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Responses of Rare and Abundant Bacterial Communities to Synergistic Phosphate Fertilization and *Trichoderma* Inoculation Meant to Improve Alfalfa Yields

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Abstract: In the field of agro-grassland, the synergism of phosphate (P) fertilization and *Trichoderma* inoculation in alfalfa production and the underlying mechanism of rare and abundant microbes that regulate rhizosphere soil processes in various environments are key but rarely studied topics. Here, we conducted field research through the inoculation/noninoculation of *Trichoderma* and five levels of phosphorus fertilizer to explore the biological relationships of rhizosphere soil properties, rare and abundant taxa, and alfalfa yields. Our results demonstrate that using *Trichoderma* inoculation and 15 g/m^2 of phosphorus fertilizer exhibited optimal alfalfa yield compared with other treatments. alfalfa yields significantly (R^2 = 0.33; $p < 0.001$) increased along with soil fertility. Both rare (R_{ANOSIM} = 0.900; p = 0.001) and abundant (R_{ANOSIM} = 0.769; p = 0.001) bacterial communities were significantly different under *Trichoderma* inoculation and P fertilization in a nonmetric multidimensional scaling (NMDS) analysis. Furthermore, different ecological processes dominated the rare and abundant bacterial community assembly. PLS-PM analysis showed that *Trichoderma* inoculation positively regulated the abundant bacteria community and P fertilization regimes manipulated the rare bacteria community, synergistically contributing to alfalfa yields. Overall, this article believes that inoculation with *Trichoderma* and appropriate application of phosphorus fertilizer can significantly increase alfalfa yield and affect soil enzyme activity, and the rhizosphere soil abundant and rare bacterial community characteristics have different responses to the synergistic effect of *Trichoderma* and phosphorus fertilizer. Our research emphasizes the fundamental role of abundant and rare microbes in maintaining crop production using *Trichoderma* inoculation and P fertilization. Therefore, distinguishing rare and abundant species is beneficial to comprehensively understanding microbial-driven processes and providing theoretical support for maintaining ecosystem productivity.

Keywords: *Trichoderma*; phosphate fertilization; alfalfa; abundant and rare bacterial communities; PLS-PM

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1. Introduction

Alfalfa (*Medicago sativa* L.) is an important perennial legume forage that possesses significant nutritional quality and plant productivity [1]. Alfalfa is widely cultivated around the world [2], with a wide range of adaptability to a variety of environmental conditions [3]. Alfalfa development conditions, especially in its establishment year, determine its winter survival and yield potential [4]. Evidence suggests that agronomic practices significantly contribute to alfalfa production [5,6]. Fertilization is an essential approach to improving nutrient absorption and guarantees alfalfa hay production [7]. As phosphorus (P) is involved in plant growth and physiology, and alfalfa is a high P-demand crop, soil with P deficiency

commonly threatens crop productivity [8]. Previous studies have shown that using P fertilizers can help alfalfa grow, regenerate, and achieve high yields [9,10]. Nevertheless, in real production, using phosphate fertilizer usually exceeds plant demands, resulting in the accumulation of soil phosphorus elements, which not only increases manufacturing costs but also leads to eutrophication and other serious environmental problems [11,12]. Therefore, it is necessary to explore suitable phosphorus application rates to improve its use, contributing to the sustainable development of agro-grasslands.

Good communication between plants and beneficial microorganisms in soil can significantly improve the performance of plants in different habitats [13]. *Trichoderma* spp. can improve nutrient utilization efficiency and plant productivity, indicating their important role in element turnover and bioavailability [14]. Bononi et al. [15] investigated the Amazon biome and found that isolated *Trichoderma* strains could produce enough organic acid to solubilize phosphate, showing positive responses in soybean growth promotion. Using such phosphate-solubilizing microbes to help plants absorb phosphorus is a feasibly sustainable strategy for managing phosphorus deficiency. Moreover, many studies have also revealed that *Trichoderma* strains can significantly improve plant biomass by regulating soil microbial taxa [16]. For instance, a previous study found that *Trichoderma* bioorganic fertilizer reduces the negative effects of monoculture in agricultural systems, such as decreased diversity of soil bacterial communities, and contributes to the growth and health of tomato plants [17]. In our earlier study [18], we explored the actual impact of *Trichoderma* fertilization on the growth of *Leymus chinensis* based on field experiments and observed that there was a close relationship between biomass changes and soil microbiomes. However, many reports have been concerned with the association between overall soil microflora and productivity.

Soil microbes hold an important role in ecosystem functions and sustainable stability [19]. Abundant taxa are generally considered to be an active and important part of the biogeochemical cycle and largely contribute to soil productivity [20], and it has traditionally attracted more attention, while rare taxa have commonly been ignored. Recently, many studies have emphasized that rare species may also play a fundamental role in maintaining ecosystem stability [21,22]. Additionally, the diversity and community composition of abundant and rare taxa respond diversely to external disturbances [23]. For example, prior studies have assessed the influences of vegetation restoration on sandy soil bacterial assemblage patterns and observed that rare soil bacteria respond more strongly to desertification restoration than abundant bacteria [24]. Secondly, the differences in the assembly processes of rare and abundant microbial communities can also lead to functional differences [25,26]. Nevertheless, in agro-grassland fields, it is still unclear how the synergistic effects of various P-fertilization regimes and beneficial microbe inoculation affect the community assembly and function of abundant and rare taxa, as well as their potential associations with the aboveground plants.

