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Effects of Chloropicrin, Dimethyl Disulfide and Metham Sodium Applied Simultaneously on Soil-Born Bacteria and Fungi

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Abstract: The area used to grow high-value crops is currently decreasing because production in the same soil for many years increases soil-borne diseases that reduce crop yield and quality as well as farmer income. Soil-borne disease is effectively controlled by soil fumigation prior to planting. In this study, the five different types of soils that had been used to grow tomatoes, watermelon, cucumber, ginseng and ginger were collected from field plots with high incidence of soil-borne diseases. This experiment adopts the indoor fumigation method to conduct triple fumigation of chloropicrin(PIC), metham sodium(MS) and dimethyl disulfide(DMDS) on different soil collected to examine changes in the soil microbial community, including pathogenic fungi and bacteria and beneficial microorganisms in order to clarify the impact on the overall structure of soil microbial community while controlling complex and multiple pathogens. High-throughput gene sequencing was used to detect bacterial and fungal taxonomic changes in the treated soils. Triple fumigation significantly reduced the abundance of at least five kinds of pathogenic fungi, *Fusarium oxysporum*, *Mortierella*, *Neocosmospora*, *Nitrospira Alternaria* and significantly increased the abundance of two kinds of beneficial species, *Bacillus* and *Trichoderma*. The research result observed increases and decreases in the biodiversity and richness of beneficial and pathogenic bacteria and fungi in response to triple fumigation of soil that had been used to grow tomatoes, watermelon, cucumber, ginseng and ginger. The most significant effect was observed in the experimental field of Panax notoginseng in Wenshan, Yunnan. Triple fumigation showed good potential to decrease pathogenic bacteria and fungi in soils and improve the disease resistance of soils, and that it has a good application prospect in the field of soil disinfestation.

Keywords: soil fumigation; high-throughput gene sequencing; soil microbial community

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1. Introduction

China is the world's largest producer of cucumber, tomato and watermelon by area planted, produced and exported [1–3]. China is also the world's largest producer of *Panax notoginseng* ("ginseng") [4] and ginger [5], both of which have medicinal qualities [6,7] that are valued in China and other countries.

Continuous planting of these crops in the same soil has led to increases in soil-borne diseases that have reduced crop yield and quality as well as farmer income [5]. Infection in the seedling stage of the roots and stems typically results in a 20~30% crop reduction, but it can lead to 50~60% crop loss when the disease is severe, or even 100% crop loss when the disease is very severe [8]. The control of plant diseases is particularly difficult in cool temperatures where fumigants are less efficacious against pests. The difficulty experienced by farmers in controlling soil-borne disease has curtailed expansion in China of the areas planted with high-value crops.

Soil fumigation is typically used to control, mainly, nematodes, bacteria and weeds that reduce crop yield and quality if left uncontrolled [9]. The broad-spectrum nature of fumigants results in beneficial and pathogenic organisms being equally targeted. In a

healthy soil ecosystem, plants secrete nutrients such as sugars for use by soil microorganisms. Microorganisms promote plant growth by changing the availability of soil nutrients. The structure of the microbial community influences the extent to which substances are recycled and the overall stability of the soil ecosystem [10]. Specialized equipment is used to inject the fumigant into the soil and to automatically cover it with plastic film shortly afterwards to minimize fumigant emissions. Fumigant efficacy is determined mainly by the properties of the fumigant and the application conditions such as temperature and soil type [11,12]. Many soil fumigants are registered in China, including chloropicrin (PIC), metham sodium (MS) and dimethyl disulfide (DMDS) [13]. These three fumigants were the focus of this study.

PIC began to be widely used as a soil fumigant in countries outside of China in 1934 [14]. It is a gaseous fumigant registered in China to control mainly *Verticillium* wilt, *Fusarium* wilt, bacterial wilt, *Phytophthora* and *Meloidogyne incognita* (RKN, root knot nematode) [5]. Previous research reported that fumigation with a low dose (10–20 g/m^2) of PIC had no persistent effect on microbial diversity and that it was restored 14–16 weeks after the application of high doses (40–70 g/m^2) [15].

MS is a liquid fumigant that generates the pesticidal compound methyl isothiocyanate (MITC) in soil. In China, MS is registered for use in the production of cucumber, tomato, tobacco and other crops to control mainly RKN, Sprout Tumble (plant diseases at seedling stage caused by *Pythium*, *Phytophthora*, *Rhizoctonia* and other fungi) and annual weeds [8]. Previous research reported that MS fumigation reduced pathogenic soil bacterial diversity and population size [16]. However, some genera beneficial to plants such as *Bacillus* and *Xanthomonas* increased significantly [16]. MS, therefore, not only reduced pathogenic fungi but also strengthened the antagonistic effect of bacteria against pathogenic fungi and indirectly enhanced the activity of some beneficial bacteria [17].

DMDS is a new type of biogenic soil fumigant [8]. Fumigation with a dose of 170 mg/kg did not reduce microbial diversity and promoted the dominance of some species. However, 14 days after DMDS fumigation, the soil microorganisms gradually recovered to the same level as the control [18].

This study examined changes in soil microbial populations when fumigated with PIC, MS and DMDS simultaneously in order to clarify the effects on soil-borne bacteria and fungi in different soils by triple fumigation and provide theoretical guidance on its application.

2. Materials and Methods

2.1. Fumigants

This study employed 99.5% chloropicrin, Dalian Lvfeng Chemical Co., Ltd. (Dalian, China); 42% Metham sodium Aqueous solutions, Jiangsu Limin Soil Remediation Co., Ltd. (Jiangsu, China).; 99% Dimethyl disulfide primary liquid, Zhejiang Linhai Jianxin Chemical Co., Ltd. (Zhejiang, China).

In the indoor fumigation test, low doses of three fumigants were used, 152.7 µL (equivalent to weight consumption 50 g/m^2) of MS were pipetted onto the soil followed immediately by 51.3 µL (equivalent to weight consumption 40g/m^2) of DMDS and 51.3 µL (equivalent to weight consumption 40 g/m^2) of PIC (Table 1.)

Table 1. Doses of fumigants used in laboratory experiments.

Fumigant (Abbreviation)	Laboratory Dose	Equivalent to Weight Consumption	Main Use
Chloropicrin (PIC)	51.3 µL	40 g/m^2	Broad-spectrum antimicrobial, fungicide, herbicide, nematicide and insecticide
Metham sodium (MS)	152.7 µL	40 g/m^2	Pesticide, herbicide, and fungicide.
Dimethyl disulfide (DMDS)	51.3 µL	50 g/m^2	Control of nematodes, weeds and soil-borne plant pathogens

2.2. Soil Physicochemical Properties

The soil for this study was taken from continuous cropping soil in five different regions of three provinces. The soil type of Fangshan (Beijing) is cinnamon soil (69.2% sand, 4.0% clay and 26.8% silt) with pH of 7.1, electrical conductivity of 918.00 (μm/cm) and organic matter 33.4g/kg. A-N, P and K were 4.3, 242.8 and 215.8 mg/kg soil, respectively. The soil type of Shunyi (Beijing) is also cinnamon soil (58.28% sand, 4.86% clay and 29.82% silt) with pH of 8.26, electrical conductivity of 175.00 (μm/cm) and organic matter 16.4g/kg. A-N, P and K were 5.16, 85.00and 142.00 mg/kg soil, respectively. Daxing (Beijing) is the main producing area of watermelon in Beijing and the soil type is mainly sandy soil (58.28% sand, 4.86% clay and 36.86% silt) with pH of 8.42, electrical conductivity of 229.00 (μm/cm) and organic matter 30g/kg. A-N, P and K were 2.55, 62.50 and 165.00 mg/kg soil, respectively. Wenshan (Yunnan) is the main planting area of Panax notoginseng. The soil types are mainly Yellowish-brown lateritic soil and red earths (12.03% sand, 14.23% clay and 73.74% silt) with pH of 5.5, electrical conductivity of 681.00 (μm/cm) and organic matter 68g/kg. A-N, P and K were 15.8, 61.00 and 255.00 mg/kg soil, respectively. Xinxiang (Henan) is one of the major provinces for ginger planting, and the soil type is mainly fluvo-aquic soil (8.32% sand, 12.78% clay and 78.9% silt) with pH of 8.3, electrical conductivity of 474.00 (μm/cm) and organic matter 8g/kg. A-N, P and K were 8.5, 41.00 and 155.00 mg/kg soil, respectively (Table 2).

Table 2. Physical and chemical properties of soil.

