plants

MDPI

Article

Biological Decline of Alfalfa Is Accompanied by Negative Succession of Rhizosphere Soil Microbial Communities

Yuanyuan Ma [1,2], Yan Shen [1,*], Xiaoping Zhou [2], Hongbin Ma [1], Jian Lan [1], Bingzhe Fu [1] and Quanhong Xue [3]

[1] College of Forestry and Prataculture, Ningxia University, Yinchuan 750021, China; 13363921675@163.com (Y.M.)
[2] Ningxia Rural Science and Technology Development Center, Yinchuan 750001, China; 15909546644@163.com
[3] College of Natural Resources and Environment, Northwest A&F University, Yangling 712100, China; xuequanhong@163.com
* Correspondence: myynczx@163.com

Abstract: The growth and biological decline of alfalfa may be linked to the rhizosphere microbiome. However, plant–microbe interactions in the rhizosphere of alfalfa and associated microbial community variations with stand age remain elusive. This study explored the successional pattern of rhizosphere microbial communities across different aged alfalfa stands and its relationship with alfalfa decline. Rhizosphere soils were collected from 2- and 6-year-old alfalfa stands. Control soils were collected from interspaces between alfalfa plants in the same stands. Soil bacterial and fungal communities were characterized by 16S and ITS rRNA gene sequencing, respectively. Specific microbial taxa colonized the rhizosphere soils, but not the control soils. The rhizosphere-specific taxa mainly included potentially beneficial genera (e.g., *Dechloromonas*, *Verrucomicrobium*) in the young stand and harmful genera (e.g., *Peziza*, *Campylocarpon*) in the old stand. Alfalfa roots regulated soil microbial communities by selective promotion or inhibition of distinct taxa. The majority of time-enriched taxa were reported as harmful fungi, whose relative abundances were negatively correlated with plant traits. Time-depleted taxa were mostly known as beneficial bacteria, which had relative abundances positively correlated with plant traits. The relative abundances of functional bacterial genes associated with vancomycin biosynthesis, zeatin biosynthesis, and amino acid metabolism trended lower in rhizosphere soils from the old stand. An upward trend was observed for fungal pathogens and wood saprotrophs with increasing stand age. The results suggest that root activity drives the negative succession of rhizosphere microbial communities during alfalfa decline in old stands.

Keywords: *Medicago sativa* L.; biological decline; rhizosphere-specific taxa; time-enriched taxa; time-depleted taxa; functional prediction

check for updates

Citation: Ma, Y.; Shen, Y.; Zhou, X.; Ma, H.; Lan, J.; Fu, B.; Xue, Q. Biological Decline of Alfalfa Is Accompanied by Negative Succession of Rhizosphere Soil Microbial Communities. *Plants* **2024**, *13*, 2589. https://doi.org/10.3390/plants13182589

Academic Editor: Won Kyong Cho

Received: 4 August 2024
Revised: 11 September 2024
Accepted: 13 September 2024
Published: 16 September 2024

Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Alfalfa (*Medicago sativa* L.) is a perennial leguminous forage crop widely grown in the United States, Europe, and Asia for hay and silage [1]. A long-term bottleneck in growing alfalfa is that biological decline occurs in old stands with increasing age. Alfalfa plants aged 2 years perform the best in yield and quality. When growing for more than four consecutive years, alfalfa plants become smaller and sparser, with reduced plant height, decreased branch number, lower root activity, and increased disease incidence. All these changes lead to a precipitous decline in the nutritional quality and yield of alfalfa [2,3]. Alfalfa decline is commonly seen across its growing areas in the loess hilly region of Northwest China, constraining the development of alfalfa production [4]. To reduce economic losses and achieve sustainable production, the alfalfa decline problem must be solved.

Many crops decline in terms of yield loss and quality reduction under long-term continuous cropping [5–7]. In most cases, crop decline is associated with abnormally structured rhizosphere microbiomes [7–9]. For example, long-term continuous cropping

of flue-cured tobacco (*Nicotiana tabacum* L.) altered the composition of rhizosphere bacterial and fungal communities. With increasing years of continuous cropping, rhizosphere microbial taxa involved in soil elemental cycling (e.g., Chloroflexi, Patescibacteria) decreased in their relative abundances, which were correlated with the yield loss and quality reduction in tobacco [10]. Additionally, potentially harmful taxa (e.g., *Fusarium*) increased in the rhizosphere soil of American ginseng (*Panax quinquefolius* L.) under continuous cropping for three years, causing severe root rot [11]. Although previous studies have characterized rhizosphere microbial communities associated with alfalfa (e.g., [12]), whether they contain unique taxa that are distinct from those in bulk soil communities is still unclear. Does the structure of rhizosphere microbial communities vary with increasing stand age of alfalfa, and if yes, how do microbial community variations relate to alfalfa decline? Answering these questions can enhance our mechanistic understanding of alfalfa decline.

Rhizosphere microbes live at the plant root–soil interface. These taxonomically and functionally diverse microbes interact with host plants and play a crucial role in plant growth, development, and stress tolerance [13]. The rhizosphere microbiome is also referred to as the second genome of plants [14], and its structure is shaped by microbial interactions with roots and soil [15]. Specific host plants and soil types allow for the formation of rhizosphere-specific microbial communities [16]. On the one hand, beneficial microbes recruited to the rhizosphere can produce microbiostatic and microbicidal compounds, or activate the plant immune system to achieve an antimicrobial effect. On the other hand, pathogenic microbes recruited by root exudates are likely to compete for nutrients and impair plant growth, causing crop failure [17]. Therefore, characterizing the diversity and composition of rhizosphere microbial communities associated with alfalfa has implications for the long-term management of alfalfa growth, stress tolerance, and disease biocontrol.

In the present study, we hypothesized that alfalfa decline is accompanied by abnormal variations in the rhizosphere microbiome. The purpose of the study was to seek evidence that rhizosphere microbial communities are abnormally structured during alfalfa decline in old stands. Rhizosphere soils were collected from alfalfa stands of different ages, and microbial communities were characterized by 16S and ITS rRNA gene sequencing. The composition of rhizosphere-specific taxa was determined by comparing them with microbial taxa in control soils obtained from interspaces between alfalfa plants in the same stands. The successional pattern of rhizosphere microbial communities from young to old stand age was analyzed, and its association with alfalfa decline was explored. The results of this study could uncover the microbial mechanisms underlying alfalfa decline and provide useful data for the development of early detection and prevention techniques.

2. Results

2.1. Rhizosphere Microbial Community Diversity

Sequence clustering yielded a total of 11,030 bacterial OTUs (40 phyla and 778 genera) and 1145 fungal OTUs (9 phyla and 232 genera). For both bacterial (Figure 1a) and fungal (Figure 1b) communities, PCA distinguished rhizosphere soil samples from the 6-year-old alfalfa stand (6R) and corresponding controls (6C), as well as rhizosphere soil samples from the 2-year-old alfalfa stand (2R) and corresponding controls (2C). Compared to 2C soils, 2R soils showed higher bacterial and fungal diversity in terms of Chao1 and Shannon indices (Figure 1c–f). Lower microbial diversity was observed for 6R soils compared to 6C soils (Figure 1d–f), except for the Chao1 index of bacteria (Figure 1c).

