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Effects of Crystal Lime Sulfur Fumigation and Application of Root-Growth-Promoting Agents on the Control of Apple Replant Disease

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Abstract: Apple replant disease (ARD) is seriously hindering the development of the apple industry. This experiment assessed the effects of two different root-growth-promoting agents (Indoleacetic acid and nutrient elements) on the microbial environment of apple-replanted soil and the growth of apple rootstock *Malus hupehensis* Rehd. seedlings after fumigation with crystal lime sulfur. The results showed that the simultaneous application of crystal lime sulfur, indoleacetic acid, and nutrient elements (T4) improved the biomass of *Malus hupehensis* Rehd. seedlings. It also enhanced the activities of soil enzymes and root antioxidant enzymes (SOD, POD, CAT). Their activities were significantly higher than in the individual treatments and resulted in a decrease in malondialdehyde (MDA) content. The T4 treatment significantly increased the net photosynthetic rate and chlorophyll content of the plant, thus effectively increasing the plant growth status. After fumigation, the amount of soil microorganisms was reduced, and the amount of bacteria and actinomycetes was increased after mixed application with the root-growth-promoting agent. The abundance of different species such as *Pseudallescheria*, *Guehomyces*, *Trichoderma*, *Bacillus*, *Gaiella*, and *Sphingomonas* was effectively increased, and the amount of *Fusarium oxysporum* was reduced. Through correlation analysis between different species and plant and soil enzymes, we found that the different species were positively correlated with root respiration rate and SOD activity and negatively correlated with MDA content. The differentially accumulated microbial species may be the key microorganism that promotes plant growth. Therefore, the simultaneous application of crystal lime sulfur, indoleacetic acid, and nutrient elements can optimize the apple replant soil environment and promote the growth of *Malus hupehensis* Rehd. seedlings, and can be used to control apple replant disease.

Keywords: replant disease; crystal lime sulfur; nutrient elements; indoleacetic acid

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1. Introduction

China is the country with the largest apple cultivation area in the world. Currently, 60% of the orchards have entered the aging stage [1]. Restricted by land resources, continuous cultivation of older apple tree plants is inevitable, and when replaced, continuous apple tree cultivation will lead to ARD [2]. ARD is phenotypically expressed with a damaged root system, shorter plants, aggravated incidence of diseases and insect pests, low yield, and poor quality [3,4]. Previous studies have shown that ARD is mainly caused by the increase in pathogenic bacteria and imbalances in the soil microbial community structure [5,6]. Numerous studies have shown that *Fusarium*, *Trichosporon*, *Cylindrocarpon*, and *Pythium* are the main causes of replanting-caused issues in apple-producing countries such as the United States, Italy, and South Africa [7,8]. Wang et al. [9] found that *Fusarium* was the

pathogen causing ARD in Bohai Bay, China. Xiang et al. [10] identified a significant positive correlation between *Fusarium* prevalence in old orchard soil and the ARD occurrence degree. Therefore, the identification of approaches to reduce the pathogens in the replanted soil and optimize soil microbial community structure has become the primary task to overcome ARD.

Current studies have shown that soil fumigation and disinfection before planting can effectively control ARD [11]. Due to the increased environmental protection awareness, methyl bromide, which has a good fumigation effect, has been completely banned due to environmental toxicity [12], so it is increasingly important to find environmentally friendly alternatives. Crystal lime sulfur is a bactericide used since antiquity with a strong bactericidal effect, and its active compound is calcium polysulfide. An appropriate lime sulfur application can control apple scab [13]. Calcium polysulfide is considered among the most effective control agents with the strongest long-term stability and can also repair Cr(VI) contaminated soils [14]. Nicola et al. [11] found that soil fumigation can significantly reduce the total amount of microorganisms and nutrient content in the soil. However, plants must maintain the absorption of certain nutrient elements to ensure their normal growth and development. Bauerle et al. [15] showed that rapid root growth and increased root meristem activity could also improve the resistance against pathogens in the soil. Chen et al. [16] demonstrated that calcium affects the normal growth and development of the vegetative system and reproductive organs, the antioxidant enzyme system, photosynthesis, and the physiological and biochemical processes of plants. When plants suffer from stress, reactive oxygen species, such as superoxide anion and hydroxyl radical, will increase sharply. During evolution, plants formed an antioxidant defense enzyme system composed of enzymes such as SOD, CAT, and POD [17]. St Clair et al. [18] found that magnesium functions as a phosphorus carrier in plants, which is conducive to promoting root growth, while magnesium is also a component of chlorophyll. Chlorophyll is the key component of plant photosynthesis, and its content is an essential physiological index reflecting the photosynthetic capacity of crops. Under stress conditions, the membrane structure of plant thylakoids is destroyed, which reduces chlorophyll synthesis and photosynthetic rate and ultimately affects plant growth [19]. Kaznina et al. [20] found that zinc can promote carbon dioxide fixation in photosynthesis and promotes auxin synthesis. Zhang et al. [21] identified that molybdenum deficiency in broccoli led to chlorophyll reduction and necrosis between the veins of the old leaves, resulting in wilting and even death of the broccoli leaves, as well flowering inhibition or delay till early autumn. Antonietti et al. [22] demonstrated that boron promoted cell elongation and cell division and was conducive to root growth and elongation. Sulfur can function as a signaling compound. Sulfur is involved in environmental stress resistance and can enhance the resistance of plants to fungal pathogens [23]. Indoleacetic acid (IAA) has auxin activity and is an indole-class plant growth regulator. It can promote cell division and differentiation, regulate root systems, etc. [24,25]. Indoleacetic acid is widely applied in plants and promotes plant growth [26].

According to previous studies, fumigation of replanted soil with crystal lime sulfur can effectively prevent and control ARD, with the optimal proportion of crystal lime sulfur added to soil found to be 1‰ [27]. On this basis, the application of plant growth regulators is conducive to the propagation of beneficial bacteria, can play a positive role in the soil microbial community structure, and promotes soil health and the growth and development of plants [28,29]. Jiang et al. [30] found that methyl bromide can effectively eliminate the pathogenic fungus *Fusarium* in replanted soil, which is currently the most effective fumigant for controlling ARD. Some studies have shown that the treatment of replanted soil with a root-growth-promoting agent can change the soil environment, significantly reducing the populations of harmful microorganisms in the soil while promoting the propagation of beneficial bacteria in the soil and effectively preventing and controlling ARD [31]. In this study, *Malus hupehensis* Rehd., a common apple rootstock, was used as the plant material. Methyl bromide was used as the high-standard control treatment. To study the effects

of adding indoleacetic acid and nutrient elements to replanted soil after fumigation with crystal lime sulfur on the soil environment and plant growth. Our results can contribute to environmentally friendly and effective measures to alleviate ARD.

2. Materials and Methods

2.1. Test Materials

The experiment was conducted in the College of Horticultural Science and Engineering of Shandong Agricultural University and the National Apple Engineering Center. The test soil was taken from Manzhuang Town, Tai'an City, Shandong Province, China. The soil was apple orchard sandy loam from an apple orchard of more than 30 years of age. The experimental period was 2021–2022. In April 2021, the aged orchard soil was taken randomly from multiple points in the orchard, from a distance of about 80 cm from the tree trunk and about 30 cm from the soil surface. The soil samples were then mixed to obtain a uniform mixture. The physical and chemical properties of the soil samples were determined. The content of organic matter was 11.28 mg·kg^{-1}, the available nitrogen was 28.2 mg·kg^{-1}, the available potassium was 78.29 mg·kg^{-1}, the available phosphorus was 10.6 mg·kg^{-1}, and the soil pH was 7.15.