We synergistically used five levels of phosphorus fertilization on *Trichoderma* (inoculat ion/non-inoculation) for 10 treatments to survey biological connections between rhizosphere soil properties, rare and abundant communities, and alfalfa yield. To answer the following questions: (1) Which agronomic practices are optimal for yielding maximum alfalfa yields? (2) How do *Trichoderma* and P fertilization regulate the community characteristics of abundant and rare taxa, thereby contributing to alfalfa yield? Research has shown that excessive application of phosphorus fertilizer can have a negative impact on plant yield [4], but *Trichoderma* bioorganic fertilizer can significantly promote plant growth [18]. In addition, considering that abundant and rare taxa have different responses to external interference [23]. Therefore, we assume that: (1) The alfalfa yield has significant advantages under the conditions of *Trichoderma* inoculation and appropriate addition of phosphorus fertilizer (P2 or P3); (2) Abundant and rare taxa have different responses to *Trichoderma* inoculation and phosphorus fertilizer application, thereby affecting alfalfa yield.

2. Materials and Methods

2.1. Alfalfa Seeds, Trichoderma, and P Fertilizers

Alfalfa seeds (*Medicago sativa* L. cv. Aurora) were supplied by Zhengzhou Kaiyuan Grass Industry Technology Co., Ltd., Zhengzhou, China, Alfalfa seeds have a germination rate of 95%, and an autumn dormancy level of 8, with many branching points, good persistence, large leaf volume, and strong recovery ability. As previously described, the seed surfaces were sterilized [27]. Bioworks, Inc., Victor, NY, USA supplied the required *Trichoderma harzianum* T-22 ($\geq 10^9$ cfu/g) for this experiment. Some 50 g of *Trichoderma* powder was thoroughly mixed with alfalfa seeds and applied to the experimental plot. Phosphorus (P) fertilizers (Calcium superphosphate) were provided by Nanjing Duoliduo Technology Co., Ltd., Nanjing, China, with $P_2O_5 \geq 12\%$.

2.2. Experimental Design

The experimental site of this study is located at the Baima Research Station of Nanjing Agricultural University (31°36′46″ N 119°8′42″ E). The area belongs to a subtropical monsoon climate, and the annual mean temperature and mean precipitation at the experimental site are 15 °C and 1000–1200 mm, respectively. The site was plowed and leveled before the experiment. The soil type is Anthrosols (http://www.fao.org, accessed on 17 June 2024). The basic soil physicochemical properties are pH 7.31, Alkaline-N 50.1 mg/kg, Olsen-P 9.23 mg/kg, SOM 9.09 mg/kg, and available K 83.6 mg/kg. The granulometric composition of the soil is >0.25 mm 17.61%, 0.05–0.25 mm 43.26%, and <0.05 mm 39.13%, respectively.

This experiment was a 2 × 5 factorial design (completely random) with phosphorus fertilizer addition and *Trichoderma* inoculation arranged. The experiment consisted of two *Trichoderma* inoculation treatments (non-*Trichoderma* control and *Trichoderma* inoculation, named −M and +M, respectively) and five phosphorus fertilizer levels (0, 5, 10, 15, and 20 g/m^2, named P0, P1, P2, P3, and P4, respectively). Among them, the actual application amounts corresponding to the five phosphorus fertilizer levels are 0, 1.25, 2.5, 3.75, and 5 kg/plot. The size of the experimental plot was 5 m × 6 m. Each treatment was repeated thrice. For inoculation treatments, *Medicago sativa* seeds were inoculated with *Trichoderma* before sowing. The experiment began on 15 October 2021, and the alfalfa grew until May 2022, when it was harvested. The planting depth for the alfalfa seeds was 2.5 cm, and planting line spacing was 30 cm, with a density of 5 g/m^2. The phosphorus fertilizers were distributed evenly in each plot, adopting the same agricultural irrigation and weeding measures.

2.3. Alfalfa and Rhizosphere Soil Sample Collection

We concurrently collected soil and plant samples from 10 different treatments. Randomly destructive sampling was conducted on three subplots (1 m × 0.25 m) within each experimental plot to generate representative composite samples. We left 5 cm of aboveground plants for harvesting and dried them for 72 h (65 °C) before weighing them. At the same time, we collected rhizosphere soil, as described in a previous study [28]. A portion of the collected soil samples was stored at 4 °C to determine soil enzyme activity, and other parts were aired-dried for soil physicochemical property analysis. Additionally, the remaining soil was stored at −80 °C to extract total soil DNA.

2.4. Rhizosphere Soil Properties Determination

Soil pH was measured using a pH meter (PB-10, Sartorius, Göttingen, Germany) with soil–water slurry (1:5, w/v). We used a B-173 conductivity meter (HORIBA, Kyoto, Japan) to measure soil electrical conductivity (EC). Soil organic carbon (SOC) was assayed using a carbon and nitrogen analyzer (Multi N/C 2100, Alytik, Jena, Germany). Alkali-hydrolyzed nitrogen (AN), available potassium (AK), and Olsen-P were measured based on Bao et al. [29]. Soil alkaline phosphomonoesterase and saccharase activities were measured by Wang et al. [30] and Wei et al. [31], respectively. Soil alkaline phosphatase activity was measured by p-nitrophenol colorimetry using p-nitrophenyl phosphate as substrate [32].

Determination of invertase activity using the 3,5-dinitrosalicylic acid colorimetric method using sucrose as substrate [33].