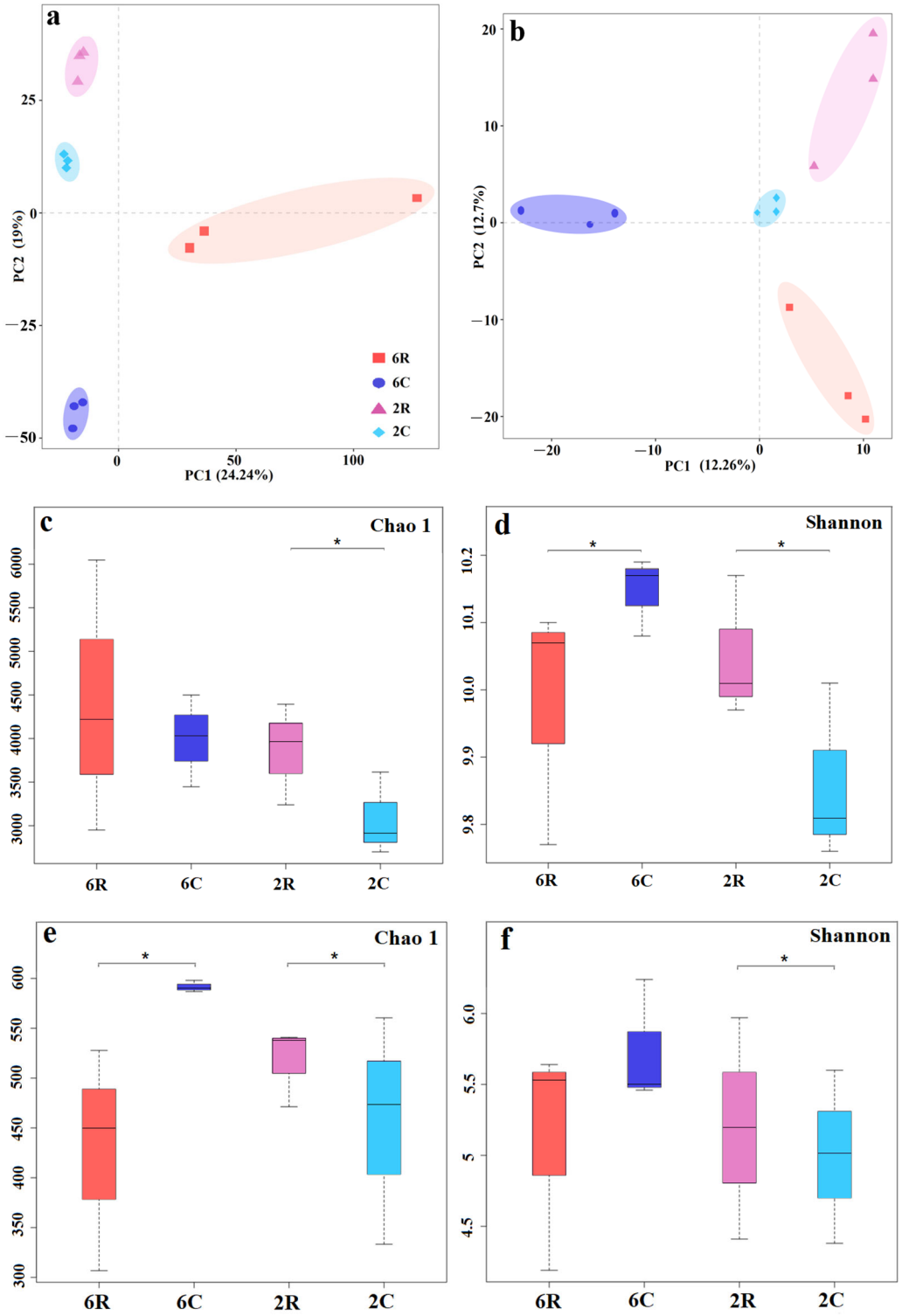


Figure 1. Soil microbial community diversity in alfalfa stands of different ages: (**a**,**b**) Principal coordinates analysis of bacterial and fungal β-diversity based on Bray–Curtis distance. (**c**,**d**) α-Diversity indices of bacteria. (**e**,**f**) α-Diversity indices of fungi. 2R and 2C are rhizosphere soils from the 2-year-old alfalfa stand and corresponding control soils, respectively; 6R and 6C are rhizosphere soils from the 6-year-old alfalfa stand and corresponding control soils, respectively. Error bars represent the standard deviation of the means (n = 3). * indicates a significant difference in α-diversity between rhizosphere and control soils ($p < 0.05$).

2.2. Identification of Rhizosphere-Specific Microbial Taxa

Comparing the microbial community composition between sample groups (2R vs. 2C; 6R vs. 6C) revealed the presence of unique taxa in the rhizosphere soils, but not in the control soils (i.e., rhizosphere-specific taxa). In 2R soils, 1486 bacterial OTUs (Figure 2a) and 165 fungal OTUs (Figure 2b) were enriched, representing 98 bacterial and 18 fungal genera, respectively. In 6R soils, 3240 bacterial OTUs (Figure 2a) and 208 fungal OTUs (Figure 2b) were enriched, representing 124 bacterial and 39 fungal genera, respectively. Among the rhizosphere-specific taxa, 435 bacterial OTUs and 27 fungal OTUs were shared between 2R and 6R soils, which represented 11 bacterial and 5 fungal genera, respectively.

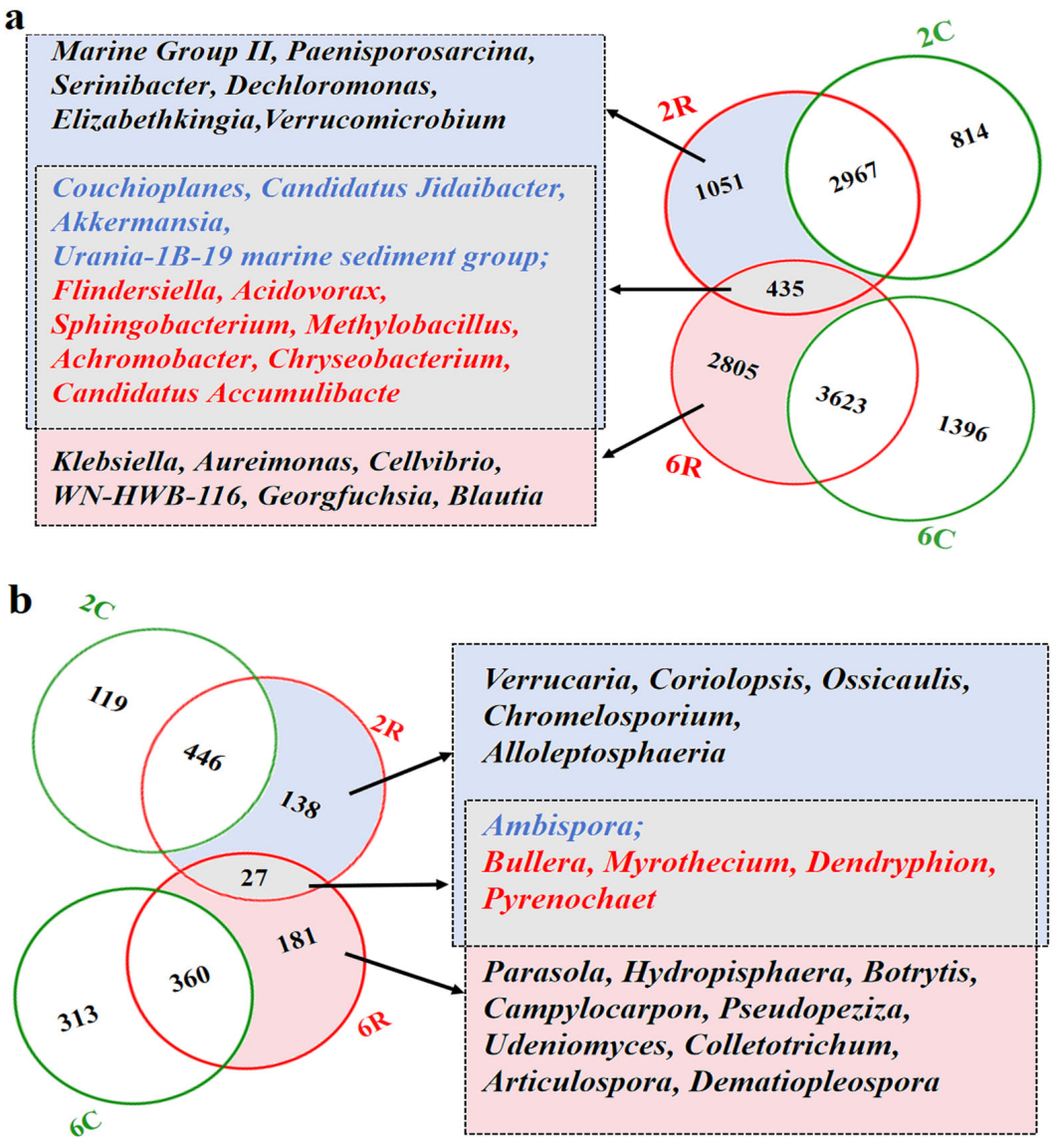


Figure 2. Microbial taxa enriched in the rhizosphere soil of alfalfa: (**a**) Number of operational taxonomic units (right) and taxonomy of genera (left) in the rhizosphere-specific bacterial community. (**b**) Number of operational taxonomic units (left) and taxonomy of genera (right) in the rhizosphere-specific fungal community. The blue color indicates genera with a higher relative abundance in rhizosphere soils from the 2-year-old alfalfa stand (2R) than in the corresponding control soils (2C), and the red color indicates genera with a higher relative abundance in rhizosphere soils from the 6-year-old alfalfa stand (6R) than in the corresponding control soils (6C).

2.3. Regulation of Rhizosphere Microbial Communities by Root Activity

To evaluate the selective effects of root activity on rhizosphere microbial communities, taxa with higher (i.e., root-upregulated taxa) or lower (i.e., root-downregulated taxa) relative abundances in rhizosphere soils than in control soils were analyzed. In both 2R and 6R soils, the bacterial community was dominated by the phylum Actinobacteriota, which accounted for 42.5% and 39.4%, respectively. The root effects on the top 20 most abundant bacterial phyla (Figure 3a) and genera (Figure 3b) were depicted. Irrespective of stand age, root activity upregulated the relative abundances of the phyla Latescibacterota and Methylomirabilota, as well as the genera *MND1* and *IMCC26256*. The opposite effect of root activity was observed for the phyla Cyanobacteria and Abditibacteriota, in addition to the genera *Blastococcus* and *Saccharomyces*. Interestingly, the phyla PCR-54 and Firmicutes, together with the genus *RB41*, were upregulated in 2R soils but downregulated in 6R soils. In contrast, the phyla Bdellovibrionota and Armatimonadota, as well as the genus *Gitt-GS-136*, were downregulated in 2R soils but upregulated in 6R soils.

