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Water-Covered Depth with the Freeze–Thaw Cycle Influences Fungal Communities on Rice Straw Decomposition

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Abstract: Rice is a staple food for the world's population. However, the straw produced by rice cultivation is not used sufficiently. Returning rice straw to the field is an effective way to help reduce labor and protect the soil. This study focused on the effect of water-covered depth with the freeze–thaw cycle on rice straw decomposition and the soil fungal community structure in a field in Northeast China. The field and controlled experiments were designed, and the fungal ITS1 region was tested by high-throughput sequencing for analyzing the fungal communities in this study. The results showed that water coverage with the freeze–thaw cycle promoted the decomposition of rice straw and influenced the fungal community structure; by analyzing the network of the fungal communities, it was found that the potential keystone taxa were *Penicillium*, *Talaromyces*, *Fusarium*, and *Aspergillus* in straw decomposition; and the strains with high beta-glucosidase, carboxymethyl cellulase, laccase, lignin peroxidase, and manganese peroxidase could also be isolated in the treated experiment. Furthermore, plant pathogenic fungi were found to decrease in the water-covered treatment. We hope that our results can help in rice production and straw return in practice.

Keywords: rice straw; fungi; soil; water-covered; freeze-thaw

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1. Introduction

Rice straw is one of the most abundant renewable resources in the terrestrial environment. Returning straw to the field effectively improves the comprehensive utilization rate of straw resources, balances the farmland ecosystem, and achieves sustainable agricultural development [1,2]. Its effective decomposition process is critical to the soil nutrient cycles in agricultural systems [3]. Straw is composed of lignin, cellulose, and hemicellulose, which intertwine and form a sturdy spatial structure [4]. Thus, decomposing rice straw requires the simultaneous action of multiple enzymes derived from decomposers [5], which act in combination during the decomposition process [6] and show noticeable succession changes in the microbial community, mainly dominated by bacterial communities in the early stages [7,8]. These have been identified as "r"-type microorganisms with a faster degradation rate that consumes the easy-to-decompose carbon source of straw and synthesizes extracellular enzymes [9]. The increased enzyme activity promotes "k" type microorganisms with a lower reproduction rate to harbor hard-to-decompose soil organic matter. Although fungi were identified as typical "k"-type microorganisms, it was found that they showed a higher degree of enrichment than the "r"-type after the input of exogenous organic matter, and the fungi were more enriched in exogenous 13C [10]. That is to say, fungal communities tend to dominate at the later stages of straw degradation, since only stubborn C-compounds (such as lignin) are available and fungal mycelia can penetrate the waxy layer of straw lignin [11,12]. Moreover, the enzyme activity produced

by fungi was reported to be higher than that of bacteria [13]. Therefore, we chose fungal communities as our research subject.

Since the fungal community plays an essential role in the decomposition progress, its composition may be a regulating factor. The fungal community significantly differs across land-use types, especially in the initial colonizing phase [14], which affects the enzyme activities related to straw degradation such as soil moisture [15]. There are countless members of the fungal world, and they are seamlessly connected. The associations among members highlights the complexity of the microbiome, which positively influences multiple ecosystem functions [16,17]. Some researchers have shown that the composition and structure of microbiome communities alone cannot represent the subtle function of the entire micro-ecology [18]. Combining that with the correlation between fungal communities may determine the results of decomposition. Interactions between fungal communities will change with the availability of resources and environmental conditions. The keystone taxa of microorganisms are highly related and will have a significant impact on the structure and function of the microbiome, regardless of their relative abundance in space and time [19]; they have a critical role in the microbial community, and their removal will lead to considerable changes in the structure and function, even total collapse [20]. Therefore, a question was raised as to which missing members of keystone taxa will affect the growth and reproduction of the surviving community and whether a synthetic community composed of keystone taxa could also be highly efficient.

According to the purpose of the application, an important principle is that more efficient and suitable strains can be screened from similar ecological environments [21]. Sampling from high-temperature compost, decaying crop stalks, and straw returned to the soil as the source, a series of strains adapted to medium to high temperature were screened, as reported, and their straw degradation rate reached 30–50%, which were used for straw return in relatively high-temperature areas in southern China [22]. The freeze–thaw cycle is an essential climatic feature in northeast China. Soils in ecosystems at the high altitudes experience freeze–thaw cycles during winter as the air temperature fluctuates above and below 0 °C, and FTs can significantly disturb biogeochemical processes by influencing soil moisture, structure, and microorganisms. In addition to being a source of significant abiotic stress, FTs can impact on the degradation of various types of litter in the farmland soil ecosystem. It is necessary to access high-efficiency decomposing strains and synthetic fungal communities to adapt to the local climate. An intriguing phenomenon was observed when soil with rice straw was covered with water through the whole winter in northeastern China, where the straw degradation effect was clear enough to be perceived or examined by the unaided eye the following spring. Fungal communities have developed various strategies to face ever-changing water supplies and rely heavily on soil moisture for survival traits such as colonization and reproduction. Therefore, water availability is one of the main factors affecting the structure of fungi and their metabolite/enzyme activities [23]. Therefore, would changes in soil water availability also affect and regulate the fungal community that functions in straw degradation?

In this study, we experimentally investigated how the freeze–thaw cycle and water coverage impact soil fungal communities and networks. We conducted an in situ paddy field-based experiment contrasting the growth of the same rice cultivar in a greenhouse and transplantation soil. The treatments were intended to create a gradient of water-induced potency shifts in rice straw degradation with a response to temperature imposed by winter to the following spring. In order to identify critical microbial participants, we used co-occurrence network analysis to identify the keystone taxa involved. Then we carried out further microbial cultivation, constructed a simple synthetic fungal community, and verified the potential of straw degradation suitable for this area. We hypothesized that (1) the decomposition rate of straw covered with water and undergoing freeze–thaw would be higher than that of straw under anhydrous or non-freeze–thaw treatment, but water content has little effect on the rate, and (2) some specific fungi secrete corresponding

enzymes during straw degradation and can continue to play a role under freeze–thaw conditions.