Malus hupehensis Rehd., a common rootstock seedling variety, was selected as the test material. In January 2021, the seeds of *Malus hupehensis* Rehd. were laminated: the seeds were placed in a 4 °C environment for 40 days until they were exposed. In March, the laminated seeds were seeded into the specific substrate for the test. At the beginning of May, seedlings with approximately 5 true leaves without the presence of diseases and pests were selected and transplanted into pot plants with different soil treatments (diameter 24 cm, height 18 cm, soil mass 7 kg).

The crystal lime sulfur fumigant used in the test was produced by Hebei Shuangji Chemical Co., Ltd. (Shijiazhuang, China).

2.2. Test Treatment

The soil treatment with crystal lime sulfur was carried out 15 days before the planting of *Malus hupehensis* Rehd. seedlings (in the middle of April 2021). The treatment was performed as follows: the crystal lime sulfur and the soil from the aged apple orchard were mixed evenly with an application concentration of 1.0 g·kg^{-1} (Jiang et al., 2020) [27]. The soil was then sealed in a plastic bag for fumigation treatment. After a week, the sealed plastic bags containing the fumigated soil were opened and dried before further treatments. The details of each treatment are shown in Table 1.

Table 1. The different treatments assessed in this study.

Abbreviation	Treatment
CK1	Replanted soil
CK2	Methyl-bromide-fumigated soil
T1	Crystal lime sulfur
T2	Crystal lime sulfur and indoleacetic acid
T3	Crystal lime sulfur with nutritional elements
T4	Crystal lime sulfur, indoleacetic acid, and nutrient elements

With the formula of Hoagland nutrient solution as the basis, the formula of nutrient elements was modified as follows: 0.05 g zinc sulfate, 0.04 g ammonium molybdate, 0.112 g boric acid, 0.4 g superphosphate, 0.1 g magnesium sulfate, 0.2 g sodium silicate, and 0.056 g manganese sulfate were added per 7 kg of soil. Indoleacetic acid was added at 37.5 mg per 7 kg of soil.

On the day before transplanting, the soil fumigated with crystal lime sulfur was thoroughly mixed with the different combinations of root-growth-promoting treatments according to the six experimental treatments and was then potted. At the beginning of May

2021, *Malus hupehensis* Rehd. seedlings with similar growth were transplanted for each treatment (10 pots for each treatment, 1 seedling for each pot), and then unified fertilizer and water management was carried out. In July, August, and September 2021, soil and plant samples from the different treatments were collected. When collecting soil samples, the surface soil was removed, and the soil attached to the main root and side root was gently shaken and placed into a sealed bag, not damaging the root system. Then, the soil was passed through a 2 mm sieve, and each sample was divided into two parts. One part was placed into a low-temperature refrigerator (to measure the number of soil microorganisms and conduct high-throughput sequencing of soil microorganisms), and the other part was dried at room temperature to measure soil enzyme activity. The *Malus hupehensis* Rehd. seedlings were washed with water, and the plant height, stem diameter, and fresh weight were measured, as well as the dry weight after air drying. Finally, the roots with vigorous growth were sampled and stored in liquid nitrogen to measure root enzyme activities.

2.3. Measured Indicators and Methods

2.3.1. Determination of *Malus hupehensis* Rehd. Seedling Biomass

Three seedlings without disease symptoms and insect pests and with little difference in growth were selected from different treatments, and the plant height and stem diameter were measured with a scale, vernier caliper, and a meter ruler. Then, the soil on the surface of the plant was washed, and after the plant was cleaned, the fresh weight was measured with an electronic weighing device. After the determination, the plants were placed at 105 °C for 30 min and then dried at 65 °C until a constant weight was reached to measure the dry weight.

2.3.2. Determination of Chlorophyll Content and Photosynthetic Parameters of *Malus hupehensis* Rehd. Seedlings

The chlorophyll content in leaves was determined by the ethanol extraction method [32]. First, 0.2 g of fresh and clean leaves was weighed and thoroughly ground in calcium carbonate powder and 5 mL of 98% ethanol. Then, 10 mL of ethanol was added until the tissues turned white. Then, the ground tissues were left to stand for 10 minutes and then were filtered through a funnel into a brown 25 mL volumetric flask while the remaining tissues were washed with a small amount of ethanol. A 20 mL volume of 80% acetone solution was added into the 25 mL volumetric flask and was left to stand for 24~36 h in the dark until the leaves turned white. Absorbance was recorded at 665 nm, 649 nm, and 470 nm, with 80% acetone used as the control. The chlorophyll content was calculated through a formulaThe specific calculation method is provided in the supplementary information.

The method of Wang et al. [32] was used to determine the photosynthetic parameters. On the sunny and windless morning of August 19, the photosynthetic parameters were measured by the CIRAS-3 portable photosynthetic instrument (PP System, UK) from 9:00 to 11:00. Three healthy functional leaves of plants with the same growth (the 3rd to 5th expanded leaves from top to bottom) were selected from each treatment, and the net photosynthetic rate (P_n) was measured. For the calculation, an internal light intensity of 450 μm^{-2} s^{-1}, a CO_2 content of 360 μL L^{-1}, and relative humidity of the leaf chamber at 25 °C was used.

2.3.3. Determination of Root Respiration Rate and Root Protective Enzyme Activity

Young root tissues from the lateral root were selected for the root respiration rate with TTC method [33]. The method was as follows: 0.5 g of plant root tip sample was weighed, placed into a 10 mL beaker, and 0.4% of TTC aqueous solution was added, as well as the equivalent mixture of phosphate buffer solution to a 10 mL final volume. The solution was immersed and was quickly sealed and then placed in the dark at 37 °C for 1~3 h. Subsequently, 2 mL of sulfuric acid of about 1 mol·L^{-1} was added, and then the reaction was stopped (at the same time, a blank control was prepared by adding sulfuric acid first, then adding the root sample, with the other steps being the same as above). Finally, the

root sample was taken out of the solution, which was removed, and 10 mL of 95% ethanol was added into each test tube, which was then sealed. The sample was extracted for 24 h until the root turned white. The spectrophotometer was used to measure the absorbance at 485 nm. The method of Singh et al. [34] was used to determine the root antioxidant enzymes. Further details are provided in the supplementary information.

2.3.4. Determination of Soil Enzyme Activity

The activity of neutral phosphatase was determined by sodium diphenyl phosphate colorimetry, the invertase activity by 3,5-dinitrosalicylic acid colorimetry, the urease activity by indophenol blue colorimetry, and catalase activity by potassium permanganate titration. The method of Chen et al. [35] was used for the determination of soil enzyme activity. Further details are provided in the supplementary information.

2.3.5. Determination of Soil Microbial Quantity

The number of soil microorganisms (bacteria and actinomycetes) was determined by the dilution plate counting method [36]. First, 10 g of the fresh soil sample was weighed, and 90 mL of distilled water was added into a triangular flask with glass beads. The sample was placed in a shaker for 30 min to fully mix the water and soil sample in the triangular flask. Then, 1 mL of the mixed sample was taken and placed into a test tube containing 9 mL of distilled water. The solution was fully mixed to obtain a 10^{-2} dilution and prepare 10^{-3}, 10^{-4} (for actinomycetes), and 10^{-5} (for bacteria) sample dilution solutions, as described by this method. The culture medium was prepared in advance. A 100 µL volume of the sample dilutions was used for plate coating, performed in triplicates for each treatment.