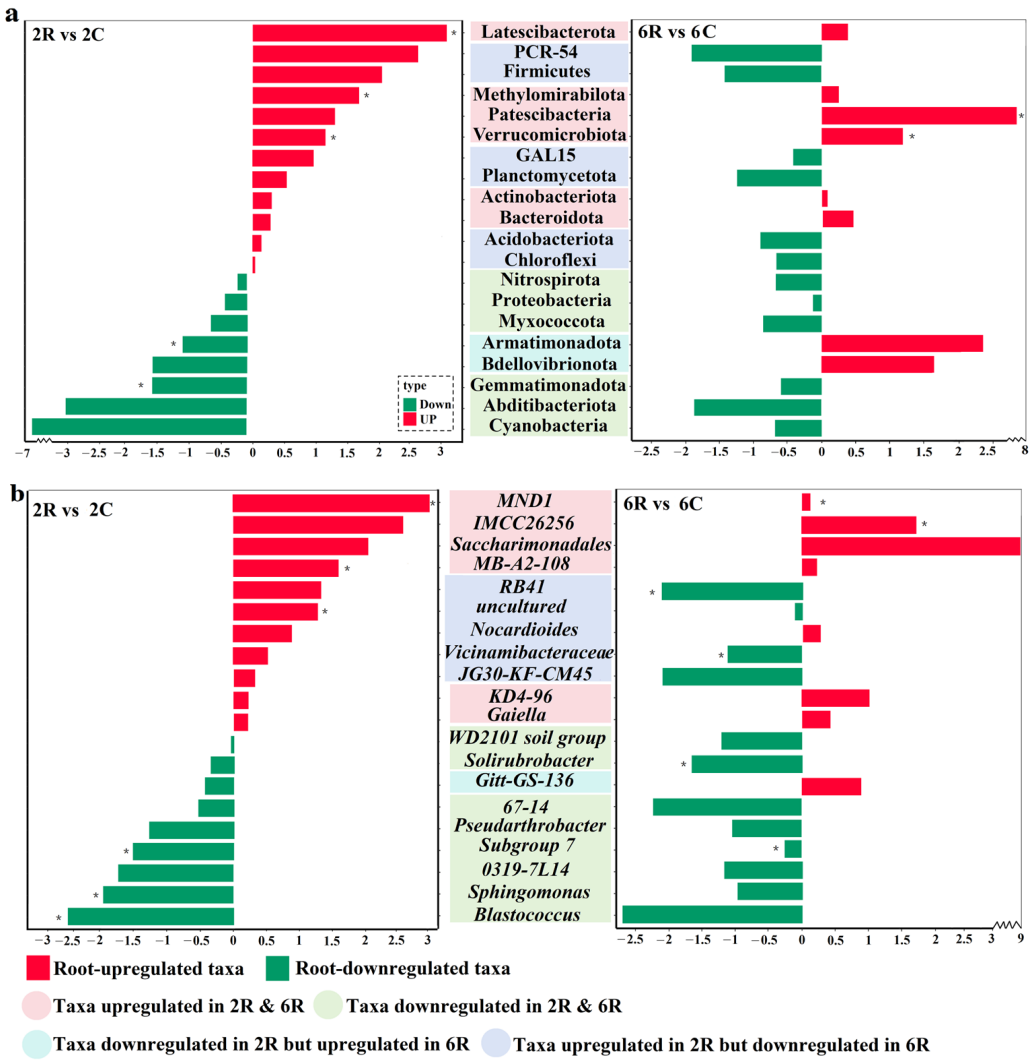


Figure 3. The selection of rhizosphere soil bacteria by root activity of alfalfa. Log_{10} fold changes in the relative abundance of the top 20 bacterial (**a**) phyla and (**b**) genera. 2R vs. 2C indicates the comparison group between rhizosphere soils from the 2-year-old alfalfa stand and corresponding control soils; 6R vs. 6C indicates the comparison group between rhizosphere soils from the 6-year-old alfalfa stand and corresponding control soils. * indicates a significant difference in taxa relative abundance between rhizosphere and control soils ($p < 0.05$).

The fungal community in 2R and 6R soils was dominated by the phylum Ascomycota, which accounted for 58.6% and 69.6%, respectively. The root effects on the nine fungal phyla (Figure 4a) and the top 19 most abundant fungal genera (Figure 4b) were analyzed. In addition to the phyla Kickxellomycota and Glomeromycota, the genera *Rhizophagus* and *Fusariella* were upregulated by root activity, irrespective of stand age. The opposite effect of root activity was observed for the phyla Rozellomycota and Ascomycota (Figure 4a), as well as the genus *Metarhizium*. Some phyla (e.g., Mortierellomycota, Chytridiomycota) and genera (e.g., *Mortierella*, *Rhizophlyctis*) were upregulated in 2R soils, while they were downregulated in 6R soils. Most of the fungal genera downregulated in 2R soils were upregulated in 6R soils (e.g., *Paramyrothecium*, *Vishniacozyma*).

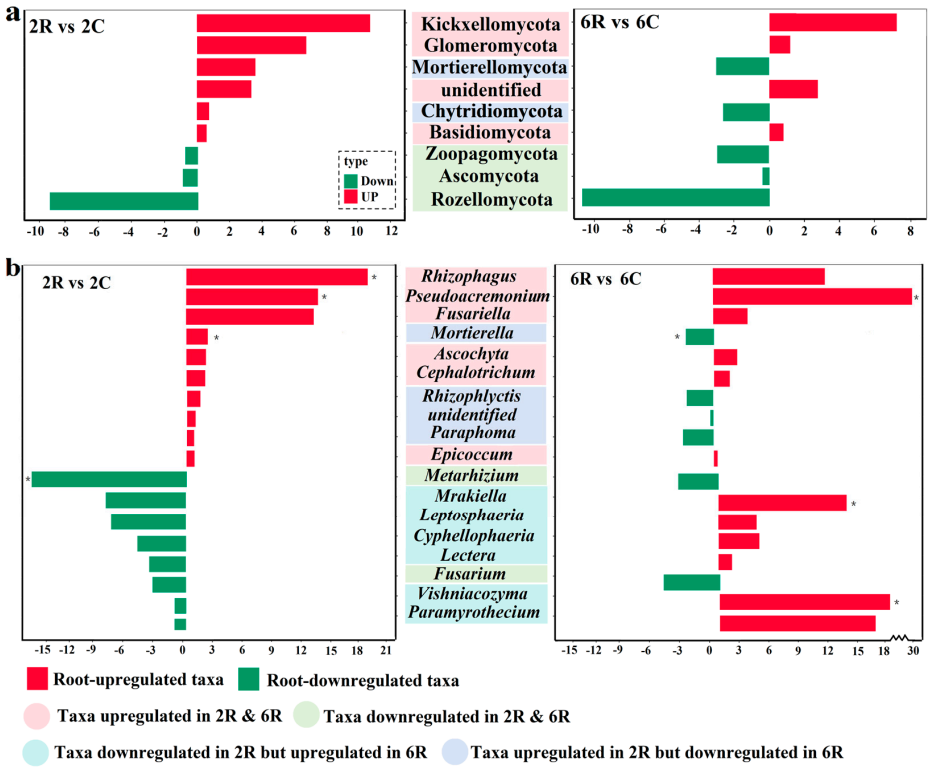


Figure 4. The selection of rhizosphere soil fungi by root activity of alfalfa. Log_{10} fold changes in the relative abundance of the nine fungal (**a**) phyla and (**b**) the top 19 most abundant genera. 2R vs. 2C indicates the comparison group between rhizosphere soils from the 2-year-old alfalfa stand and corresponding control soils; 6R vs. 6C indicates the comparison group between rhizosphere soils from the 6-year-old alfalfa stand and corresponding control soils. * indicates a significant difference in taxa relative abundance between rhizosphere and control soils ($p < 0.05$).

2.4. Effects of Stand Age on Rhizosphere Microbial Communities

The response of rhizosphere microbial communities to stand age was characterized by focusing on taxa with higher (i.e., time-enriched taxa) or lower (i.e., time-depleted taxa) relative abundance in 6R soils than in 2R soils. Among the top 20 bacterial and fungal genera in terms of relative abundance, the time-enriched taxa were mainly fungi (Figure 5a), whereas the time-depleted taxa were mostly bacteria (Figure 5b). At the species level, pathogenic microbes, such as *Paramyrothecium roridum*, *Fusarium oxysporum*, and *Pseudopeziza medicaginis*, emerged as time-enriched taxa (Figure 5c). Beneficial microbes, represented by *Ensifer meliloti*, *Pararhizobium giardinii*, and *Bacillus* spp. were identified as time-depleted taxa (Figure 5d).

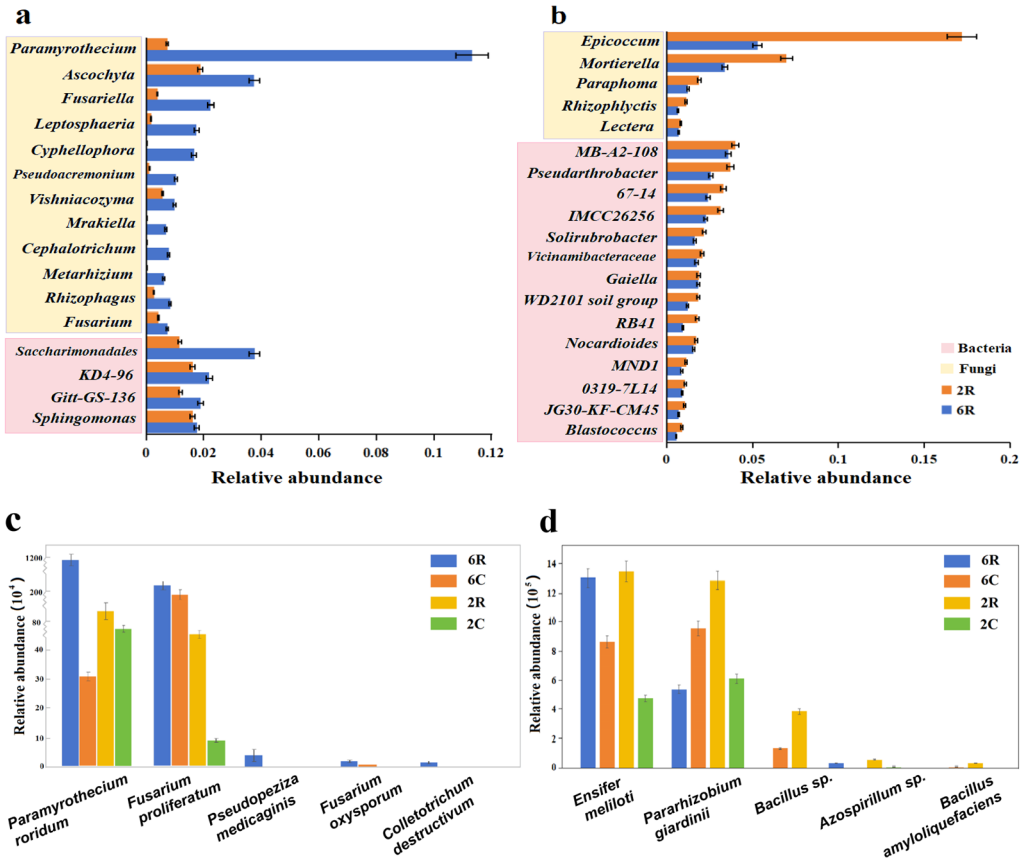


Figure 5. Relative abundances of time-enriched (**a**), time-depleted (**b**), pathogenic (**c**), and beneficial (**d**) microbial taxa in the rhizosphere soil of alfalfa. 2R and 2C represent rhizosphere soils from the 2-year-old alfalfa stand and corresponding control soils, respectively; 6R and 6C represent rhizosphere soils from the 6-year-old alfalfa stand and corresponding control soils, respectively. Error bars represent the standard deviation of the means (n = 3).

