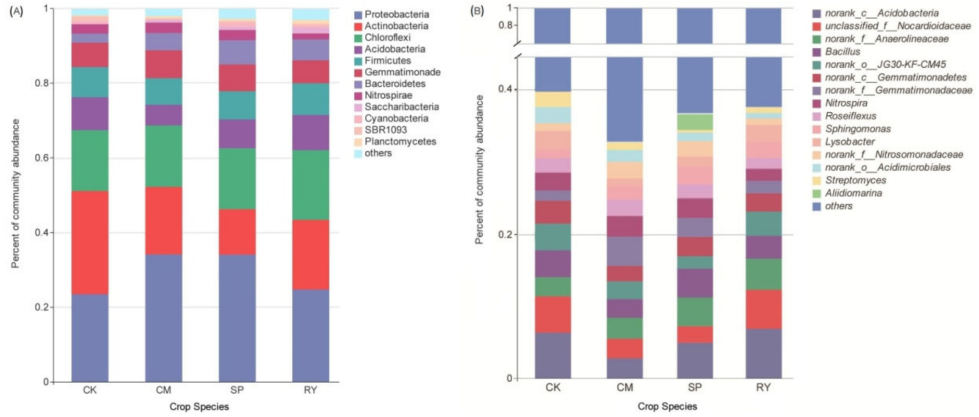

Figure 4. Relative abundance maps of the dominant bacterial taxa in the soils of the different crop treatments based on 16S rRNA sequences at the phylum (**A**) and genus (**B**) levels. CK, control; CM, *Astragalus sinicus*; SP, *Spinacia oleracea*; RY, *Lolium perenne*.

3.4. Relationships between the Relative Abundance of Soil Microorganisms and Crop Nutrient Accumulation

The relative abundances of 16S rRNA and ITS sequences in the soils were significantly correlated with crop nutrient accumulation. The accumulation of all the nutrients by the crops was positively correlated with the relative abundance of soil bacterial and fungal sequences (Figure S6).

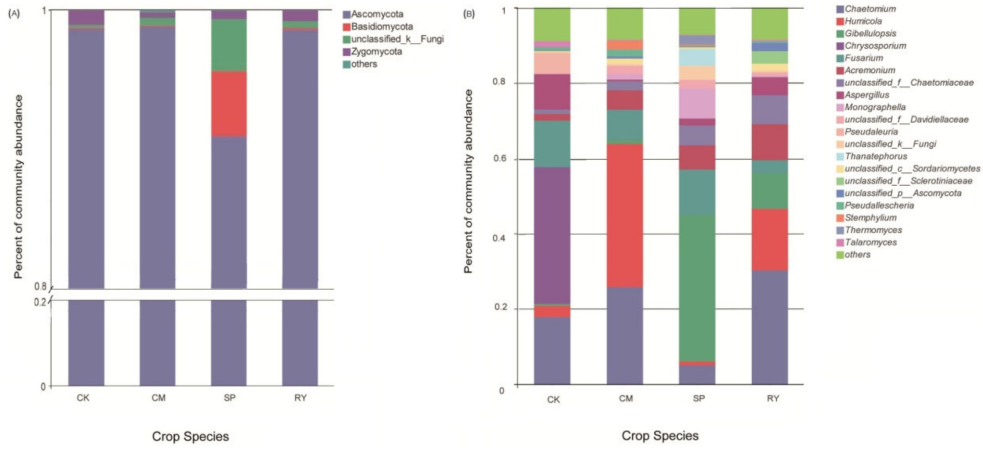

Figure 5. Relative abundance maps of the dominant fungal taxa in the soils of the different crop treatments based on internal transcribed spacer sequences at the phylum (**A**) and genus (**B**) levels. CK, control; CM, *Astragalus sinicus*; SP, *Spinacia oleracea*; RY, *Lolium perenne*.

