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Microbiological and Mechanism Analysis of Novel Wheat Seed Coating Agents-Induced Growth Promotion of Wheat Seedlings

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Abstract: TFC (10% thifluzamide–fludioxonil–clothianidin) is a novel wheat seed-coating agent. In the field, we confirmed that 10% TFC plays a positive role in preventing soil-borne diseases and promoting wheat seedling growth. However, its effects on rhizosphere microecology and the underlying molecular mechanism are not fully understood. Field trials revealed a positive effect on the biomass, plant height, and root length of wheat sharp eyespots in a Yingshang field, with 95.3% control efficiency. The effects of 10% TFC on the rhizosphere soil microbiome of young wheat plants were evaluated using high throughput sequencing technology. The results demonstrated that seed-coating agents significantly changed bacterial and fungal communities, and reduced the number of bacteria but increased the number of fungi. Sequence analysis revealed that the abundance of Proteobacteria, Actinobacteria, and Patescibacteria in bacteria and Ascomycota, Mortierellomycota, and Basidiomycota in fungi were significantly enriched, which have been reported as being beneficial for plant growth and pathogen resistance. In contrast, the abundance of Mucoromycota in fungi was reduced, and most of the related genera identified were pathogenic to plants. In this study, 15-day-old wheat plant tissues treated with 10% TFC were subjected to global transcriptome analysis by RNA sequencing to provide insights into the effects of 10% TFC on seedling growth. The comparative analysis of *Triticum aestivum* L. libraries identified 8286 differentially expressed genes (DEGs), of which 2290 and 5996 genes were up- and downregulated in seedling growth in the presence of 10% TFC, respectively. Gene ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) functional analyses were performed for up- and downregulated DEGs separately, showing that these DEGs were enriched for terms related to the phenylpropanoid biosynthesis pathway, the protein products of which promote cell differentiation and seedling growth. This research provides comprehensive insights into its effects on wheat seedling growth and the rhizosphere microecology of seed coatings and provides important insights into their regulation and into understanding the potential benefits of seed coatings in disease management and plant growth promotion.

Keywords: seed-coating agents; growth; microbial diversity; wheat

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1. Introduction

Wheat (*Triticum aestivum* L.) is one of the most important food crops in the world, thereby playing a significant role in global food security [1–3]. Soil-borne wheat diseases caused by various pathogens are common, seriously affecting seed germination and subsequent seedling growth [4–6]. Coating seeds with agents containing fungicides offers a potential control measure to prevent seed-borne and/or soil-borne fungal diseases [7]. In China, fungicide seed treatments are currently widely used to control wheat seedling

diseases such as *Rhizoctonia cerealis* and *Bipolaris sorokiniana* [8]. The extensive use of seed-coating agents may lead to the accumulation of specific pesticides in the soil, which may negatively impact soil biodiversity [9].

Soil microbes are the most active part of the soil, as the driving force for material transformation and nutrient cycling in the soil, and they contain a large number of microorganisms, such as fungi, archaea, and bacteria [10–12]. Soil microbes have recently received increasing attention due to their perceived effects on plant health and productivity [13]. Soil microbes may have functions in plant growth promotion, biocontrol, and phytoremediation [14–16]. Recent studies have revealed that the application of chemical fertilizers and pesticides impact the soil microbial community structure [17,18]. Nevertheless, our understanding of the effects of chemical seed-coating agents on soil microbes is still limited.

Additionally, due to the rapid response of microorganisms to environmental changes, microbial community structure changes are considered to be effective biomarkers for soil conditions and land quality changes. Maintaining a diverse soil microbial community is important for sustainable agriculture; however, the application of chemical fungicides adversely affects non-target microbial communities [19]. Following the application of fungicides, we can frequently observe the loss of several fungal species and the ecological succession of other species of microorganisms [20]. While these studies provide some information on the effects of fungicides on soil microorganisms, they do not simultaneously focus on the diversity and composition of bacterial, fungal, and archaeal communities. In addition, few studies have been published on the effects of coating seeds with fungicides on soil microbial communities. Therefore, it is necessary to systematically study the possible influence of seed coatings on rhizosphere microbial communities, in order to improve safety, sustainability, and product quality.

With the development of gene sequencing technology, the effect of rhizosphere soil microorganism diversity on fresh plants has been determined in recent years. The 16S/ITS rRNA gene fragments obtained directly from soil samples were sequenced by employing this method to study the microbial community's structure and diversity in soil samples. Since this method has various advantages, including high flux, a large amount of information, and simple operation, it has been widely used in the study of soil microbial diversity with remarkable outcomes [21,22]. Although there has been some work carried out that studied the effects of soil amendments on rhizosphere bacterial populations, to the best of our knowledge, no studies have yet been published on mapping using 16S/ITS rRNA to profile the possible modifications of the rhizosphere microbial community as a result of applying chemical seed coatings to wheat seeds [23,24].

A further consequence of soil microbial community changes is the changes in plant tissues, which will better reveal the mechanism of seed-coating agent effects on seedling growth. Transcriptome techniques can reveal changes in plant tissues after the application of chemical fungicides, and can be combined with microbiome analysis to provide insights into the effects of 10% TFC on seedling growth. Therefore, the analysis of rhizosphere soil microbial community and transcriptome sequencing can help us gain insight into the complex biological processes in seed-coating agents-induced growth promotion of wheat seedling growth.

The specific objectives of this study were to study the effects of seed-coating agents on soil microecology and reveal the mechanism of seed-coating agents' effects on seedling growth. On the one hand, wheat plants were grown from seeds treated with 10% TFC and, on the other hand, from untreated seeds in four sites to test the effects on plant growth and disease infections. Then, a preliminary pot trial in a greenhouse was conducted, to investigate the influence of 10% TFC in the rhizosphere soils of young wheat plants using 16S/ITS rRNA sequencing, and gene expressions in 15-day-old wheat seedlings, cultured in the presence or absence of 10% TFC, were compared through transcriptome sequencing.

