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Investigating Changes in the Soil Fungal Community Structure, Functions, and Network Stability with Prolonged Grafted Watermelon Cultivation

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Abstract: Grafting is a commonly employed technique for enhancing the yield and improving resistance to biotic and abiotic stress of cultivated plants. However, whether and how continuous cropping of grafted plants affects the composition, function, and stability of the soil fungal community remain poorly understood. In this study, we investigated the effects of planting years (including 0 years (Y0), 2 years (Y2), 10 years (Y10), and 18 years (Y18)) of grafted watermelon on the structure and functional composition of the soil fungal community under field conditions. Compared with the Y0 soil, the Y2, Y10, and Y18 soils exhibited a significant ($p < 0.05$) decrease in the richness, Shannon index, and evenness (56.8–65.7%, 22.4–46.3%, and 3.8–38.1%, respectively) in the alpha diversity of the fungal community, but a significant ($p < 0.05$) increase (0.4–1.3 times) in the fungal population. The structure, core and unique microbiomes, and functional composition of the soil fungal community differed significantly across different planting years. The Y2, Y10, and Y18 soils exhibited significant increases ($p < 0.05$) in relative abundances of Ascomycota and saprophytic fungi and the proportion of core OTUs, but significantly decreased abundances of Basidiomycota, Chytridiomycota, Rozellomycota, pathogenic and symbiotic fungi, and the proportion of unique OTUs when compared with the Y0 soil. The types of potential plant pathogens and their relative abundance were also significantly increased alongside the planting years (among Y2, Y10, and Y18 soils). Furthermore, the results indicated that the continuous cropping of grafted watermelon altered the co-occurrence networks, leading to a reduction in the complexity and stability of the fungal community networks. Overall, our findings suggest that continuous cropping of grafted watermelon may adversely affect the structure and functioning of soil microbial community, eventually decreasing the effectiveness of grafting technology disease control.

Keywords: grafting cultivation; continuous cropping; microbial diversity; co-occurrence network

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1. Introduction

Watermelon (*Citrullus lanatus*) is a crucial horticultural crop with various economic benefits and high nutritional value. It is widely cultivated in East and Southeast Asia. China is among the chief watermelon producers, with an annual output of up to 60 million tons that accounts for approximately 60% of the global production [1]. Because of increasing demands and limited availability of arable areas, watermelon is usually cultivated in monoculture systems. However, this practice has made watermelon highly vulnerable to various soil-borne diseases, such as *Fusarium* wilt, leading to substantial economic losses worldwide [2,3]. Thus, mitigating the incidence of these diseases in watermelon plants has become a major challenge in horticulture.

Grafting is a promising technology for controlling soil-borne diseases. It has been successfully used to promote healthy growth of various economically crucial crops worldwide, such as melons, solanaceae family fruits, and fruit trees, significantly contributing

to the sustainable development of the horticultural industry [2,4,5]. In the 1970s, China started using grafting technology to control soil-borne diseases of watermelon, and by 2008, its application was extended to more than 20% of the watermelon cultivation areas [6]. The disease suppressive mechanism of grafting is attributable mainly to the utilization of disease-resistant rootstocks, which enable the scion to acquire the disease resistance genes from the rootstock plant, leading to the enhanced overall disease resistance of plants [7]. For example, many studies have reported the beneficial effects of using crops with strong resistance (such as pumpkin, winter melon, and gourd) as the rootstock in reducing the incidence of diseases such as *Fusarium* wilt, gummy stem blight, and root-knot nematode in watermelon [8–10]. However, according to some studies, long-term use of the same rootstock may lead to the development of resistance in soil-borne pathogens, thereby weakening the effectiveness of disease control [11,12]. For instance, continuous planting of *Meloidogyne*-resistant tomato rootstocks over three consecutive seasons increased *Meloidogyne* population density in the soil [12]. Moreover, our previous fieldwork showed that the severity of soil-borne diseases of grafted watermelon increased with the increasing planting years. Overall, these results indicate that the long-term cultivation of specific rootstocks can alter the effectiveness of grafting in managing soil-borne diseases; however, the underlying reasons remain unknown.

Soil microorganisms play a vital role in maintaining soil nutrient circulation and promoting crop growth [13]. To cause soil-borne diseases, pathogens must endure in the "struggle" with other microorganisms, during which plant root secretions serve as a "bridge" [14,15]. Many studies have reported that the root secretions from grafted crops can increase the population density of beneficial bacteria and reduce the colonization efficiency of pathogens, thereby improving the structure of rhizosphere bacterial communities and increasing the resistance of crops [9,10,16]. Being an integral part of soil microbes, the fungal community and its functional composition serve as the major indicator of plant health. For example, in our previous study involving the use of reductive soil disinfestation strategy for reducing the incidence of *Fusarium* wilt in watermelon, the improvement in the fungal community structure and its functional composition was identified as a key factor for disease suppression [17]. Moreover, while studying the causal relationship between the soil microbiome and plant health, fungal communities were found to be more crucial in predicting plant health than bacterial communities [18]. The characteristics of bacterial communities in grafted plants cultivation remain a research hotspot; however, studies investigating the characteristics of fungal communities, especially focusing on their composition and functions under long-term continuous cropping of grafted plants, are limited. Understanding the fungal community and its functional succession in soils under prolonged cultivation of grafted plants may help reveal the mechanism underlying the decline in disease resistance in grafted watermelon after long-term cultivation.