2.5. Rhizosphere Soil DNA Extraction, PCR Amplification, and Sequencing Data Processing

We extracted total DNA (0.25 g of fresh soil) using FastDNA SPIN Kit (MP Biomedicals, Santa Ana, CA, USA), referring to the manufacturer's directions. We used the Nanodrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) to assess the concentration and purity of genomic DNA (A260/A280 ratio). We amplified the V4-V5 region of the 16S rRNA gene by employing the 515F/907R primers [34]. The PCR amplification system (25-μL) involved the following: 2 μL of 2.5 mM dNTPs, 5 × FastPfu Buffer (5 μL), both forward and reverse primers are 1 μL 5 μM, 10 ng DNA template, FastPfu polymerase (0.4 μL), and the addition of ddH_2O to 25 μL. The PCR amplification involved the following parameters 3 min at 95 °C, 15 s at 95 °C (30 cycles, denaturation), 30 s for annealing at 55 °C, 45 s at 72 °C elongation, and 72 °C for 10 min (extension). We constructed a library of purified PCR amplification products and sequenced them in paired-end (2 × 250) on the Illumina HiSeq platform (Illumina, Foster City, CA, USA) based on the standard protocols of Genesky Biotechnologies Co., Ltd., Shanghai, China.

2.6. Bioinformatic Analysis

Raw reads were processed based on QIIME2, using the cutadapt plugin to trim adapters and primer sequences and the DADA2 plugin to control and detect the quality of amplified subsequence variants (ASVs) [35]. RDP classifier (version 11.5) was used for bacterial sequence classification and representative bacterial sequences were annotated using the SILVA128 database with a confidence threshold of 0.7. Raw sequence data can be obtained from the NCBI with the registration number PRJNA907136.

We conducted leveling according to 21,420 sequences (minimum number) and subsequent analysis, which were clustered into 18,425 ASVs. ASVs with mean relative abundance >0.1% and <0.01% in all samples were viewed as "abundant" taxa and "rare" taxa, respectively [22,36].

2.7. Statistical Analysis

Data were log-transformed where appropriate to meet normality criteria. We employed the SPSS 22.0 (IBM, Armonk, NY, USA) software for statistical analyses. We used a two-way analysis of variance (ANOVA) for data processing, and the multiple comparisons method was applied based on Tukey HSD tests ($p < 0.05$). The difference between the two groups was tested using the independent sample *t*-test. We selected the appropriate function curve and inflection point to identify the value of each parameter based on the data characteristics. Rhizosphere soil pH, EC, SOM, AK, AN, and AP were chosen as soil fertility assessment parameters. We calculated the correlation coefficients according to Spearman analysis and determined the weights for each parameter (Table S1). Then we calculated the rhizosphere soil fertility index with reference to previous studies [37].

We calculated multiple alpha-diversity indices in the "vegan" R package and estimated bacterial community compositions in different treatments according to NMDS and analysis of similarities (ANOSIM). We estimated beta diversity based on the Bray–Curtis distance between samples. We used the method of inferring community assembly mechanisms by the phylogenetic-bin-based null model (iCAMP) analysis to evaluate the assembly process of bacterial communities, which was coupled with the beta net relevance index (betaNRI) and the amended Raup-Crick metric (RC) [38]. This method is based on considering selection and other ecological processes at the level of individual taxonomic groups/lineages. $BetaNRI_k$ values > +1.96 were regarded as being dominated by heterogeneous selection, while beta NRI_k values < −1.96 were regarded as the main contributions of homogeneous selection. For $|betaNRI_k|$ values ≤ 1.96 and RC_k values < −0.95 or > +0.95, homogenizing dispersal or dispersal limitation were considered as dominating the community assembly. However, when $|betaNRI_k|$ was ≤1.96 and $|RC_k|$ was ≤0.95, "undominant" or "drift"

dominated the community assembly. Moreover, we extracted PCoA1 and PCoA2 axes according to Bao [39]. Community composition (or β diversity) measures the rate of change in species composition at a regional scale along a certain gradient direction from one community to another, with a focus on exploring changes at the taxonomic level. However, the production and maintenance of microbial diversity and function are known as community assembly processes, which reflect the combination of spatiotemporal processes that determine community composition, with a focus on exploring changes at the phylogenetics level. Complex multivariate relationships between rhizosphere soil fertility index, enzyme activity, bacterial diversity, community composition, and alfalfa yields were identified through partial least squares path modeling (PLS-PM) [40,41]. This is a particularly useful statistical method for proving the causal relationship between measured variables and latent variables. This method is similar to structural equation modeling but uses the partial least squares method instead of the maximum likelihood method as the evaluation criterion. It allows for obtaining robust explanatory models without any variables or error distribution assumptions. According to the coefficient of determination (R^2) and goodness of fit (GoF) of the potential variables explained, a suitable framework was selected and evaluated with the "plspm" (1000 bootstraps) R package [42]. The above analysis was based on R software (v.4.1.0).