2.3.6. DNA Extraction and Real-Time Quantitative Analysis of Fusarium

The extraction and purification of total genomic DNA from the sampled soil were performed using the E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA), following the manufacturer's instructions. The CFX Connect system (BIO-RAD, Hercules, CA, USA) was used to analyze the copy number of the *Fusarium oxysporum* gene in the soil by real-time quantitative PCR. The primers used were the FR (5′-GGCCTGAGGGTTGTAATG-3′) and FF (5′-CGAGTTATACAACTCATCAACC-3′). The reactions were performed according to the instructions of the SYBR Premix Ex Taq Kit (TaKaRa Biotech Co., Ltd., Dalian, China). Each reaction in the 25 µL PCR system included 1.5 µL of DNA template, 12.5 µL of SYBR Premix Ex Taq II (TaKaRa), 1 µL of each primer, and 9 µL of double-distilled water. The reaction procedures were as follows: pre-denaturation at 95 °C for 30 s, denaturation at 95 °C for 5 s, and annealing at 60 °C for 30 s, for a total of 40 cycles.

2.3.7. High-Throughput Sequencing of Soil Fungi and Bacteria

Total DNA was extracted from each soil sample using the E.Z.N.A. Soil DNA Kit (Omega Bio-tek Inc., Norcross, GA, USA) according to the manufacturer's protocols. The final DNA concentration and purification were determined using a NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, NC, USA), and DNA quality was checked by 1% agarose gel electrophoresis. PCR amplification was carried out using a Thermocycler PCR system (GeneAmp 9700, ABI, Los Angeles, CA, USA). The V3–V4 hypervariable regions of the 16S rRNA gene were amplified with bacterial primers 338F 5′-ACTCCTACGGGAGGCAGCAG-3′ and 806R 5′-GGACTACHVGGGTWTCTAAT-3′ [37]. Fungal rRNA gene amplification was performed in the fungal ITS sequence region using the barcode primers ITS1F5′-CTTGGTCATTTAGAGGAAGTAA-3′ and ITS2R5′-GCTGCGTTCTTCATCGATGC-3′ [36]. The resulting PCR products of bacteria and fungi were extracted from a 2% agarose gel, further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and quantified using QuantiFluor-ST (Promega, Madison, WI, USA) according to the manufacturer's protocol.

2.4. Statistical Analysis

The data were analyzed using the R software suite (Version 3.3.1, Robert Gentleman and Ross Ihaka, New Zealand) and Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA). SPSS 23.0 (IBM SPSS Statistics, IBM Corporation, Armonk, NY, USA) was used to compare the mean values, and one-way ANOVA and Duncan's new complex range method were used to identify significant differences ($p < 0.05$).

3. Results

3.1. Effect of Different Treatments on the Growth of Malus hupehensis Rehd. Seedlings

As demonstrated in Table 2, it was evident that the growth of *Malus hupehensis* Rehd. seedlings increased after treatment with crystal lime sulfur (T1), crystal lime sulfur and indoleacetic acid (T2), crystal lime sulfur with nutrient elements (T3), crystal lime sulfur, indoleacetic acid, and nutrient elements (T4) in comparison to the CK1 treatment.

Table 2. Plant biomass of *Malus hupehensis* Rehd. seedlings under different treatments.

Time	Treatment	Plant Height (cm)	Stem Diameter (mm)	Fresh Weight (g)	Dry Weight (g)
July	CK1	30.40 ± 0.31 d	3.67 ± 0.17 d	6.76 ± 1.66 d	3.13 ± 0.42 d
	CK2	67.67 ± 3.71 a	7.84 ± 0.41 a	35.25 ± 7.11 b	17.86 ± 1.96 b
	T1	46.00 ± 0.58 c	5.01 ± 0.17 c	17.10 ± 2.73 c	10.09 ± 1.22 cd
	T2	53.67 ± 1.76 b	6.28 ± 0.43 b	23.68 ± 5.90 c	11.21 ± 2.02 bc
	T3	56.00 ± 1.73 b	7.70 ± 0.24 a	23.12 ± 1.72 c	18.53 ± 4.24 ab
	T4	66.33 ± 1.20 a	7.83 ± 0.28 a	43.80 ± 3.46 a	25.79 ± 2.69 a
August	CK1	49.97 ± 1.36 b	6.14 ± 0.02 c	25.02 ± 0.20 d	6.36 ± 0.10 f
	CK2	66.37 ± 2.12 a	8.16 ± 0.12 a	105.00 ± 0.58 a	43.82 ± 1.26 a
	T1	55.77 ± 3.24 b	4.88 ± 0.17 d	28.36 ± 0.51 d	13.71 ± 0.34 e
	T2	53.60 ± 1.46 b	6.13 ± 0.10 c	40.24 ± 1.80 c	18.02 ± 0.83 d
	T3	53.17 ± 0.64 b	6.34 ± 0.22 c	42.03 ± 3.37 c	23.88 ± 0.31 c
	T4	65.07 ± 1.43 a	7.00 ± 0.03 b	70.22 ± 1.80 b	32.70 ± 0.91 b

Notes: CK1: Replanted soil; CK2: Methyl-bromide-fumigated soil; T1: Crystal lime sulfur; T2: Crystal lime sulfur and indoleacetic acid; T3: Crystal lime sulfur with nutrient elements; T4: Crystal lime sulfur, indoleacetic acid, and nutrient elements. Data are means ± SE (n = 3); values marked with the same letter within a sampling date are not significantly different at $p < 0.05$ according to Duncan's new multiple range test; ANOVA = analysis of variance.

In the July 2021 measurements, the fresh weight, stem diameter, plant height, and dry weight of the seedlings in the T1 treatment increased by 152%, 36%, 51%, and 222%, respectively, compared with the replanted soil (CK1). When compared with the CK1 treatment, the T2 treatment increased fresh weight by 240%, the stem diameter by 71%, the plant height by 77%, and the dry weight by 258%. Moreover, the T3 treatment increased fresh weight, stem diameter, plant height, and dry weight by 242%, 109%, 84%, and 492%, respectively. The growth of *Malus hupehensis* Rehd. seedlings increased the most in the T4 treatment, with their fresh weight, ground diameter, plant height, and dry weight increasing by 547%, 113%, 118%, and 724%, respectively, when compared to the CK1 treatment. In the August 2021 measurements, the T4 treatment resulted in the most significant increase in the biomass of *Malus hupehensis* Rehd. seedlings, when compared with the CK1 treatment. The increase in fresh weight, stem diameter, plant height, and dry weight of potted seedlings was 181%, 14%, 30%, and 414%, respectively. The growth-promoting effects of the different treatments on plants were as follows: CK2 > T4 > T3 > T2 > T1 > CK1.

3.2. Effect of Different Treatments on Soil Enzyme Activity

As shown in Figure 1, in July, August, and September 2021, all treatments effectively improved soil enzyme activity compared to CK1. Among them, the activities of invertase,

phosphatase, catalase, and urease increased most significantly in the T4 treatment, with 145%, 103%, 66%, and 64% in July, an increase of 50%, 152%, 45%, and 74% in August, and an increase of 69%, 47%, 3%, and 25% in September.