2. Materials and Methods

2.1. Wheat Seeds and Chemicals

The seeds of wheat used in this study were free from chemicals and were stored at room temperature (20–24 °C). Cultivar Yangmai 20 was used in the experiment, one of the most popular winter cultivars in China. Pesticides, including thifluzamide, fludioxonil, and clothianidin were purchased from ANPEL Laboratory Technologies Inc. (Shanghai, China), and all had >96% purity. Agricultural additives obtained from Haian Petrochemical Factory (Nantong, China), and the details are as shown in Table S1. All solvents and other chemicals used in the study were of analytical grade. The 27% difenoconazole–fludioxonil–thiamethoxam (DFT) seed-coating agents (suspension) were purchased from Syngenta Nantong Crop Protection Co., Ltd. (Nantong, China).

2.2. Preparation of 10% TFC for Wheat

The preparation of the seed-coating agent was prepared by the wet sand processing and superfine grinding method [25]. The optimal formula for the seed-coating agent was determined using an orthogonal test. The procedure conditions were as follows: all the ingredients, such as thifluzamide, fludioxonil, clothianidin, NNO, LAE-9, S-20, XG, magnesium aluminum silicate, and pigment, were mixed according to certain proportions to obtain the aqueous solution of the desired consistency. Then, other additives (film-forming auxiliaries, plant growth regulators, dispersants, colorants, etc.) were added to the aqueous solution according to a certain ratio, and the solution was continuously stirred at 25 °C under normal pressure for 4–5 h until completely dissolved. At this point, the preparation of a novel seed-coating agent was completed, and the stability was acceptable.

2.3. Field Experiments

In order to evaluate the effects of 10% TFC on plant growth, a field trial was conducted between the years 2021 and 2022 at four sites, namely Fengtai, Feixi, Fengyang, and Yingshang in Anhui province (China); these four sites are the main wheat production areas of southern and northern Anhui. The experimental sites have loam soil with medium fertility, and the soil's nutrient properties were as follows: soil organic matter, nitrate nitrogen, alkaline nitrogen, available potassium, available potassium, and the pH for the Fengtai site soil were 0.45 g/kg, 20.4 mg/kg, 12.5 mg/kg, 28.9 mg/kg, 8.40 g/kg, and 8.60, respectively; the corresponding values for Fengyang site soils were 26.8 g/kg, 98 mg/kg, 56.5 mg/kg, 75 mg/kg, 32.6 g/kg, and 8.14, the corresponding values for Feixi site soils were 23.8 g/kg, 102 mg/kg, 65.6 mg/kg, 85 mg/kg, 45.9 g/kg, and 8.64, and the corresponding values for Yingshang site soils were 46.8 g/kg, 105 mg/kg, 36.5 mg/kg, 95 mg/kg, 35.6 g/kg, and 8.24. The climate is a continental monsoon climate with a mean annual precipitation (MAP) of 1149 mm, and the annual average temperature is 17.0 °C; the average temperature ranged from 14 to 17 °C during the wheat-growing season. The amount of rainfall in 2021 was 1240 mm, and, in 2022, it was 979.8 mm. In each location, the treated and control seeds were assigned to the experimental plots using a completely randomized block design, with 3 replicates. Each plot consisted of 10 five-meter-long rows, separated by 1.5 m of bare cultivated ground. All test seeds were film-coated by mechanical planting; 10% TFC was applied at a rate of 1 mL per 100 g of seed, and 27% DFT was applied at the recommended dosage of 1:300. At sowing, no starter fertilizers were applied to the seed furrow. A random sample of 100 plants from each plot was selected to determine the root and shoot lengths and fresh and dry weights under each treatment (100 days). After heading, 100 plants were randomly selected to determine the control effect for soil-borne disease infections.

2.4. Test Soil and Wheat Seed-Coating Treatments

The soil was sifted and then sieved through a 2 mm round-hole sieve after air-drying. Each plastic pot (10.8 cm height, 12 cm diameter) contained 500 g soil [26]. There were two treatments: 10% TFC (the active ingredient content is 10%), and CK (uncoated wheat

seeds as a blank control). After air-dried, disinfected wheat seeds were coated following the recommended dosage for 10% TFC (10% TFC, 10 mL/kg seeds). The chemicals were diluted with water in the ratio of 1:100 (chemical: seed by weight) and added into the seeds, which were fully mixed until the chemical suspension was 'uniformly' distributed on the seed surface. Treated seeds were air-dried for 20 min, and 100 seeds were then sampled per group and placed evenly into a pot containing soil. Plants were regularly watered during the whole trial period. All pots were incubated under greenhouse conditions, i.e., at 25 ± 3 °C with natural illumination for 16 h, and at 20 ± 3 °C for 8 h per day. The water content in the initial pot soil was maintained at close to 70% of the water-holding capacity, as described in Chen et al. (2017) [24]. Young wheat plants were harvested after 15 days, and samples of the rhizosphere soil were collected. To collect the rhizosphere soil, plants were gently extracted from the ground and, after removing most of the soil by shaking the remaining rhizosphere, soil adhering to the roots was carefully collected with a small sterile brush. Samples were immediately stored at −80 °C for microbiome analysis.

2.5. DNA Extraction from Rhizospheric Soils

Soil samples were used to extract DNA using the OMEGA Soil DNA Kit (D5625-01) (Omega Bio-Tek, Norcross, GA, USA), following the manufacturer's instructions. After DNA extraction, the purity and concentration of the DNA were determined using agarose electrophoresis. The DNA was diluted to 1 ng μL^{-1} in sterilized ultra-pure water and stored at −80 °C for next-generation sequence analysis and polymerase chain reaction.