2. Materials and Methods

2.1. Experimental Site

The *Oryza sativa* subsp. *japonica* S. Kato plantation is located at Songyuan, Jilin (45°21′23″ N/124°50′38″ E), in the Songnen plain. The area has distinct seasonal freezes and thaws (FTs). The seasonal FT on the plateau extend from October to April of the next year. FTs in the area can occur every month from November to March of the next year, especially in November, February, and March. The test plots were set as 2 × 2 m repeating plots, with interspacing of more than 3 m and the use of 1 m high steel material for rain barriers to ensure consistency of the test. Thus, this freeze–thaw area was FT. The second sampling site was conducted in the constant temperature greenhouse at the Northeast Institute of Geography and Agroecology, China (43°59′54″ N, 125°23′55″ E), with the same soil and rice cultivar as our HT treatment. The area of interest at the plantation was characterized by dark clay soil with the following values: pH 7.38 ± 0.07, electrical conductivity (EC) 240.93 ± 14.15, soil organic matter 17.18 ± 0.38 g/kg, total nitrogen 2.13 ± 0.12 mg/kg, total carbon 10.19 ± 0.17, total phosphorus 7.48 ± 0.24 mg/kg, and available potassium 59.50 ± 0.01 mg/kg. Rice occupied the field before the plantation. Both field location tests (FT) and homoiothermal experiments (HT) were conducted to study the effects of direct and whole straw manuring methods on increasing yield of rice crop. The whole straw was returned after the harvest season (October 2018) and then irrigated with different water content, as the irrigation water level was controlled at 0, 10, and 20 cm depth, and straw fragments were collected in April 2019. In each sample plot, five soil sample ("S" distribution) were collected randomly in the 0–20 cm soil layer and mixed as one soil sample. Soil samples were collected along with the straw litters. The samples were preserved in dry ice and transported to the lab. In addition, part of the soil was air-dried and sieved with a 2 mm diameter screen to determine the physicochemical properties.

2.2. Soil DNA Extraction, Gene Amplification, and Sequencing

Soil DNA from each sample was extracted using a FastDNA Spin Kit for Soil™ (MP Biomedicals, Irvine, CA, USA) from 0.5 g of soil previously sieved through 2 mm mesh. The DNA samples were stored at −80 °C for further analysis. The quality and concentration of extracted DNA samples were examined by a SynergyTM H1 microplate reader (BioTek Instruments, Winooski, VT, USA).

For all samples (6 treatment × 4 replicates), the internal transcribed spacer 1 (ITS1) region of fungi was PCR amplified using the high-taxonomic-coverage primers ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2-R (5″-GCTGCGTTCTTCATCGATGC-3′) [24]. The PCR amplicons were purified with Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN, USA) and subsequently quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). The PCR reaction was conducted using the improved DNA polymerase system with a temperature profile of 95 °C for 10 min; followed by 35 cycles at 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 50 s; and a final extension at 72 °C for 5 min. Then, samples were stored at 4 °C for further sequencing.

2.3. Analysis of Soil and Straw Properties

A scanning electron microscope (SEM) characterized the degraded residual from the plot and the surface morphology, split structure, and inner surface structure of the tube cavity (Hitachi High-Tech, Shanghai, China). The straw was pasted on the copper with conductive glue, and vacuum gold spraying was performed. The observation conditions were 85% relative humidity and acceleration voltage of 15 kV. Soil pH and electrical conductivity (EC) were measured by diluting the soil in water (1:5). Samples were allowed to equilibrate for 30 min before pH and EC were measured. Total carbon and total nitrogen were determined by an element analyzer (Heraeus Elementary Vario EL, Hanau, Germany),

SOM was determined by the method used in [25], total phosphorus was determined by the Auto Analyzer (Skalar Analytical, Breda, The Netherlands), and available potassium was determined by a flame photometer (Jingke Technology, Shanghai, China).

2.4. Illumina HiSeq Sequence Processing

The 250 bp paired-end sequencing was performed by using Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA) at Biomarker Technologies Co., Ltd. (Beijing, China), the Illumina MiSeq platform. The raw sequences were processed using the pipeline established for Illumina amplicon sequencing data. Raw sequences were divided into sample libraries using unique barcodes and were shortened by removing the primer and barcode sequence and merged using Flash v1.2.11 (https://sourceforge.net/projects/flashpage/, accessed on 6 June 2021). Higher-quality and more accurate statistical and bioinformatics analysis data were obtained by using Trimmomatic software (v0.33) to remove shorter tags (less than 75%) and unique (i.e., singleton) sequences and conduct quality filtering and abandon to obtain clean tags. Chimeras were removed with UCHIME (v4.2) if 2 parents had more than 80% similarity to the query sequence.

The 1,689,365 reads that passed the filtering processes were clustered with a cutoff sequence similarity of 97% using the UPARSE consensus taxonomic assigner algorithm as implemented in USEARCH v10.0 software [26], then it was used as operational taxonomic units (OTUs) in the subsequent statistical analyses. OTUs with fewer than 10 sequencing reads in all samples were removed. The functional group of fungal OTUs was inferred using FUNGuild v1.0.

Taxonomy was assigned for each phylotype using the RDP classifier based on the UNITE database for fungi. The raw sequencing data were submitted to the NCBI and are accessible under accession number PRJNA648822 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA648822, accessed on 28 November 2020).

2.5. Statistical Analysis

Based on our raw data, fungal diversity analysis was performed were assessed via one-way analysis of variance (ANOVA) using IBM SPSS v20.0 statistical software (SPSS, Chicago, IL, USA) to compare diversity (Shannon) and richness (Chao1) between different treatments.

To elucidate the effects of freeze–thaw (FT) and homoiothermal (HT) treatments with water coverage (0, 10, and 20 cm) on the structure of fungal communities. Principal coordinate axis analysis (PCoA) with the Bray–Curtis distance and permutational multivariate analysis (PERMANOVA), based on the normalized OTU tables, was performed by using the "vegan" package in the R platform v3.6.2 (https://www.r-project.org/, accessed on 6 June 2021) and linear discriminant analysis effect size (LEfSe) was determined based on normalized OTU tables using STAMP software v2.1.3 with Welch's *t*-test and Storey's FDR adjustment [27]. The R platform v3.6.2 (https://www.r-project.org/, accessed on 6 June 2021) was used for experimental data processing. Redundancy analysis (RDA) was performed using the "vegan" package in the R platform to measure the correlation relationship between soil properties and the fungal community at the genus level. The data underwent ANOVA, and the analysis method was the Kruskal–Wallis test and the Bartlett test with $p < 0.05$ as adjustment [28].

Means and standard errors were calculated for soil parameters and the microbial indices of each site. One-way analysis of variance was used to test for significant differences in soil properties and relative abundance of various microbial groups among different sites. The significance was determined using Tukey's HSD test under the significance level of 0.05. Analyses were performed using the software package IBM SPSS statistics v19.0 for Windows.