Considering the high incidence of soil-borne diseases, including *Fusarium* wilt and gummy stem blight, primarily caused by fungal pathogens, in watermelon, we propose the following hypotheses: (1) the soil fungal community and its functionality may deteriorate with the long-term continuous cropping of grafted watermelon, and (2) the extent of deterioration of the fungal community and its functionality may vary in soils with different years of continuous cropping, that is, the effects of planting years on the fungal community and its functionality in soils with grafted watermelon may be significant. To address these hypotheses, we collected the soils with grafted watermelon (pumpkin rootstock) across different continuous cropping years (i.e., 0 year, 2 years, 10 years, and 18 years) under field conditions. Furthermore, we investigated and compared the fungal diversities, community structures, core and unique microbiomes, co-occurrence networks, and functionalities across different cropping years by using the Illumina MiSeq platform.

2. Materials and Methods

2.1. Study Site Description and Soil Sample Collection

The study was conducted in a region, termed as the "Hometown of Watermelons", located in Changle County (36°19′ N–36°16′ N, 118°13′ E–119°10′ E), Weifang City, Shandong Province, China. This region is characterized by a typical temperate monsoon conditions (average annual temperature: 12.6 °C; average annual precipitation: 549.6 mm). The watermelon cultivation area has surpassed 10,000 hm^2, with wide distribution of grafted watermelon greenhouses that use pumpkin as the rootstock.

Within watermelon plantations under consistent water and fertilizer management conditions in greenhouses, we selected 25 representative plants each with 2 years (Y2), 10 years (Y10), and 18 years (Y18) of grafted watermelon cultivation. Samples of the soil surrounding the roots were collected using a 2.5-cm-diameter stainless steel soil borer. As a control (Y0), we collected soil samples from adjacent open cornfields where watermelons were never cultivated. Subsequently, soil samples from five plants under each duration were randomly mixed to obtain a combined sample, which was transported to the laboratory in a cooler. Consequently, four treatments were used in total, each with five biological replicates. Each soil sample was sieved using a 2-mm mesh for homogenization, and the impurities, such as plant residues and stones, were removed. The resulting soil samples were divided into two parts. One part was kept in fresh soil at 4 °C or air-dried at room temperature (25 °C) for the analysis of physicochemical properties, including water content (WC), pH, electrical conductivity (EC), total organic carbon (TOC), total nitrogen (TN), available potassium (AK), and available phosphorus (AP). The other part was frozen at −20 °C for DNA extraction, fungal quantification, and sequencing analysis.

2.2. Analysis of Physicochemical Properties

WC was determined based on the dry weight by subjecting the soil samples to oven drying at 105 °C for 12 h. Soil pH and EC were determined in the soil-to-deionized water ratios of 1:2.5 and 1:5 by using the S220K and DDS-320 electrodes (Metter, Greifense, Switzerland), respectively. The contents of soil TOC and TN were measured using the wet digestion method with H_2SO_4-$K_2Cr_2O_7$ and semi-micro Kjeldahl digestion, respectively. Soil AK and AP were extracted using 1 mol L^{-1} ammonium acetate and 0.5 mol L^{-1} $NaHCO_3$, followed by measurement through flame photometry and molybdenum-antimony resistance colorimetry, respectively.

2.3. DNA Extraction and Fungal Quantification

Soil DNA (0.5 g from each replicate) was extracted using the FastDNA® Spin Kit (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's instructions. The concentration and purity of the extracted DNA were determined based on the A260/A280 and A230/A260 ratios, calculated using values obtained from the DS-11 ultra-micro spectrophotometer (Denovix, Wilmington, DE, USA). The soil fungi were quantified using QuanStudio3 Real-Time PCR (Applied Biosystems, Waltham, MA, USA), with ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and 5.8S (5′-CGCTGCGTTCTTCATCG-3′) as the primers. The amplification mixes, protocol, and standard curve were in accordance with our previous study [19].

2.4. MiSeq Sequencing and Data Processing

According to the methods of a previous study [19], the ITS region of fungi was amplified using the paired primers ITS1 (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′). After successful amplification, the PCR products of each soil were purified using AMPure XP Beads (Beckman Coulter, Brea, CA, USA). The equimolar concentrations of the amplicons were sequenced using an Illumina MiSeq platform at Genesky Biotechnologies Inc. (Shanghai, China).

The raw sequencing data were processed using the QIIME software package (version 1.9.1), with the codes described in a previous study [20]. Briefly, the raw data were

merged and quality controlled using the scripts multiple_join_paired_ends.py and multiple_split_libraries_fastq.py with the default arguments, respectively. The chimeras hidden in the quality sequences were identified and removed using the default argument in Usearch-uchime2 and filter_otus_from_otu_table.py, respectively. The obtained sequences were clustered into operational taxonomic units (OTUs) and annotated into taxonomic orders by using the default argument in pick_open_reference_otus.py and the UNITE reference database at 97% sequence similarity. The high-quality sequences across all soil samples were rarefied to 67,824, and alpha diversities of the soil fungal communities were calculated using the script alpha_diversity.py with the default argument.

2.5. Bioinformatics and Statistical Analysis

The fungal community structures and their differences were determined using non-metric multidimensional scaling (NMDS) and permutational multivariate analysis of variance (PERMANOVA) within the "phyloseq" and "vegan" packages in R software (version 4.0.4) [21,22], respectively. The co-occurrence networks among fungal OTUs (relative abundance > 0.01%) in each soil were analyzed using the "psych" package [23] based on Spearman correlation ($|r| > 0.8$, $p < 0.01$). Their visualization, topology, and stability analyses were performed according to previous studies [24,25]. Core and unique microbiomes for different soil samples were identified, as previously described [26]. Additionally, OTU sequence-based fungal community functions were annotated into the ecological guild functional profiles by using the FunGuild tool [27].