2.6. Illumina NovaSeq Sequencing

Soil bacterial, fungal, and archaeal microbial abundance and composition in the rhizosphere soil samples were analyzed using a high-throughput sequencing technique, which was assessed using an Illumina HiSeq 2500 instrument (Personalbio, Shanghai, China) [27,28]. PCR amplification of the rhizosphere soil bacterial 16S rRNA V3–V4 region was carried out with the forward primer 338 F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806 R (5′-GGACTACHVGGGTWTCTAAT-3′). The fungal ITS region was amplified via the forwarding primer ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and reverse primer ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′). PCR amplification of the rhizosphere soil archaeal 16S rRNA V4–V5 region was carried out with the forward primer 524 F (5′-TGYCAGCCGCCGCGGTAA-3′) and the reverse primer 958R (5′-YCCGGCGTTGAVTCCAATT-3′). PCR was performed under the following conditions: 30 s of denaturation at 98 °C, followed by 30 cycles, 15 s of denaturation at 98 °C, 30 s of annealing at 50 °C, 30 s of extension at 72 °C, and a final extension step at 72 °C for 5 min. The PCR products were extracted and combined in equimolar ratios with the quantitative DNA binding method in order to create a DNA pool that was further used for sequencing. Amplicons were pooled in equimolar amounts and sequenced using the 2 × 250 bp pair-end strategy on the Illumina HiSeq platform Personalbio (Shanghai, China). QIIME (Quantitative Insights into Microbial Ecology) was used to analyze the sequencing data. Barcodes were removed from the sequences, and any sequences < 150 bp or with ambiguous bases were removed [29]. The sequences were clustered de novo into operational taxonomic units (OTUs), based on sequence similarity at a threshold of 97% [30]. According to its algorithm principle, the screened sequence with the highest frequency in OTUs was considered to be the representative sequence of OTUs. The Alpha diversity index analysis and the Beta diversity index were conducted. Principal component analysis (PCA) was used to analyze the differences in the community structures of the different groups.

2.7. Wheat Transcriptome Analysis

The young wheat plant tissues were collected 15 days after sowing, and all the samples for each treatment were subjected to global transcriptome analysis. Three biological replicates were paired for each sample, immediately frozen in liquid nitrogen, and stored at −80 °C. The wheat transcriptome was determined at Personalbio Company (Shanghai,

China). Briefly, total RNA was isolated via the isolation kit (MOBIO Laboratories Inc., Carlsbad, CA, USA). Quality and integrity were determined with a Nano Drop ND-1000 UV-vis Spectrophotometer (Thermo Scientific, Rockwood, TN, USA). After concentration, three micrograms of RNA were analyzed. Sequencing libraries were built with the NEB-Next Ultra Directional RNA Library Prep Kit (Illumina, San Diego, CA, USA). Briefly, the Oligo (dT) magnetic beads were used to enrich the mRNA with a polyadenylic acid (polyA) structure in the total RNA. The RNA was interrupted to a fragment of about 300 bp in length by means of ion interruption. After the library construction procedure, PCR amplification was used to enrich the library fragments. Then, the library was selected in accordance with the fragment size. The library size was 450 bp, and the quality of the library was checked using bioinformatics analysis (QIIME2 2019.4). After RNA extraction and purification, and the library building of the samples, the second-generation sequencing technology (Next-Generation Sequencing, Pico green) was used to perform paired-end (PE) 150 sequencing on these libraries via the Illumina sequencing platform. The expression level in each gene was measured by fragments per kilobase per million reads (FPKM) [31]. Additionally, HTSeq (0.9.1) statis tics were performed to compare the read count values on each gene to the original expression of the gene. FPKM was applied to standardize the expression. DESeq (1.20.0) was used to analyze the genes with different expressions, using the following screened conditions: expression difference multiple $|\log2FoldChange| > 1$, significant $p < 0.05$. Gene ontology (GO) terms were retrieved using iNAP (integrated Network Analysis Pipeline), and gene function annotation was conducted through comparison to the Kyoto Encyclopedia of Genes and Genomes (KEGG).

2.8. Bioinformatics and Statistical Analysis

Sequence data analyses were mainly performed using QIIME2 (version 1.7.0) and R packages (v3.2.0). Alpha diversity was determined using the QIIME suite of programs, including the Chao1, Shannon, Simpson, and ACE indicators, and the R software was used to draw the dilution curve. Beta diversity analysis was performed to investigate the structural variation in microbial communities across samples using Jaccard metrics. Nonmetric multidimensional scaling (NMDS) and the unweighted pair group method with arithmetic mean (UPGMA) were used for hierarchical clustering.

A one-way analysis of variance (ANOVA) and an LSD multiple comparison test were used to analyze differences in microbial diversity or functional gene abundance between treatments, and statistical significance was defined as $p < 0.05$. Pearson's correlation analysis was used to determine relationships, and a correlation analysis of community diversity was also performed. Favorable model fits were determined based on the criteria described in Schermelleh-Engel et al. (2013) [32].

3. Results

3.1. Effects of 10% TFC on Plant Growth

The effect of seed-coating agents (10% TFC) on the germination and growth of wheat in a field trial was investigated. The results of this study showed that the seed-coating agent (10% TFC) had a significant effect on the biomass, the stem, and root length of wheat in the field trial (Table 1). Differences in wheat biomass were also observed between the different field trials (Figure 1a–e). Briefly, the fresh and dry weights ranged from 8.59 to 17.42 and from 3.96 to 6.86 g following treatment with DFT and TFC in Fengtai, while the corresponding values in Feixi ranged from 34.34 to 48.56 g and from 8.84 to 19.82 g, respectively. In addition, the fresh and dry weights ranged from 18.67 to 34.75 g and from 3.01 to 5.55 g following treatment with DFT and TFC in Yingshang, while the corresponding values in Fengyang ranged from 16.16 to 33.86 g and from 2.98 to 7.7 g, respectively. This was possibly due to the effect of farming methods; wheat–maize rotations are the dominant cropping systems in Yingshang and Fengyang, whereas the farming methods of Fengtai and Feixi consist of wheat after rice. Previous studies also showed that there are differences among different farming methods in root system biomass and root system volume [33,34].