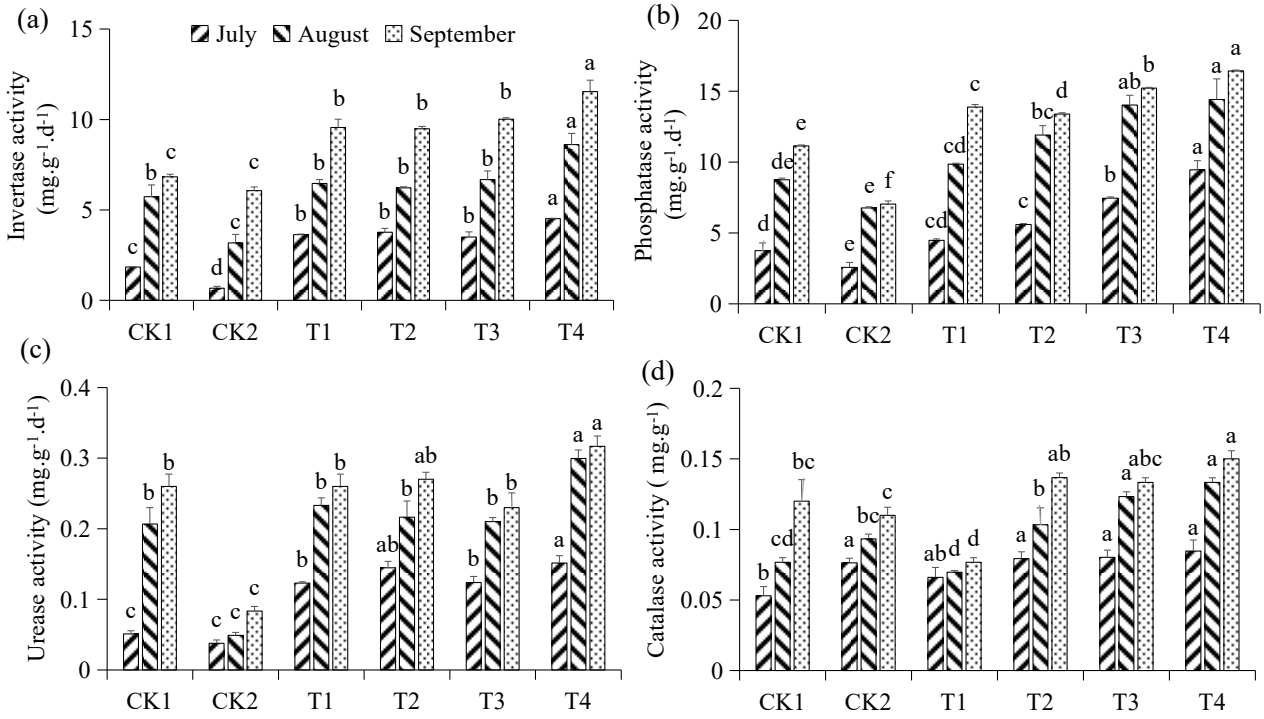

Figure 1. Effects of different treatments on soil enzyme activities. (**a**) Invertase activity; (**b**) Phosphatase activity; (**c**) Urease activity; (**d**) Catalase activity. CK1: Replanted soil; CK2: Methyl-bromide-fumigated soil; T1: Crystal lime sulfur; T2: Crystal lime sulfur and indoleacetic acid; T3: Crystal lime sulfur with nutrient elements; T4: Crystal lime sulfur, indoleacetic acid, and nutrient elements. Data are means ± SE (n = 3); values marked with the same letter within a sampling date are not significantly different at $p < 0.05$ according to Duncan's new multiple range test; ANOVA = analysis of variance.

3.3. Effects of Different Treatments on Activities of Antioxidant Enzymes and the MDA Content in the Roots of Malus hupehensis Rehd. Seedlings

As shown in Figure 2, after fumigating the replanted soil with crystal lime sulfur, the addition of a root-growth-promoting agent effectively improved the activity of antioxidant enzymes (SOD, POD, CAT) in seedling roots and reduced the content of MDA. Among them, the T4 treatment had the most significant effect. The SOD, POD, and CAT activities in the T4 treatment were 5.46-, 3.85-, and 16.4-fold higher compared to CK1, respectively.

3.4. Effects of Different Treatments on Root Respiration Rate of Malus hupehensis Rehd. Seedling

The effects of the different soil treatments on the root respiration rate of *Malus hupehensis* Rehd. seedlings are shown in Figure 3. The root respiration rate of the seedling roots in the replanted soil was higher than that after fumigation with crystal lime sulfur and the application of different combinations of root growth promoters. Those treatments greatly promoted the root vitality of the seedlings. The effect of the different treatments on root respiration was as follows: T4 > T3 > T2 > T1 > CK1. The T4 treatment had the greatest impact on respiration, which was 1.40-, 1.24-, and 1.05-fold higher than the T1, T2, and T3 treatments, respectively.

3.5. Inhibitory Effect of Different Treatments on Fusarium oxysporum in the Seedling Rhizosphere Soil

As shown in Figure 4, the growth of *F. oxysporum* in the soil was inhibited after the replanted soil was fumigated with crystal lime sulfur and the application of root-growth-promoting agents. Compared with CK1, the content of *F. oxysporum* in T1, T2, T3 and T4 treatments decreased by 78%, 80%, 79% and 88%, respectively. The T4 treatment resulted in the most significant inhibitory effect on *F. oxysporum* among the four treatments.

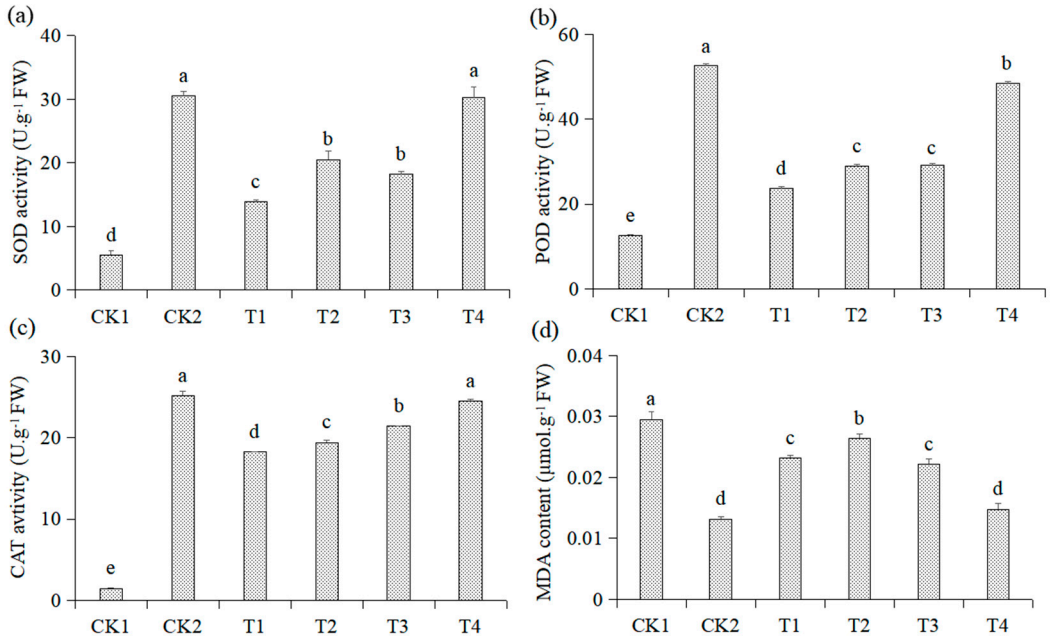

Figure 2. Effects of different treatments on the activities of root antioxidant enzymes and the MDA content in *Malus hupehensis* Rehd. seedlings. (**a**) SOD activity; (**b**) POD activity; (**c**) CAT activity; (**d**) MDA content. CK1: Replanted soil; CK2: Methyl-bromide-fumigated soil; T1: Crystal lime sulfur; T2: Crystal lime sulfur and indoleacetic acid; T3: Crystal lime sulfur with nutrient elements; T4: Crystal lime sulfur, indoleacetic acid, and nutrient elements. Data are means ± SE (n = 3); values marked with the same letter within a sampling date are not significantly different at $p < 0.05$ according to Duncan's new multiple range test; ANOVA = analysis of variance.