Soil	Sand	Clay	Silt	A-N	K	P	OM	ES	PH
					mg/kg		g/kg	μm/cm	1:2.5
Fangshan, Beijing (N 39°6″, E 115°9″)	69.2	4.0	26.8	4.3	215.8	242.8	33.4	918	7.1
Shunyi, Beijing (N 40°13″, E 116°65″)	66.65	3.53	29.82	5.16	142	85	16.4	175	8.26
Daxing, Beijing (N 39°73″, E 116°33″)	58.28	4.86	36.86	2.55	165	62.5	30	229	8.42
Wenshan, Yunnan (N 23°06″, E 103°43″)	12.03	14.23	73.74	15.8	255	61	68	681	5.5
Xinxiang, Henan (N 35°31″, E 113°85″)	8.32	12.78	78.9	8.5	155	41	8	474	8.3

2.3. Triple Fumigation Method

Soils from a depth of 5–20 cm were collected from multi-year continuous cropping plots in Beijing that were used to produce tomatoes (Fangshan district, enclosed facility), cucumber (Shunyi district, enclosed facility), watermelon (Daxing district, field), ginseng (Yunnan field and Yunnan-Wenshan field) and ginger (Henan province, field). Plant residues, stones and other materials were manually removed from the soils. The soils were passed through a 2 mm sieve [19]. The water content was adjusted to 60% with sterilized water. The soils were placed in labelled plastic bags and stored in the dark at 25 °C for one week before use [20].

Precisely 500g of soil were placed in a desiccator. Then, 152.7 μL of MS were pipetted onto the soil followed immediately by 51.3 μL of DMDS and 51.3 μL of PIC. The soil was quickly mixed and then the lid was sealed onto the desiccator using Vaseline™. The desiccator was stored at room temperature at about 28 °C in the dark for 10 d prior to removal of the cover and aeration for 3 d [20].

Twelve treatments were established. Treatments (1)–(6) comprised the triple fumigation. Treatments (7)–(12) were the controls for the triple fumigation. The treatments were (1) FS_ T: combined fumigation of Fangshan soil used to produce tomato; (2) SY_ T: combined fumigation of Shunyi soil used to produce cucumber; (3) DX_ T: combined fumigation of Daxing soil used to produce watermelon; (4) YN1_ T: combined fumigation

of Yunnan soil used to produce ginseng; (5) YN2_ T: combined fumigation of Yunnan (Wenshan) soil used to produce ginseng; (6) HN_ T: combined fumigation of Henan province soil used to produce ginger; (7) FS_ CK: unfumigated Fangshan soil with 255.3 µL sterile water; (8) SY_ T: unfumigated Shunyi soil with 255.3 µL sterile water; (9) DX_ CK: unfumigated Daxing soil with 255.3 µL sterile water; (10) YN1_ CK: unfumigated Yunnan soil with 255.3 µL sterile water added; (11) YN2_ CK: unfumigated Wenshan/Yunnan soil with 255.3 µL sterile water added; (12) HN_ CK: unfumigated Henan soil with 255.3 µL sterile water added. Each treatment was replicated three times. About 1 g of soil from each desiccator was refrigerated at −80 °C for later analysis.

2.4. DNA Extraction, PCR Amplification, and High-Throughput Sequencing

Total genomic DNA was extracted from 0.25 g of soil using the method provided in the DNeasy®PowerSoil®Pro Kit (Qiagen Com., USA). The extracted DNA was verified by 0.1% (*w*/*v*) agarose gel electrophoresis, and the concentration and purity of the DNA was determined using a NanoDrop™ 2000 UV-Visible spectrophotometer (Thermo Fisher Scientific, USA). To monitor taxonomic changes in the bacteria and fungi in each soil sample, we used two primers, 338F(5′-ACTCCTACGGGAGCAGCAG-3′)-806R(5′-GGATACHGGGTWTCTAAT-3′) [21] and ITS1F(5′-CTTGGTCATTTAGGAAGTAA-3′)-ITS2R(5′-GCTGCGTTCTTCATCATGATGC-3′) [22], to target the bacterial 16S rRNA and fungal ITS regions, respectively.

PCR amplification reactions were simultaneously carried out in three 20 µL mixtures consisting of 4 µL of 5 × FastPfu Buffer (16S v3–v4)/2 mL of 10 × Buffer (ITS), 2 µL dNTPs, 0.8 µL each of forward and reverse primers, 0.4 µL of FastPfu Polymerase (16S v3–v4)/0.2 µL of rTaq Polymerase (ITS), 0.2 µL of BSA and 10 ng of template DNA [23].

The PCR amplification conditions used were: initial denaturation at 95 °C for 3 min, followed by 28 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 45 s and a final extension at 72 °C for 10 min [23].

PCR products were detected by gel electrophoresis (2% agarose) and further purified by AxyPrep™ DNA Gel Extraction Kit (Axygen BioSciences Inc., USA), following the manufacturer's instructions. The recovered and purified PCR products were sequenced by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) using the MiSeq™ System (Illumina Inc., USA).

2.5. Statistical Analysis of Data

The original 16S rRNA gene sequences were demultiplexed and quality-filtered with Trimmomatic (Dell Laboratory, USA). Truncated reads shorter than 50 bp and those containing ambiguous characters were discarded. The reserved sequences were analyzed using the UPARSE pipeline to cluster Operational Taxonomic Units (OTUs) containing representative sequences and compared with the Silva 16S rRNA Database at a confidence threshold of 0.7. All samples were extracted according to the minimum number of sample sequences. A one-way analysis of variance (ANOVA) and Duncan's multiple range test were performed on the means of each treatment. Additional correlation analyses were performed using SPSS v25.0 statistical software (IBM, USA).

3. Results

3.1. Analysis of Changes to Species Diversity and Species Richness

Chao1 and ACE indices were used to indicate changes in bacterial and fungal species richness, while the Shannon and Simpson indices were used to indicate changes in bacterial and fungal species diversity; biodiversity is positively correlated with Shannon, ACE and Chao1 index, and negatively correlated with Simpson index in response to triple fumigation of soil that had been used to grow tomatoes, watermelon, cucumber, ginseng and ginger.

3.1.1. Bacterial and Fungal Taxonomic Changes in Triple-Fumigated Tomato Soil

Compared with the control, there was no significant difference in the Simpsom, Shannon, Ace and Chao1 of fungal and bacterial microbial communities after triple fumigation of soil that had been used to grow tomatoes (Figure 1).

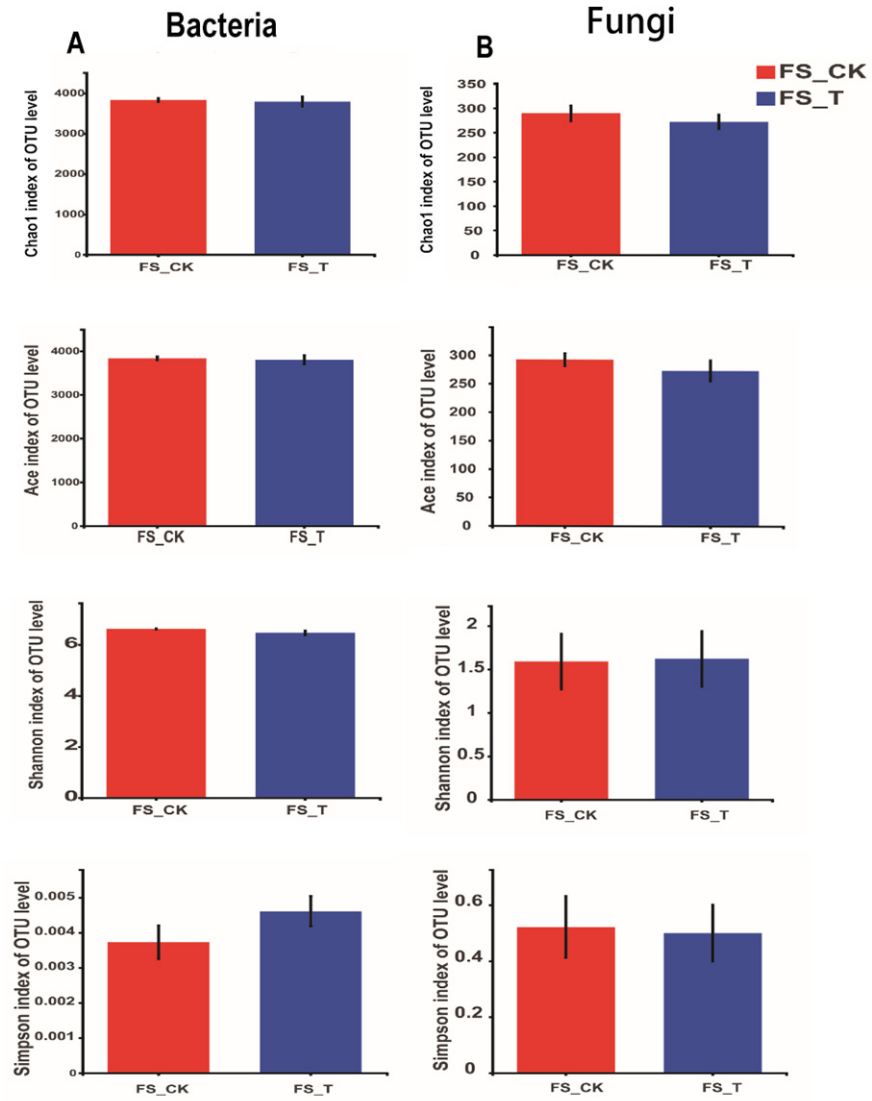


Figure 1. Chao1, ACE, Shannon and Simpson indices showing taxonomic changes in (**A**) bacteria and (**B**) fungi in triple-fumigated soil that had been used to produce tomatoes. The *t*-test reported the statistical differences (asterisked) between the means of three replicates within each graph. 'Triple fumigation' refers to the simultaneous application of chloropicrin, dimethyl disulfide and metham sodium.