2.5. Predicted Functions of Rhizosphere Microbial Communities

Picrust2 predicted 176 functional categories of bacterial communities in all soil samples. Some bacterial functional genes related to important metabolic pathways showed significantly different relative abundances among various samples (Figure 6a). In 6R soils, bacterial functional genes related to environmental adaptation increased compared to those of other soils, whereas genes involved in polyketide metabolism and amino acid metabolism decreased in 6R soils. FunGuild-based functional prediction of fungi yielded three trophic modes (pathotroph, saprotroph, and symbiotroph). Using the Kruskal–Wallis rank sum test, 14 fungal guilds were detected at different relative abundances among samples (Figure 6b). Compared to 6C and 2R soils, pathogens and wood saprotrophs increased in 6R soils, accompanied by decreased plant saprotrophs, soil saprotrophs, and epiphytes. Ectomycorrhizal fungi also increased in 6R soils compared to 2R soils, whereas lichenized fungi almost disappeared in 6R soils.

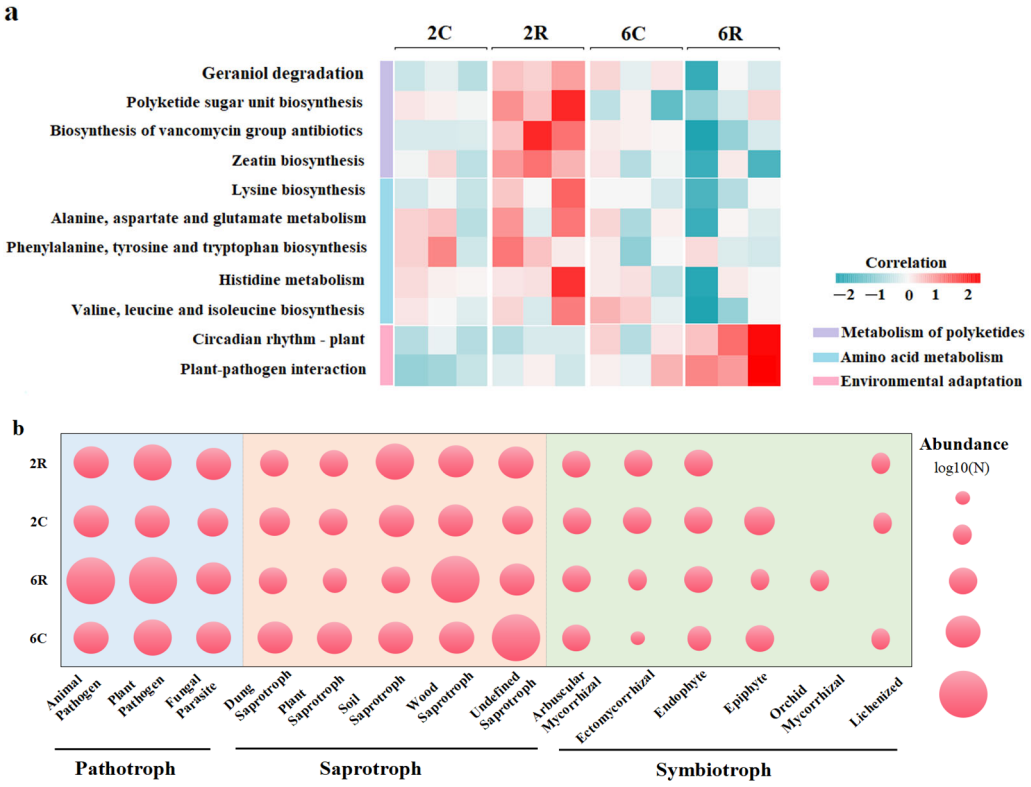


Figure 6. Functional shifts of rhizosphere bacterial (**a**) and fungal (**b**) communities associated with alfalfa. 2R and 2C denote rhizosphere soils from the 2-year-old alfalfa stand and corresponding control soils, respectively; 6R and 6C represent rhizosphere soils from the 6-year-old alfalfa stand and corresponding control soils, respectively.

2.6. Linking Rhizosphere Microbial Taxa to Plant Traits and Soil Properties

To clarify the relationship of rhizosphere-specific taxa, time-enriched taxa, and time-depleted taxa to plant traits and soil properties, Spearman's correlation heatmaps were created (Figures 7 and S1). At the genus level, taxa unique to 2R soils (Figures 7a and S1a) and time-depleted taxa (Figures 7b and S1b) mostly showed positive correlations with plant traits and soil properties. For example, *Paenisporosarcina* and *Verrucomicrobium* (rhizosphere-specific bacteria) were significantly positively correlated with plant height, fresh weight, and leaf-to-stem ratio ($p < 0.05$). *Paenisporosarcina* was also significantly positively correlated with soil alkali-hydrolyzable nitrogen and available phosphorus contents ($p < 0.05$). Additionally, a significant positive correlation emerged between *Blastococcus* (time-depleted bacteria) and plant height ($p < 0.05$). *Epicoccum* and *Rhizophlyctis* (time-depleted fungi) were significantly positively correlated with plant fresh weight and leaf-to-stem ratio, respectively ($p < 0.05$). Generally, taxa unique to 6R soils (Figures 7a and S1a) and time-enriched taxa (Figures 7b and S1b) showed negative correlations with plant traits and soil properties. For example, *Botrytis*, *Campylocarpon*, and *Colletotrichum* (rhizosphere-specific fungi) were significantly negatively correlated with crude protein content in shoots ($p < 0.05$). *Campylocarpon* and *Alloleptosphaeria* (rhizosphere-specific fungi) were significantly negatively correlated with soil total potassium content. *Cyphellophora* (time-enriched fungi) was significantly negatively correlated with plant height, as well as crude protein and neutral detergent fiber contents in shoots ($p < 0.05$).

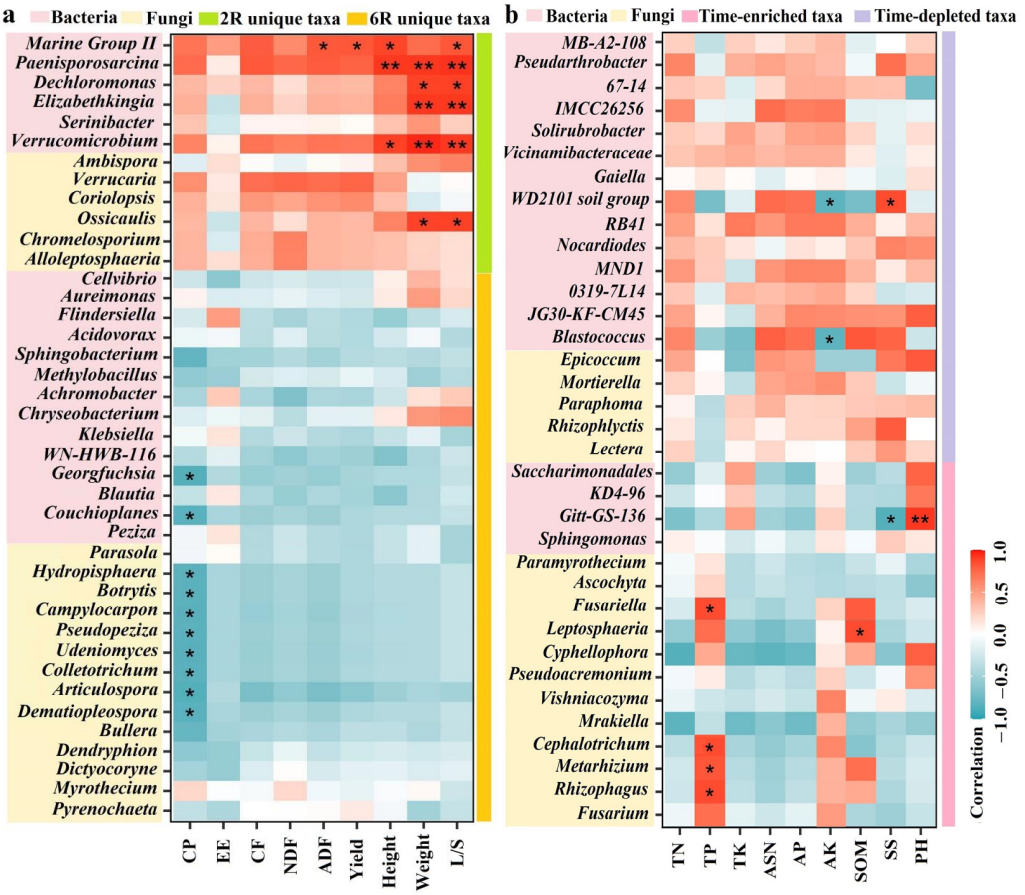


Figure 7. Correlation heatmaps between rhizosphere microbial taxa and plant traits of alfalfa: (**a**) Rhizosphere-specific taxa; (**b**) Time-dependent taxa. 2R and 6R denote rhizosphere soils from the 2- and 6-year-old alfalfa stands, respectively. CP, crude protein; EE, ester extract; CF, crude fiber; NDF, neutral detergent fiber; ADF, acid detergent fiber; L/S, leaf-to-stem ratio. * $p < 0.05$; ** $p < 0.01$.

3. Discussion

This study indicated that alfalfa root activity exhibited selective promoting or inhibitory effects on rhizosphere microbial communities. Consequently, the rhizosphere soil of alfalfa was colonized by unique microbial taxa, and their composition varied with stand age. While some rhizosphere taxa were enriched in the old stand compared to the young stand, others were depleted. The relative abundance and function of time-enriched and time-depleted taxa were closely linked to plant growth, yield, and quality traits of alfalfa. These rhizosphere microbial taxa may serve as potential biomarkers for early detection or even prediction of biological decline in alfalfa. The results also provide useful data to select potential functional microbes for the prevention of alfalfa decline by manipulation of the rhizosphere microbiome.