3. Results

3.1. Comparison of Alfalfa Yield and Rhizosphere Soil Fertility in Various P Fertilization and Trichoderma Treatments

Two-way ANOVA showed that there were significant ($F = 65.17$, $p < 0.001$) interaction effects on alfalfa yields between the P fertilizer and *Trichoderma* treatments (Table 1). The largest alfalfa yield was observed in the +M+P3 treatment (6.15 t/hm^2). We observed a substantial difference in yields between +M+P3 and −M+P3 (4.20 t/hm^2). Specifically, in −M, the yields of P0 (CK, 2.56 t/hm^2) were significantly ($p < 0.05$) smaller than those of the P1-P4 treatments. Although the yields of P4 (4.05 t/hm^2) were not significantly higher than P2 (3.94 t/hm^2), they were significantly higher than those of P1 (2.93 t/hm^2). In +M, compared with other phosphate fertilizer treatments, P3 had significant advantages regarding alfalfa yields. Nevertheless, we observed no substantial difference in alfalfa yields between P2 (4.20 t/hm^2) and P4 (3.92 t/hm^2), but they were significantly higher than those of P0 (2.68 t/hm^2) and P1 (3.54 t/hm^2). The alfalfa yields of P1 were only more advantageous than those of the P0 treatment (Table 1). Then, there is a significant interaction between phosphatase and invertase under the conditions of *Trichoderma* inoculation ($F = 4.8$, $p < 0.01$) and phosphorus fertilizer application ($F = 5.5$, $p < 0.01$) (Table 1). The P fertilization and *Trichoderma* treatments generated largely different physicochemical properties (Table S2). Additionally, least squares regression analysis revealed that alfalfa yields increase with the rhizosphere soil fertility index ($R^2 = 0.33$, $p < 0.001$) (Figure 1).

Table 1. Variations in alfalfa yields and rhizosphere soil enzyme activity in diverse P fertilization and *Trichoderma* treatments.

Trichoderma	P-Fertilizer	Yield	Phosphatase	Invertase
−M	P0	2.56 (0.02) e	112.17 (2.52) ab	0.98 (0.04) abcd
	P1	2.93 (0.06) d	196.27 (51.48) ab	0.91 (0.12) abcd
	P2	3.94 (0.12) b	92.45 (27.39) b	1.01 (0.03) abc
	P3	4.2 (0.09) b	169.99 (23.68) ab	0.74 (0.04) cd
	P4	4.05 (0.08) b	143.33 (29.4) ab	0.83 (0.02) bcd
+M	P0	2.68 (0.04) de	178.21 (38.48) ab	0.8 (0.09) cd

Table 1. *Cont.*

Trichoderma	P-Fertilizer	Yield	Phosphatase	Invertase
	P1	3.54 (0.01) c	138.34 (22.71) ab	1.11 (0.16) ab
	P2	4.2 (0.09) b	211.34 (29.37) a	0.69 (0.12) d
	P3	6.15 (0.04) a	128.8 (15.78) ab	1.17 (0.1) a
	P4	3.92 (0.07) b	152.71 (42.39) ab	1.01 (0.11) abc
Two-way analysis of variance				
T		361.38 ***	0.16 ^{ns}	0.93 ^{ns}
P		153.25 ***	0.93 ^{ns}	1.13 ^{ns}
T × P		65.17 ***	4.8 **	5.5 **

Notes: P0, P1, P2, P3, and P4 represent different phosphate fertilizer levels (0, 5, 10, 15, 20 g/m^2), respectively; +M and −M represent whether there is *Trichoderma* inoculated in the treatment. T represents the main effect of *Trichoderma* inoculation, P represents the main effect of phosphate fertilizer levels, and T × P represents the interaction effect between the two treatments. ^{ns} represents no significance, ** $p < 0.01$, *** $p < 0.001$. Besides, different lowercase letters (a, b, c, d, e) indicate significant differences among different treatments ($p < 0.05$, multiple comparisons with Tukey HSD test). The values in the table represent the average (standard error).

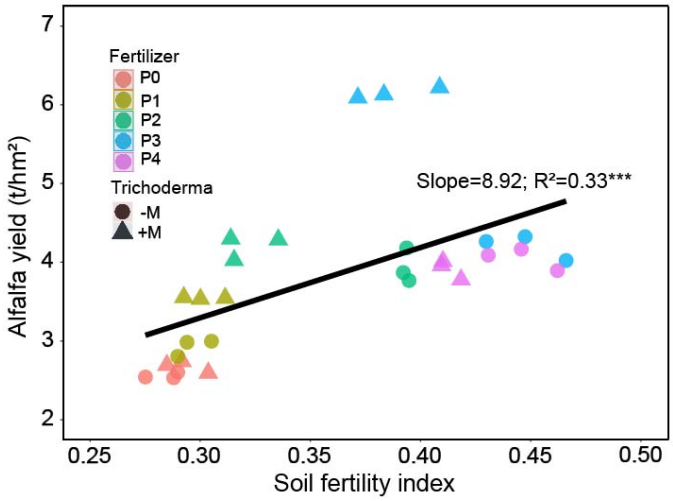


Figure 1. The relationship between alfalfa yields and the rhizosphere soil fertility index according to least-squares regression analysis. P0, P1, P2, P3, and P4 represent different phosphate fertilizer levels (0, 5, 10, 15, and 20 g/m^2, respectively); +M and −M indicate whether *Trichoderma* is inoculated in the treatment. ***, $p < 0.001$.

3.2. Abundant and Rare Bacterial Alpha Diversity and Community Composition Response to Different P Fertilization and Trichoderma Treatments

Abundant bacterial communities identified nine bacterial phyla, with dominant phyla including Proteobacteria, Acidobacteria, Chloroflexi, and Actinobacteria, etc. The total relative abundance of the four dominant phyla under all treatments was greater than 85% (Figure 2a). A total of 27 bacterial phyla were identified as rare bacterial communities, with dominant phyla, including Proteobacteria, Bacteroidetes, Acidobacteria, Actinobacteria, and Chloroflexi. The total relative abundance of the five dominant phyla under all treatments was greater than 68% (Figure 2b).