The best agent for promoting the germination and growth of wheat in Feixi and Fengtai was 10% TFC, which provided a better wheat biomass than the positive control, e.g., 27% DFT (difenoconazole + fludioxonil + thiamethoxam, 2.2 + 2.2 + 22.6%, respectively).

Table 1. Effects of seed-coating treatments on germination and growth of wheat in field trial.

Experimental Sites	Coating	Main Performance Indexes of Wheat				Control Efficiency of Sharp Eyespot
		Root Length (cm)	Stem Length (cm)	Fresh Weight (g)	Dry Weight (g)	Control Effect (%)
Fengtai	TFC	12.06 ± 0.48 a	17.50 ± 0.73 a	17.42 ± 3.65 a	6.863 ± 1.76 a	83.7
	DFT	11.46 ± 0.33 a	15.20 ± 0.20 b	13.16 ± 0.49 ab	5.57 ± 0.25 ab	87.3
	CK	9.96 ± 0.18 b	12.96 ± 0.75 c	8.59 ± 0.31 b	3.96 ± 0.19 b	-
Feixi	TFC	30.86 ± 0.38 a	12.13 ± 0.88 a	48.56 ± 3.56 b	19.82 ± 3.91 b	72.2
	DFT	23.30 ± 1.98 b	10.83 ± 0.16 a	32.83 ± 1.70 a	10.81 ± 1.37 a	77.8
	CK	22.96 ± 1.49 b	11.96 ± 0.13 a	34.34 ± 5.32 a	8.84 ± 1.57 a	-
Yingshang	TFC	12.49 ± 1.00 a	22.74 ± 0.52 ab	34.75 ± 0.75 b	5.55 ± 0.03 b	95.3
	DFT	12.68 ± 0.144 a	24.50 ± 0.97 a	42.95 ± 1.29 a	6.19 ± 0.10 a	91.7
	CK	12.93 ± 0.26 a	20.59 ± 0.57 b	18.67 ± 0.57 c	3.01 ± 0.11 c	-
Fengyang	TFC	12.39 ± 0.71 a	23.76 ± 1.10 a	33.86 ± 2.08 a	7.70 ± 0.24 a	90.3
	DFT	12.72 ± 1.20 a	17.86 ± 0.64 ab	26.87 ± 2.14 b	6.40 ± 0.18 b	87.3
	CK	11.50 ± 0.74 a	15.51 ± 1.17 b	16.16 ± 1.32 c	2.98 ± 0.07 c	-

This table presents the means and the standard error of three replicates to compare the difference of three treatments at the same time, and different letters indicate a significant difference ($p < 0.05$, Tukey's test). CK: uncoated seeds; TFC: 10% TFC-coated seeds; DFT: 27% DFT-coated seeds.

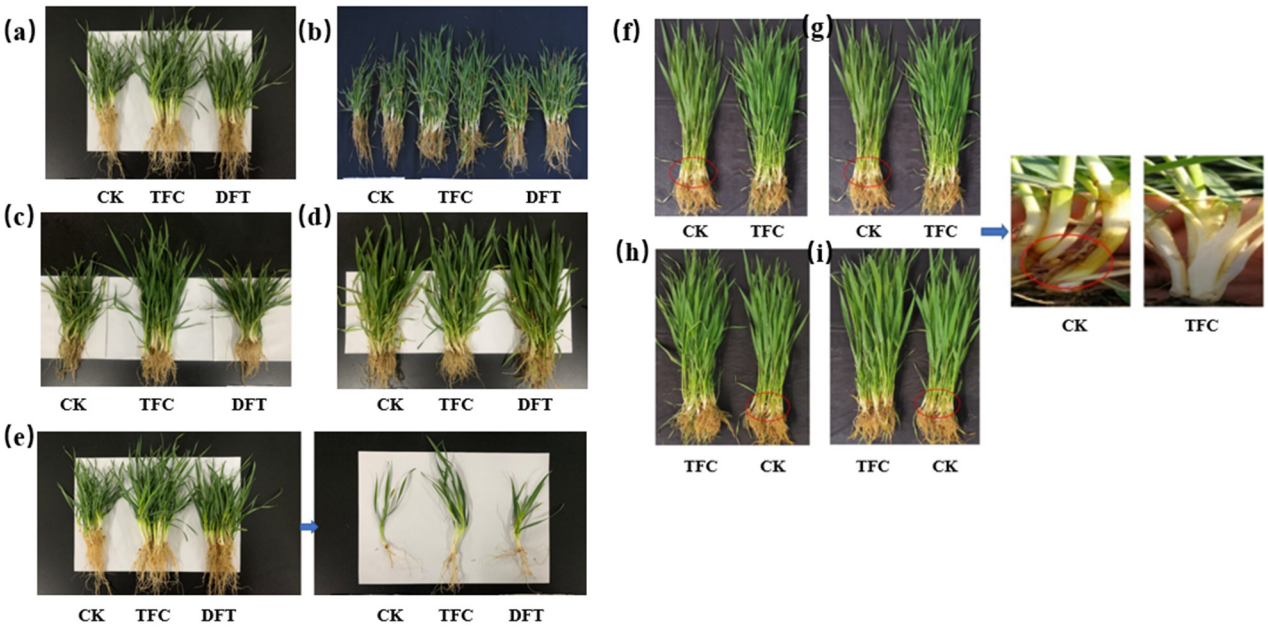

Figure 1. Effects of seed-coating treatments on plant growth ((**a**,**e**) Fengtai; (**b**) Fengyang; (**c**) Feixi; and (**d**) Yingshang) and soil-borne diseases ((**f**) Fengtai; (**g**) Fengyang; (**h**) Feixi; and (**i**) Yingshang). CK: uncoated seeds; TFC: 10% TFC-coated seeds; DFT: 27% DFT-coated seeds.