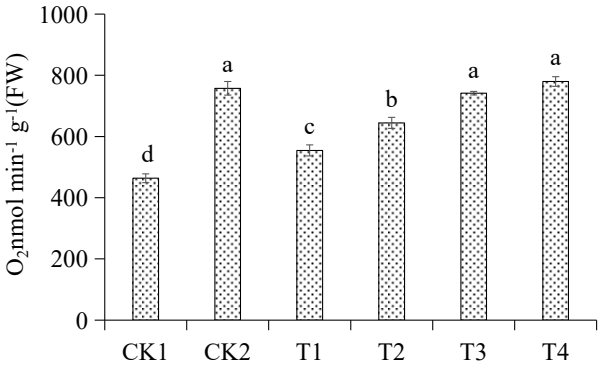

Figure 3. Effects of the different treatments on the root respiration rate of *Malus hupehensis* Rehd. seedlings. CK1: Replanted soil; CK2: Methyl-bromide-fumigated soil; T1: Crystal lime sulfur; T2: Crystal lime sulfur and indoleacetic acid; T3: Crystal lime sulfur with nutrient elements; T4: Crystal lime sulfur, indoleacetic acid, and nutrient elements. Data are means ± SE (n = 3); values marked with the same letter within a sampling date are not significantly different at $p < 0.05$ according to Duncan's new multiple range test; ANOVA = analysis of variance.

3.6. Effects of Different Treatments on Photosynthesis and Chlorophyll Content of Malus hupehensis Rehd. Seedling

As shown in Figure 5, the addition of two root-growth-promoting agents after the treatment with crystal lime sulfur significantly increased the net photosynthetic rate, effectively increasing the growth of the seedlings. Compared with the CK1 treatment, the net photosynthetic rate of the T4 treatment was about two-fold higher compared to CK1. Moreover, the net photosynthetic rate of the T2 and T3 treatments was lower compared to the T4 treatment. At the same time, each treatment increased the chlorophyll content in the plant leaves, with a relatively large increase in chlorophyll a and chlorophyll b and a relatively small increase in carotenoids.

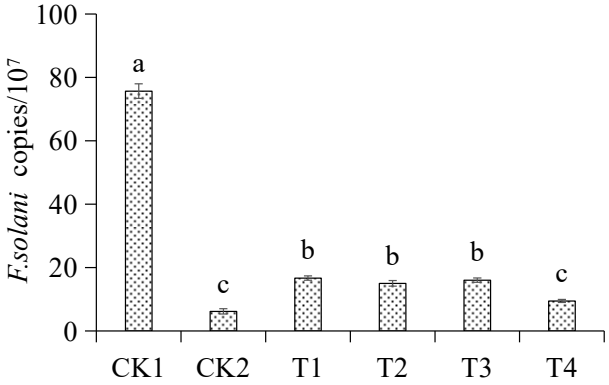

Figure 4. Effects of different treatments on the number of soil *F. oxysporum*. CK1: Replanted soil; CK2: Methyl-bromide-fumigated soil; T1: Crystal lime sulfur; T2: Crystal lime sulfur and indoleacetic acid; T3: Crystal lime sulfur with nutrient elements; T4: Crystal lime sulfur, indoleacetic acid, and nutrient elements. Data are means ± SE (n = 3); values marked with the same letter within a sampling date are not significantly different at $p < 0.05$ according to Duncan's new multiple range test; ANOVA = analysis of variance.

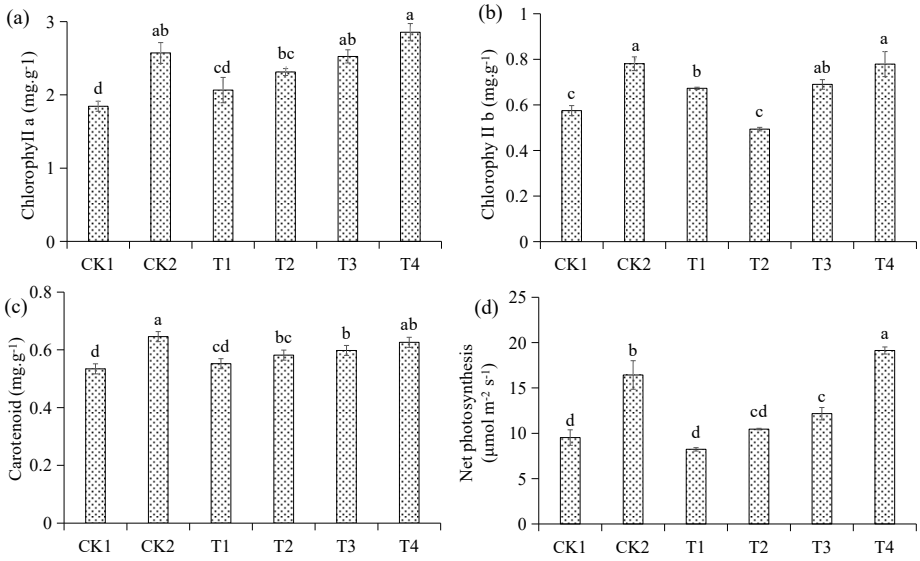

Figure 5. Effects of different treatments on the photosynthesis and chlorophyll content of *Malus hupehensis* Rehd. seedlings. (**a**) Chlorophyll a; (**b**) Chlorophyll b; (**c**) Carotenoid; (**d**) Net photosynthesis. CK1: Replanted soil; CK2: Methyl-bromide-fumigated soil; T1: Crystal lime sulfur; T2: Crystal lime sulfur and indoleacetic acid; T3: Crystal lime sulfur with nutrient elements; T4: Crystal lime sulfur, indoleacetic acid, and nutrient elements. Data are means ± SE (n = 3); values marked with the same letter within a sampling date are not significantly different at $p < 0.05$ according to Duncan's new multiple range test; ANOVA = analysis of variance.

3.7. Effects of Different Treatments on the Number of Culturable Microorganisms in the Soil

As shown in Table 3, the number of fungi and microorganisms in the soil after fumigation with crystal lime sulfur significantly decreased, and the fumigation and application of root-growth-promoting agents significantly promoted the bacteria and actinomycetes' growth and reproduction. The T4 treatment resulted in the highest number of bacteria and actinomycetes compared to all other treatments. Specifically, when compared with CK1 treatment, the number of bacteria and actinomycetes increased by 71% and 27%, respectively, in T4.