The significance of differences ($p < 0.05$) among planting years was determined through ANOVA and the Duncan's multiple range test using SPSS 22.0. Relationships between the planting years, physicochemical properties, and fungal community compositions were determined through random forest analysis.

3. Results

3.1. Soil Physicochemical Properties

Compared with the Y0 soil, the Y18 soil exhibited a significant ($p < 0.05$) increased in pH of 0.44, whereas the Y2 soil showed a remarkable decrease of 0.56. The pH of the Y10 and Y18 soils were significantly higher ($p < 0.05$) than that of the Y2 soil (Table 1). In addition, EC of the Y2, Y10, and Y18 soils significantly ($p < 0.01$) increased by 2.54, 1.05, and 1.39 times, respectively, with the Y2 soil exhibiting the highest EC, differing significantly ($p < 0.01$) from those of the Y10 and Y18 soils (Table 1). TOC content of the Y10 and Y18 soils and the AP content of the Y18 soil were significantly ($p < 0.05$) higher than those of the Y2 soil. The AK content of the Y2 soil was remarkably ($p < 0.01$) higher than that of the other soils (Table 1). Both the WC and TN contents in the Y18 and Y10 soils were significantly ($p < 0.05$) higher than those in the Y0 and Y2 soils. Taken together, these results showed that despite the similar soil types and greenhouse conditions of Y2, Y10, and Y18 soils, the physicochemical properties among these soils varied greatly, which may be related to the extension of planting years (Table 1).

Table 1. Effects of planting years on soil physicochemical properties.

Planting Years	Physicochemical Properties						
	pH	EC ($\mu S \cdot cm^{-1}$)	TOC ($g \cdot kg^{-1}$)	TN ($g \cdot kg^{-1}$)	AP ($mg \cdot kg^{-1}$)	AK ($mg \cdot kg^{-1}$)	WC (%)
Y0	6.42 ± 0.31 b	88.8 ± 54.9 c	11.01 ± 2.00 ab	0.82 ± 0.21 c	106.7 ± 121.6 b	208.5 ± 21.9 d	5.06 ± 1.79 b
Y2	5.86 ± 0.16 c	314.7 ± 54.9 a	9.46 ± 0.23 b	0.99 ± 0.07 c	137.8 ± 11.6 b	367.8 ± 31.3 a	5.76 ± 1.58 b
Y10	6.53 ± 0.33 ab	182.1 ± 26.9 b	12.75 ± 3.59 a	1.22± 0.11 b	197.6 ± 63.7 ab	260.9 ± 44.2 c	11.35 ± 1.57 a
Y18	6.86 ± 0.07 a	211.9 ± 12.9 b	14.04 ± 1.66 a	1.68 ± 0.09 a	259.0 ± 38.6 a	305.5 ± 28.6 b	10.41 ± 2.70 a

Note: Y0, Y2, Y10, and Y18 indicate the soils of grafted watermelons planted for 0, 2, 10, and 18 years, respectively. Different letters in the same column indicate significant differences at 0.05 level according to Duncan's multiple range test.

3.2. Soil Fungal Population and Alpha Diversity

In terms of fungal population, the Y2 (5.74×10^8 copies·g^{-1}), Y10 (3.49×10^8 copies·g^{-1}), and Y18 (3.80×10^8 copies·g^{-1}) soils exhibited a significant ($p < 0.05$) increase trend (1.3, 0.4, and 0.5 times, respectively) compared with the Y0 (2.45×10^8 copies·g^{-1}) soil. Fungal populations of the Y2, Y10, and Y18 soils exhibited no significant differences ($p > 0.05$; Table 2).

Table 2. Effects of planting years on the soil fungal population and alpha diversity.

Planting Years	Population of Fungi (lg ITS Copies·g^{-1})	Richness	Shannon Index	Evenness
Y0	8.31 ± 0.27 b	848 ± 160 a	4.28 ± 0.52 a	0.63 ± 0.06 a
Y2	8.75 ± 0.06 a	366 ± 35 b	2.30 ± 0.18 c	0.39 ± 0.02 d
Y10	8.53 ± 0.07 a	339 ± 26 b	3.32 ± 0.10 b	0.57 ± 0.02 b
Y18	8.55 ± 0.17 a	291 ± 27 b	2.67 ± 0.21 c	0.47 ± 0.03 c

Note: Y0, Y2, Y10, and Y18 indicate the soils of grafted watermelons planted for 0, 2, 10, and 18 years, respectively. Different letters in the same column indicate significant differences at 0.05 level according to Duncan's multiple range test.

The richness, Shannon index, and evenness of alpha diversity of fungal communities in the Y2, Y10, and Y18 soils decreased significantly ($p < 0.05$) by 56.8%–65.7%, 22.4%–46.3%, and 25.4%–38.1%, respectively, compared with those of the Y0 soil (Table 2). Notably, the Shannon index and evenness of the Y2 soil were the lowest ($p < 0.05$), whereas those of the Y10 soil were the highest (Table 2).