3.1.2. Bacterial and Fungal Taxonomic Changes in Triple-Fumigated Watermelon Soil

Compared with the control, Ace and Chao1 in the soil of continuous cropping watermelon after triple fumigation decreased significantly, and Simpson and Shannon indexes did not change significantly; therefore, the species diversity did not change significantly, but the species abundance decreased significantly (Figure 2).

3.1.3. Bacterial and Fungal Taxonomic Changes in Triple-Fumigated Cucumber Soil

Bacterial and fungal species richness was unchanged by triple fumigation of soil that had been used to grow cucumber; however, bacterial and fungal species diversity was reduced in bacteria, and both increased and reduced in fungi (Figure 3).

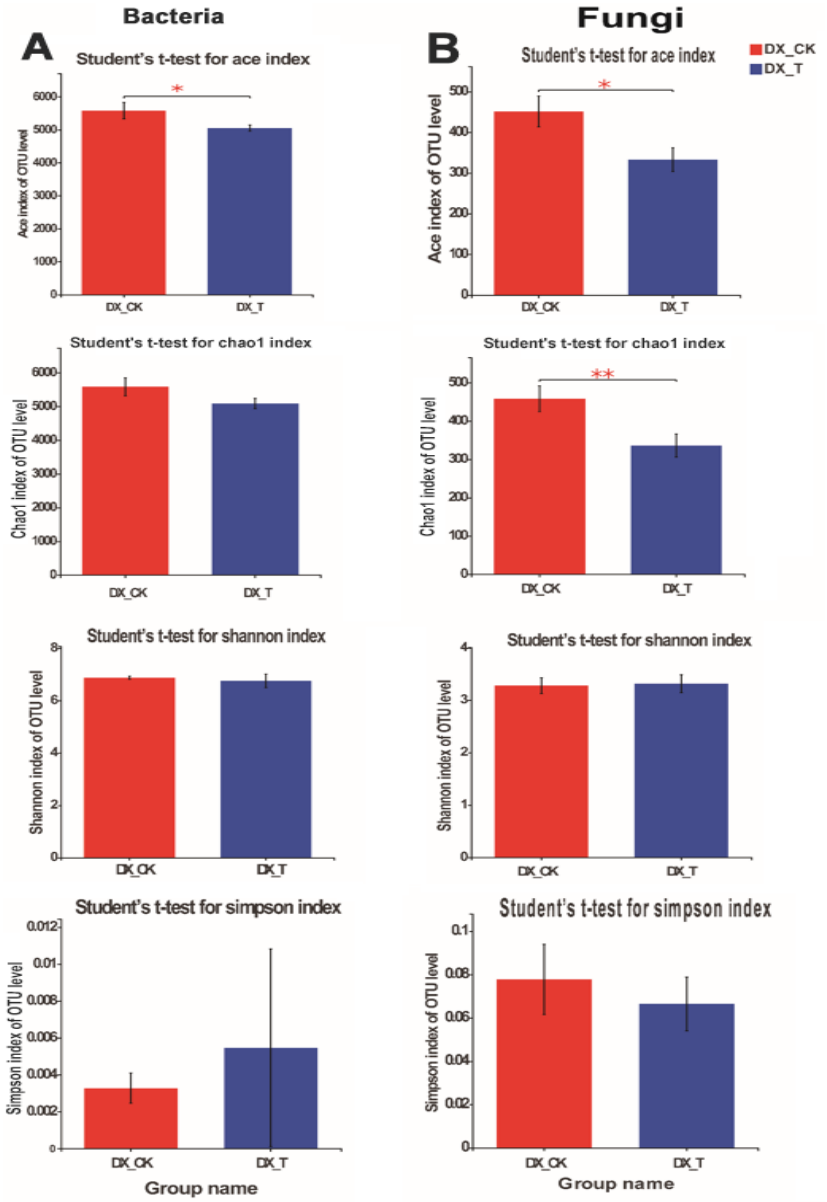


Figure 2. Chao1, ACE, Shannon and Simpson indices showing taxonomic changes in (**A**) bacteria and (**B**) fungi in triple-fumigated soil that had been used to produce watermelon. 'Triple fumigation' refers to the simultaneous application of chloropicrin, dimethyl disulfide and metham sodium. The *t*-test reported the statistical differences (asterisked, * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$) between the means of three replicates within each graph.

3.1.4. Bacterial and Fungal Taxonomic Changes in Two Triple-Fumigated Soil Types Used to Produce Ginseng

Bacterial species richness was reduced in both soil types used to grow ginseng; however, fungal species richness was reduced in one soil type (Figure 4). Bacterial species diversity was reduced in both soil types used to grow ginseng, but fungal species diversity remained unchanged in both soil types.

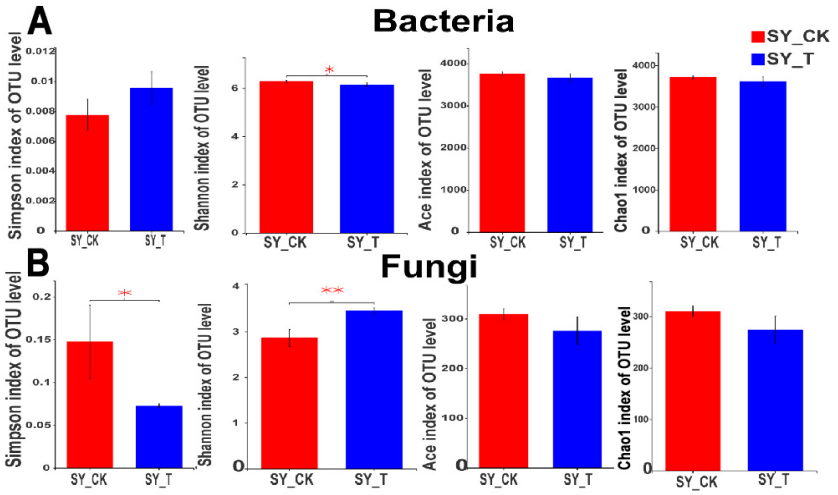


Figure 3. Chao1, ACE, Shannon and Simpson indices showing taxonomic changes in (**A**) bacteria and (**B**) fungi in triple-fumigated soil that had been used to produce cucumber. 'Triple fumigation' refers to the simultaneous application of chloropicrin, dimethyl disulfide and metham sodium. The *t*-test reported the statistical differences (asterisked, * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$.) between the means of three replicates within each graph.

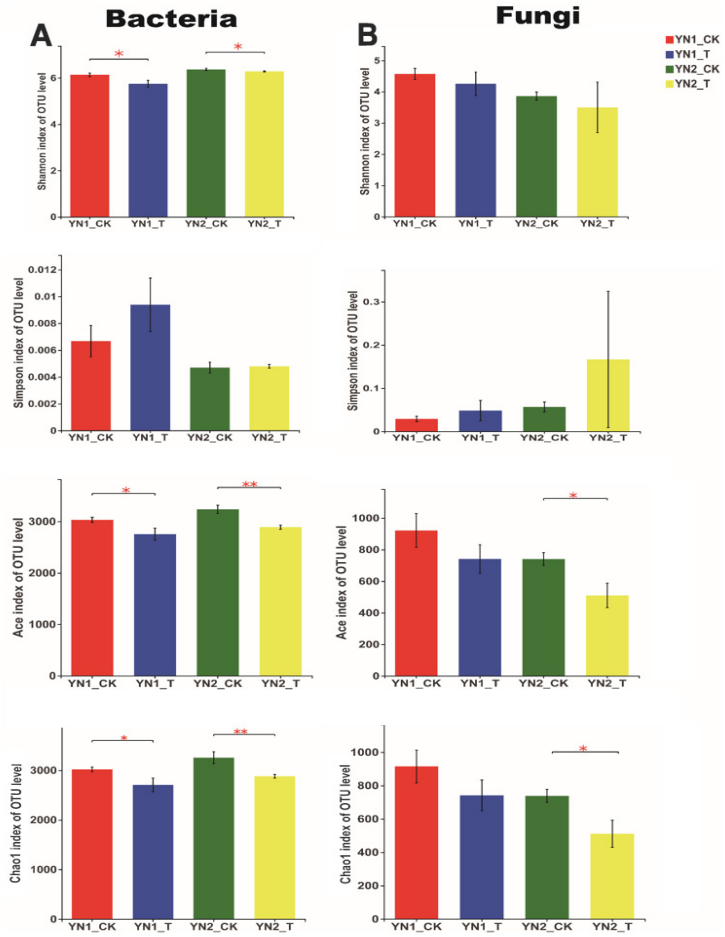


Figure 4. Chao1, ACE, Shannon and Simpson indices showing taxonomic changes in (**A**) bacteria and (**B**) fungi in triple-fumigated soil types (YN1 and YN2) that had been used to produce ginseng. 'Triple fumigation' refers to the simultaneous application of chloropicrin, dimethyl disulfide and metham sodium. The *t*-test reported the statistical differences (asterisked, * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$.) between the means of three replicates within each graph.