3.1. Variations in Rhizosphere-Specific Microbial Communities with Stand Age

In the present study, specific microbial taxa were found to colonize the rhizosphere of alfalfa, depending on stand age. In the young stand, the rhizosphere-specific taxa were represented by the genera *Marine Group II*, *Dechloromonas*, and *Verrucomicrobium* (bacteria), as well as *Verrucaria* and *Coriolopsis* (fungi). These taxa showed beneficial effects on plant growth, yield, and quality traits, and some of them were closely related to soil nutrient availability. *Dechloromonas* is a group of denitrifying bacteria, which can also inhibit plant pathogens [18]. *Verrucomicrobium* has been reported to facilitate the establishment of beneficial plant–microbe interactions by maize roots in a rainfed agroecosystem [19]. In the old stand, the rhizosphere-specific taxa were mainly identified as *Peziza* (bacteria),

Campylocarpon, *Pseudopeziza*, *Colletotrichum*, and *Botrytis* (fungi). Most of these taxa exhibited adverse effects on alfalfa quality in terms of crude protein content. Notably, the four fungal taxa comprise pathogens commonly found in crops [20–23]. *P. medicaginis* of the genus *Pseudopeziza* is the principal pathogen causing common leaf spot in alfalfa [24].

To sum up, the rhizosphere-specific taxa detected in the young alfalfa stand played a positive role in enhancing plant growth. In the old alfalfa stand, more harmful taxa were unique to the rhizosphere soil. This community shift driven by stand age could be attributed to the selective effects of alfalfa root activity.

3.2. Selective Promotion and Inhibition of Rhizosphere Microbial Taxa by Root Activity

Root activity promoted a set of bacteria to colonize the rhizosphere of alfalfa across stand ages, as demonstrated by their upregulated relative abundances. The root-upregulated bacteria mainly included the phyla Latescibacterota, Methylomirabilota, Patescibacteria, Verrucomicrobiota, and Actinobacteriota, as well as the genera *MND1*, *Saccharimonadales*, and *KD4-96*. Methylomirabilota methanotrophs play a role in plant nitrogen fixation [25]. Patescibacteria species can enhance cadmium tolerance in *Dahlia pinnata* Cav. [26]. The phylum Actinobacteriota has been reported to facilitate plant growth and reduce the harm of environmental stresses [27]. *MND1* is a genus associated with suppression of cucumber wilt [28]. The genus *Saccharimonadales* is a major contributor to the availability of soil phosphorus [29]. *KD4-96* is a genus of aerobic beneficial fungi [30]. Root activity also promoted rhizosphere colonization by some fungi, including the phyla Kickxellomycota, Glomeromycota, and Basidiomycota, as well as the genera *Rhizophagus*, *Pseudoacremonium*, and *Fusariella*. Glomeromycota is a phylum of arbuscular mycorrhizal fungi, providing essential nutrient support for plant growth [31]. The genus *Rhizophagus* can establish mutualistic symbiosis with legume roots to form root nodules and fix nitrogen. *Rhizophagus* spp. are also able to alleviate leaf spot and bolster plant resistance to pea aphids in alfalfa [32]. The genus *Fusariella* contains a variety of plant pathogens [33], and several species of the genus *Ascochyta* are known as plant pathogens [34].

Some bacteria were found to be inhibited by alfalfa root activity across stand ages, as indicated by their downregulated relative abundances. The root-downregulated bacteria were represented by the phyla Cyanobacteria and Myxococcota, and the genera *Blastococcus*, *Sphingomonas*, and *Pseudarthrobacter*. The phylum Myxococcota plays a prominent role in the rhizosphere biological process of rice allelopathy, and its proportion is higher in the rhizosphere of rice plants with stronger allelopathy [35]. The genus *Blastococcus* shows higher relative abundance in maize rhizosphere under continuous cropping than under rotational cropping [36]. *Sphingomonas* spp. perform beneficial functions, such as the degradation of complex organic matter and antagonism of plant pathogenic fungi [37]. *Pseudarthrobacter* spp. show plant growth-promoting effects [38]. The fungi inhibited by alfalfa root activity mainly belonged to the phyla Rozellomycota and Ascomycota, in addition to the genus *Metarhizium*. Most known plant pathogens belong to the phylum Ascomycota [39,40], whereas *Metarhizium* spp. can colonize roots to promote plant growth [41].

In summary, alfalfa root activity regulated rhizosphere microbial communities by selectively promoting or inhibiting the colonization of distinct taxa. Most of the root-upregulated taxa conveyed beneficial effects on host plants, although there were also some pathogens. While the root-downregulated taxa mainly exhibited harmful effects on host plants, some were known as beneficial taxa. The selection of soil microbes by root activity could be related to the stand age and growth environment of alfalfa.

3.3. Rhizosphere Microbial Community Succession in Relation to Alfalfa Decline

As the stand age of alfalfa increased, the rhizosphere-specific microbial communities changed to be dominated by potentially harmful taxa, and the root effects on microbial taxa varied. For the bacterial community, the phyla Firmicutes and Planctomycetota, and the genera *Nocardiodes*, *RB41*, and *JG30-KF-CM45* were promoted in the young stand but inhibited in the old stand. The same pattern was observed for the phylum Chytridiomycota

and the genera *Mortierella* and *Rhizophlyctis* in the fungal community. It has been reported that *Nocardiodes* can enhance the availability of soil iron and potassium, improving plant nutrition [42]. *JG30-KF-CM45* is a group of denitrifying bacteria [43]. *Mortierella* can facilitate crop growth by increasing the contents of soil available phosphorus, potassium, calcium, magnesium, and boron [44]. The majority of taxa inhibited in the young stand were promoted in the old stand, including the phyla Bdellovibrionota and Armatimonadota and the genus *Gitt-GS-136* (bacteria), as well as the genera *Paramyrothecium* and *Vishniacozyma*, *Leptosphaeria*, *Cyphellophaeria*, and *Mrakiella* (fungi). *Paramyrothecium* spp. are reported as plant pathogens and cause leaf spot or leaf blight on many commercial crops worldwide [45]. *Leptosphaeria* spp. cause phoma leaf spot and stem cancer in many plant species [46,47]. This means that old alfalfa plants created favorable conditions for rhizosphere colonization by harmful microbes, rather than beneficial microbes.

As time-enriched species, a range of pathogenic bacteria (e.g., *P. medicaginis*, *C. destructivum*, *P. roridum*, and *Fusarium* spp.) was negatively related to plant traits. *C. destructivum* and *F. oxysporum* are important pathogens responsible for root rot of alfalfa [48]; *P. roridum* is the primary pathogen causing common leaf spot of alfalfa [49]. As time-depleted species, some beneficial bacteria (e.g., *E. meliloti*, *P. giardinii*, and *Bacillus amyloliquefaciens*) were positively related to plant traits. *E. meliloti* and *P. giardinii* are major nitrogen-fixing rhizobia associated with alfalfa [50]. Both of the species can promote plant growth and mediate rhizosphere communities [51]. Additionally, *B. amyloliquefaciens* is a crucial biocontrol bacterium, which has the ability to increase crop yield, inhibit pathogen growth, and reduce plant diseases [52].

3.4. Rhizosphere Microbial Community Function Associated with Alfalfa Decline

An increase in bacteria related to environmental adaption was observed in rhizosphere soils from the old stand. This indicates deterioration of the rhizosphere microenvironment and consequent enrichment of bacteria with stronger adaptive capacity. Amino acid-metabolizing bacteria also decreased in rhizosphere soils from the old stand. Amino acids are raw materials for protein biosynthesis; these compounds play a decisive role in plant growth by enhancing physiological and biochemical processes, such as photosynthesis, chlorophyll biosynthesis, nutrient uptake, and root development [53]. As amino acid metabolism is involved in plant nitrogen cycling [54], the decrease in amino acid-metabolizing bacteria is indicative of a potential decline in nitrogen cycling function in the rhizosphere microenvironment of old alfalfa plants.

Plant–pathogen interactions are regulated by the genotype and biochemical traits of both the plant and the pathogen [55]. Polyketide-metabolizing bacteria decreased in rhizosphere soils from the old stand, as evidenced by the lower relative abundances of taxa related to vancomycin biosynthesis, zeatin biosynthesis, and geranylgeraniol degradation. This provides evidence that the old alfalfa stand is less resistant to pathogen stress and more susceptible to pathogen attack. Polyketides are a class of natural secondary metabolites with biological activity produced by microbes and plants. These compounds are of biological significance in plant–environment interactions, as well as in plant growth and development [56]. In particular, vancomycin is a glycopeptide antibiotic that prevents the biosynthesis of bacterial cell walls and shows bactericidal effects [8]. As a cell growth regulator, zeatin performs functions in promoting lateral bud growth, stimulating cell differentiation, accelerating callus and seed germination, and preventing leaf senescence [57].

Fungal pathogens, wood saprotrophs, and orchid mycorrhizal fungi preferentially colonized rhizosphere soils from the old stand. Fungal pathogens impair host root cells to acquire nutrients, causing soil-borne diseases; a higher diversity of fungal pathogens leads to a lower stability of ecosystem productivity [58]. Wood saprotrophs thrive in plant litter, degrading lignin and cellulose [59]. The higher abundance of wood saprotrophs in the old alfalfa stand may be attributed to the accumulation of fallen stems and leaves, together with aged and dead roots in the rhizosphere soil. Plant saprotrophs, soil saprotrophs, and ectomycorrhizal fungi were inhibited to some extent in rhizosphere soils from the old stand.

Saprophytic fungi decompose and utilize organic matter, acting as one of the key drivers of soil nutrient transformation [58]. Ectomycorrhizal fungi help plants to absorb nutrients and resist pathogen attack [60]. Therefore, the possible reasons for alfalfa decline include the increase in pathogenic microbes and the decrease in beneficial microbes in the rhizosphere soil, coupled with the weakening of important metabolic functions.