3.3. Soil Fungal Community Structure and Composition

The fungal community structure at the OTU level changed significantly ($p < 0.01$) in the Y2, Y0, and Y18 soils compared with the Y0 soil. Fungal communities varied significantly ($p < 0.01$) among the Y2, Y10, and Y18 soils (Figure 1A). At the phylum level, Ascomycota, Mortierellomycota, Basidiomycota, Chytridiomycota, Rozellomycota, Glomeromycota, Olpidiomycota, and Kickxellomycota were identified as the dominant phyla in the soils with different planting years. Of note, the relative abundance of Ascomycota in the Y2, Y10, and Y18 soils increased significantly ($p < 0.05$) by 32.4%, 18.7%, and 26.6%, respectively, compared with that in the Y0 soil. The relative abundance of Ascomycota was significantly ($p < 0.05$) higher in the Y2 soil than in the Y10 soil (Figure 1B). An opposite trend was noted in the relative abundances of Basidiomycota, Chytridiomycota, Rozellomycota, Olpidiomycota, and Kickxellomycota in the Y2, Y10, and Y18 soils, all of which were significantly ($p < 0.05$) decreased compared with those in the Y0 soil (Figure 1B).

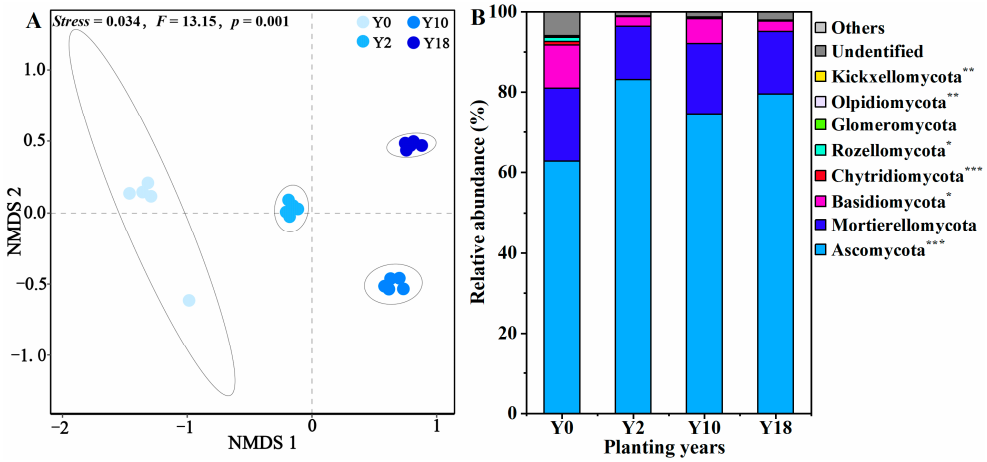

Figure 1. Effects of planting years on the structure (**A**) and dominant phylum composition (**B**) of the soil fungal community. The fungal community structure was assessed by computing Bray–Curtis distance based on the OTU level, and the confidence ellipses indicate 95% confidence interval. Stress

value (<0.2) represents the better fit of the NMDS ordination. F and *p* value indicate the impact of the planting year on fungal community structure using PERMANOVA. *, **, and *** indicate significant differences in the relative abundances of the microbes among the soils with different planting years at 0.05, 0.01, and 0.001 levels, respectively. Y0, Y2, Y10, and Y18 represent the soils where grafted watermelons were cultivated for 0, 2, 10, and 18 years, respectively.

3.4. Compositions of Core and Unique Microbiomes

To determine the effect of planting years on the soil generalists and specialists, we identified the core and unique microbiomes at the OTU level for different soil samples. The core microbiome was defined by the OTUs that consistently appeared in at least three replicates of all soils. In contrast, the unique microbiome was defined by the OTUs that appeared in a minimum of three replicates from a specific soil type.

The number of retained OTUs in the Y0, Y2, Y10, and Y18 soils was 655, 308, 270, and 241, respectively. A total of 79 core OTUs were identified in all soil types, accounting for 12.1%, 25.6%, 29.2%, and 32.8% of the retained OTUs in the Y0, Y2, Y10, and Y18 soils, respectively. An increasing trend was noted in the number of core OTUs with the increase in planting years (Figure 2A–C). The number of unique OTUs in the Y0, Y2, Y10, and Y18 soils was 417, 56, 85, and 80, respectively, which accounted for 63.7%, 18.2%, 31.5%, and 33.2% of the retained OTUs. A decreasing trend was noted in the number of unique OTUs as the planting year increased (Figure 2A–C). The proportion of core OTU sequences was significantly ($p < 0.05$) higher in the Y2 and Y10 soils than in the Y0 and Y18 soils (Figure 2C).