3.1.5. Bacterial and Fungal Taxonomic Changes in Triple-Fumigated Soil Used to Produce Ginger

Bacterial species richness and diversity were reduced by triple fumigation of soil that had been used to grow ginger; however, fungal species richness and diversity remained unchanged (Figure 5).

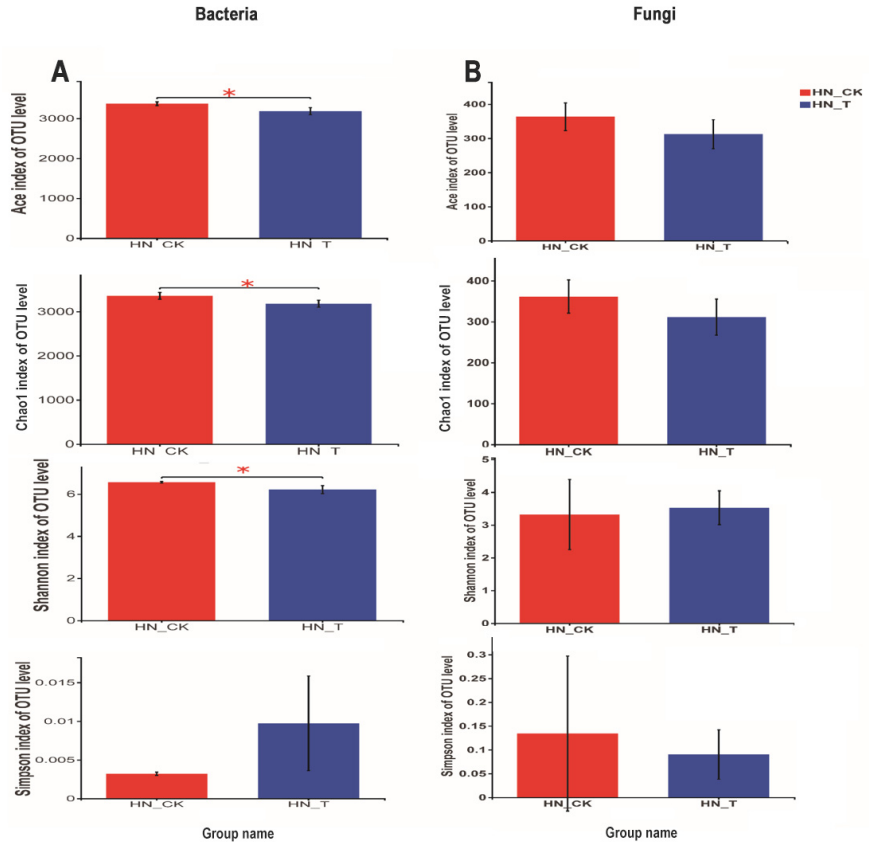


Figure 5. Chao1, ACE, Shannon and Simpson indices showing taxonomic changes in (**A**) bacteria and (**B**) fungi in triple-fumigated soil that had been used to produce ginger. 'Triple fumigation' refers to the simultaneous application of chloropicrin, dimethyl disulfide and metham sodium. The *t*-test reported the statistical differences (asterisked, * $0.01 < p \leq 0.05$) between the means of three replicates within each graph.

3.2. Bacterial Community Taxonomic Analysis

The student's *t*-test was used to determine the statistical significance of relative changes at the phylum and genus levels in bacteria present in triple-fumigated soil that had been used to grow tomatoes, watermelon, cucumber, ginseng and ginger.

3.2.1. Bacterial Taxonomic Change in Triple-Fumigated Soil Used to Produce Tomatoes

The relative abundance of *Bacteroidota* and *Elusimicrobiota* phyla in soil that had been used to produce tomatoes was significantly decreased after triple fumigation (Figure 6). The relative abundance of *Bacillus*, *Virgibacillus* and *Lysinibacillus* genera increased significantly after triple fumigation, whereas the genus *Haloactinopolyspora* decreased signficantly.

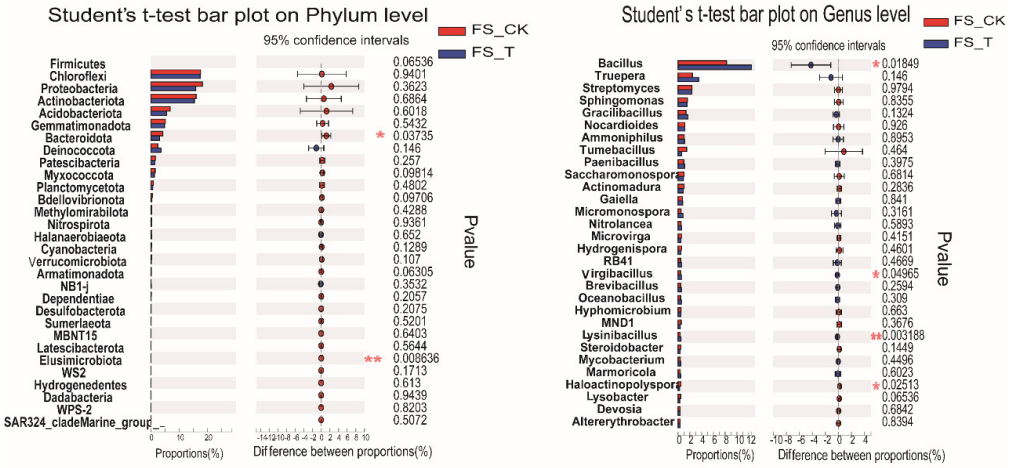


Figure 6. Changes in the relative abundance of bacterial (**A**) phyla and (**B**) genera in triple-fumigated soil that had been used to produce tomatoes. 'Triple fumigation' refers to the simultaneous application of chloropicrin, dimethyl disulfide and metham sodium. The *t*-test reported the statistical differences (asterisked, * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$) between the means of three replicates within the phylum or genus levels.

3.2.2. Bacterial Taxonomic Change in Triple-Fumigated Soil Used to Produce Watermelon

The relative abundance of *Firmicutes* phylum in soil that had been used to produce watermelons was increased significantly after triple fumigation; however, the relative abundance of *Myxococcota*, *Nitrospirota*, *Methylomirabilota* and *Bdellovibrionota* decreased significantly (Figure 7). The relative abundance of *Bacillus* genus increased significantly after triple fumigation, whereas *Nitrospira*, *Bryobacter* and *Galbitalea* genera decreased significantly.