Table 3. Effects of different treatments on the number of soil microorganisms.

Treatment	Bacteria ($\times 10^5$ CFU·g^{-1})	Actinomyces ($\times 10^5$ CFU·g^{-1})
CK1	8.67 ± 0.33 cd	15.00 ± 0.58 b
CK2	3.00 ± 0.58 e	8.67 ± 0.88 d
T1	7.00 ± 0.58 d	11.33 ± 0.88 c
T2	10.33 ± 0.88 c	15.00 ± 0.58 b
T3	12.67 ± 0.33 b	18.00 ± 0.58 a
T4	15.00 ± 0.58 a	19.00 ± 0.58 a

Notes: CK1: Replanted soil; CK2: Methyl-bromide-fumigated soil; T1: Crystal lime sulfur; T2: Crystal lime sulfur and indoleacetic acid; T3: Crystal lime sulfur with nutrient elements; T4: Crystal lime sulfur, indoleacetic acid, and nutrient elements; Data are means ± SE (n = 3); values marked with the same letter within a sampling date are not significantly different at $p < 0.05$ according to Duncan's new multiple range test; ANOVA = analysis of variance.

3.8. Effects of Different Treatments on Soil Microbial Community Diversity

Based on the above results, the difference between the T4 and CK1 treatments was the most significant. To further explore the impact on the microbial community structure, we used beta diversity to study the differences in microbial diversity between the T4 and CK1 treatments, with CK2 (methyl bromide) as a high-standard control. As shown in Figure 6, hierarchical clustering and PCoA analysis were carried out on the bacterial and fungal sequences, respectively. We found that the T4 treatment and CK1 treatment were far apart, indicating a significant difference in the composition of fungi and bacterial communities between these treatments. In the PCA analysis of the fungal community, PC1 explained 65.55% and PC2 16.05%, of the total variance, with both explaining a cumulative 81.60%. For the bacterial community, the PC1 value explained 79.05%, and PC2 explained 13.75% of the total variance, with both explaining a cumulative 92.80% of the total variance. Therefore, the soil microbial community composition after the T4 treatment was significantly different from that of the CK1 treatment.

3.9. Effects of Different Treatments on Species Composition of Soil Microbial Community

As shown in Figure 7a,b, when compared with the CK1 treatment, the T4 treatment increased the abundance of fungi beneficial to plant growth, such as *Pseudallescheria*, *Guehomyces*, and *Trichoderma*. In contrast, the abundance of *Fusarium*, which plays a key role in the replant disease, decreased. The *Ascomycota* and *Basidiomycota* fungi increased in abundance. As shown in Figure 7c,d, when compared with CK2 treatment, the T4 treatment increased the abundance of bacteria beneficial to plant growth, such as *Sphingomonas*, *Bacillus*, *Gailla*, *Actinobaciota*, and *Firmicutes*.

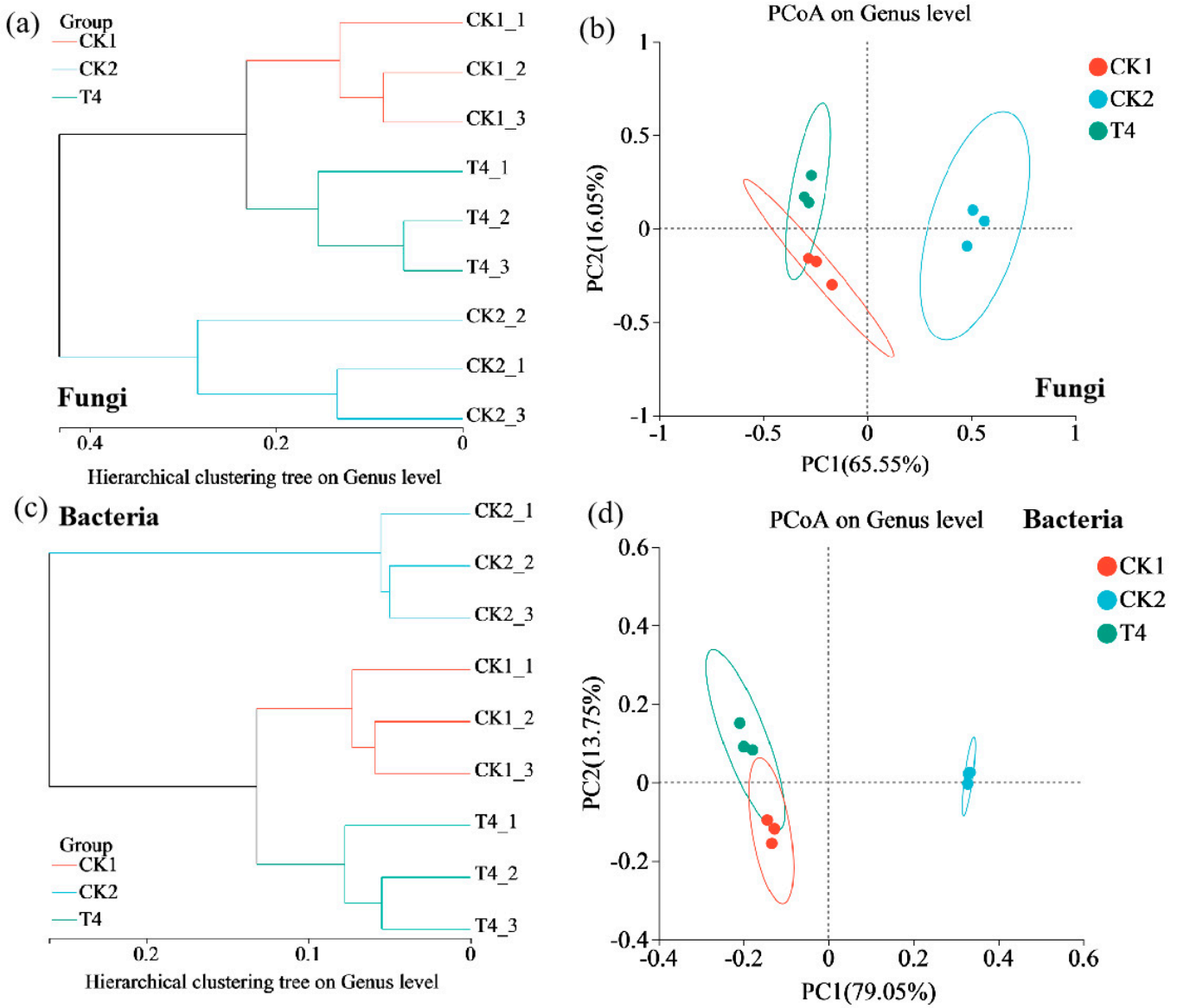

Figure 6. Beta diversity analysis of the soil fungal and bacterial communities under different treatments. (**a**) Hierarchical cluster analysis of fungi; (**b**) PCoA analysis of fungi; (**c**) Hierarchical cluster analysis of bacteria; (**d**) PCoA analysis of bacteria. A single-factor ANOVA was used to test the differences in beta diversity of the soil samples from different treatments, with the *y*-axis showing the average value of each index. CK1: Replanted soil; CK2: Methyl-bromide-fumigated soil; T4: Crystal lime sulfur, indoleacetic acid, and nutrient elements.