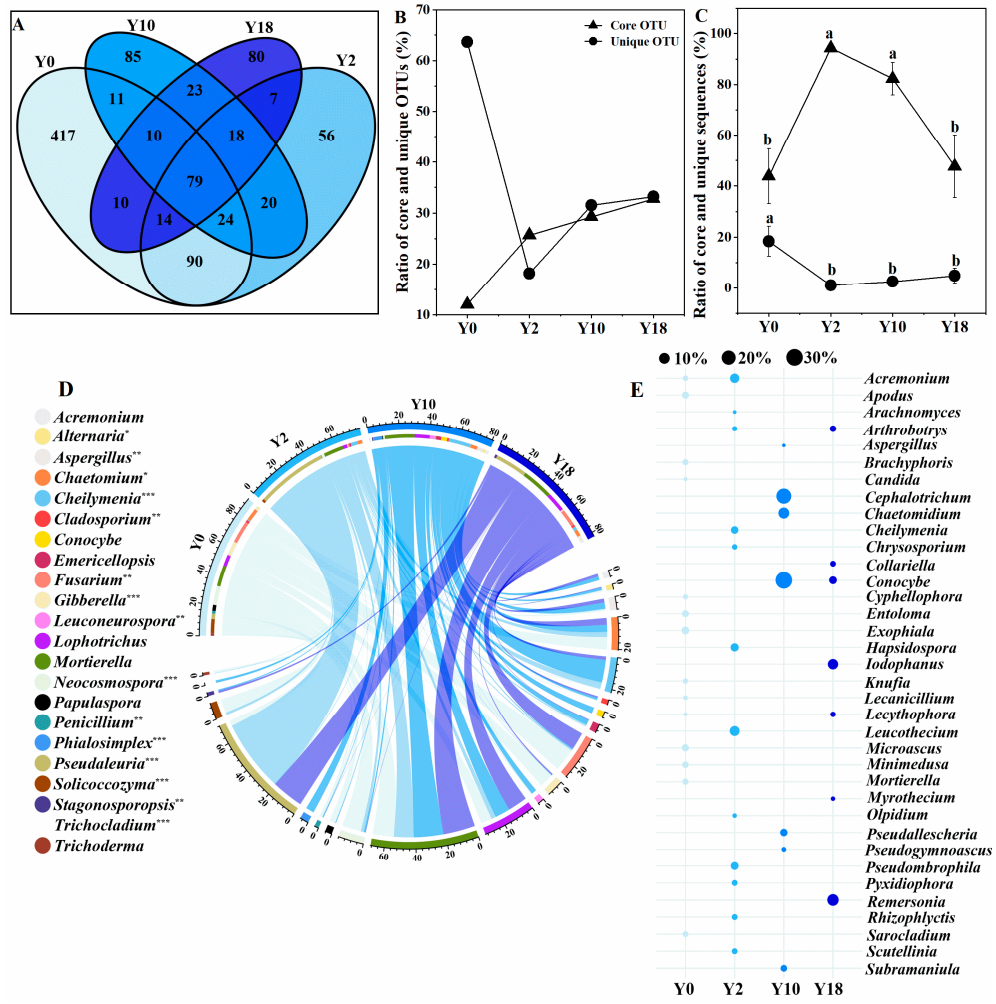

Figure 2. Effects of planting years on the core and unique microbiomes of the soil fungal community. (**A**) Venn diagram for the numbers of core and unique OTUs among soils with different planting years.

(**B**,**C**) The proportion of core and unique OTUs in the total number of OTUs (**B**) and sequences (**C**), respectively. Different lower case letters in (**C**) indicate significant ($p < 0.05$) differences according to Duncan's multiple range test. (**D**,**E**) The relative abundance of core (**D**) and unique (**E**) microbiomes in soils with different planting years at the genus level. *, **, and *** indicate significant differences in the relative abundances of the core microbiomes among the soils with different planting years at 0.05, 0.01, and 0.001 levels, respectively.

Most core OTUs obtained from these soils with different planting years were clustered into 22 genera (Figure 2D). Specifically, the core genera in the Y0 soil were *Chaetomium*, *Gibberella*, *Neocosmospora*, *Penicillium*, and *Solicoccozyma*; those in the Y10 soil were *Alternaria*, *Aspergillus*, *Cheilymenia*, *Leuconeurospora*, *Phialosimplex*, and *Trichocladium*; and those in the Y18 soil were *Fusarium*, *Cladosporium*, and *Stagonosporopsis*. The Y2 soil comprised *Pseudaleuria* as the single core genus. Additionally, most of the unique OTUs found in these soils were classified into 36 genera, with the Y0, Y2, Y10, and Y18 soils harboring 14, 12, 7, and 7 genera, respectively (Figure 2E). The genera *Acremonium*, *Apodus*, *Brachyphoris*, *Candida*, *Cyphellophora*, *Entoloma*, *Exophiala*, *Knufia*, *Lecanicillium*, *Lecythophora*, *Microascus*, *Minimedusa*, *Mortierella*, and *Sarocladium* were unique to the Y0 soil. The unique genera observed in the Y2 soil were *Arachnomyces*, *Arthrobotrys*, *Chrysosporium*, *Leucothecium*, *Olpidium*, *Pseudombrophila*, *Pyxidiophora*, *Rhizophlyctis*, and *Scutellinia*. The unique genera observed in the Y10 soil were *Aspergillus*, *Cephalotrichum*, *Conocybe*, *Pseudallescheria*, *Chaetomidium*, *Pseudogymnoascus*, and *Subramaniula*. Finally, *Arthrobotrys*, *Collariella*, *Iodophanus*, *Lecythophora*, *Myrothecium*, and *Remersonia* appeared only in the Y18 soil.

3.5. Soil Fungal Community Stability

We further constructed co-occurrence networks and calculated the topological parameters to evaluate the complexity and stability of soil fungal community at the OTU level among different planting years. The results indicated significant differences ($p < 0.05$) in fungal co-occurrence networks among the soils with different planting years (Figure 3A). Specifically, network nodes in the Y0 fungal community were composed of the aforementioned dominant phyla (Figure 1B); however, in the Y2 soil fungal network, the phyla Rozellomycota, Glomeromycota, and Kickxellomycota were not included; Rozellomycota and Kickxellomycota were excluded from the Y10 soil fungal network; and Glomeromycota and Kickxellomycota were excluded from the Y18 soil fungal network. Furthermore, the co-occurrence network parameters, such as number of nodes and edges, average weighted degree, network diameter, average path length, and modularity, were significantly ($p < 0.05$) decreased in the Y2, Y10, and Y18 soils compared with those in the Y0 soil (Figure 3B). The number of nodes and edges, average weighted degree, and modularity were higher in the Y10 soil than in the Y0 and Y18 soils, with these parameters being the lowest in the Y18 soil (Figure 3B). After 50% of the nodes were removed from each network, maximum vulnerability increased significantly ($p < 0.05$) in the Y2, Y10, and Y18 soils by 2.8, 4.2, and 3.9 times compared with the Y0 soil, respectively; however, robustness decreased significantly ($p < 0.05$) in the Y2, Y10, and Y18 soils by 47.2%, 44.5%, and 47.7%, respectively (Figure 3B).