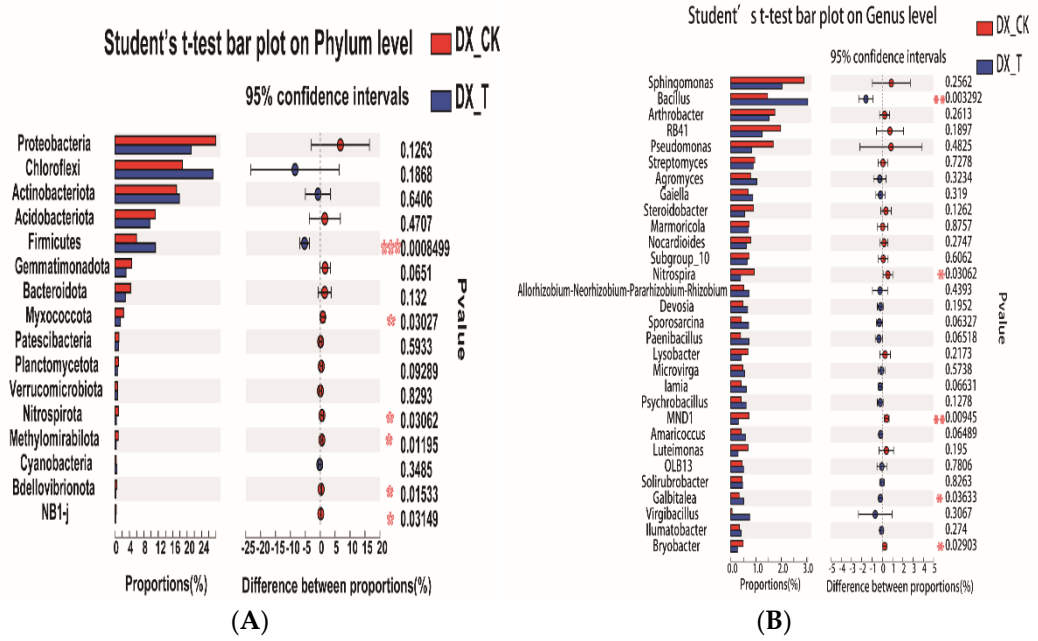


Figure 7. Changes in the relative abundance of bacterial (**A**) phyla and (**B**) genera in triple-fumigated soil that had been used to produce watermelon. 'Triple fumigation' refers to the simultaneous application of chloropicrin, dimethyl disulfide and metham sodium. The *t*-test reported the statistical differences (asterisked, * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$.) between the means of three replicates within the phylum or genus levels.

3.2.3. Bacterial Taxonomic Change in Triple-Fumigated Soil Used to Produce Cucumber

The relative abundance of *Actinobacteriota*, *Bacteroidetes* and *Nitrospirobacteria* phyla in soil that had been used to produce cucumber were reduced significantly after triple fumigation (Figure 8). The relative abundance of *Paenibacillus* genus increased significantly after triple fumigation, whereas *Steroidobacter*, *Bryobacter*, *Nitrospira*, *Nocardioides*, *Mycobacterium*, *Gemmatimonas* and *Streptomyces* genera decreased significantly.

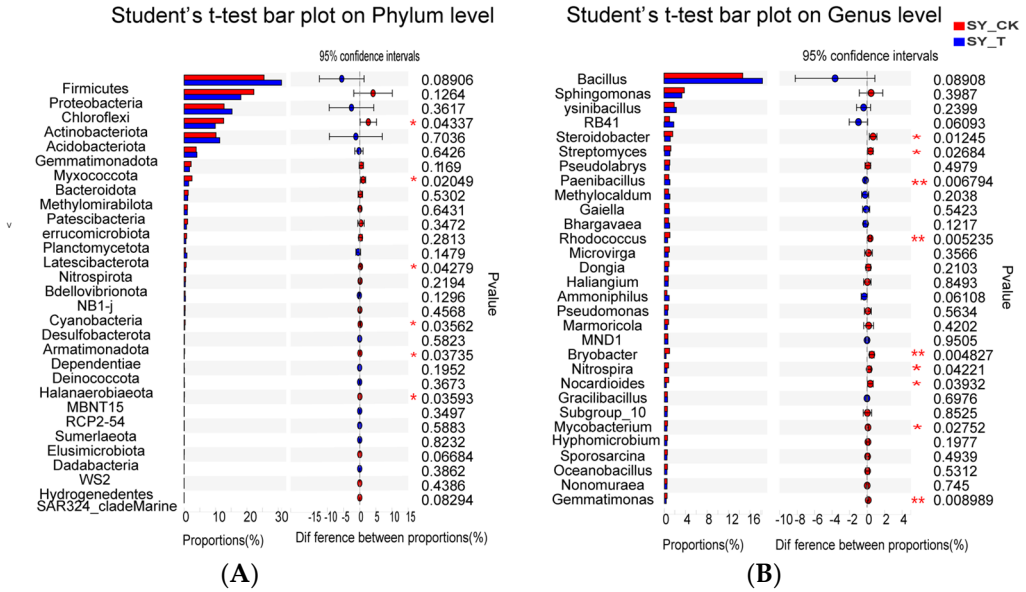


Figure 8. Changes in the relative abundance of bacterial (**A**) phyla and (**B**) genera in triple-fumigated soil that had been used to produce cucumber. 'Triple fumigation' refers to the simultaneous application of chloropicrin, dimethyl disulfide and metham sodium. The *t*-test reported the statistical differences (asterisked, * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$) between the means of three replicates within the phylum or genus levels.

3.2.4. Bacterial Taxonomic Change in Two Types of Triple-Fumigated Soil Used to Produce Ginseng

The relative abundance of *Actinobacteria*, *Chloroflexi* and *Firmicutes* phyla in YN1 soil used to grow ginseng increased significantly after triple fumigation, while the relative abundance of *Proteobacteria* and *Acidobacteria* phyla decreased significantly (Figure 9A). The relative abundance of *Chloroflexi* phylum in YN2 soil used to grow ginseng increased significantly after triple fumigation, whereas *Patescibacteria*, *Myxococcota*, *Bacteroidota*, *Armatimonadota*, *Errucomicrobiota*, *Nitrospirota* and *Patescibacteria* phyla decreased significantly (Figure 9B).

The relative abundance of *Acidothermus* and *Conexibacter* genera in YN1 soil used to grow ginseng increased significantly after fumigation, whereas *Sphingomonas*, *Bradyrhizobium*, *Bryobacter* and *Pseudolaris* phyla decreased significantly (Figure 9C). The relative abundance of *Gaiella*, *Conexibacter*, *Bacillus*, *Acidothermus* and *Paenibacillus* genera in YN2 soil increased significantly after fumigation, while *Bradyrhizobium* decreased significantly (Figure 9D).

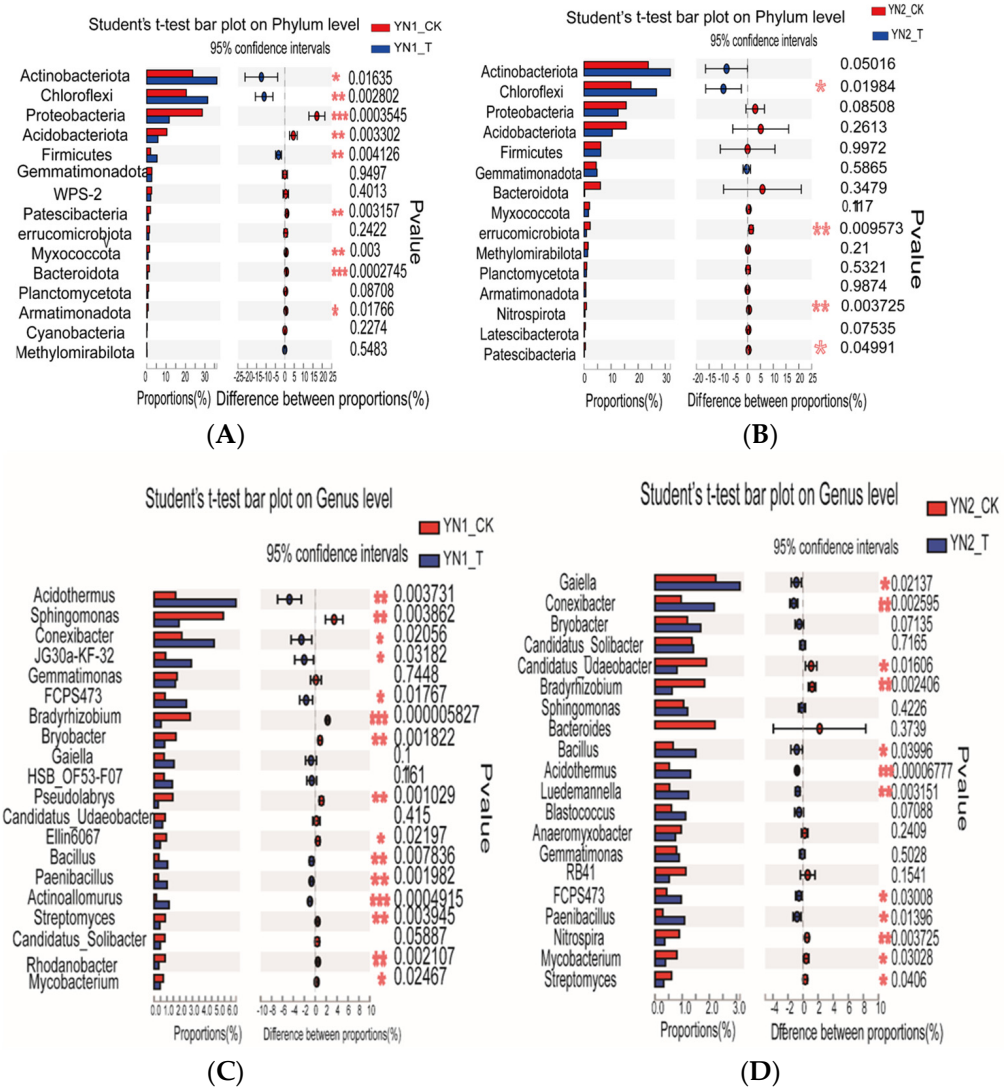
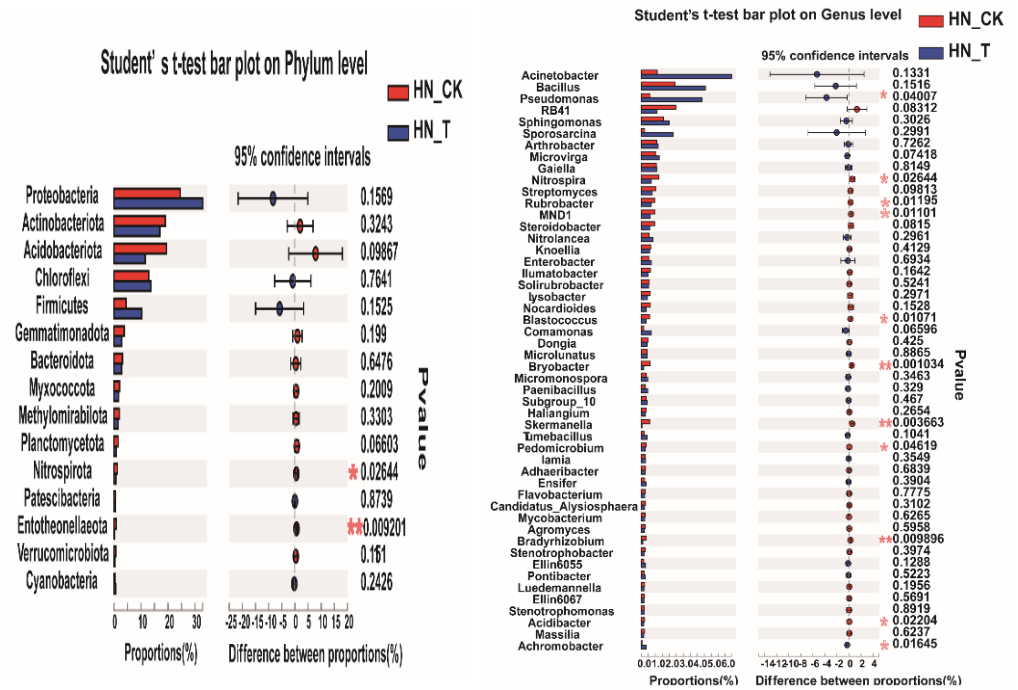