4. Materials and Methods

4.1. Study Area

The study was conducted in Pengyang County (106°38′ E, 35°41′ N), a rainfed agricultural area in the loess hilly region of southern Ningxia, China. This area is located in the central hilly gully area of the Loess Plateau, with a typical temperate semi-arid continental monsoon climate. The mean annual temperature ranges from 7.4 °C to 8.5 °C, and the mean annual precipitation is 429.8 mm. The major soil type is classified as loessial soil (Entisol). Since the national policy of returning farmland to forest and grassland was implemented in 2003, alfalfa has been widely grown in this area. The experiment began in April 2017 and lasted until May 2023. We selected two field plots (48 m × 12 m each) with the same elevation and spaced ~100 m apart in the study area. One plot was grown with alfalfa in 2017, after 2 years of maize cropping; this plot was referred to as the 6-year-old alfalfa stand (old stand). The other plot was grown with alfalfa in 2021, after 6 years of maize cropping; this plot was referred to as the 2-year-old alfalfa stand (young stand). The alfalfa cultivar used in the experiment was Gannong No. 4. The plots were managed under the same cropping system and practices, including annual harvest time, fertilization, and irrigation. Sampling was carried out at the first full-bloom stage of alfalfa in May 2023. Tables S1 and S2 provide details for the alfalfa plant traits and soil properties in the experimental plots.

4.2. Soil Sampling and Chemical Analysis

Three 1 m × 1 m quadrats were randomly selected from each stand. In each quadrat, soil profiles were dug to 30 cm depth using a shovel, within 50 cm from the stem of three randomly selected alfalfa plants. Alfalfa roots were exposed as completely as possible, and the loose soil adhering to fibrous roots was removed with a soil knife. Then, the fibrous roots were gently shaken and the fallen soil was collected (50 g per plant). Soils from the roots of three plants were thoroughly mixed and used for chemical analysis. The remaining soil adhering to roots (i.e., rhizosphere soil) was brushed off with a sterilized hairbrush and passed through a 20-mesh sterilized sieve. After removing impurities with sterilized forceps, rhizosphere soils from three plants were thoroughly mixed, then sealed in sterile centrifuge tubes (15.00 g per tube) and immediately stored at −80 °C until DNA extraction. To collect control soils, three patchy areas with no weeds and no alfalfa plants were selected in each stand. After removing ~1 cm thick topsoil, soils were collected from the 0–30 cm depth with a 7.5 cm diameter soil auger. The control soils were prepared as described for the rhizosphere soils.

Soil samples were air-dried, ground, and passed through a 20-mesh sieve before chemical analysis. The potassium dichromate volumetric method was used to determine soil organic matter [61]. For quantification of alkali-hydrolyzable nitrogen and total nitrogen, the alkaline hydrolysis and semi-micro Kjeldahl methods were respectively used [62]. Soil total phosphorus and potassium were respectively analyzed by molybdenum-antimony anti-colorimetric assay and flame photometry with samples fused using sodium hydroxide. Ultraviolet spectrophotometry was conducted to analyze soil available phosphorus in sodium bicarbonate extracts [63]. Flame photometry was adopted to determine soil-available potassium in ammonium acetate extracts [64]. Soil pH was measured in 1:2 (w/v) soil suspensions using a digital pH meter (PHST-5; Merweather Biotechnology Co., Ltd., Shanghai, China). Soil total salt was determined by conductometry [65].

4.3. Soil Microbial Community Analysis

Genomic DNA extraction from soil samples (5.00 g each) was performed using the E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Inc., Doraville, GA, USA). After quality check, the extracted DNA served as a template for amplifying the V3–V4 region of the bacterial 16S rRNA gene and the internal transcribed spacer 1 (ITS1) region of the fungal 18S nuclear RNA gene. The PCR primers used were 338F (5′-ACTCCTACGGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) for bacteria [66], and SSU0817F (5′-TTAGCATGGAATAATRRAATAGGA-3′) and 1196R (5′-TCTGGACCTGGTGAGTTTCC-3′) for fungi [67]. PCR products were purified automatically using the Agencourt AMPure XP Kit (Beckman Coulter, Inc., Brea, CA, USA). Library construction was performed using the NEB Next Ultra II DNA Library Prep Kit (New England Biolabs, Inc., Ipswich, MA, USA). The libraries were sequenced on Illumina Novaseq 6000 (Illumina, Inc., San Diego, CA, USA) to generate 2 × 250 bp paired-end reads. The raw data were obtained in FASTQ format with truncated adapter sequences and have been deposited in the NCBI Sequence Read Archive database (https://www.ncbi.nlm.nih.gov/; accessed on 17 June 2024) under project numbers PRJNA1124538 (bacteria) and PRJNA1124542 (fungi).

4.4. Plant Sampling and Analysis

In each quadrat, six alfalfa plants were randomly selected and completely dug out for growth analysis. The shoots of alfalfa plants remaining in the six quadrats were harvested and used for yield and quality evaluation. All plant samples were sealed in plastic bags and brought back to the laboratory. The fresh weight per plant and the total fresh weight of all plants from six quadrats were determined using an electronic scale (accuracy: 0.01 g). Alfalfa yield was calculated by dividing the total fresh weight of all plants by the quadrat area. Plant height was measured using a ruler, and stem thickness was obtained using digital Vernier calipers. The stems and leaves were separated, deactivated at 105 °C for 1 h, and oven-dried at 70 °C for 48 h until constant weight. The dry weight of stems and leaves was determined to calculate the leaf-to-stem ratio. After deactivation (105 °C, 24 h) of shoot samples, crude protein content was analyzed according to AOAC standards [68]. Crude fiber content was analyzed using the filter bag technique [69]. Neutral and acid detergent fiber contents were measured using an ANKOM A200i fiber analyzer (ANKOM Technology, Macedon, NY, USA) based on the method of Soest et al. [70]. Crude fat (ether extract) was determined using an ANKOM AXT15i automatic fat analyzer (ANKOM Technology) as described by Li et al. [71].

4.5. Data Analysis

Trimmomatic was used to denoise and assemble the raw reads; then, FLASH was used to perform precise denoising of the assembled reads [72]. Sequence analysis was carried out using QIIME v1.9.1 [73], and high-quality reads were obtained for downstream analysis. The high-quality reads were clustered into operational taxonomic units (OTUs) at 97% similarity using Uparse software (Uparse v7.0.1001) [74]. A representative sequence of OTUs was selected based on the principle of its algorithm, the sequence with the highest frequency of occurrence in OTUs was selected as the representative sequence of OTUs. Species annotation of OTUs sequences was analyzed using the Mothur method with the SSUrRNA database from SILVA1384 (setting a threshold of 0.8 to 1) [75] to obtain taxonomic information and at each taxonomic level separately: kingdom, phylum, class, order, family, genus, species, and the community composition of each sample. Rapid multiple sequence alignment was performed using MUSCLE (Version 3.8.31) software to obtain phylogenetic relationships for all OTUs representative of the sequences. Based on OTU abundance data, the α- and β-diversity of bacterial and fungal communities were calculated and plotted using QIIME v1.9.1 [73]. Wilcoxon rank sum test of α-diversity indices and principal coordinates analysis (PCoA) of β-diversity were created using the ggplot2 package of R v3.6.0, and Venn diagrams of microbial communities were plotted using the Venn package of R v3.6.0 [76]. Bar charts of taxa relative abundance (Log_{10} fold changes) were plotted using

Sigmaplot v15.0 (Systat Software Inc., San Jose, CA, USA). PICRUSt2 was adopted to predict bacterial community functions [77], and the FUNGuild database was used for prediction of fungal community functions [78]. Significant differences in the relative abundances of predicted functional pathways among samples were analyzed by the Kruskal–Wallis rank sum test [79]. The relationships of rhizosphere microbial taxa-to-plant traits and soil properties were determined using Spearman's correlation analysis.

5. Conclusions

This study clarified the pattern of microbial community succession in the rhizosphere soil of alfalfa with stand age. The relationship between rhizosphere-specific microbial taxa and alfalfa biological decline was deciphered. It was found that root activity altered the composition of rhizosphere microbial communities by inhibiting or promoting colonization of distinct taxa. The selective effect of alfalfa roots varied with stand age. Overall, long-term continuous cropping of alfalfa led to a negative succession of rhizosphere microbial communities. This was evidenced by the enrichment of harmful fungi and the depletion of beneficial bacteria in the rhizosphere soil, coupled with the weakening of amino acid metabolism and antibiotic biosynthesis. The variations in the rhizosphere microbial community structure and function were linked to alfalfa decline in the old stand. The results of this study underscore the role of plant–microbe interactions in alfalfa decline. The findings could be useful for the development of biotechniques to detect and prevent alfalfa decline.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants13182589/s1, Figure S1: Correlation heatmaps between rhizosphere microbial taxa and soil properties in alfalfa stands; Table S1: Plant traits of alfalfa; Table S2: Soil properties; Table S3: Relative abundance of unique microbial taxa in the rhizosphere soil of alfalfa ($\times 10^6$); Table S4: Relative abundance of bacterial phyla in 2R and 2C ($\times 10^4$); Table S5: Relative abundance of bacterial phyla in 6R and 6C ($\times 10^4$); Table S6: Relative abundance of bacterial genera in 2R and 2C ($\times 10^4$); Table S7: Relative abundance of bacterial genera in 6R and 6C ($\times 10^4$); Table S8: Relative abundance of fungal phyla in 2R and 2C ($\times 10^4$); Table S9: Relative abundance of fungal phyla in 6R and 6C ($\times 10^4$); Table S10: Relative abundance of fungal genera in 2R and 2C ($\times 10^5$); Table S11: Relative abundance of fungal genera in 6R and 6C ($\times 10^5$); Table S12: Relative abundances of pathogenic microbial taxa in the rhizosphere soil of alfalfa ($\times 10^6$); Table S13: Relative abundances of beneficial microbial taxa in the rhizosphere soil of alfalfa ($\times 10^6$).

Author Contributions: Conceptualization, Y.M. and Q.X.; methodology, Y.M. and Y.S.; software, Y.M.; validation, Y.M., Y.S. and H.M.; formal analysis, Y.M.; investigation, Y.M. and J.L.; resources, B.F.; data curation, Y.M.; writing—original draft preparation, Y.M.; writing—review and editing, Y.S. and Q.X.; visualization, Y.M.; supervision, Y.S. and H.M.; project administration, X.Z.; funding acquisition, X.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Key Research & Development Project of Ningxia Autonomous Region, China, grant number 2023 BCF01022, and Natural Science Foundation of Ningxia Autonomous Region, China, grant number 2024AAC03066.