2.6. Network Analyses and Keystone Taxa

Network analyses were performed to evaluate the complexity and identify potential keystone taxa for each sampling point based on normalized OTUs with predicted functions. Co-occurrence network analyses were conducted based on a SparCC correlation matrix using the psych package in R [29]. Correlation coefficients were more significant than 0.8, with a corresponding *p*-value less than 0.05 considered statistically rigorous. The network structure was visualized by Gephi, choosing the undirected network and the Fruchterman-Reingold layout [30]. The top 20 OTUs with high degree and high closeness centrality were chosen to identify the keystone taxa [31].

2.7. Constructing Synthetic Fungal Communities

We tried to isolate the keystone taxa from the straw residue using the standard serial dilution plating technique. After incubation at 28 °C for 7 days with constant purification and identification, only 5 fungal suspected isolates corresponded to the keystone taxa. After pairwise antagonism testing [32], 4 non-antagonistic strains were maintained for further characterization. To assess the impact of the 4 suspected isolates on networks, we constructed specific networks (OTUs corresponding to the identified isolates), which showed that microbial networks were less complex and even collapsed without the suspected isolates (Supplementary Materials, Table S1) as modularity declined rapidly. We assessed these 4 stains as our materials to represent critical functional components in communities.

The total DNA of the strains was extracted according to the instructions in the Ezup Column Fungi Genomic DNA Purification Kit (Sangon Biotech, Shanghai, China), and the PCR amplification products were sent to Sangon Biotech (Shanghai) Co., Ltd., for sequencing. The results of the ITS sequences of four strains were compared via BLAST in the NCBI database (https://ncbi.nlm.nih.gov/, accessed on 6 June 2021) and using the neighbor-joining method for constructing a phylogenetic tree in MEGA v7.0 [33], and the strains were stored in the refrigerator at −80 °C with 20% glycerol.

The preserved strains were inoculated into Rose Bengal medium and placed in a constant temperature incubator at 30 °C for 3–4 days. The hyphae were picked and transferred and subcultured at least 3 times [34]. The activated fungal spores were inoculated into a potato-dextrose agar medium, cultured at 30 °C for spore formation for at least 6 days, and then filtered with sterile gauze to remove the hyphae and part of the part the medium, and the number of spores per unit was counted with a hemocytometer. A fungal spore liquid was prepared with a spore concentration of 1.0×10^7 CFU/mL with sterile water, synthetic fungal communities were constructed and inoculated onto minimal medium at pH 7.0 ± 0.1 composed of 200 g/L rice straw with 2 g/L $NH_4H_2PO_4$, 0.6 g/L KH_2PO_4, 0.1 g/L $CaCl_2$, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 1.0 g/L K_2HPO_4, 0.1 g/L NaCl, 0.05 g/L $FeSO_4{\cdot}7H_2O$, and 0.014 g/L $ZnSO_4{\cdot}7H_2O$. According to the related literature [35], the freezing temperature was set to −10 °C, the melting temperature was 10 °C, and the duration of each stage was 12 h; that is, the whole freeze–thaw cycle was 24 h. Measurements were taken once a week. The activity of the straw-degrading enzyme lasted until the 8th week [36].

2.8. Rice Straw Degrading Enzyme Activity

The beta-glucosidase, carboxymethyl cellulase, laccase, lignin peroxidase, and manganese peroxidase activity in the straw residuals were detected weekly. The samples were centrifuged at 4 °C (12,000 rpm, 10 min). The supernatant was filtered through a 0.45 μm membrane to remove cells and kept at 4 °C until enzyme activity analysis within 24 h. Beta-glucosidase and carboxymethyl cellulase activity was determined using methods adapted from a previous study [37]. One enzyme activity unit was defined as the enzyme releasing one μmol of reducing sugar per minute per milliliter of supernatant. According to the instruction manual, laccase, lignin peroxidase, and manganese peroxidase were evaluated using Solarbio Reagent Kits (Solarbio Science & Technology. Beiing, China). One

enzyme activity unit was defined as the amount of enzyme required to oxidize 1 nmol of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), veratrol, or guaiacol per minute per milliliter of supernatant, respectively.

3. Result

3.1. Degradation Degree of Straw

The surface morphologies and structures of the degraded material collected from homoiothermal (HT) and freeze–thaw (FT) treatments are shown in Figure 1. It is shown that the degree of damage of straw fiber and the number of fiber fragments produced were slightly higher in the FT treatment than the HT treatment, and the degree of fiber breakage was proportional to the increasing moisture. For example, litter from 0 cm had a smooth surface with a compact and neat structure (Figure 1a,b). The structure of straw under 10 cm was destroyed (Figure 1c,d), with the bundle-like structure of fiber loosened, and the surface of the straw was relatively rough with some fibers broken into fragments. The fibers in the 20 cm treatment were utterly broken in the middle layer of the lumen (Figure 1e,f). The addition of water and the occurrence of freeze–thaw cycles can accelerate the disintegration of the fiber structure in the straw, thereby speeding up the degradation process.

3.2. Study on the Beta Diversity of Microorganisms in the Plants' Rhizospheres

PERMANOVA based on the Bray–Curtis similarity matrix was performed (Figure 2). The results show that the fungal communities in FT and HT treatments differed significantly (F-value: 4.7204; R^2: 0.17666; $p < 0.001$) and were distinctly clustered with different water gradients (F-value: 1.9166; R^2: 0.15436; $p < 0.008$); the percentage of variation explained by axis 1 and 2 was 20 and 13%, respectively. To identify the effects of fungal diversity, we investigated the Chao1 index (Figure 3c) and Shannon index (Figure 3d) of the fungal community in all treatments. The result of the Chao1 index showed no significant difference between HT and FT treatments (Figure 3c; $p > 0.05$). However, the Shannon index of HT treatment without water was significantly higher than that of other treatments, especially HT with 10 cm water coverage (Figure 3d; $p < 0.05$)

The main proportions of fungal phyla across HT and FT treatments were subsequently examined, and the dominant phyla were Aphelidiomycota, Basidiomycota, and Ascomycota (Figure 3a). The relative abundance of Aphelidiomycota and Basidiomycota in FT treatment was higher than in HT treatment (Figure 3a). On the other hand, the relative abundance of Ascomycota in HT was higher than in FT. Furthermore, according to the relative abundance of phyla level, Mucoromycota and Zoopagomycota only existed in HT, while Blastocladiomycota only existed in FT. Since we focused on cultivable microorganisms, the OTU relative abundance data were further analyzed at the genus level using STAMP (Figure S1). *Plectosphaerella*, *Microdochium*, *Coprinopsis*, *Holtermanniella*, and *Didymella* were significantly enriched under the freeze–thaw cycle (Figure S1). *Torula* and *Acaulopage* led to significant differences between HT and FT (Figure S1). The fungal genus showed no significant statistical enrichment under different levels of moisture coverage (Figure 3b).