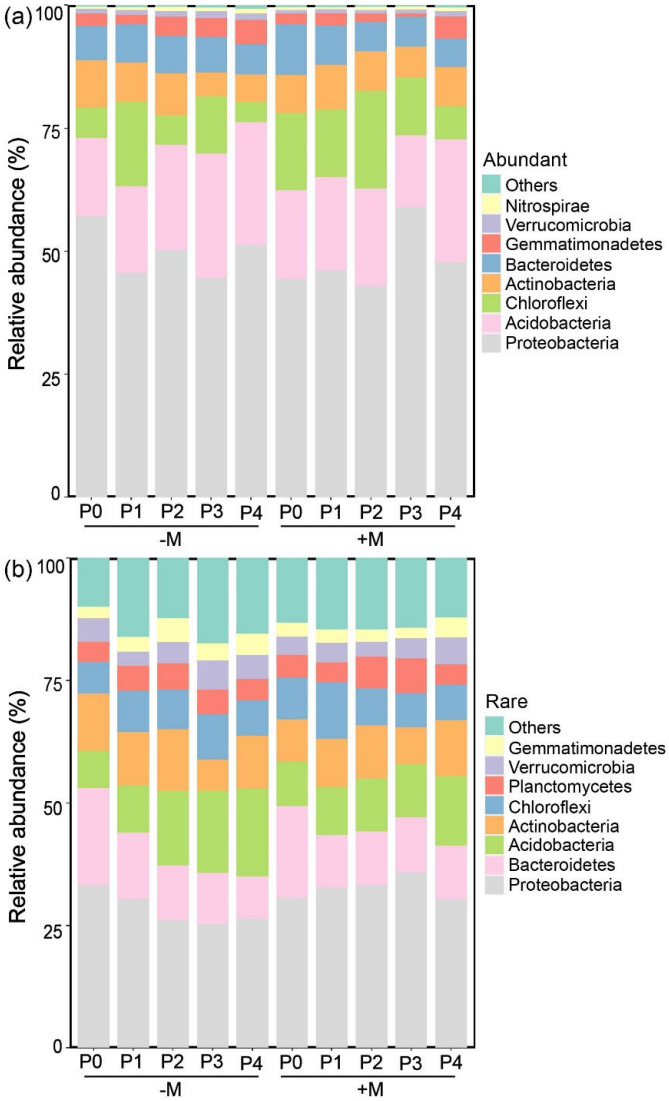


Figure 2. Taxonomic composition of abundant (**a**) and rare (**b**) bacterial communities at phylum levels under different P fertilization and *Trichoderma* treatments. P0, P1, P2, P3, and P4 represent different phosphate fertilizer levels (0, 5, 10, 15, and 20 g/m^2, respectively); +M and −M indicate whether *Trichoderma* is inoculated in the treatment.

The alpha diversity of the rare and abundant bacterial species was significantly ($p < 0.05$) different under the P fertilization and *Trichoderma* treatments (Table S3, Figure S1). NMDS analysis and the ANOSIM test showed that rare (R = 0.900, $p = 0.001$) and abundant (R = 0.769, $p = 0.001$) bacterial community structures exhibited significant differences under various P fertilization and *Trichoderma* treatments (Figure 3a,b). The β diversity (Bray–Curtis distance) of rare bacterial communities is higher than that of abundant taxa under the P fertilization and *Trichoderma* treatments (Figure S2).

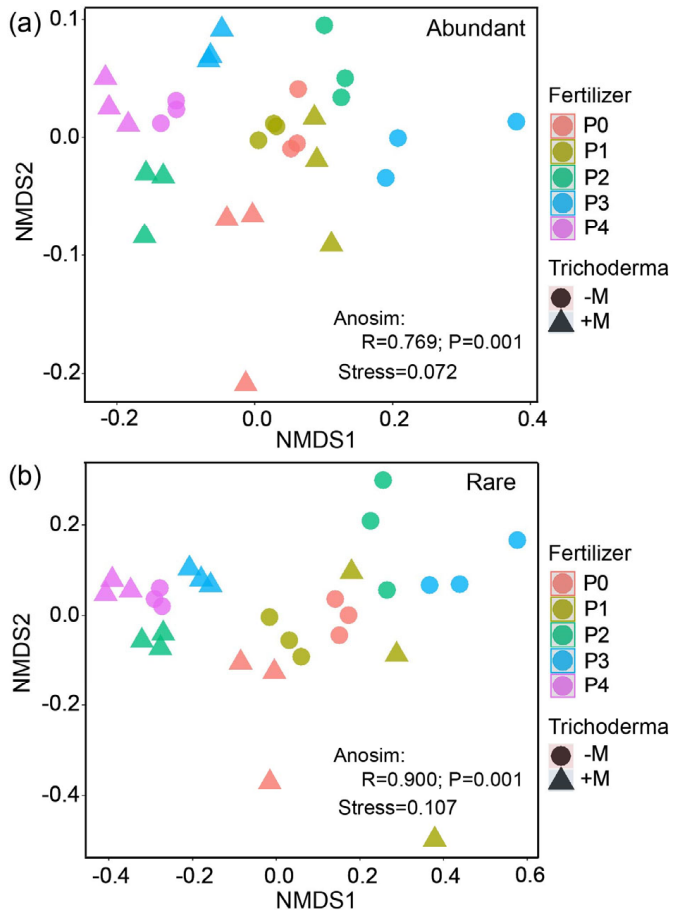


Figure 3. Nonmetric multidimensional scaling (NMDS) indicated rhizosphere soil abundant (**a**) and rare (**b**) bacterial community compositions in different P fertilization and *Trichoderma* treatments (ANOSIM test). P0, P1, P2, P3, and P4 represent different phosphate fertilizer levels (0, 5, 10, 15, and 20 g/m^2, respectively); +M and −M indicate whether *Trichoderma* is inoculated in the treatment.