RDA based on the soil biochemical properties explained 85.13% of the variation in the first two components of the 16S rRNA community diversity (Figure 6A). The first component (RDA1) represented 58.31% of the variability and was dominated by pH and MBN. The second component (RDA2) represented 26.82% of the variability and was dominated by AK and TDS. With respect to the ITS community diversity, the first two trait axes of the RDA accounted for 50.86% and 43.03% of the total variation, respectively; AMN scored high on the first axis, and MBN, SOC, AP, TDS, pH and MBC scored high on the second axis (Figure 6B). For the bacterial 16S rRNA community, most bacterial genera were clustered and scattered in the directions of pH, TDS and AK. Meanwhile, most fungal genera were clustered and scattered in the directions of TDS, AMN, pH and AK.

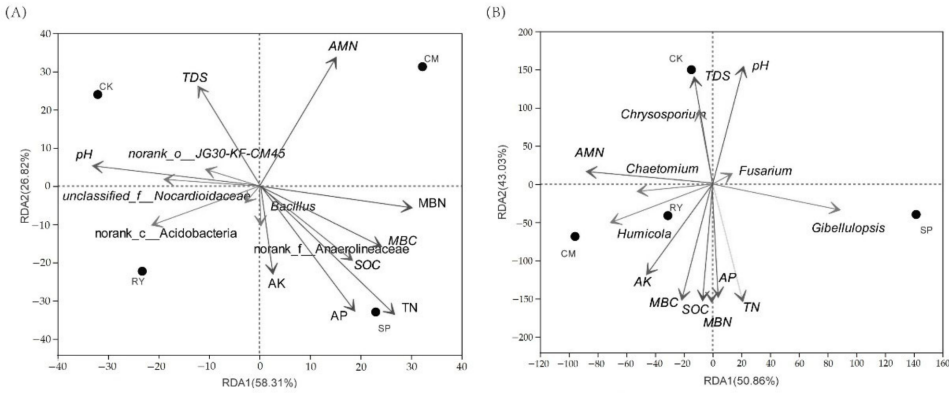

Figure 6. Redundancy analysis (RDA) of soil microbial community diversity and soil biochemical properties using the five most dominant genera according to bacterial 16S rRNA (**A**) and fungal internal transcribed spacer (**B**) sequences. TN, total nitrogen; SOC, soil organic carbon; AMN, alkali-hydrolyzable nitrogen; AP, available phosphorus; AK, available potassium; TDS, total dissolved salts; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen. CK, control; CM, *Astragalus sinicus*; SP, *Spinacia oleracea*; RY, *Lolium perenne*.

4. Discussion and Conclusions

4.1. Crop Biomass Accumulation and Nutrient Absorption in Secondary Salinized Soil

Depending on the ability of a crop to adapt to the stress of secondary salinization, crop growth and nutrient absorption differs. In this study, consistent with the yields of the crops, the order of the total amount of nutrient absorption and accumulation of the three crops was as follows: RY > SP > CM (Figures 1 and 2). Previous studies have shown that when the saline conditions of the soil are aggravated, the growth of legumes is inhibited, and the amount of biomass and nutrient absorption and accumulation decreases [17]. CM, which is a legume used as green manure, can obtain the nutrients required for crop growth through biological nitrogen fixation even in soils with low fertility. However, milk vetch is sensitive to the soil pH and salt content, which restricts its growth and nitrogen-fixing ability in the face of saline-alkali adversity [9,18]. In the present study, the fresh biomass of CM was 30.8 kg ha^{-1}, which was only 1/3 of the average yield of RY.