Data Availability Statement: All data generated or analyzed during this study are included in this published article [and its Supplementary Information files].

Acknowledgments: We thank Hong Bai and Yuan Mao for their assistance in field data collection.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Singer, S.; Lehmann, M.; Zhang, Z.; Subedi, U.; Burton, H.; Lim, N.; Ortega, P.; Chen, G.; Acharya, S.; Hannoufa, A. Elucidation of physiological, transcriptomic and metabolomic salinity response mechanisms in *Medicago sativa*. *Plants* **2023**, *12*, 2059. [CrossRef] [PubMed]
2. Rong, S.; Shi, S.; Sun, C. Determination of coumarins and major phenolic acids in plant and rhizosphere soil of alfalfa (*Medicago sativa* L.). *Soils* **2016**, *48*, 931–938.
3. Yin, G. Soil Microecological Characteristics and Reduction of Autotoxicity in Alfalfa-Wheat-Maize Rotation System. Ph.D. Thesis, Gansu Agricultural University, Lanzhou, China, 2019.

4. Wang, R.; Liu, J.; Jiang, W.; Ji, P.; Li, Y. Metabolomics and microbiomics reveal impacts of rhizosphere metabolites on alfalfa continuous cropping. *Front. Microbiol.* **2022**, *13*, 833968. [CrossRef] [PubMed]
5. Ma, L.; Ma, S.; Chen, G.; Lu, X.; Chai, Q.; Li, S. Mechanisms and mitigation strategies for the occurrence of continuous cropping obstacles of legumes in China. *Agronomy* **2024**, *14*, 104. [CrossRef]
6. Li, X.; Lewis, E.; Liu, Q. Effects of long-term continuous cropping on soil nematode community and soil condition associated with replant problem in strawberry habitat. *Sci. Rep.* **2016**, *6*, 30466. [CrossRef] [PubMed]
7. Zhang, Y.; Sun, S.; Luo, D.; Mao, P.; Rosazlina, R.; Martin, F.; Xu, L. Decline in morel production upon continuous cropping is related to changes in soil mycobiome. *J. Fungi* **2023**, *9*, 492. [CrossRef]
8. Yu, Y.; Qi, Z.; Zhang, Z.; Zhou, S.; Jin, M. Plants select antibiotic resistome in rhizosphere in early stage. *Sci. Total Environ.* **2023**, *858*, 159847. [CrossRef]
9. Zeeshan, U.; Yu, J.; Yao, G.; Yang, H. Systematic Review on the continuous cropping obstacles and control strategies in medicinal plants. *Int. J. Mol. Sci.* **2023**, *24*, 12470. [CrossRef]
10. Li, G.; Gong, P.; Zhou, J.; Wang, L.; Song, X.; Ding, P.; Jin, Y.; Zhang, Y.; Zhou, X.; Yang, J.; et al. The succession of rhizosphere microbial community in the continuous cropping soil of tobacco. *Front. Environ. Sci.* **2024**, *11*, 1251938. [CrossRef]
11. Bi, Y.; Zhang, X.; Jiao, X.; Li, J.; Peng, N.; Tian, G.; Wang, Y.; Gao, W. The relationship between shifts in the rhizosphere microbial community and root rot disease in a continuous cropping American ginseng system. *Front. Microbiol.* **2023**, *14*, 1097742. [CrossRef]
12. Zhou, Z.; Zhang, Y.; Zhang, F. Community assembly correlates with alfalfa production by mediating rhizosphere soil microbial community composition in different planting years and regimes. *Plant Soil.* **2022**, *479*, 355–370. [CrossRef]
13. Liu, Y.; Xu, Z.; Chen, L.; Xun, W.; Shu, X.; Chen, Y.; Sun, X.; Wang, Z.; Ren, Y.; Shen, Q.; et al. Root colonization by beneficial rhizobacteria. *FEMS Microbiol. Rev.* **2024**, *48*, 66. [CrossRef] [PubMed]
14. Roeland, L.; Corné, M.; Peter, A. The rhizosphere microbiome and plant health. *Trends Plant Sci.* **2012**, *17*, 478–486. [CrossRef]
15. Zhou, Y.; Coventry, D.; Gupta, V. The preceding root system drives the composition and function of the rhizosphere microbiome. *Genome Biol.* **2020**, *21*, 89. [CrossRef]
16. Gabriele, B.; Kornelia, S. Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiol. Ecol.* **2009**, *68*, 1–13. [CrossRef]
17. Zhou, Q.; Wang, Y.; Yue, L. Impacts of continuous cropping on the rhizospheric and endospheric microbial communities and root exudates of Astragalus mongholicus. *BMC Plant Biol.* **2024**, *24*, 340. [CrossRef]
18. Zhao, W.; Bi, X.; Peng, Y.; Bai, M. Research advances of the phosphorus-accumulating organisms of *Candidatus Accumulibacter*, *Dechloromonas* and *Tetrasphaera*: Metabolic mechanisms, applications and influencing factors. *Chemosphere* **2022**, *307*, 135675. [CrossRef]
19. Aguirre, W.; Rocha, E.; Shapiro, L. Enrichment of Verrucomicrobia, Actinobacteria and Burkholderiales drives selection of bacterial community from soil by maize roots in a traditional milpa agroecosystem. *PLoS ONE* **2018**, *13*, e0208852. [CrossRef]
20. Abeywickrama, P.; Zhang, W.; Li, X.; Jayawardena, R.; Hyde, K.; Yan, J. Campylocarpon fasciculare (Nectriaceae, Sordariomycetes); Novel emergence of black-foot causing pathogen on young grapevines in China. *Pathogens* **2021**, *10*, 1555. [CrossRef]
21. Ogawa, T.; Chen, J.; Mise, K.; Takano, Y. Multiple Colletotrichum species commonly exhibit focal effector accumulation in a biotrophic interface at the primary invasion sites in their host plants. *Plant Signal Behav.* **2021**, *16*, 1935604. [CrossRef]
22. Qin, F.; Liu, D.; Sun, B.; Ruan, L.; Ma, Z.; Wang, H. Identification of alfalfa leaf diseases using image recognition technology. *PLoS ONE* **2016**, *11*, e0168274. [CrossRef]
23. Veloso, J.; van Kan, J. Many Shades of Grey in Botrytis-Host Plant Interactions. *Trends Plant Sci.* **2018**, *23*, 613–622. [CrossRef] [PubMed]
24. Li, Y.; Huang, H.; Wang, Y. Transcriptome characterization and differential expression analysis of disease-responsive genes in alfalfa leaves infected by *Pseudopeziza medicaginis*. *Euphytica* **2018**, *214*, 126. [CrossRef]
25. Cui, J.; Zhang, M.; Chen, L.; Zhang, S.; Luo, Y.; Cao, W.; Zhao, J.; Wang, L.; Jia, Z.; Bao, Z. Methanotrophs contribute to nitrogen fixation in emergent macrophytes. *Front. Microbiol.* **2022**, *13*, 851424. [CrossRef] [PubMed]
26. Li, X.; Li, B.; Jin, T.; Chen, H.; Zhao, G.; Qin, X.; Yang, Y.; Xu, J. Rhizospheric microbiomics integrated with plant transcriptomics provides insight into the Cd response mechanisms of the newly identified Cd accumulator Dahlia pinnata. *Front. Plant Sci.* **2022**, *13*, 1091056. [CrossRef]
27. Elshafie, H.; Camele, I. Rhizospheric actinomycetes revealed antifungal and plant-growth-promoting activities under controlled environment. *Plants* **2022**, *11*, 1872. [CrossRef] [PubMed]
28. Yang, F.; Jiang, H.; Chang, G.; Liang, S.; Ma, K.; Cai, Y.; Tian, B.; Shi, X. Effects of rhizosphere microbial communities on cucumber fusarium wilt disease suppression. *Microorganisms* **2023**, *11*, 1576. [CrossRef]
29. Wang, Z.; Zhang, H.; Liu, L. Screening of phosphate-solubilizing bacteria and their abilities of phosphorus solubilization and wheat growth promotion. *BMC Microbiol.* **2022**, *22*, 296. [CrossRef]
30. Lei, H.; Jin, C.; Xiao, Z. Relationship between pepper (*Capsicum annuum* L.) root morphology, inter-root soil bacterial community structure and diversity under water–air intercropping conditions. *Planta* **2023**, *257*, 98. [CrossRef]
31. Kitahara, R.; Ikeda, Y.; Shimura, H. A unique mitovirus from Glomeromycota, the phylum of *Arbuscular mycorrhizal* fungi. *Arch. Virol.* **2014**, *159*, 2157–2160. [CrossRef]