Based on function prediction, with increased water content, the fungal community evolved from plant pathogenic dominated to saprotroph decomposer dominated (Figure S2); saprotrophs were significantly correlated with other fungi in FT at 10 and 20 cm (Figure 4c,e) and HT at 10 and 20 cm (Figure 4d,f), whereas plant pathogens were significantly correlated with other fungi in FT at 0 cm (Figure 4a) and HT at 0 cm (Figure 4b). From the functional point of view, with increased water content, plant pathogenicity decreased gradually, and saprophytic fungi increased gradually, especially in the HT group.

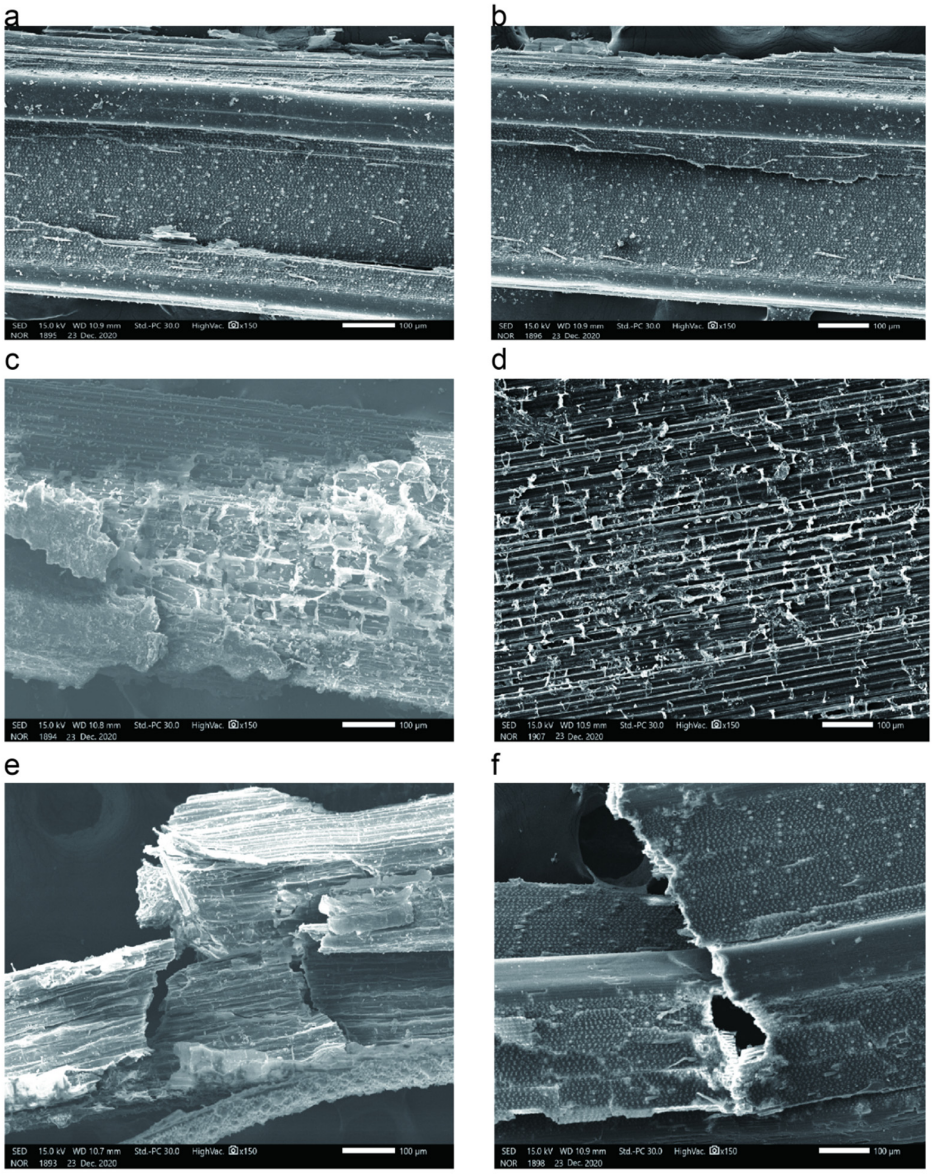

Figure 1. Scanning electron microscope (SEM) analysis results of rice straw surface structure at a 150× resolution in freeze–thaw (FT) (**a**,**c**,**e**) and homoiothermal (HT) (**b**,**d**,**f**) groups: (**a**,**b**) drought; (**c**,**d**) 10 cm water coverage; (**e**,**f**) 20 cm water coverage.

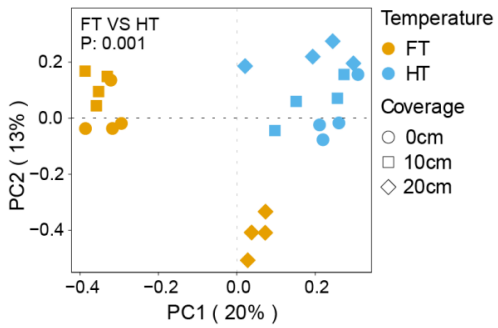

Figure 2. For the PERMANOVA of origins factor, all samples were separated into two groups: freeze–thaw (FT) and homoiothermal (HT) treatments in 0 cm (drought), 10 cm water coverage, and 20 cm water coverage. Principal coordinates analysis (PCoA) was performed based on the Bray–Curtis dissimilarity matrix at operational taxonomic units (OTUs) level across all the samples.