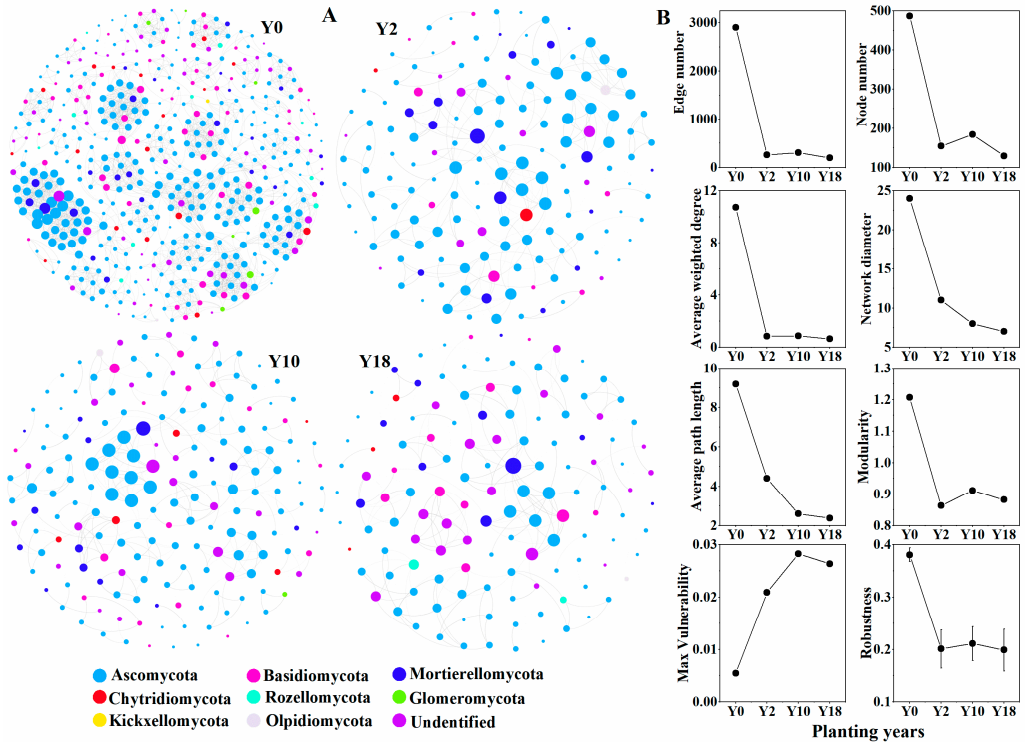

Figure 3. Effects of planting years on the co-occurrence network (**A**) and topological parameters (**B**) of the soil fungal community. The colors of nodes indicate different dominant phyla, and the size of each node is proportional to the connectivity of OTUs in the co-occurrence network. The error line indicates the standard deviation.

3.6. Functional Composition of Fungal Community

The functional annotations of two confidence levels, namely "highly probable" and "probable", were retained for further analysis. The microbial taxa with multiple combined nutrition modes were collectively categorized as "others". Here, the functional composition of the fungal community differed significantly ($p < 0.01$) among soils with different planting years (Figure 4A). Compared with the Y0 soil, the relative abundances of pathotrophic and symbiotrophic fungi in the Y2, Y10, and Y18 soils decreased significantly ($p < 0.05$), whereas those of saprotrophic fungi exhibited an opposite trend (Figure 4B). Specifically, the relative abundances of animal and plant pathogens among pathotrophic fungi, endophytes, lichenized, arbuscular mycorrhizae, and ectomycorrhizae of symbiotrophic fungi were significantly ($p < 0.05$) higher in the Y0 soil than in the other soils. The relative abundance of unidentified saprotrophic fungi was significantly higher ($p < 0.05$) in the Y2, Y10, and Y18 soils than in the Y0 soil. Notably, the dung/wood saprotrophic, dung saprotrophic, and dung/wood/plant saprotrophic fungi were remarkably ($p < 0.05$) enriched in the Y10 soil. Finally, the Y18 soils exhibited significant enrichment of dung/plant saprotrophic, plant/wood saprotrophic, and dung/plant/wood saprotrophic fungi ($p < 0.05$; Figure 4C).