Figure 9. Changes in the relative abundance of (**A**) bacterial phyla in YN1 soil; (**B**) bacterial phyla YN2 soil; (**C**) bacterial genera in YN1 soil; (**D**) bacterial genera in YN2 soil. All soils were triple-fumigated and had been used to produce ginseng. 'Triple fumigation' refers to the simultaneous application of chloropicrin, dimethyl disulfide and metham sodium. The *t*-test reported the statistical differences (asterisked, * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$) between the means of three replicates within the phylum or genus levels.

3.2.5. Bacterial Taxonomic Change in Triple-Fumigated Soil Used to Produce Ginger

The relative abundance of *Nitrospirota* and *Entheonellaeota* phyla in soil that had been used to produce ginger were reduced significantly after triple fumigation (Figure 10). The relative abundance of *Pseudomonas* and *Achromobacter* genera increased significantly after triple fumigation, whereas *Rubrobacter*, *Blastococcus*, *Bryobacter*, *Skermanella*, *Pedomicrobium*, *Bradyrhizobium* and *Acidobacter* genera decreased significantly.



(**A**) (**B**)

Figure 10. Changes in the relative abundance of bacterial (**A**) phyla and (**B**) genera in triple-fumigated soil that had been used to produce ginger. 'Triple fumigation' refers to the simultaneous application of chloropicrin, dimethyl disulfide and metham sodium. The *t*-test reported the statistical differences (asterisked, * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$) between the means of three replicates within the phylum or genus levels.

3.3. Fungal Community Taxonomic Analysis

The student's *t*-test was used to determine the statistical significance of relative changes at the phylum and genus levels in fungi present in triple-fumigated soil that had been used to grow tomatoes, watermelon, cucumber, ginseng and ginger.

3.3.1. Fungal Taxonomic Change in Triple-Fumigated Soil Used to Produce Tomatoes

The relative abundance of *Mortierella* phylum in soil that had been used to produce tomatoes was significantly decreased after triple fumigation (Figure 11). The relative abundance of *Gymnacella*, *Microaccus*, *Arthrographis* and *Arthroderma* genera in soil that had been used to produce tomatoes increased significantly after triple fumigation, whereas *Mortierella*, *Neocosmospora* and *Fusarium* genera decreased significantly.

3.3.2. Fungal Taxonomic Change in Triple-Fumigated Soil Used to Produce Watermelon

There were no significant differences in the dominant fungal phyla present in soil used to produce watermelon before and after triple fumigation, whereas the relatively small population of *Rozellomycota* fungal phylum increased significantly (Figure 12). The relative abundance of *Microaccus*, *Acaulium* and *Acremonium* fungal genera increased significantly after triple fumigation in soil that had been used to produce watermelon. The relatively small populations of *Cephaliophora* and *Alternaria* fungal genera decreased significantly after triple fumigation. The relatively small population of the fungus *Cephalotrichum* increased significantly after triple fumigation.

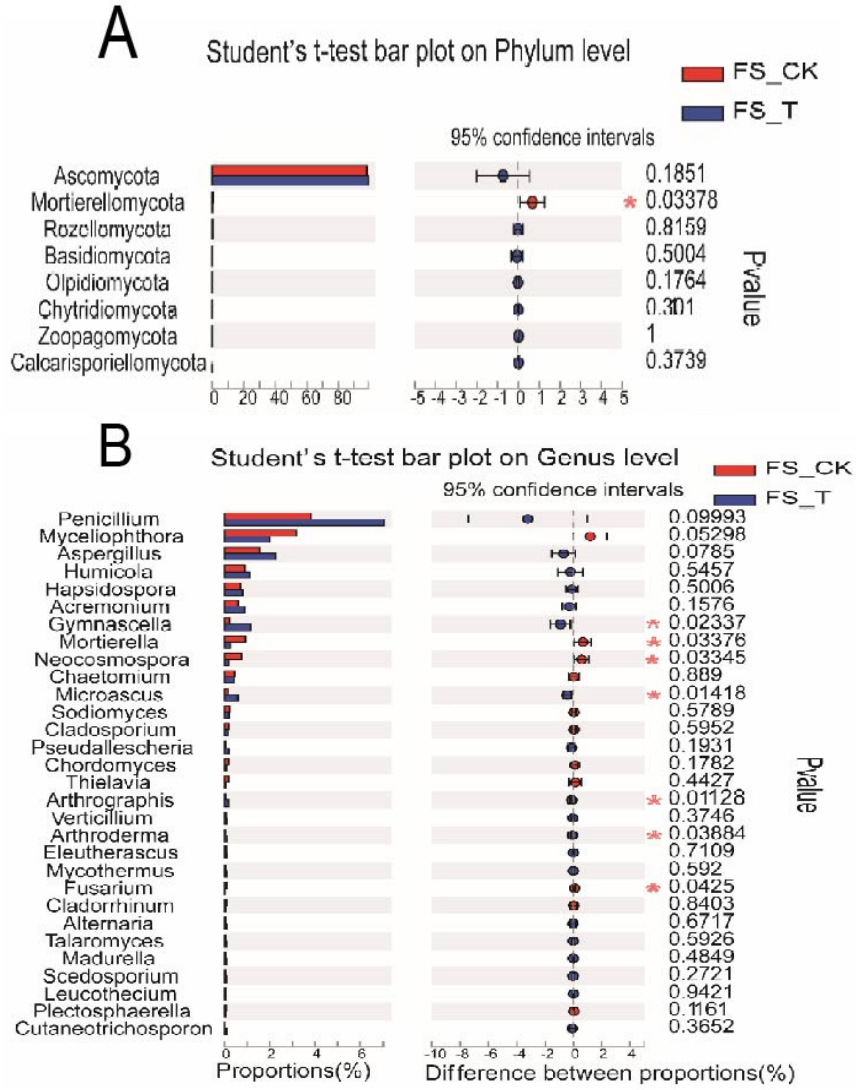
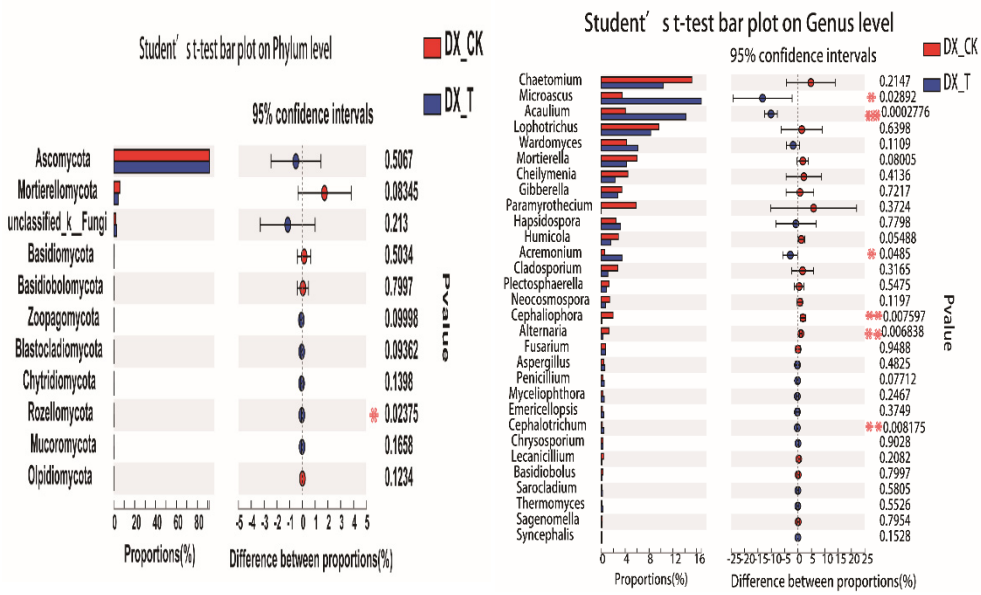