The control efficiency of the seed-coating agents on soil-borne diseases in the field trial was also measured (Figure 1f–i). In this study, we only investigated wheat sharp eyespot, due to this being only the disease that occurred during the year in the fields. The results showed that 10% TFC and 27% DFT effectively controlled and reduced disease severity in the four field sites, and the control efficiency reached 72.20% and 95.30%, respectively. In Yingshang field, the control efficiency of 10% TFC was the highest (95.30%), followed by that of 10% TFC in Fengyang field (81.57%).

These results indicate that the use of 10% TFC as the seed-coating agent appears to enable the effective control of wheat sharp eyespot, while having a positive impact on the

germination and growth of the wheat in the field. In summary, 10% TFC can achieve certain economic benefits, and its introduction and application in agriculture is meaningful.

3.2. Changes in Rhizosphere Soil Fungal Community

The microbial community diversity (fungal) in 10% TFC-coated and CK samples was characterized using partial ITS rRNA gene sequencing obtained from DNA directly extracted from rhizosphere soil samples of wheat coated with 10% TFC. After filtering and removing the chimaeras, a total of 121,846 high-quality fungal sequences and 106,016 high-quality fungal sequences were obtained from the CK and treatment group soil samples after quality filtering, respectively (Table S1). Dilution curves (Figure S1) directly reflect the species richness in the samples. The dilution curves of the six samples tended to level off as the sequencing amount increased, suggesting that the diversity in the libraries was representative of the community, and the sequencing data reflected the actual situation of the fungal community in the samples.

The seed-coating treatments significantly affected fungal alpha diversity. Compared with the control group, the seed-coating treatments resulted in significantly higher fungal alpha diversity indices (Simpson and Shannon) and evenness index (Pielou's) results (Figure 2a). This indicates that the 10% TFC treatments in this experiment had a positive effect on the community diversity and the evenness of rhizosphere soil fungal community. However, the seed-coating treatments did not significantly affect the fungal Chao1 diversity index (Figure 2a). PCoA, based on the Bray–Curtis distance, showed that the control samples were separated from coating-treated samples (Figure 2b). It can be observed that the contribution rates of the two principal components were 74.9% and 12.8%. Compared with the CK, for the first principal component (PCo1) and the second principal component (PCo2), there was significant change with the seed-coating treatment, indicating that the coating treatment can change the structure of the soil fungal community.

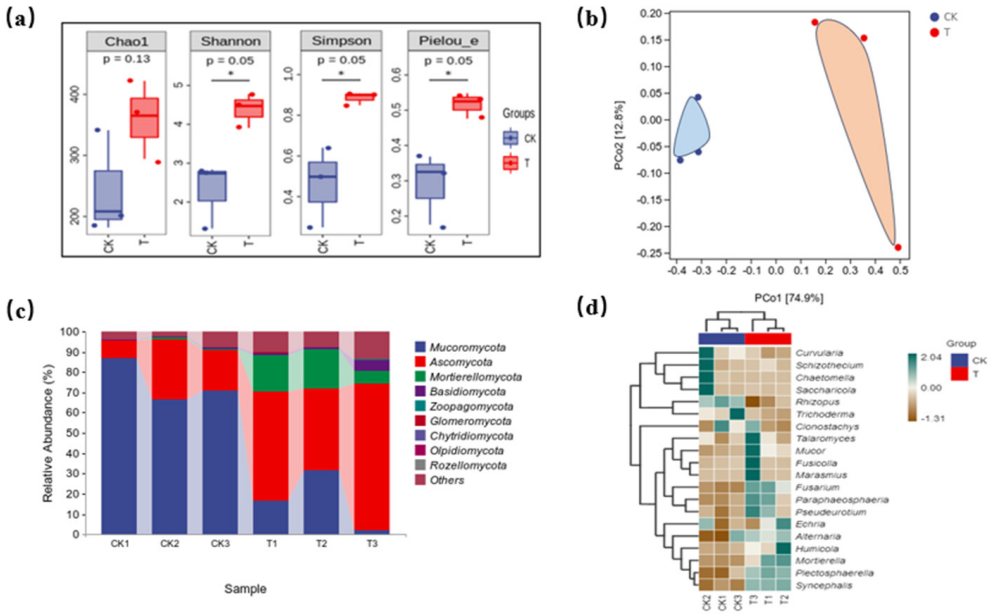

Figure 2. Effects of seed-coating treatment on fungal Chao1, Shannon, Simpson, and Pielou's diversity indices (**a**), PCoA of fungal community structure (the points with different colors indicate different groups) (**b**), phylum level fungal community composition (**c**), and heatmap of species clustering at the genus level of fungi in the different soil samples (**d**). CK: uncoated seeds; T: 10% TFC-coated seeds. Asterisks indicate significant differences (* $p < 0.05$).

The relative abundances of fungal taxa were examined at the phylum (Figure 2c) and genus (Figure S2) levels, to assess whether there were any significant shifts in the taxonomic composition of the microbial communities between coating treatments. As shown in

Figure 2c, the dominant phyla of soil fungi under both treatments were Mucoromycota and Ascomycota, and their relative abundances were different from each other. Among the top 10 most abundant phyla, there were higher relative abundances of Ascomycota and Mortierellomycota, and a lower relative abundance of Mucoromycota in coated seeds than in uncoated seeds. In the CK control group, Mucoromycota was the most dominant (74.79%), followed by Ascomycota and Mortierellomycota (19.28% and 0.76%, respectively) (Figure 2c). In contrast, the relative abundances of Ascomycota (55.45%) were highest under the 10% TFC treatment; the phyla showing significant increases were Mortierellomycota (14.61%) and Basidiomycota (5.67%), while Mucoromycota decreased (16.73%).