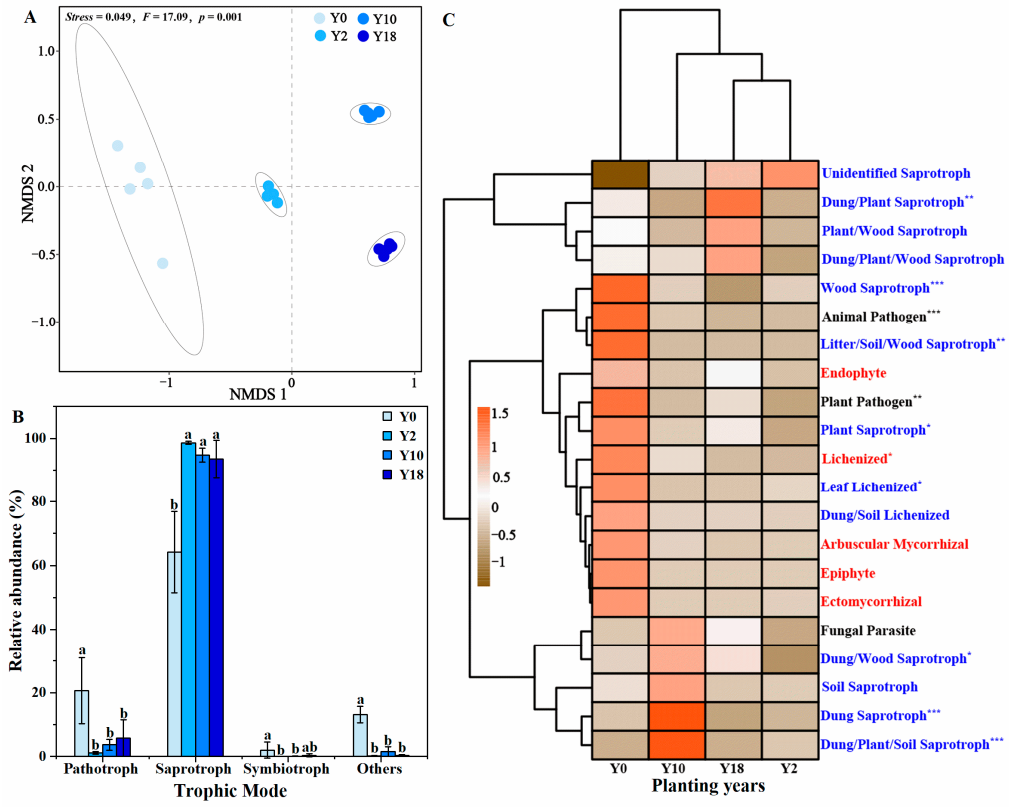

Figure 4. Effect of planting years on the fungal community functional composition of the soils. (**A**) Nonmetric multidimensional scaling (NMDS) ordinations of the fungal community functional composition based on Bray–Curtis distance of functional groups. Confidence ellipses indicate 95% confidence interval. Stress value (<0.2) represents the better fit of the NMDS ordination. F and *p* value indicate the impact of the planting year on fungal community structure using PERMANOVA. (**B**) The relative abundance of different trophic functional groups among varying planting years. Different letters indicate significant ($p < 0.05$) differences according to Duncan's multiple range test. (**C**) Heat map for the relative abundance (Z-score) of functional groups that varied among different planting years. *, **, and *** indicate significant differences in the relative abundances of the functional groups among the soils with different planting years at 0.05, 0.01, and 0.001 levels, respectively.

3.7. Driving Factors Affecting the Soil Fungal Community and Functional Composition

Random forest analysis was used to further identify the main predictors of fungal communities and their function composition. The results indicated that the planting years and soil physicochemical factors, specifically EC and WC, significantly ($p < 0.05$) influenced the fungal community structure and function, with planting years being the most predominant contributor (Figure 5A,B). Further, the regression analysis revealed a strong fitting correlation of planting years with the fungal community compositions and functions (Figure 5C,D).

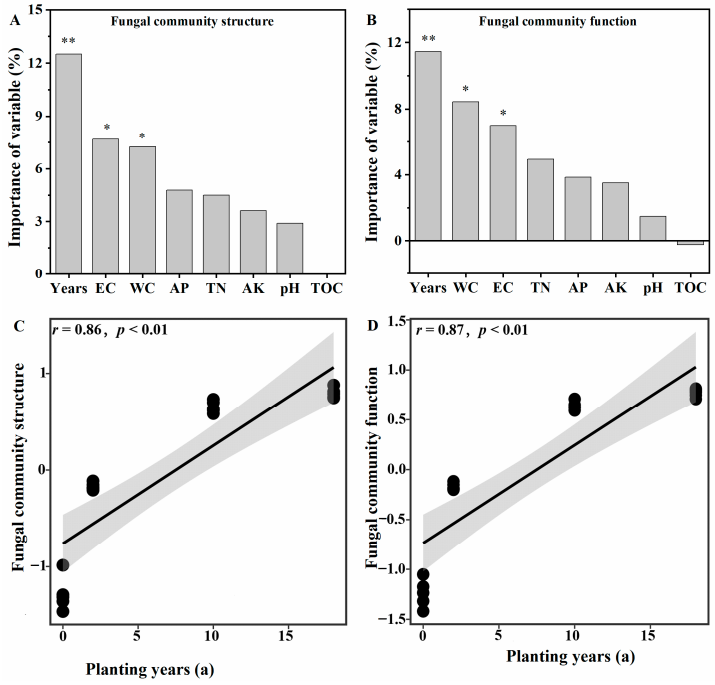

Figure 5. Effects of soil physicochemical properties and planting years on the soil fungal community structure (**A**,**C**) and functional (**B**,**D**) compositions. The community structure and functional composition of fungi represent the first axis value of the NMDS analysis. * and ** indicate a significant difference (at $p < 0.05$ level) and an extremely significant difference (at $p < 0.01$ level), respectively.

4. Discussion

Fungi are the main components of the soil microbiome, with various symbiotic, parasitic, and pathogenic fungal species playing a vital role in the plant–soil feedback system [28,29]. For example, during long-term continuous cropping, soil microorganisms usually develop into the "fungus-type" soil profile, characterized by an increased abundance of fungal pathogens and a decreased abundance of beneficial microbes. This significantly increases the risk of soil-borne disease outbreaks [30,31]. Although the relevant plant pathogens were not determined in this study, a significant upward trend was noted for the fungal population of the soils with grafted watermelon planted for different years compared with that of the Y0 soil. This suggested that the cultivation of grafted plants may shift the soil microbial community toward a "fungus-type" profile. Therefore, clarifying succession of the soil fungal community during long-term cultivation of grafted plants is crucial for further optimizing their disease resistance.