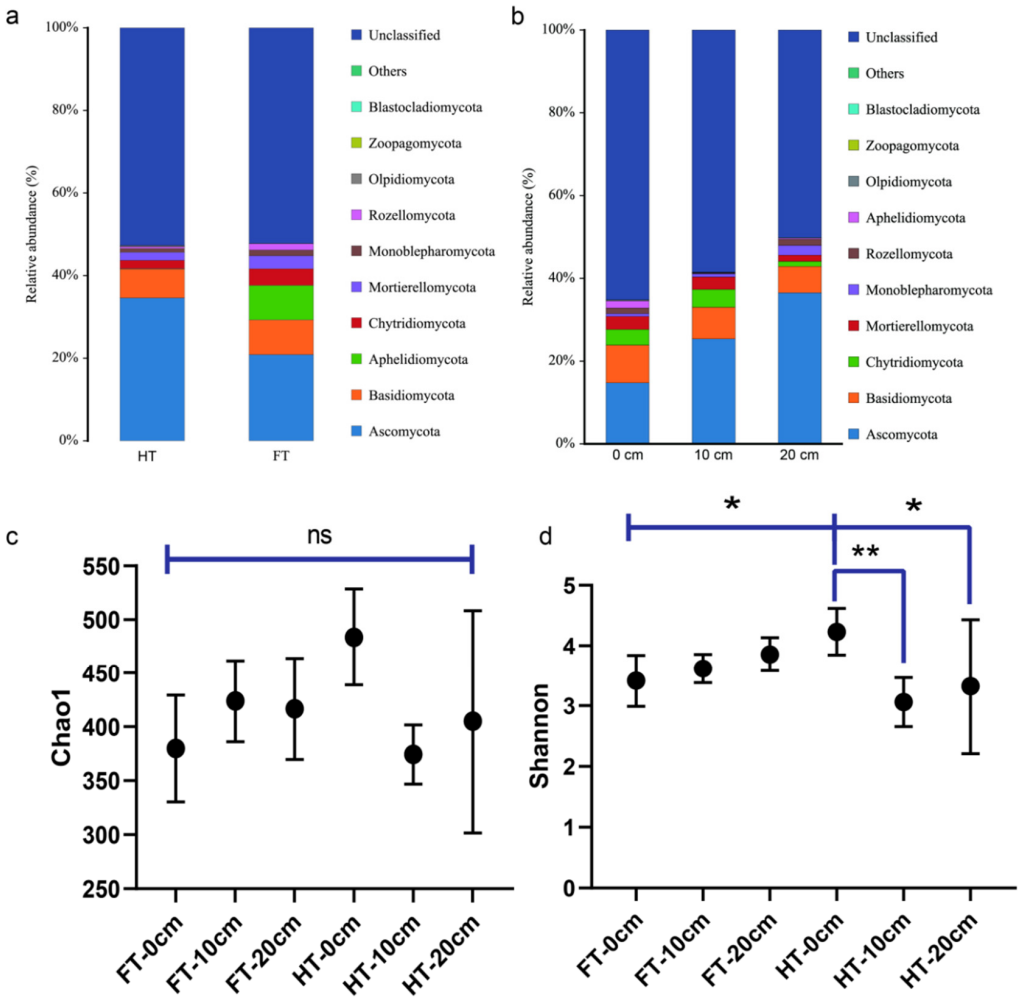

Figure 3. Distribution of phyla in soil fungal communities in (**a**) freeze–thaw; (FT) treatment; (**b**) homoiothermal (HT) treatment using a normalized OTU table: 0 cm (drought), 10 cm water coverage, and 20 cm water coverage. Alpha diversity compared soil fungal communities between groups based on (**c**) Chao1 and (**d**) Shannon indices. Error bars represent standard errors (n = 4). Displayed significant scores are ** $p < 0.01$, and * $p < 0.05$.

The higher average weighted degree and modularity of the nodes in the FT network indicated that the connectivity among nodes was higher than in HT (Table 1). The connected fungi' positive–negative connection ratio in FT treatment was lower than in HT treatment, indicating that the fungal communities in FT were stable. Furthermore, compared with the HT treatment group, the yeast in the FT treatment group occupied the central position (Figure 4a,c,e). With progressing decomposition, the dominance of *Penicillium*, *Aspergillus*, *Penicillium*, *Talaromyces* (saprotroph), and *Fusarium* (plant pathogen) was noted across all networks.

Table 1. Network parameters and potential keystone matching with culturable isolates under all treatments.

Treatments	Water Coverage	Nodes/Edges	Average Degree	Average Weighted Degree	Modularity	Keystone Taxa
FT	0 cm	74/151	4.081	2.892	1.071	OTU_433(*Penicillium oxalicum* J27); OTU_127(*Fusarium* sp.J23)
	10 cm	76/165	4.342	2.237	1.2	
	20 cm	74/154	4.162	1.514	1.619	
HT	0 cm	95/189	3.979	2.8	1.002	OTU_318(*Aspergillus* sp.L61); OTU_409(*Talaromyces* sp.J542)
	10 cm	78/161	4.128	3.256	0.894	
	20 cm	86/198	4.605	3.814	0.842	