On the other hand, the application of 10% TFC had an obvious positive effect on fungal genera, such as changing the predominant fungal composition in the wheat rhizosphere. The test results demonstrate that the 10% TFC treatment restricted the development of *Rhizopus* while partly promoting the growth of the beneficial fungi *Mortierella*, *Fusicolla*, and *Fusarium* (Figure S2). At the genus level, a cluster heatmap was used to show the abundance of the top 10 genera in the sample (Figure S2). *Rhizopus* had higher relative abundance in uncoated seeds. *Fusarium* and *Mortierella* were more abundant in 10% TFC-coated seeds. More specifically, the relative abundances of *Rhizopus* were significantly lower in treatment group (0.02–0.25%) than in the CK group (0.70–0.95%) ($p < 0.05$). A similar pattern was observed for *Chaetomella*. The relative abundance of *Fusarium*, *Mortierella*, and *Fusicolla* were significantly higher in the treatment group (0.2–0.35%, 0.2–0.25%, and 0.01–0.2%, respectively) than in the CK group (0.1–0.15%, 0.01–0.03%, and 0.01–0.02%, respectively). Additionally, the treated group also had an impact on the abundance of the other four dominant fungal genera (*Paraphaeosphaeria*, *Talaromyces*, *Trichoderma*, and *Pseudeurotium*), but the difference was not significant. Notably, the 10% TFC treatment also increased the variability in the abundance of the dominant fungal genera. For example, there were more than seventy fungal species in the treatment group, compared with seven species in the blank control group. This increase was statistically significant in the control ($p < 0.05$) and treatment groups ($p < 0.001$). The top 20 most abundant fungi genera, in terms of relative abundance, were selected for heat map analysis (Figure 2d). Specifically, in T3, the relative abundances of *Talaromyces*, *Mucor*, *Fusicolla*, and *Marasmius* were high; in T2, *Humicola* and *Echria* had high relative abundance; in CK3, *Trichoderma* and *Alternaria* were high in relative abundance. Meanwhile, in CK2, the five genera with high relative abundance were *Curvularia*, *Schizothecium*, *Chaetomella*, *Saccharicola*, and *Rhizopus*. It can be observed that the 10% TFC treatment did not have genera in common with the CK soil, indicating that 10% TFC affected the abundance of the soil fungal community. In addition to differences at the phylum and genus levels, ten and five taxonomy units that differed the most between treatments (with a log10 LDA (linear discriminant analysis) threshold of >2.0) were identified for fungi, respectively, at the phylum, class, order, family, and genus levels (Figure S3).

3.3. Changes in Rhizosphere Soil Bacterial Community

To analyze the composition of the bacterial communities, the amplicon libraries for Illumina HiSeq 2500 sequencing 16S rRNA were constructed and sequenced (Table S2). A total of 93,375, 93,192, 10,3472, 59,075, 99,616, and 71,925 high-quality 16S rRNA sequences, respectively, were obtained from the control and treatment group samples (three replicates of each). Dilution curves can directly reflect the species richness in the samples (Figure S4). The dilution curves of the six samples tended to level off as the sequencing amount increased, indicating that the sampling was reasonable and the sequencing data reflected the actual situation of the bacterial community in the samples.

Unlike fungal diversity, seed-coating treatments did not significantly affect bacterial alpha diversity (Figure 3a). This study's analysis of the bacterial community richness indices, Chao1, showed that there were no significant differences between the treatments (*p*-value). Compared with the control group, the seed-coating treatments reduced the Simpson and Shannon index scores of bacterial diversity in the rhizosphere soil, but the difference

was insignificant (*p*-value) (Figure 3a). This indicates that the 10% TFC treatments in this experiment had a negative effect on the bacterial community diversity of rhizosphere soil; the specific reasons for this need to be further investigated, due to the complexity of the soil bacterial structure. In the bacterial PCoA plots, the control samples were separated from the seed-coating-treated samples (Figure S5). As shown in Figure 3b, the results revealed that the dominant bacterial phyla in all rhizosphere samples were Proteobacteria, Actinobacteria, Firmicutes, Patescibacteria, Acidobacteria, Chloroflexi, Bacteroidetes, and Gemmatimonadetes, accounting for >95% of all sequence reads (Figure 3b). The test results demonstrate that 10% TFC restricted the development of the harmful bacteria Gemmatimonadetes, while partly promoting the growth of the beneficial bacterial genera Proteobacteria, Actinobacteria, and Patescibacteria. Compared with the control group, the relative abundances of Actinobacteria, Firmicutes, and Patescibacteria, with 10% TFC application, were increased by 27.8%, 11.4%, and 8.0%, respectively. For Proteobacteria, its relative abundance was increased under 10% TFC treatment, but this was not significantly different to the control group. In the soil, seed coating reduced the relative abundance of Acidobacteria compared to the control. The relative abundances of Chloroflexi, Bacteroidetes, and Gemmatimonadetes following 10% TFC treatment decreased, compared to those under the control group conditions, representing a significant difference ($p < 0.05$). In addition to differences at the phylum and genus levels, one and five taxonomy units that differed the most between treatments (with a log10 LDA (linear discriminant analysis) threshold of >2.0) were identified for bacterial diversity, respectively, at the phylum, class, order, family, and genus levels (Figure S6).

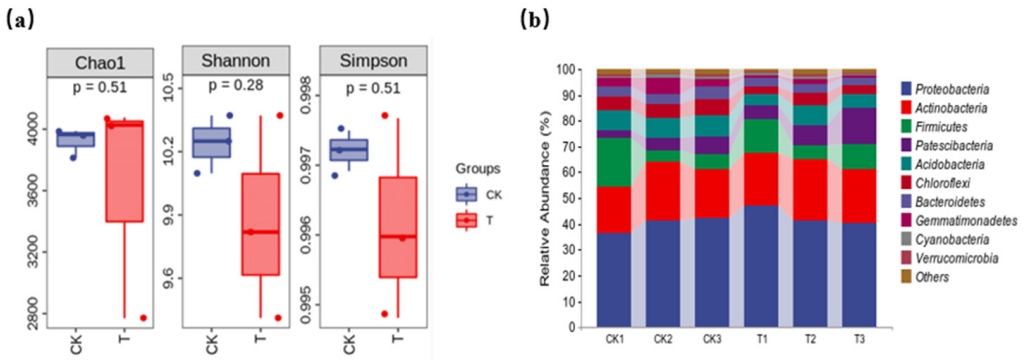

Figure 3. Effects of seed-coating treatment on bacterial Chao1, Shannon, and Simpson diversity indices (**a**) and phylum-level bacterial community composition (**b**). CK: uncoated seeds; T: 10% TFC-coated seeds.