Figure 11. Changes in the relative abundance of fungal (**A**) phyla and (**B**) genera in triple-fumigated soil that had been used to produce tomatoes. 'Triple fumigation' refers to the simultaneous application of chloropicrin, dimethyl disulfide and metham sodium. The *t*-test reported the statistical differences (asterisked, * $0.01 < p \leq 0.05$) between the means of three replicates within the phylum or genus levels.

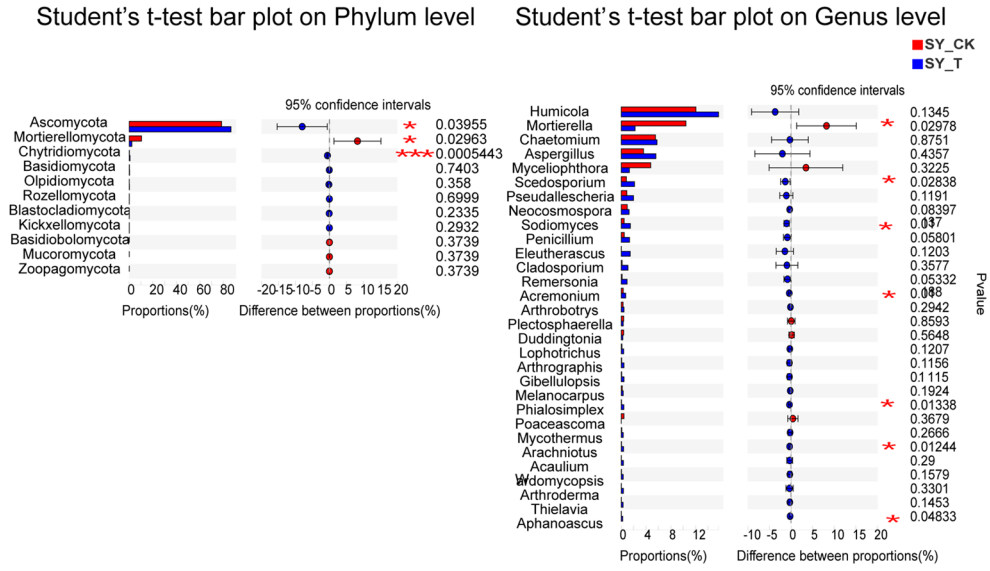
3.3.3. Fungal Taxonomic Change in Triple-Fumigated Soil Used to Produce Cucumber

The relative abundance of *Mortierellomycota* fungal phylum in triple-fumigated soil that had been used to produce cucumber decreased significantly, whereas the relative abundance of *Ascomycota* and *Chytridiomycota* fungal phyla in triple-fumigated soil that had been used to produce cucumber increased significantly (Figure 13). The relative abundance of *Mortierella* fungal genus in triple-fumigated soil that had been used to produce cucumber decreased significantly, whereas the relative abundance of *Scedosporium*, *Sodiomyces*, *Acremonium*, *Arachnotus* and *Aphanoascus* fungal genera increased significantly.



(**A**) (**B**)

Figure 12. Changes in the relative abundance of fungal (**A**) phyla and (**B**) genera in triple-fumigated soil that had been used to produce watermelon. 'Triple fumigation' refers to the simultaneous application of chloropicrin, dimethyl disulfide and metham sodium. The *t*-test reported the statistical differences (asterisked, * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$) between the means of three replicates within the phylum or genus levels.



(**A**) (**B**)

Figure 13. Changes in the relative abundance of fungal (**A**) phyla and (**B**) genera in triple-fumigated soil that had been used to produce cucumber. 'Triple fumigation' refers to the simultaneous application of chloropicrin, dimethyl disulfide and metham sodium. The *t*-test reported the statistical differences (asterisked, * $0.01 < p \leq 0.05$; *** $p \leq 0.001$.) between the means of three replicates within the phylum or genus levels.

3.3.4. Fungal Taxonomic Change in Two Types of Triple-Fumigated Soil Used to Produce Ginseng

The relative abundance of the dominant fungal phylum *Basidiomycota* in triple-fumigated YN1 soil that had been used to produce ginseng, and the dominant phylum *Ascomycota* in YN2 soil that had also been used to produce ginseng, both increased significantly

(Figure 14). However, the relative abundance of the fungal phylum *Mortierellomycota* in both YN1 and YN2 soil decreased significantly.

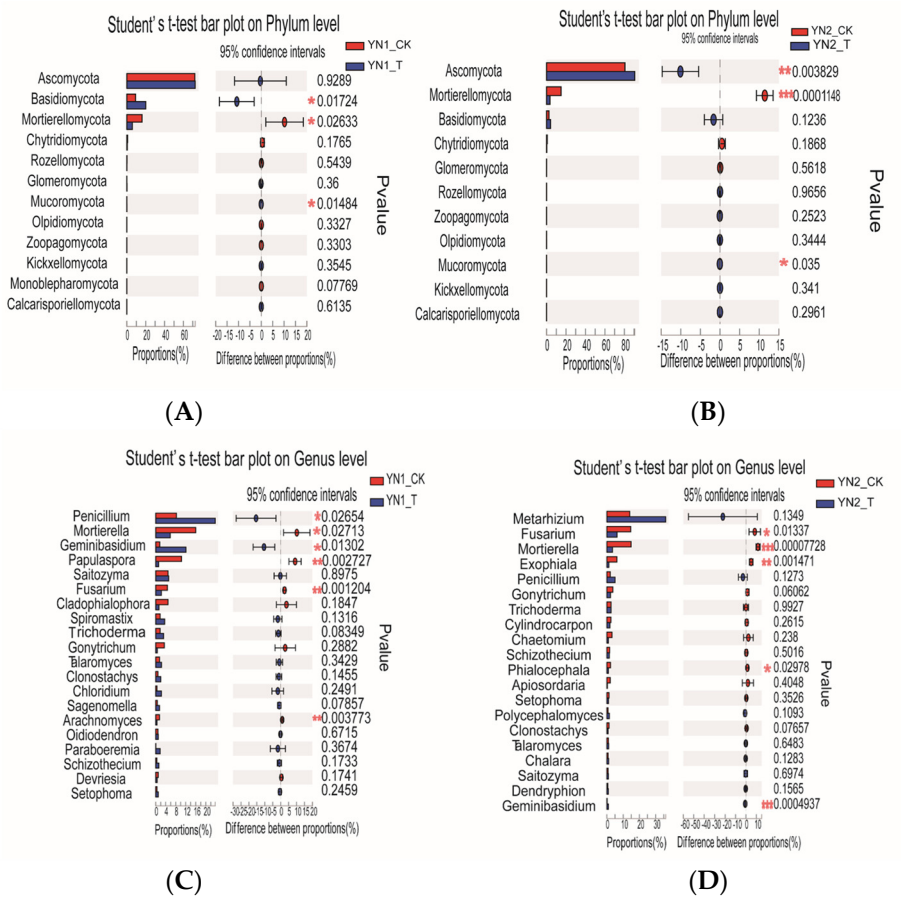


Figure 14. Changes in the relative abundance of (**A**) fungal phyla in YN1 soil; (**B**) fungal phyla YN2 soil; (**C**) fungal genera in YN1 soil; (**D**) fungal genera in YN2 soil. All soils were triple-fumigated and had been used to produce ginseng. 'Triple fumigation' refers to the simultaneous application of chloropicrin, dimethyl disulfide and metham sodium. The *t*-test reported the statistical differences (asterisked, * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$.) between the means of three replicates within the phylum or genus levels.