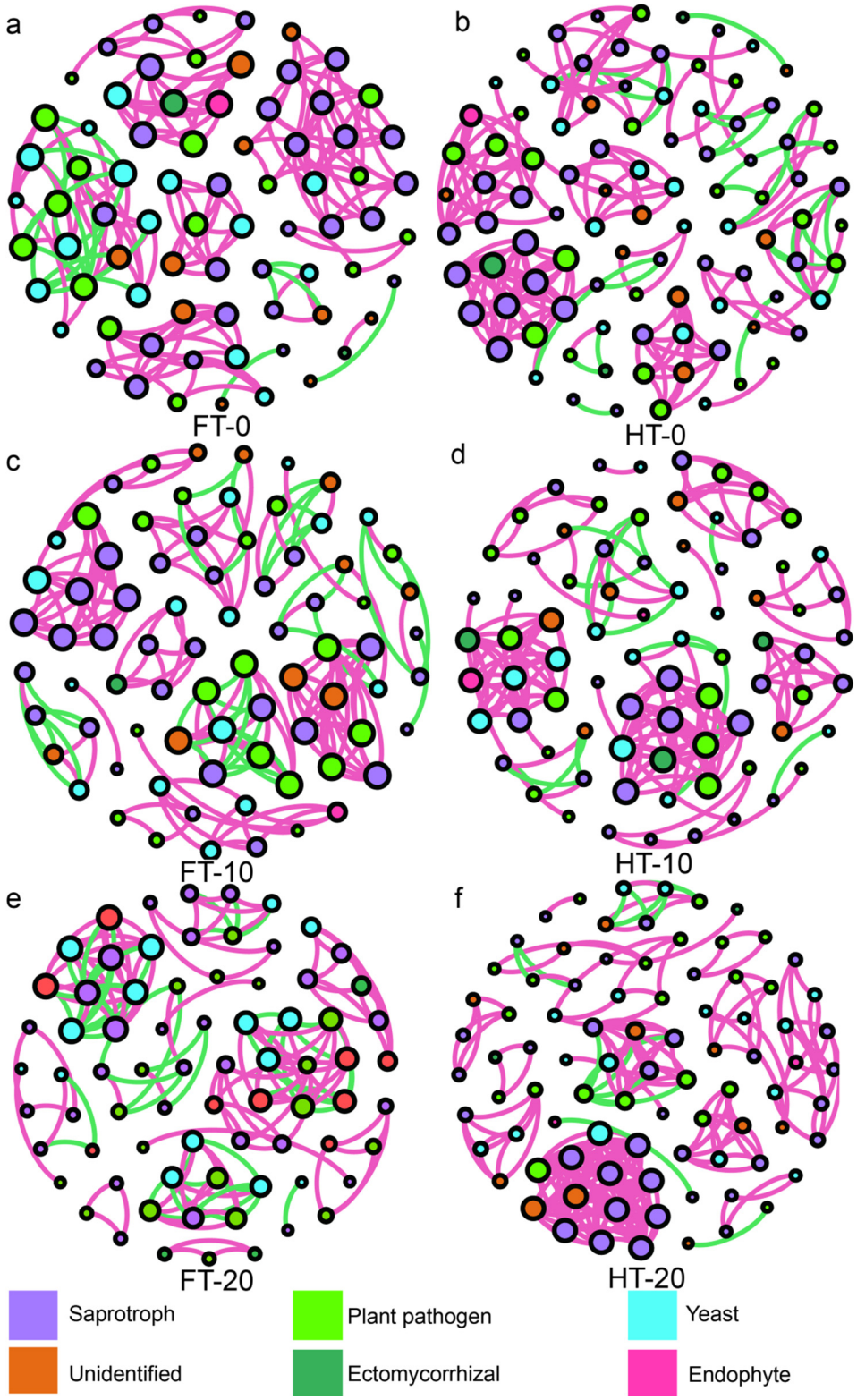

Figure 4. Co-occurrence networks of fungal microbiomes of soil fungal communities in (**a**) freeze–thaw treatment without water coverage, (**b**) homoiothermal treatment without water coverage, (**c**) freeze–thaw treatment with 10 cm water coverage, (**d**) homoiothermal treatment with 10 cm water coverage, (**e**) freeze–thaw treatment with 20 cm water coverage, and (**f**) homoiothermal treatment with 20 cm water coverage. Nodes are colored according to potential functions. Each node is labeled at the OTU level, node size is proportional to degree (number of connections), and red and green edges indicate positive and negative correlation.

OTU-specific networks without four fungal isolates were also constructed. The results show that co-occurrence networks were less complex without the keystone OTUs (Table S1). For example, essential parameters, such as average degrees and modularity, decreased after removing OTUs corresponding to the identified isolates.

3.3. RDA Analysis of Fungal Communities and Soil Properties

Correlations between fungal succession (genus level) and soil properties in each treatment were shown by RDA (Figure 5). The same water potential with FT showed distinct correlations compared with HT treatment. The different moisture gradients under FT treatment showed distinct correlations under HT treatment and were separated along the second axis1, the percentage of which was 40.63%. In addition, *Mrakia* and *Massarina* seemed to be mainly influenced by TN, and *Mrakiella* seemed to be mainly influenced by pH, with a higher positive correlation at the second axis, with a percentage of 23.22%. Interactive forward selection RDA showed that TC (pseudo-F: 2.5; $p < 0.05$) significantly affected the soil fungal community structure at the genus level, which explained the 10.10% during the fungal succession process. Therefore, TC is the primary parameter that can influence soil fungal communities under all plots.

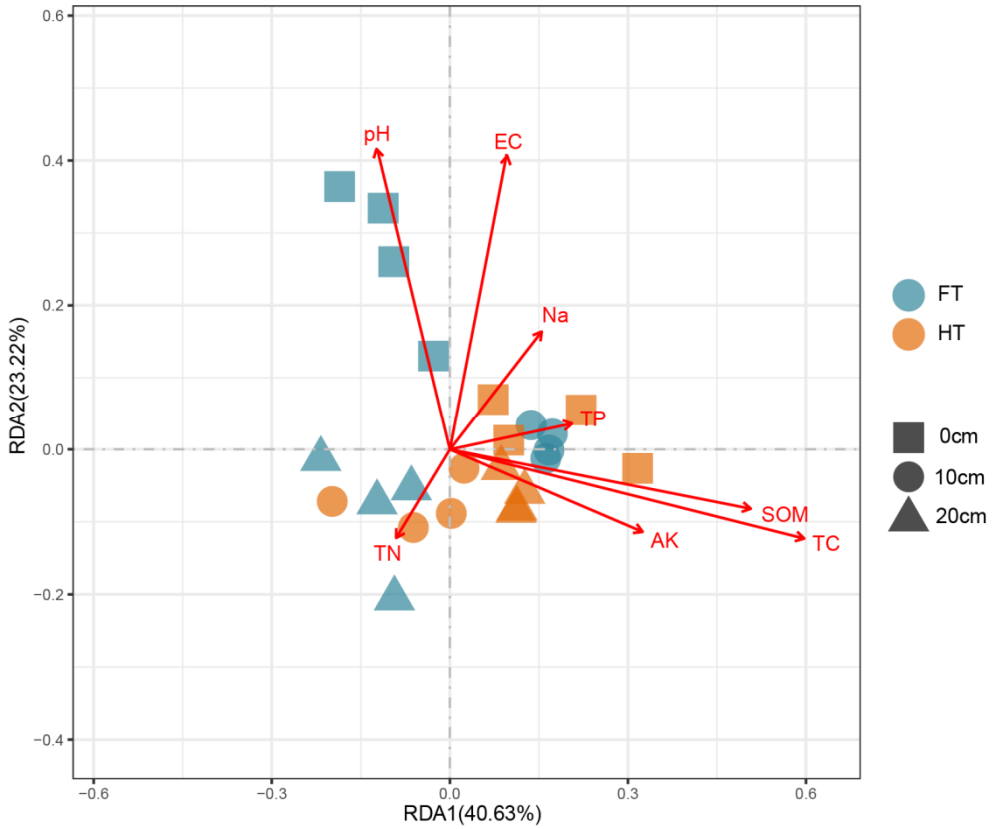

Figure 5. Redundancy analysis (RDA) of soil microbial structure in 24 samples using soil properties as explanatory variables. Shown are scores of samples, microbial community indicators, and soil properties on the first two axes. FT, freeze–thaw treatment; RT, homoiothermal treatment; SOM, soil organic matter; TN, total nitrogen; TC, total carbon; Na, sodium ion; EC, electrical conductivity; TP, total phosphorus; AK, available potassium.