3.4. Changes in Rhizosphere Soil Archaeal Community

To analyze the composition of the archaeal communities, the amplicon libraries for Illumina HiSeq 2500 sequencing 16S rRNA were constructed and sequenced. A total of 52,144, 58,986, 55,212, 47,905, 44,602, and 50,445 high-quality 16S rRNA sequences, respectively, were obtained from the control and treatment group samples (three replicates of each) (Table S3). Dilution curves (Figure S7) can directly reflect the species richness in the samples. The dilution curves of the six samples tended to level off as the sequencing amount increased, indicating that the sampling was reasonable and the sequencing data reflected the actual situation of the archaeal community in the samples.

There were no significant differences in archaeal alpha diversity indices (Chao1, Simpson, and Shannon) between treatments (Figure 4a). However, the Faith's PD index score for 10% TFC treatment was significantly higher than that of the control (Figure 4a). The PCoA of Bray–Curtis beta diversity indices showed significant differences in the archaeal plots between coating treatments (Figure 4b). The dominant archaeal phyla (Figure 4c) and genera (Figure S8) were used to assess the changes in the taxonomic composition of archaeal communities between coating treatments. In all seed samples, the dominant archaeal phyla were Thaumarchaeota, Euryarchaeota, and Crenarchaeota, accounting for

>90% of the total reads (Figure 4c). Among them, Thaumarchaeota occupies the largest proportion, followed by Euryarchaeota and Crenarchaeota. The relative abundances of dominant archaeal genera are shown in Figure S8. The control and 10% TFC treatments had high levels of similarity. The dominant archaeal genera were *Nitrososphaeraceae*, *Methanobacterium*, and *Bathyarchaeia* [35]. *Nitrososphaeraceae* and *Methanobacterium* had higher relative abundance in uncoated seeds. *Methanobacterium* and *Methanosarcina* were more abundant in 10% TFC-coated seeds. In addition, higher abundances of *Rice-Cluster-II*, *Candidatus Nitrocosmicus*, *Candidatus Nitrososphaera*, *Methanosaeta*, *Methanocella*, and *Rice-Cluster-II* were found in the 10% TFC-coated seeds. In addition to differences at the phylum and genus levels, ten and five taxonomy units that differed the most between treatments (with a log10 LDA (linear discriminant analysis) threshold of >2.0) were identified for archaea, respectively, at the phylum, class, order, family, and genus levels (Figure S9).

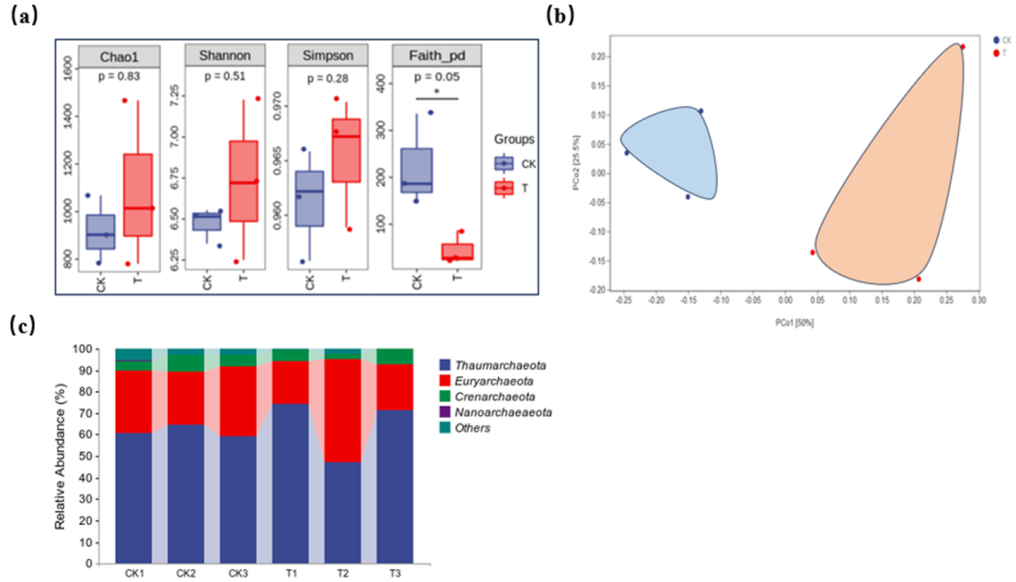

Figure 4. Effects of seed-coating treatment on archaeal Chao1, Shannon, Simpson, and Faith's PD diversity indices (**a**), PCoA of archaeal community structure (the points with different colors indicate different groups) (**b**), and phylum-level archaeal community composition (**c**). CK: uncoated seeds; T: 10% TFC-coated seeds. Asterisks indicate significant differences (* $p < 0.05$).