3.10. Differences between Soil Microbial Species and Their Correlation with Physical and Chemical Properties

We used the Student's T-test to detect microbial species with differential abundance between the CK1 and T4 treatments (Figure 8a,b). We found that in the fungal community, the *Pseudallescheria* and *Trichoderma* species were significantly higher in the T4 treatment compared to the CK1 treatment. In the bacterial community, the *Bacillus*, *Gaiella*, and *Sphingomonas* species were significantly higher in the T4 treatment compared to the CK1 treatment. Then, we analyzed the above different species and the soil's physical and chemical properties (Figure 8c). We found that the different species were positively correlated with the root resorption rate, SOD activity, chlorophyll b, and carotenoids, and negatively correlated with MDA content.

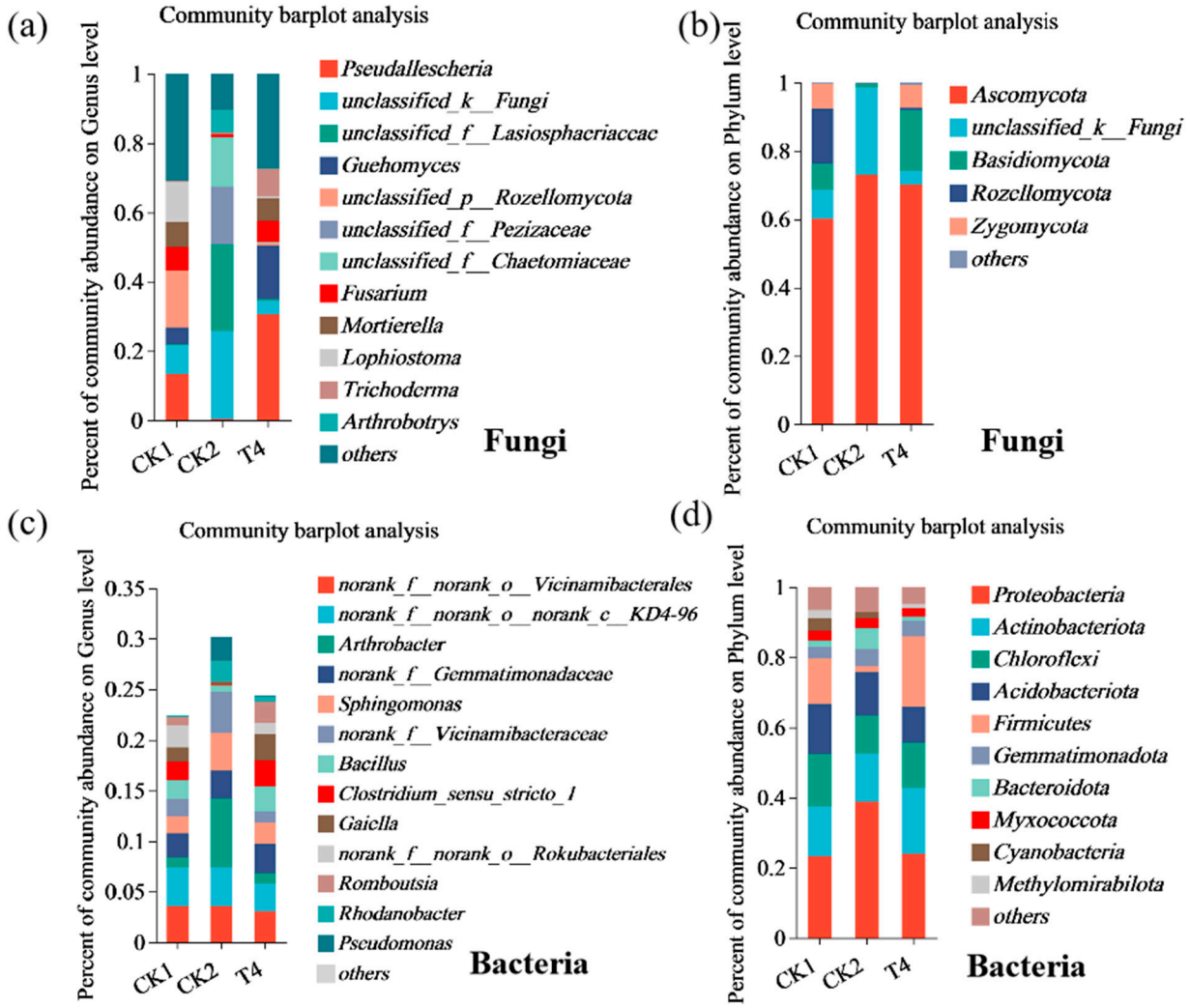

Figure 7. Relative abundance of the soil fungal and bacterial communities at the phylum and genus level under different treatments. (**a**) Fungal community abundance percentages at the genus level; (**b**) Fungal community abundance percentages at the phylum level; (**c**) Bacterial community abundance percentages at the genus level; (**d**) Bacterial community abundance percentages at the phylum level. CK1: Replanted soil; CK2: Methyl-bromide-fumigated soil; T4: Crystal lime sulfur, indoleacetic acid, and nutrient elements.