In this study, it was confirmed that crop yield and nutrient content together determine the amount of nutrient accumulation. For example, the yield of RY was significantly higher than that of the other crops (Figure 1), meanwhile it also performed better with respect to nutrient accumulation. Overall, compared to CK, all cultivation treatments increased soil available nutrients except for soil AMN in RY and SP (Table 1), which may be due to high N accumulation and low N fertilizer application. The results also showed that the root-to-shoot ratio was another key factor affecting crop nutrient accumulation. Previous studies on soil salinization in cultivation facilities have noted that aboveground plant parts were less sensitive to environmental changes than belowground parts [19,20]. Similar variation trends were observed for the root-to-shoot ratios of dry weight and the aboveground N contents of the three crops in the present study. This suggests that the variation in root-to-shoot ratio of dry weight could be a good indicator of aboveground plant N uptake status in cultivation facility soils subjected to secondary salinization.

4.2. Plant–Soil Feedback in Soil Subjected to Secondary Salinization

The mechanisms underlying plant–soil feedback in agrosystems are complex. Previous studies have reported that when exposed to salt stress, plants actively change their strategy for the absorption of inorganic ions, and synthesize proline and other substances to osmotically adjust the cytoplasmic microenvironment [21,22]. Through these changes, the plants can resist the damage caused by saline-alkali stress. The results of the present study show that when crops are grown on secondary salinized soil, the absorption of K^+ by

crops increases. In addition, the content of AP in the soil solution increased, which was possibly related to the pH value and K^+ saturation in the soil solution.

Furthermore, the results of the present study confirmed that phytoremediation increased the B/F ratio in the secondary salinized soil of the facility, especially for SP (Table 2). Previous studies have reported that SP had strong salt tolerant and antifungal ability, compared with other vegetable species on the aspects of phytoremediation and food safety [23,24]. It was found in the present study that leguminous green manure (CM) increased the relative abundances of the bacterial groups *norank_f_Gemmatimonadaceae*, and of the fungal genera *Chaetomium* and *Humicola*. *Gemmatimonadaceae* has been reported for the capacity of accumulating polyphosphate [25]. *Chaetomium* and *Humicola* have been found to be major groups of biological control agents, which not only reduce the incidence of soil-borne pathogens and plant disease, but also degrade a wide range of recalcitrant compounds [26]. These fungi possess a variety of genes that produce high-value enzymes, including chitinase and glucanase. The present study revealed that the nutrient accumulation of the crops was positively correlated with soil microbial communities, and soil pH, MBN, AK and TDS play important roles in maintaining microbial flora balance. However, we recommend future studies using dependency analysis of accuracy methods to create a holistic view of soil microbial succession and crop nutrient accumulation in the cultivation facility soils subjected to secondary salinization.

The utilization of phytoremediation in the form of planting salt-tolerant crops can alleviate the secondary salinization of soils in cultivation facilities. Such bioremediation can optimize the structure of the soil microbial community by increasing the soil microbial biomass, AP, AK and soil pH, and by reducing the soil salt content. The bacterial and fungal community compositions differed among the soils planted with the different salt-tolerant crop species. This study stresses the importance of phytoremediation for soils subjected to secondary salinization, and confirms that the crop species influences the correlations between crop nutrient accumulation and soil microbial community compositions.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture12020212/s1, Figure S1: qPCR standard curve for the 16S rRNA gene; Figure S2: qPCR amplification curve for the 16S rRNA gene; Figure S3: qPCR standard curve for the ITS gene; Figure S4: qPCR amplification curve for the ITS gene; Figure S5: Shoot and root nutrient contents of the three different crop species; Figure S6: Correlations (Pearson's *P*-value) between crop nutrient accumulation and soil microbial abundance for Chinese milk vetch (A,B); spinach (C,D) and ryegrass (E,F); Table S1: Biomass accumulation and root-to-shoot ratio of the bioremediation crops; Table S2: Bacterial (16S rRNA) and fungal (ITS) α-diversity in soil samples.

Author Contributions: Data curation and writing-original draft preparation, S.C.; investigation, S.X. and Z.F.; formal analysis, D.Z.; visualization and writing—review and editing, H.Z. (Hanlin Zhang); supervision, H.Z. (Haitao Zhu). All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest in this manuscript.

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