3.4. Changes in Enzyme Activities of Synthetic Fungal Community

Five of the 20 OTUs were highlighted from the isolates: OTU_433 (*Penicillium oxalicum* J27) and OTU_127 (*Fusarium* sp. J23) from FT, OTU_318 (*Aspergillus* sp. L61) and OTU_409 (*Talaromyces* sp. J542) from HT, and *Penicillium oxalicum* L312, which was the only one that was abandoned, since this fungus had antagonistic responses with others. We constructed

synthetic fungal communities (SFCs) using *Penicillium oxalicum* J27 and OTU_127 (*Fusarium* sp. J23) from FT, OTU_318 (*Aspergillus* sp. L61), and OTU_409 (*Talaromyces* sp. J542). The inoculum for SFCs in a concentration of 10% (v/v) was placed into a sterile container containing rice straw as a strict carbon source and cultured under repeated freezing and thawing conditions.

The activity of beta-glucosidase, carboxymethyl cellulase, laccase, lignin peroxidase, and manganese peroxidase differed significantly across all culturing times (Figure 6). In the later stages (5–8 weeks), enzyme activity was significantly higher than in the earlier stage (1–4 weeks). In general, the activity of each enzyme at different times showed different trends. For example, beta-glucosidase and carboxymethyl cellulase activity had a continuously increasing trend, and each week was more significant than the previous week until the end (Figure 6a,b). The beta-glucosidase activity was initially low (before the fourth week) but increased sharply (Figure 6a). Laccase and lignin peroxidase activity increased before the seventh week and dropped rapidly at the eighth week (Figure 6c,d). Manganese peroxidase activity increased rapidly before the fifth week, then decreased afterward (Figure 6e). These results show that SCF had promising enzyme production during freeze–thaw cycles in vitro.

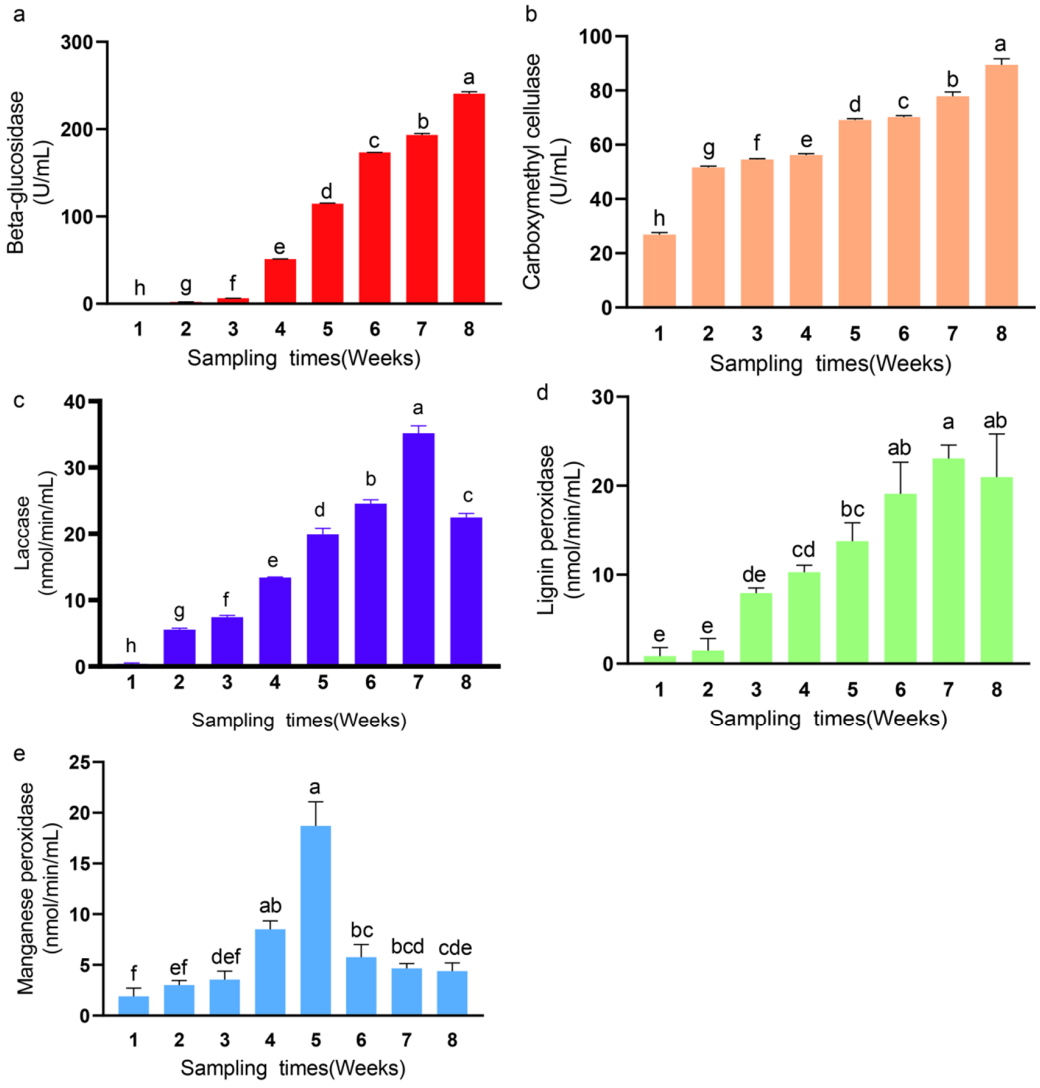

Figure 6. Changes in (**a**) beta-glucosidase, (**b**) carboxymethyl cellulase, (**c**) laccase, (**d**) lignin peroxidase, and (**e**) manganese peroxidase in synthetic fungal community (SFC) treatment. A unit of enzyme activity was calculated as nmol/min/mL (dry weight of straw). Error bars represent standard errors (n = 3). Different lowercase letters shows statistically significant difference.