3.3. Rare and Abundant Bacterial Community Assembly Processes in Different P Fertilization and Trichoderma Treatments

We used iCAMP to assume community assembly processes and observed that dispersal limitation (DL) was the critical process driving the abundant bacterial community (Figure 4a) in −M+P0 (47.72%) and +M+P3 (50.53%), while drift (DR) accounted for a relatively high proportion in other treatments. In addition, homogenizing dispersal (HoS) dominated in −M+P1 (28.96%), −M+P3 (21.18%), −M+P4 (6.19%), +M+P0 (28.60%), +M+P1 (26.09%) and +M+P2 (31.21%). The lowest proportions belonged to heterogeneous selection (HeS) and homogenizing dispersal (HD) for all treatments. For rare bacterial communities (Figure 4b), DR processes took up a majority of the proportion (>66%) in all treatments, followed by DL and HoS, but HD and HeS possessed small proportions. Furthermore, we found different proportions of rare and abundant taxa in the same treatments (Figure S3).

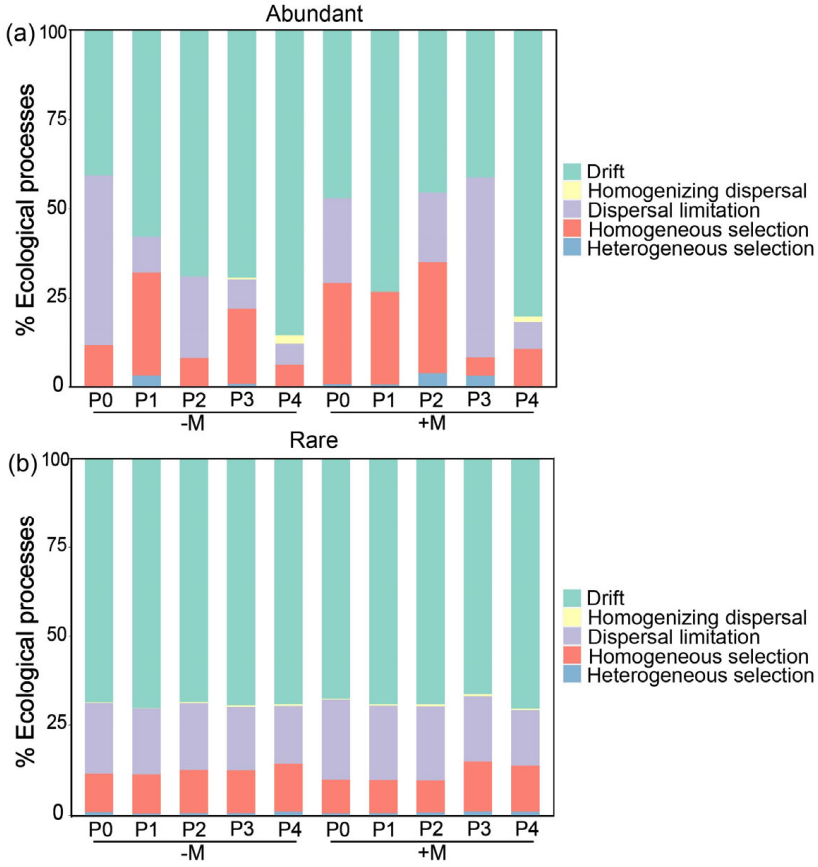


Figure 4. Ecological processes of rare and abundant rhizosphere soil abundant (**a**) and rare (**b**) bacterial communities under different P fertilization and *Trichoderma* treatments. P0, P1, P2, P3, and P4 represent different phosphate fertilizer levels (0, 5, 10, 15, and 20 g/m^2, respectively); +M and −M indicate whether *Trichoderma* is inoculated in the treatment.

3.4. Associations between Rhizosphere Soil Properties and Diversity of Rare and Abundant Bacterial Communities

We used correlation and best multiple regression modeling to assess the biological associations between bacterial diversity and soil properties under different P fertilization and *Trichoderma* treatments (Figure 5). Available phosphorus (AP) can best explain the changes in the diversity of the rare and abundant bacterial communities. Spearman correlation analysis demonstrated that EC and AP were positively connected with abundant bacterial community diversity, while soil pH had the opposite trend. In addition, the interpretation degree of soil properties to changes in NMDS1 was higher than that of NMDS2 (Figure 5).

3.5. Ecological Relationships between Rhizosphere Soil Fertility, Enzyme Activity, Bacterial Diversity, Community Assembly, and Alfalfa Yield

PLS-PM analysis was used to determine the effects (direct and indirect) of the soil fertility index, enzyme activity, microbial assembly, and microbial diversity on alfalfa yields under different P fertilization and *Trichoderma* treatments (Figure 6a). The fertilizers positively affected soil fertility (0.90 of the direct effects) and influenced alfalfa yields (0.56). Furthermore, soil fertility affected rare bacterial community assembly (0.52) and positively influenced rare bacterial community composition (0.81) and alfalfa yields (0.43). In addition, soil fertility positively affected abundant bacterial community assembly (0.47) but negatively affected abundant bacterial diversity (−0.45). *Trichoderma* positively affected alfalfa yield (0.23). Moreover, *Trichoderma* positively affected abundant bacterial community composition (0.38) and influenced alfalfa yields (0.43).