In this study, both the fungal community structures and functions varied greatly across different planting years. The random forest analysis further demonstrated the planting years as the most dominant predictor of the fungal community. Consistent with the findings of previous studies [32,33], our results unveiled that the soil fungal alpha diversity significantly decreased following different years of grafted watermelon cultivation compared with the Y0 soil, indicating that prolonged cultivation of grafted watermelon might negatively affect the soil quality. Specifically, the fungal community richness significantly decreased with the extended cultivation years (Y2, Y10, and Y18), primarily due to soil metabolic changes induced by grafted watermelon monoculture, which potentially attracted specific taxa [16,34]. For instance, Ascomycota were enriched in the soils with watermelon grafted through monocropping, aligning with the finding of a previous study [35]. In addition, soil physicochemical properties play an important role in shaping the soil microbial community under monoculture conditions [36]. In the present study, soil EC increased with the duration of grafted watermelon cropping, indicating that EC is as a major factor associated with the changes in the fungal community composition, consistent with the findings of other studies [37,38]. This result can be

explained by the drastic increase in the contents of AK and AP observed after extensive applications of chemical fertilizer during grafted watermelon planting. In addition, studies have reported the profound effect of WC on the fungal community structure [39,40], implying that fluctuations in the soil moisture content resulting from continuous cropping led to substantial changes in the fungal community structure. Thus, alterations in the fungal community composition may be attributed to the specific root exudates released from grafted watermelon and alterations in the physicochemical environment under long-term monocropping conditions.

Core microbiomes are recognized as ubiquitous species that likely play essential roles in structuring the entire microbial community [26]. Our findings showed that prolonging the duration of grafted watermelon cultivation led to an increase in the proportion of core OTUs and a gradual decrease in the proportion of unique OTUs. These results indicated that the monoculture of grafted watermelon increases the taxonomic or functional similarities among fungal communities over time. Monocultures or excessive tillage were reported to cause resource homogenization [41], resulting in the gradual replacement of locally distinct communities with ubiquitous communities [42,43]. Similarly, our results demonstrated that the fungal community was dominated by saprotrophic fungi under monoculture conditions, consistent with the result of a previous study [44]. Furthermore, the relative abundance of core taxa varied across different planting years. Specifically, *Pseudaleuria*, a beneficial microorganism that can curb disease propagation and augment crop growth, was enriched in the Y2 soil [45]. Conversely, core OTUs in the Y10 and Y18 soils potentially comprised the pathogens of watermelon, belonging to mainly *Fusarium*, *Cladosporium*, *Alternaria*, and *Stagonosporopsis* genera [30,46–48]. These results suggest that extending planting years may gradually decrease disease resistance in the soils with grafted watermelon cultivation. The Y0 soil was markedly enriched with plant pathogenic fungi such as *Fusarium* and *Cladosporium*, and the abundances of these pathotrophic fungi were significantly higher in the Y0 soil than in the other soil types. This is attributable to the long-term cultivation of corn, a known host crop for these pathogens, in this soil type.

Interactions among microbial groups play a key role in soil suppressiveness and plant health [49]. In general, complex microbial networks exhibit greater resilience to environmental disturbances and provide more benefits to plants compared with simpler networks [50]. Recent studies observed a recurring trend within the rhizosphere microbial co-occurrence network during soil-borne disease outbreaks, during which the overall network structure is frequently simplified [51,52]. This trend disrupts the stability of the microbial community and engenders an environment conducive for pathogen invasion [52]. Conversely, healthy plants have more intricate co-occurrence networks of rhizosphere microorganisms, with high connectivity, modularity, and negative correlations among microbes [53]. We found that compared with the Y0 soil, soils with the grafted watermelon monoculture exhibited a notable decrease in the connectivity, modularity index, and average weighting degree of fungal networks, with the Y18 soil having the least number of nodes and edge connections. These results indicated that the stability of the soil fungal community networks progressively decreased with the farming years, which may be because of a gradual decrease in the rhizosphere chemodiversity over time [53,54]. Furthermore, after selectively removing 50% of nodes from each fungal network, the peak vulnerability values of the networks in the Y2, Y10, and Y18 soils notably increased, with amplifications 2.8, 4.2, and 3.9 times higher than that in the Y0 soil, respectively. Conversely, the robustness decreased by 47.2%, 44.5%, and 47.7%, respectively, thus reinforcing the evidence that the stability and anti-interference capacity of the soil fungal community decrease with prolonged grafted watermelon cultivation.

This study examined and compared the fungal communities and their functional compositions where grafted watermelons were being cultivated for 0, 2, 10, and 18 years. Based on the evaluation, the following conclusions were derived: (1) Fungal communities were mainly impacted by planting years; (2) The unique taxa were replaced by core taxa over the cultivation years, leading to an increase in the abundance of potential plant pathogens

and saprotrophic nutrient fungi; (3) Throughout extended periods of grafted watermelon cultivation, the alpha diversity continued to decrease markedly, accompanied by a progressive decrease in the complexity and stability of fungal community networks. These findings highlight that the continuous application of homologous grafting technologies under field conditions is associated with certain vulnerabilities in soil fungal community succession, eventually destabilizing the soil microbiological system. This might primarily account for the continued presence of a substantial number of plant pathogens and incidence of soil-borne diseases in the rhizosphere of crops after prolonged cultivation of grafted plants. Therefore, future studies must investigate strategies for maintaining the microbial diversity and stability of the microbial community in soils with grafted plant cultivation. Investigations in this direction can provide a theoretical basis for developing grafting technologies, thereby ensuring their effectiveness in long-term prevention and control of soil-borne diseases.

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Data Availability Statement: The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA1129469.

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