4. Discussion

The freeze–thaw cycle consists of soil freezing and thawing caused by changes in soil temperature above and below 0 °C. During the freeze–thaw cycle, changes in soil properties are essentially caused by the mutual transformation of the phase state of water in the soil between liquid and solid [38]. Microbial responses to freeze–thaw cycles have generally been studied in bacterial communities in different climate areas [39,40]. Soil microorganisms were hard to decipher, since they could be opportunistic (sensitive or tolerant) to freezing–thawing cycles [41,42]. Sensitive fungi have been sabotaged during rapid climate change [43], and recovery of these sensitive microorganisms is difficult since available water can be constrained [44]. Fungi with desiccation tolerance can remain active and produce biomass during water limitations and sub-zero temperatures [45]. The fungi can enter a dormant state until the environment turns more favorable [46]. Fungi have evolved survival characteristics in harsh environments, can take advantage of empty microhabitat niches, and increase the availability of substrates produced by necrotic microbes [47,48]. These early colonists benefited from the "priority effect" [49] and ensured the resiliency of the community even when encountering environmental fluctuations [50–52]. Our results confirm that the fungi are drought-tolerant, which may be due to the structure of their hyphae, which can access water remotely along the hydrological gradient in the soil [53].

Nevertheless, fungi are more sensitive to freeze–thaw cycles [41,54] since our results show a succession of fungal communities. However, different water contents (FT 10 and 20) have little effect on the community structure, which may be due the change in the soil aggregate structure caused by the FT process combined with water saturation, so as to increase the nutrient from black soil and enables the fungal community to consume straw continuously and effectively.

In our study, the increase in soil organic carbon after freeze–thaw cycles may be due to the carbon released by microorganism cadavers in the soil, which changed the soil structure and increased the availability of organic matter. It was previously reported that soil water availability largely determines the diffusion degree of SOC and affects the availability of nutrients [55]. When the soil moisture is too low, the thickness of the soil surface water film and the diffusion rate of C will decrease [56]. After adding straw, the availability of nutrients for microorganisms and the C reaction rate is further decreased, which is consistent with our FT 0 treatment. In addition to water, soil fungal community structure is usually closely related to soil nutrient content [14], and the mineralization rate of C and exogenous additives in the non-flooded state was higher than in the flooded state. Flooding reduces C mineralization [57], which is consistent with our findings. In addition to water, soil fungal community structure is usually closely related to soil nutrient content. As the soil carbon and nitrogen cycles are closely coupled, nitrogen levels can significantly affect the carbon cycle process. Straw input leads to a short-term decrease in soil nitrogen availability and an imbalance in the carbon–nitrogen ratio. In this situation, microorganisms need to mineralize SOC to obtain additional nitrogen for growth and reproduction [58], which is an important reason why exogenous nitrogen sources are added when verifying synthetic fungal communities during straw degradation.

Our results indicate that Ascomycota plays a vital role in the process of straw decomposition under freeze–thaw conditions, which is consistent with previous findings [59]. For example, *Penicillium* sp. from Ascomycota was a high-frequency genus in a paddy field [60]. Previous studies also found that *Penicillium* could produce a series of enzymes that efficiently degrade straw [61,62]. It was also reported that *Talaromyces*, *Fusarium*, and *Aspergillus* produced a series of enzymes, such as beta-glucosidase, carboxymethyl cellulase, laccase, lignin peroxidase, and manganese peroxidase, which are essential for straw degradation [63–67], and this may explain the identification of the keystone taxa in our study. Though Aphelidiomycota was the most abundant in and only existed in FT, its members were not culturable at this time. Thus, studies should focus on sorting technology for Aphelidiomycota isolates in the future.

No species can exist alone; they form complex ecological networks with other species [68]. By analyzing the ecological co-occurrence network's topological results, we can reveal the complex relationships between taxa and characterize the stability of the ecological structure [69]. The low modularity of the closely connected HT network also makes it more susceptible to environmental fluctuations [70]. The decreased stability of the network structure caused by the lack of water saturation was likely the key to the structure's simplification [51]. This may affect the health of the soil microbial ecosystem, especially the significant accumulation of plant pathogenic fungi (*Fusarium*), which will adversely affect agricultural production in the next year.

Along with the water saturation, the abundant fungal OTUs drove plant pathogens to saprotroph-dominated communities, and compositional shifts in fungal communities following freeze–thaw cycles can lead to lasting community changes [71]. The fungal mycobiome and its functional component guilds are shaped mainly by environmental selection over water spatial dimensions and freeze–thaw cycles. The functional behaviors of fungal communities highlight the underestimated diversity of ecological patterns in seasonal community assembly.

It should be pointed out that co-occurrence networks cannot summarize all the true biological interactions in the community [72], and differences in statistical methods often lead to conceptual differences in keystone taxa [73]. It is highly recommended that statistical analysis and identification of keystone taxa should undergo subsequent laboratory verification [16,31]. So we first determined the potential keystone taxa OTUs (*Penicillium*, *Talaromyces*, *Fusarium*, and *Aspergillus*) in straw decomposition based on the connectivity and central positions in the networks and then verified the straw decomposition ability through subsequent synthetic cultures [73].

Our research confirmed that the synthetic community combined with keystone taxa obtained from the network exhibited high straw decomposition-related enzyme activity during the freeze–thaw cycle. However, we adopted -10 °C as our freezing temperature in the enzymology experiment, which may have substantially impacted the fungal synthesis community compared to natural conditions. Some scholars have simulated the natural freezing temperature of soil microorganisms at -4 and -15 °C [74,75]. The lower freezing temperature will reduce microbial activity more because of water deficiency, which causes more physiological stress [76]. Our freeze–thaw cycles may have caused sudden changes in water activity without allowing soil fungi more time to adapt to the changed osmotic pressure [23], leading to a dormant state or lack of enzyme activity for the first four weeks. Although cultivable synthetic fungal communities may be a realistic choice, they cannot fully simulate the proper ecological functions of natural fungal communities. Our research only took the first step in investigating rice straw decomposition; field application and verification will be the next goal.

5. Conclusions

In conclusion, we summarize that water coverage with the freeze–thaw cycle promoted the decomposition of rice straw and influenced the fungal community structure; by analyzing the network of the fungal communities, it was found that the potential keystone taxa were *Penicillium*, *Talaromyces*, *Fusarium*, and *Aspergillus* in straw decomposition. These taxa displayed strong decomposition abilities and enzyme activities, indicating their importance for straw decomposition. Furthermore, plant pathogenic fungi were found to be decreased in the water-covered treatment. We hope that our result can help in rice production and straw return in practice.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture11111113/s1, Figure S1: Welch's *t*-test and Storey's FDR were used to evaluate biological importance ($p < 0.05$) and differences between FT and RT treatment on genus level. Figure S2: Potential function succes-sion of soil fungal communities in (A) freeze–thaw treatment and (B) homoiothermal treatment. Table S1: Network parameters without potential keystone.

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