32. Li, Y.; Nan, Z.; Matthew, C.; Wang, Y.; Duan, T. Arbuscular mycorrhizal fungus changes alfalfa (*Medicago sativa*) metabolites in response to leaf spot (*Phoma medicaginis*) infection, with subsequent effects on pea aphid (*Acyrthosiphon pisum*) behavior. *New Phytol.* **2023**, *239*, 286–300. [CrossRef] [PubMed]
33. Tello, A.; Jiménez, M.; Sánchez, G. Management of sewage sludge by composting using fermented water hyacinth. *Environ. Sci Pollut. Res. Int.* **2015**, *22*, 14781–14792. [CrossRef] [PubMed]
34. Foresto, E.; Carezzano, M.; Giordano, W.; Bogino, P. Ascochyta blight in chickpea: An update. *J. Fungi* **2023**, *49*, 203. [CrossRef] [PubMed]
35. Fang, C.; Li, Y.; Li, C.; Li, B.; Ren, Y.; Zheng, H. Identification and comparative analysis of microRNAs in barnyardgrass in response to rice allelopathy. *Plant Cell Environ.* **2015**, *38*, 1368–1381. [CrossRef]
36. Fu, Y.; Ma, K.; Li, Q.; Li, G.; Cui, H. Effects of potato intercropped with maize on soil bacterial diversity. *Chin. J. Eco-Agric.* **2020**, *28*, 1715–1725. [CrossRef]
37. Li, P.; Zhu, Z.; Zhang, Y. The phyllosphere microbiome shifts toward combating melanose pathogen. *Microbiome* **2022**, *10*, 56. [CrossRef]
38. Ham, S.; Yoon, A.R.; Oh, H.; Park, Y. Plant growth-promoting microorganism *Pseudarthrobacter* sp. NIBRBAC000502770 enhances the growth and flavonoid content of *Geum aleppicum*. *Microorganisms* **2022**, *10*, 1241. [CrossRef]
39. Mary, L. The phylogeny of plant and animal pathogens in the Ascomycota. *Physiol. Mol. Plant P* **2001**, *59*, 165–187. [CrossRef]
40. Maharachchikumbura, S.; Chen, Y.; Ariyawansa, H. Integrative approaches for species delimitation in Ascomycota. *Fungal Divers.* **2021**, *109*, 155–179. [CrossRef]
41. Almario, J.; Fabiańska, I.; Saridis, G.; Bucher, M. Unearthing the plant-microbe quid pro quo in root associations with beneficial fungi. *New Phytol.* **2022**, *234*, 1967–1976. [CrossRef]
42. Shelobolina, E.; Roden, E.; Benzine, J.; Xiong, M.Y. Using phyllosilicate-Fe (II)-oxidizing soil bacteria to improve Fe and K plant nutrition. *U. S. Pat. Appl.* **2014**, *14*, 509.
43. Bai, Y.; Feng, P.; Chen, W.; Xu, S.; Liang, J.; Jia, J. Effect of three microbial fertilizer carriers on water infiltration and evaporation, microbial community and alfalfa growth in saline-alkaline soil. *Commun. Soil Sci. Plan.* **2021**, *52*, 2462–2470. [CrossRef]
44. Ning, Q.; Chen, L.; Li, F. Effects of mortierella on soil nutrient availability and straw degradation. *Acta Pedol. Sin.* **2022**, *59*, 206–217.
45. Withee, P.; Haituk, S.; Senwanna, C.; Karunarathna, A.; Tamakaew, N.; Pakdeeniti, P.; Suwannarach, N.; Kumla, J.; Suttiprapan, P.; Taylor, P. Identification and pathogenicity of *Paramyrothecium* species associated with leaf spot disease in northern Thailand. *Plants* **2022**, *11*, 1445. [CrossRef]
46. Bingol, E.; Qi, A.; Karandeni-Dewage, C.; Ritchie, F.; Fitt, B.; Huang, Y. Co-inoculation timing affects the interspecific interactions between phoma stem canker pathogens *Leptosphaeria maculans* and *Leptosphaeria biglobosa*. *Pest Manag. Sci.* **2024**, *80*, 2443–2452. [CrossRef]
47. Yang, J.; Han, J.; Jing, Y.; Li, S.; Lan, B.; Zhang, Q.; Yin, K. Virulent fusarium isolates with diverse morphologies show similar invasion and colonization strategies in alfalfa. *Front. Plant Sci.* **2024**, *15*, 1390069. [CrossRef]
48. Punja, Z.; Chandanie, W.; Chen, X.; Rodríguez, G. Phoma leaf spot of wasabi (*Wasabia japonica*) caused by *Leptosphaeria biglobosa*. *Plant Pathol.* **2016**, *66*, 480–489. [CrossRef]
49. Guo, Y.; Chen, B.; Zhang, S. Research progress of alfalfa root disease and its control. *J. Henan Agric. Univ.* **2022**, *56*, 914–923. [CrossRef]
50. Marek, K.; Wielbo, J.; Pawlik, A.; Skorupska, A. Nodulation competitiveness of *Ensifer meliloti* alfalfa nodule isolates and their potential for application as inoculants. *Pol. J. Microbiol.* **2014**, *63*, 375–386. [CrossRef]
51. Debnath, S.; Das, A.; Maheshwari, D.; Pandey, P. Treatment with *Atypical rhizobia*, *Pararhizobium giardinii* and *Ochrobactrum* sp. modulate the rhizospheric bacterial community, and enhances Lens culinaris growth in fallow-soil. *Microbiol. Res.* **2023**, *267*, 127255. [CrossRef]
52. Luo, L.; Zhao, C.; Wang, E.; Raza, A.; Yin, C. *Bacillus amyloliquefaciens* as an excellent agent for biofertilizer and biocontrol in agriculture: An overview for its mechanisms. *Microbiol. Res.* **2022**, *259*, 127016. [CrossRef] [PubMed]
53. Galili, G.; Amir, R.; Fernie, A. The Regulation of essential amino acid synthesis and accumulation in plants. *Annu. Rev. Plant Biol.* **2016**, *67*, 153–178. [CrossRef] [PubMed]
54. Yang, Q.; Zhao, D.; Liu, Q. Connections between amino acid metabolisms in plants: Lysine as an example. *Front. Plant Sci.* **2020**, *11*, 928. [CrossRef]
55. Chang, J.; Tabima, J. A covert operation by a plant pathogen. *Cell Host Microbe* **2016**, *20*, 413–415. [CrossRef]
56. Das, D.; Paries, M.; Hobecker, K. Phosphate starvation response transcription factors enable arbuscular mycorrhiza symbiosis. *Nat. Commun.* **2022**, *13*, 477. [CrossRef]
57. Paula, E.J. Zeatin: The 60th anniversary of its identification. *Plant Physiol.* **2023**, *192*, 34–55. [CrossRef]
58. Liu, S.; García, P.; Tedersoo, L. Phylotype diversity within soil fungal functional groups drives ecosystem stability. *Nat. Ecol. Evol.* **2022**, *6*, 900–909. [CrossRef]
59. Ferraro, V.; Venturella, G.; Cirlincione, F.; Mirabile, G.; Gargano, M.L.; Colasuonno, P. The checklist of sicilian macrofungi: Second edition. *J. Fungi* **2022**, *8*, 566. [CrossRef]

60. Gil, M.M.; López, G.; Domínguez, M.T.; Navarro, F.; Carmen, M.; Kjøller, R.; Tibbett, M.; Marañón, T. Ectomycorrhizal fungal communities and their functional traits mediate plant–soil interactions in trace element contaminated soils. *Front. Plant Sci.* **2018**, *9*, 1682. [CrossRef]
61. Wu, Y.; Chen, J. The potassium dichromate volumetric method for the determination of soil organic matter-heating method. *China High Technol. Enterp.* **2016**, *26*, 11–12.
62. Zhang, W.; Fu, Y.; Li, J. Comparative study on Kjeldahl method and Dumas combustion method for total Nitrogen measurement in soil. *Chin. Agric. Sci. Bull.* **2015**, *31*, 172–175.
63. Wang, Q.; Xu, Q.; Yao, Z. Determination of available phosphorus in soil. *Instrum. Anal. Monit.* **2009**, *4*, 36–38.
64. Dai, Z.; Li, M. Study on determination methods for available potassium in upland field. *Acta Pedol. Sin.* **1997**, *34*, 336–343.
65. Gu, X.; Zhang, F.; Xie, X. Effects of N and P addition on nutrient and stoichiometry of rhizosphere and non-rhizosphere soils of alfalfa in alkaline soil. *Sci. Rep.* **2023**, *13*, 12119. [CrossRef] [PubMed]
66. Sun, D.; Jiang, X.; Wu, Q.; Zhou, N. Intragenomic heterogeneity of 16S rRNA genes causes overestimation of prokaryotic diversity. *Appl. Environ. Microbiol.* **2013**, *79*, 5962–5969. [CrossRef]
67. Rousk, J.; Baath, E.; Brookes, P.C. Soil bacterial and fungal communities across a pH gradient in an arable soil. *ISME J.* **2010**, *4*, 1340–1351. [CrossRef]
68. AOAC. *Official Methods of Analysis*, 14th ed.; Association of Official Analytical Chemists: Washington, DC, USA, 1995. Available online: http://lib3.dss.go.th/fulltext/scan_ebook/aoac_1995_v78_n3.pdf (accessed on 18 August 2024).
69. Yang, Z.; Chen, Q.; Ai, D. Determination of crude fiber content in alfalfa by filter bag method. *Hubei Agric. Sci.* **2017**, *56*, 1739–1741. [CrossRef]
70. Soest, P.; Robertson, J.; Lewis, B. Methods of dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* **1991**, *74*, 3583–3597. [CrossRef]
71. Li, T.; Peng, L.; Wang, H.; Zhang, Y.; Wang, Y.; Cheng, Y.; Hou, F. Multi-cutting improves forage yield and nutritional value and maintains the soil nutrient balance in a rainfed agroecosystem. *Front. Plant Sci.* **2022**, *13*, 825117. [CrossRef]
72. Reyon, D.; Tsai, S.Q.; Khayter, C. FLASH assembly of TALENs for high-throughput genome editing. *Nat. Biotechnol.* **2012**, *30*, 460–465. [CrossRef]
73. Caporaso, J.; Kuczynski, J.; Stombaugh, J. QIIME allows analysis of high-throughput community sequencing data. *Nat. Met-Hods* **2010**, *7*, 335–336. [CrossRef] [PubMed]
74. Edgar, R.C. UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* **2013**, *10*, 996–998. [CrossRef] [PubMed]
75. Quast, C.; Pruesse, E.; Yilmaz, P.; Gerken, J.; Schweer, T.; Yarza, P. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* **2012**, *41*, 590–596. [CrossRef] [PubMed]
76. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2021.
77. Douglas, G.; Maffei, V.; Zaneveld, J. PICRUSt2 for prediction of metagenome functions. *Nat. Biotechnol.* **2020**, *38*, 685–688. [CrossRef]
78. Nguyen, N.; Song, Z.; Bates, S.; Branco, S.; Tedersoo, L.; Menke, J. FUNGuild: An open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecol.* **2016**, *20*, 241–248. [CrossRef]
79. Kruskal, W.; Wallis, W. Use of ranks in one-criterion variance analysis. *J. Am. Stat. Assoc.* **1952**, *47*, 583–621. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.