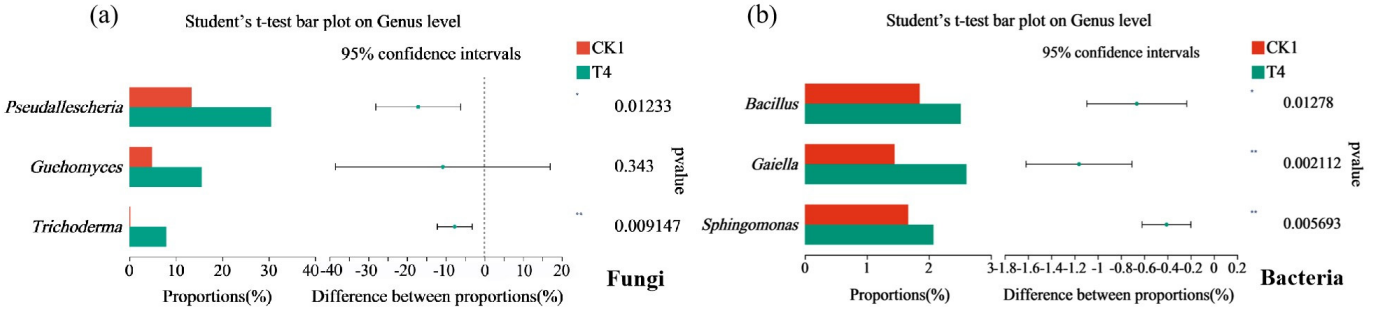

Figure 8. *Cont.*

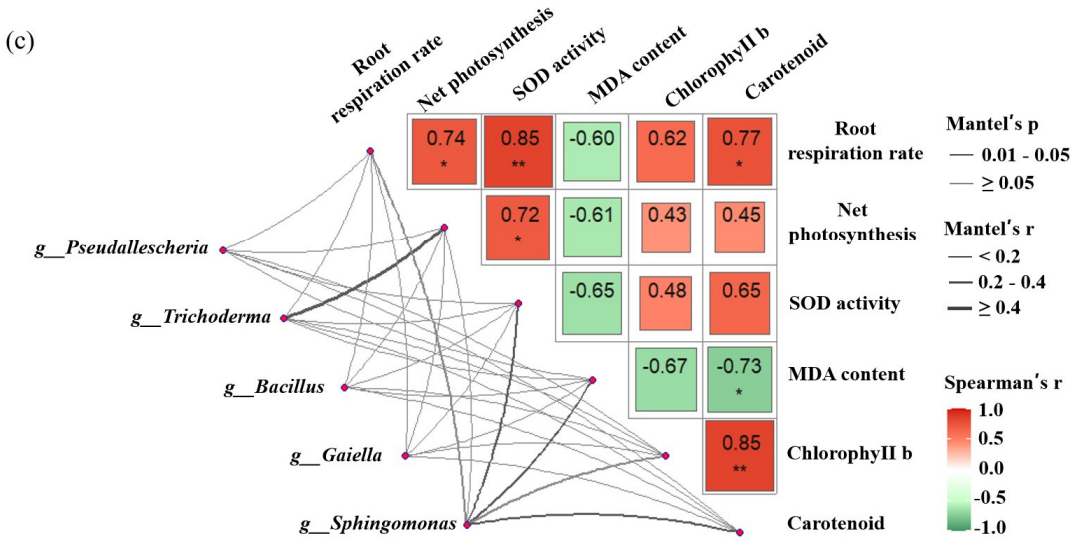

Figure 8. Analysis of fungal and bacterial species differences between the CK1 and T4 treatments and correlation between different species and physicochemical properties. (**a**) Fungal differential species between CK1 and T4 treatments; (**b**) Bacterial differential species between CK1 and T4 treatments; (**c**) Correlation analysis between different species and soil physicochemical properties. Significant differences are indicated as follows: * $0.01 < p \leq 0.05$, ** $0.001 < p \leq 0.01$.

4. Discussion

Roots are the metabolic center of the below-ground part of plants, and root vitality generally corresponds to the absorption and metabolic capacity of roots, whose size directly affects the acquisition of water and nutrients required to support plant growth [38]. Wang et al. [39] found that plant root vitality was significantly reduced under replanting conditions, leading to decreased plant biomass. Our experiments demonstrated that crystal lime sulfur could significantly promote the biomass increase of *Malus hupehensis* Rehd. seedlings after soil fumigation, but this growth-promoting effect was lower than that of methyl bromide treatment, which might be due to the methyl bromide being more effective in killing pathogenic microorganisms in soil than crystal lime sulfur [27,30]. After soil fumigation with a crystal sulfur mixture, the seedlings' biomass also increased in different degrees after adding indoleacetic acid and nutrient elements, in agreement with previous studies. Jiang et al. [40] found that a synthetic strain with indoleacetic acid added exogenously could significantly promote the growth of seedlings in replanted soil. Margaux et al. [41] confirmed that ARD severity was partly related to the reduced supply of nutrient elements. In this study, it was found that the activities of SOD, CAT, and POD of the seedlings were significantly increased after fumigation with crystal lime sulfur, which may be due to the improvement of the replanted soil environment and the resulting reduction of stress on plants [42]. The exogenous addition of indoleacetic acid and nutrient elements increased the activities of root antioxidant enzymes to different degrees, and their combined application showed the best results. The addition of indoleacetic acid and nutrient elements can possibly promote plants' growth and help them resist the stress conditions of the replanted soil. Gull et al. [43] found that the exogenous addition of indoleacetic acid could significantly enhance the activity of antioxidant enzymes and reduce the effects of salt stress in potato. Pan et al. [44] found that the exogenous addition of nutrients could significantly enhance the activity of antioxidant enzymes and reduce the incidence of gray mold in lettuce. We found that after fumigation with crystal lime sulfur, the chlorophyll content and net photosynthetic rate of plants were increased. The supplementation with indoleacetic acid and nutrient elements further increased the chlorophyll content and net photosynthetic rate of plants to a greater extent. Potentially, the fumigation of crystal lime

sulfur reduced the stress of replanted soil on plants, and the addition of indoleacetic acid and nutrient elements promoted the growth of plants [42].

Soil enzymes have been recognized as an important indicator of soil health and fertility [45]. Changes in their activities can alter the availability of nutrients absorbed by crops [46]. Invertase and phosphatase activities are closely associated with soil nutrient metabolism, and catalase activity can provide an indication of the soil's total biological activity and fertility status [47]. Previous studies found that long-term replanting decreased soil enzyme activity [40]. In this study, we found no significant differences between soil enzyme activities after fumigation with crystal lime sulfur in July and the replanted soil control. As time progressed, in September, certain soil enzyme activities after fumigation with crystal lime sulfur were significantly higher than that of replanted soil control. This may be because fumigation with crystal lime sulfur initially reduced the number of soil microorganisms, thus reducing soil enzyme activities [35]. The soil microbial community continued to recover, contributing to the increasing soil enzyme activity. However, methyl bromide has a highly lethal effect on soil microorganisms, and the recovery of microbial community structure is slow. Generally, soil enzyme activity can be restored to the level of the replanted soil control only 1–2 years after fumigation [30]. The addition of indoleacetic acid and nutrient elements after fumigation with crystal lime sulfur can improve soil enzyme activities to different degrees, and their combined effect was shown to be the strongest. On the one hand, indoleacetic acid and nutrient elements may promote the plant root growth and activate the rhizosphere soil environment, thus increasing soil enzyme activities; on the other hand, they may directly promote the recovery of the microbial community directly, thus having a synergistic effect [48].

Optimal soil microbial community structure can promote a balance in soil microecology and ensure the normal growth of plants [49]. Long-term replanting results in changes in the soil microbial community structure, shifting the soil microbial communities from being "bacteria-dominated" to "fungi-dominated" and seriously damaging the health of the soil environment [50]. In this study, the addition of indoleacetic acid and nutrient elements after fumigation with crystal lime sulfur reduced the content of *F. oxysporum* in replant soil to the greatest extent. This may be because the addition of indoleacetic acid and nutrient elements was conducive to the reproduction of bacteria and actinomycetes and inhibiting the growth of the pathogenic *F. oxysporum*. It was also found that the addition of indoleacetic acid and nutrient elements, after fumigation with crystal lime sulfur, changed the diversity of the microbial community to varying degrees. This may be because the treatment disrupted the original microecological balance of replanted soil and promoted the reorganization of the microbial community [51]. In this study, we analyzed the microbial species after treatment with indoleacetic acid and nutrient elements after fumigation of crystal lime sulfur, which were significantly higher and significantly different compared to the control treatment. It was found that *Bacillus*, *Gaiella*, and *Sphingomonas* were enriched in the treatment of indoleacetic acid and nutrient elements after fumigation with crystal lime sulfur. Numerous studies have shown that microorganisms such as *Bacillus*, *Gaiella*, and *Sphingomonas* can optimize the soil microecological environment and promote plant growth [52–55].

5. Conclusions

Fumigation with crystal lime sulfur and application of root-growth-promoting agents (Indoleacetic acid and nutrient elements) was shown to increase the biomass of *Malus hupehensis* Rehd. seedlings, alter the soil enzyme activities and effectively improve the activity of plant antioxidant enzymes (SOD, POD, CAT) in the roots of *Malus hupehensis* Rehd. seedlings. It improved the net photosynthetic rate of the plants, optimized the soil microbial community structure, reduced the number of the plant pathogen *F. oxysporum*, and increased the relative abundance of beneficial microorganisms such as *Bacillus*, *Gaiella*, and *Sphingomonas*. Our results provide theoretical and practical insights to promote the growth of apple plants in replanted soils and effectively alleviate apple replant disease.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9080901/s1, References [56–59] are cited in the Supplementary Materials.

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