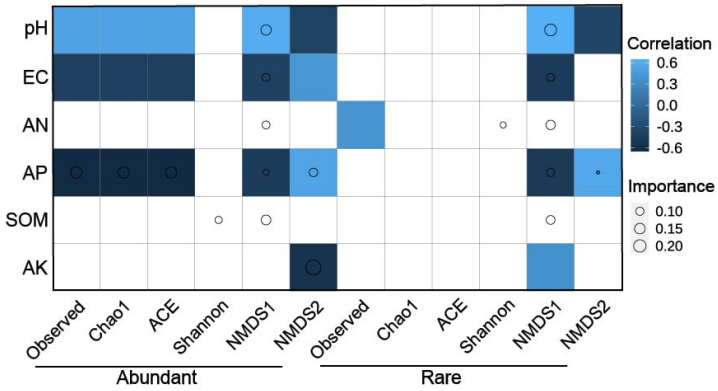


Figure 5. Contributions of rhizosphere soil physicochemical properties to rare and abundant bacterial community structures through correlation and best multiple regression models. The color grid represents significant differences ($p < 0.05$), while the blank grid indicates $p > 0.05$; the size of the circle in the figure indicates the proportion of explanatory changes, which was calculated based on multiple regression modeling and variance decomposition analysis. EC, soil conductivity; SOM, soil organic matter; AK: soil available potassium; AN: alkali-hydrolyzable nitrogen; AP: soil available phosphorus.

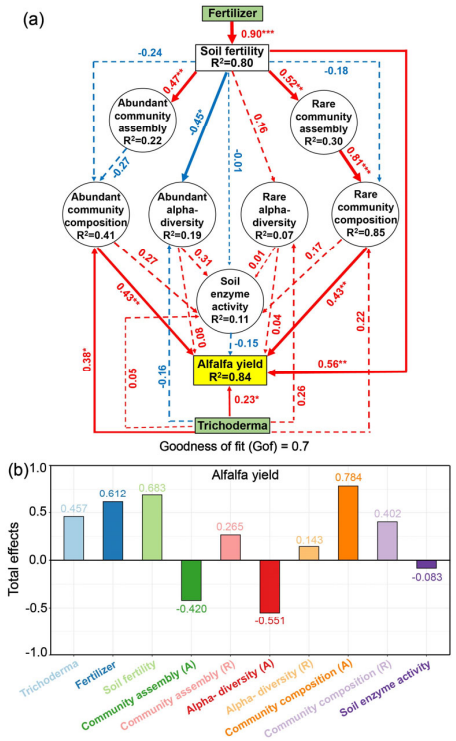


Figure 6. Cascading relationships of alfalfa yield with rhizosphere soil fertility index, enzyme activity and bacterial diversity, community composition, and community assembly. (**a**) PLS-PM shows the main pathways of the effects of the *rhizosphere* soil fertility index, enzyme activity, microbial assembly, and microbial diversity on alfalfa yield under different P fertilization and *Trichoderma* treatments. Red arrows imply positive flows of causality, while blue arrows are the opposite. Dotted and solid lines imply non-significant ($p > 0.05$) and significant levels (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), respectively. In addition, the values on the arrows represent standardized path coefficients. R^2 shows the variance of the dependent variable based on model interpretation. (**b**) Total effects on alfalfa yields for fertilizer, *Trichoderma*, rhizosphere soil fertility, enzyme activity, microbial assembly, and microbial diversity, where, A and R represent abundant and rare bacteria, respectively.

Collectively, we observed that soil fertility (0.683 of the total effects) had a greater influence on alfalfa yields than fertilizer (0.612), *Trichoderma* (0.457), and soil enzyme activity (−0.083). Furthermore, rare bacterial community assembly (0.265) and alpha diversity (0.143) had positive total effects on alfalfa yields, while abundant bacterial community assembly (−0.420) had negative total effects on alfalfa yields. The total effect of alpha-diversity (−0.551) on alfalfa yields was the opposite. Moreover, the total effect of rare community structure (0.402) was smaller than that of abundant community composition (0.784) (Figure 6b).

4. Discussion

Our results show that *Trichoderma* and P fertilization had significant positive interaction effects on alfalfa yield. Previous studies have demonstrated that *Trichoderma* and fertilizer co-application can significantly improve plant productivity [43]. Crop growth increases with increases in fertilization, but excessive use of inorganic phosphorus fertilizer might reduce plant phosphorus-use efficiency and affect crop productivity [4,44], which was also well supported in our results. In addition, excessive phosphorus fertilizer is an unnecessary cost for growers and may pose potential environmental hazards, leading to soil health issues [45,46]. Alfalfa yields in the *Trichoderma* inoculation groups were higher than those without *Trichoderma* inoculation, especially +M+P3, which possessed significant advantages, indicating that the application of *Trichoderma* and appropriate inorganic fertilizer could improve crop productivity and reduce the use of phosphorus fertilizer to a certain extent [47]. This is because bio-organic fertilizer can be an operational additional strategy for sustaining or improving crop production and decreasing the use of inorganic fertilizer residuals in soil [48]. *Trichoderma* has the characteristics of fast growth, high spore production, large colonization in the rhizosphere of plants, no interference from soil antibacterial activity, broad-spectrum antibacterial activity, and wide adaptability [49]. Therefore, it can be widely used for pathogen biological control in sustainable agriculture and is generally used in combination with inorganic or organic fertilizers. Additionally, we found that the +M+P0 values were not significantly higher than those of –M+P0 (CK), which agrees with previous results. They found that using *Trichoderma* inoculants alone or manure alone reduced crop yields to a certain extent compared with the control [50]. Furthermore, soil fertility is the foundation of the sustainability of agricultural ecosystems, as it can maintain plant productivity and minimize the use of synthetic fertilizers [51]. The comprehensive soil fertility index can evaluate soil quality [52], and our results showed that improving soil fertility can increase crop yield, as previously reported [6].