The relative abundance of the dominant genera *Penicillium* and *Geminibasidium* fungi in triple-fumigated YN1 soil that had been used to produce ginseng increased significantly, while *Mortierella*, *Papulaspora* and *Fusarium* fungal genera in triple-fumigated YN1 soil decreased significantly. The relative abundance of *Fusarium*, *Mortierella* and *Exopiala* fungal genera in triple-fumigated YN2 soil that had been used to produce ginseng decreased significantly.

3.3.5. Fungal Taxonomic Change in Triple-Fumigated Soil Used to Produce Ginger

There were no significant differences in the dominant fungal phyla present in soil used to produce ginger before and after triple fumigation (Figure 15). The fungal genus *Gibberella* that was present at a low abundance in triple-fumigated soil that had been used to produce ginger decreased significantly after fumigation, while the fungal genera *Aspergillus* and *Trichoderma* increased significantly.

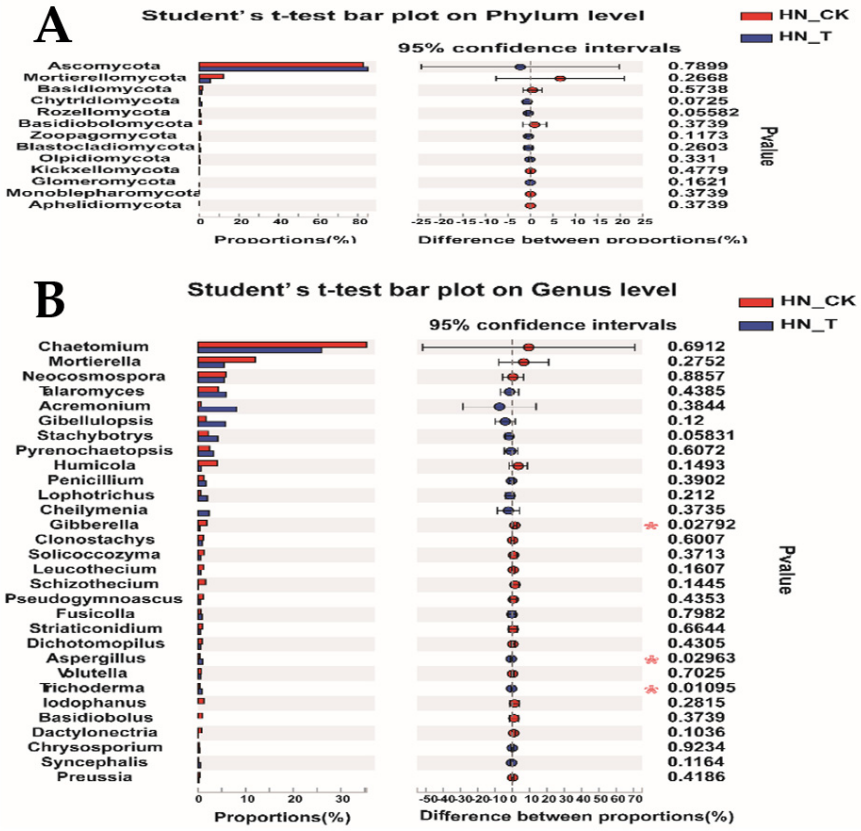


Figure 15. Changes in the relative abundance of fungal (**A**) phyla and (**B**) genera in triple-fumigated soil that had been used to produce ginger. 'Triple fumigation' refers to the simultaneous application of chloropicrin, dimethyl disulfide and metham sodium. The *t*-test reported the statistical differences (asterisked, * $0.01 < p \leq 0.05$) between the means of three replicates within the phylum or genus levels.

4. Discussion

In this experiment, the different soils collected from five provinces were all sites where soil-borne diseases were extremely serious, including many diseases caused by fungi, bacteria and nematodes. Low doses of three fumigants were used at the same time, which effectively expands the control spectrum of the fumigant, improves the control effect on pathogens, saves time, and enhances the potential disease resistance of the soil by increasing the abundance of beneficial bacteria. Ren et al. reported that, compared with the binary combination of Pic and MS, Pic and DMDS and the use of single fumigant, the ternary combination of Pic+MS+DMDS can maximize the control effect of ginger soil-borne diseases, reduce the rate of dead seedlings and increase the yield [24]. Zhang et al. reported that 1,3-dichloropropene and Pic in a ratio of 1:1 could treat pepper Phytophthora blight and root knot nematode simultaneously [25].This is similar to the results observed in this study. Multiple fumigants can reduce the number of soil-borne pathogens. The change in soil microbial community may be due to the toxicity of triple fumigation, which can kill most microorganisms, although a few resistant colonies survived. In the soil environment, those colonies then receive more living space and nutrients, and then multiply in large quantities, returning the soil to a balanced state.

4.1. Changes in Bacterial and Fungal Species Richness and Diversity

The results showed that species richness and diversity in response to triple fumigation varied according to the soil type. For example, there were no statistical differences in bacterial or fungal species richness or diversity in response to triple fumigation of soil that had been used to grow tomatoes. However, bacterial and fungal species richness was reduced by triple fumigation of soil that had been used to grow watermelon; however,

bacterial and fungal species diversity remained unchanged. Bacterial species richness was reduced in both YN1 and YN2 soil types that were used to grow ginseng (Figure 9); however, fungal species richness was reduced in one soil type (Figure 14). Bacterial species diversity, however, was reduced in both YN1 and YN2 soil types used to grow ginseng, whereas fungal species diversity remained unchanged in both soil types.

The variability in the bacterial and fungal response to triple fumigation might be due to physicochemical differences in the composition of the soils. Further investigation is required to determine the cause of the variable response.

4.2. Changes in Bacterial Taxonomic Composition in the Soil

The results showed that *Bacillus* and *Virgibacillus* bacteria increased significantly in soil used to grow tomatoes that was triple-fumigated. Previous research reported that *Bacillus* promoted the yield and quality of sweet potato [26]. *Bacillus amylolyticus* inhibited the population growth of the pathogenic fungus *Fusarium oxysporum* [27]. Haas and Defago (2005) reported a negative correlation between *Pseudomonas* and the abundance of soil-borne pathogens [28]. We observed that the relative abundance of *Pseudomonas* bacteria increased significantly after triple fumigation of soil used to produce ginger. Previous research reported that *Pseudomonas* may have contributed significantly to the observed reduction in soil-borne disease in response to PIC and biofumigation [29].

4.3. Changes in Fungal Taxonomic Composition in the Soil

The results showed changes in the fungal taxonomic composition in different types of soil that were triple fumigated. For example, the relative abundance of the genus *Fusarium* and *Neocosmospora* decreased in triple-fumigated soil that had been used to produce tomatoes. Although we did not identify it to the species level, previous research reported that *Fusarium oxysporum* is a typical and widespread soil-borne pathogen, which can adhere to the diseased body or survive in the soil for a long time through chlamydospores or hyphae, and spread through irrigation, causing vascular bundle disease of tomato and seriously affecting its yield and quality [19]. *Neocosmospora* is reported to cause peanut base rot at rates as high as 30% of the planted crop in severe infections [30].

It was discovered through data analysis that the fungal genus *Trichoderma*, which had a low abundance initially, actually increased significantly in triple-fumigated soil that had been used to produce ginger. *Trichoderma harzianum* is a beneficial fungus, widely existing in soil that has been commercialized for the control of soil-borne pests in greenhouses [31] and, in particular, Fusarium Wilt [32].

5. Conclusions

Through observation, increases and decreases were found in the biodiversity and richness of beneficial and pathogenic bacteria and fungi in response to triple fumigation of soils that had been used to grow tomatoes, watermelon, cucumber, ginseng and ginger with PIC, MS and DMDS. The application of PIC, MS and DMDS simultaneously ("triple fumigation") was used to control soil-borne pathogens because, when the three fumigants are used together, the complete prevention and control of complex and multiple pathogens can be realized once and for all. In this experiment, the low dosage of three fumigants used at one time effectively improved the control effect on pathogenic fungi such as *Fusarium oxysporum*, saving the control time, significantly increasing the abundance of beneficial species such as *Bacillus* and *Trichoderma* and improving the potential disease resistance of soil. Triple fumigation is an effective soil treatment method, which has the potential to be used in a variety of crops and protected land types.

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