We found that both abundant and rare bacterial communities share the same dominant phyla, such as Proteobacteria and Actinobacteria, which is consistent with a previous study [53]. Secondly, soil microbial diversity drives various ecosystem processes, such as the biogeochemical cycles [19]. Previous studies show that different fertilization systems could significantly alter soil microbial community biodiversity [54,55], which agrees with our findings. This might be closely linked to the direct or indirect regulation of soil microbial communities by root exudates [56]. Root exudation is a crucial component of soil active carbon and is affected by plant metabolic changes and phosphorus fertilizer addition changes soil phosphorus fertility and directly affects the growth competitiveness of soil microorganisms [57,58]. In addition, different fertilization regimes and fertilizer dosages lead to unique soil environments and specific bacterial or fungal communities [59]. Similarly, we observed considerable differences in community structure between the various treatments, and this observation agrees with previous research reports [60]. Additionally, the nutrient availability of soil phosphorus can significantly influence the growth and activity of microbes, thereby affecting community diversity and composition [61]. We also found that available phosphorus was a major factor in abundant and rare community diversities. Perhaps because alfalfa is a typical crop that requires water and phosphorus, rhizosphere microorganisms are more sensitive to changes in soil available phosphorus [62].

In the process of ecological succession, the assembly process mediates changes in microbial community composition, thereby affecting sustainable ecological functions [63]. Therefore, it is essential to assess the relative contributions of stochastic and deterministic processes [64]. A change in the environment can lead to fluctuation in the intensity of stochastic and deterministic assembly processes [65]. Different fertilization amounts can change the gradient of environmental conditions, including different niches and survival adaptation [13], which partly explains the diverse processes of community assembly between different phosphate fertilizer treatments. For example, compared with the low-phosphate fertilizer treatment (P0–P2), there was a higher proportion of stochastic processes in the abundant bacterial communities at higher phosphate fertilizer levels (P3–P4). This might be due to the intense competition between microbes for the nutrients needed for survival under nutrient-constraint conditions, which leads to the dominance of the deterministic process; however, the microorganisms were also in an appropriate environment with abundant resources, so few alliances occurred among them [66,67]. Previous reviews also suggest that the deterministic process can be manipulated in extreme conditions, while stochasticity leads to more assembly processes in mild environmental situations [68]. Microbial community assembly processes exhibit significantly different behaviors in various agricultural management schemes [69], which explains the difference in the community assembly processes between *Trichoderma* inoculation (+M) and non-inoculation (−M) in our study. We also found differences in the assembly processes between the abundant and rare bacterial communities, which agrees with previous results [70]. This might lead to a difference in environmental adaptability, community coverage, and niche competitiveness between rare and abundant microorganisms [21,71,72].

PLS-PM showed important pathways for the effects of the soil fertility index, enzyme activity, microbial assembly, and microbial diversity on alfalfa yield under different P fertilization and *Trichoderma* treatments. *Trichoderma* inoculation has significant positive effects on alfalfa growth [60]. Furthermore, reasonable fertilization can considerably enhance crop yield and fertilizer utilization efficiency, and it can improve soil quality [73]. Evidence suggests that crop productivity increases with increased soil fertility [6]. Additionally, the complexity of the microbial community assembly process depends on soil fertility [74]. The community assembly process could explain how microbial community diversity is generated and maintained; this process controls changes in soil microbial community composition during ecosystem succession, thereby affecting ecosystem resilience and ecosystem function [75]. In addition, some studies show that the microbial community's structure is closely related to plant productivity [76]. As soil microorganisms play a vital role in carbon and nitrogen cycling and other biological processes, maintaining and improving soil microbial diversity is essential for ensuring plant productivity [13].

5. Conclusions

Our study indicates that *Trichoderma* inoculation and P fertilization regimes synergistically contribute to improving alfalfa yield and changing soil enzyme activity. Alfalfa yields were excellent in +M+P3 (*Trichoderma* incubation and 15 g/m^2 phosphorus fertilizer). Soil fertility was closely related to the alfalfa yield. Proteobacteria and actinomycetes are dominant phyla shared by abundant and rare taxa. Rare and abundant bacterial community diversities and assembly processes were diverse under *Trichoderma* inoculation and P fertilization regimes. AP is the main factor affecting the diversity of abundant and rare bacterial communities. Additionally, *Trichoderma* inoculation and phosphorus fertilizer addition could improve alfalfa yield by regulating abundant and rare bacterial community composition, respectively. Our results highlighted the prominence of rare and abundant microorganisms in maintaining crop yields, suggesting that it is possible to increase plant productivity by mediating rare and abundant taxa in the process of strengthening agricultural ecosystems.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy14071453/s1, Figure S1: Comparison of α-diversity between abundant and rare bacterial communities under different P fertilization and *Trichoderma* treatments; Figure S2: Comparison of β-diversity between abundant and rare bacterial communities under different P fertilization and *Trichoderma* treatments; Figure S3: Sum of relative contributions of ecological processes to the assemblies of abundant (a) and rare (b) soil bacterial communities under different P fertilization and *Trichoderma* treatments; Table S1: Scoring function values and weights assigned to selected soil fertility parameters; Table S2: Comparison of soil physicochemical properties under different P fertilization and *Trichoderma* treatments; Table S3: Comparison of soil α-diversity under different P fertilization and *Trichoderma* treatments.

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