3.5. Microbial Taxa Sensitive to Seed-Coating Agents

To assess whether the relative abundance of each taxon differed between the seed-coating-treated and the control samples in the two soils, the linear discriminant analysis effect size (LEfSe) method was used (Figure 5a–c). The relative abundances of a total of 82 fungal and 99 bacterial taxa differed between the treated and the control samples in the soil; the corresponding value for the archaeal taxa was six. The operational taxonomic units (OTUs) of a total of 985 fungal, 16,764 bacterial, and 4076 archaeal taxa were obtained for the treated and the control samples (Figure 5d–f); namely, the numbers of OTUs in the control group were 540, 8475, and 1910, while those of the treatment group were 664, 8289, and 2166, respectively. Notably, ten fungal taxa were significantly enriched in all seed samples (Figure 5g), namely *Pyrenochaetopsis*, *Paraphaeosphaeria*, *Septoria*, *Didymella*, *Setophoma*, *Fusarium*, *Myrmecridium*, *Mortierella*, *Mucor*, and *Syncephalis*. Eight bacterial taxa were significantly enriched in all seed samples (Figure 5h), namely *Kutzneria*, *Niastella*, *Bacillus*, *Clostridium_sensu_stricto_3*, *Saccharimonadales 0319-6G20*, *Archangium*, *Aquabacterium*, *Massilia*, and *Dyella*. Ten archaea taxa were significantly enriched in both treatments (Figure 5i); they are *Bathyarchaeia*, *Rice_Cluster_I*, *Methanobacterium*, *Methanocella*, *Rice_Cluster_II*, *Methanosaeta*, *Methanosagoian*, *Candidatus_Nitrososphaera*, *Candidatus_Nitrocosmicus*, and *Nitrososphaeraceae*.

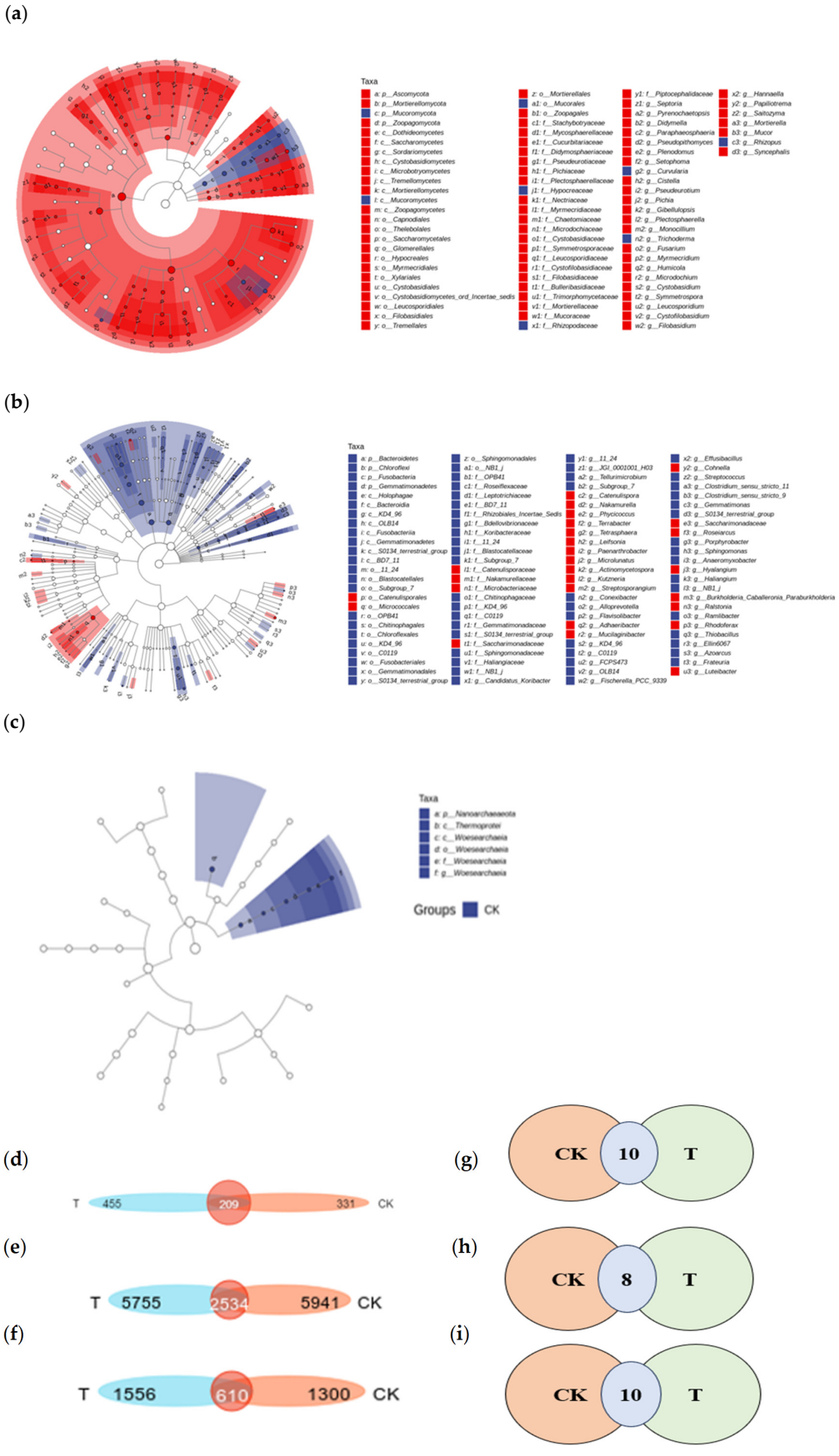

Figure 5. The linear discriminant analysis effect size (LEfSe) results for CK and 10% TFC treatments in the soils. (**a**) Cladogram of fungal taxa in the soil; (**b**) cladogram of bacterial taxa in the soil; (**c**) cladogram of archaeal taxa in the soil; (**d**) Venn diagram of fungal biomarkers in each treatment in

the soils; (**e**) Venn diagram of bacterial biomarkers in each treatment in the soils; (**f**) Venn diagram of archaeal biomarkers in each treatment in the soils; (**g**) Venn diagram of fungal taxonomic distributions for treatments among different combinations; (**h**) Venn diagram of bacterial taxonomic distributions for treatments among different combinations; (**i**) Venn diagram of archaeal taxonomic distributions for treatments among different combinations. CK: uncoated seeds; T: 10% TFC-coated seeds. Only taxa that had met the linear discriminant analysis significance threshold of >2.0 are shown. Every circle indicates a treatment; the numbers of OTUs shared between different treatments is interpreted using the number in the overlapping circles, while the number in the non-overlapping area represents the number of unique OTUs of the specific treatment.

3.6. Transcriptional Change Responding to 10% TFC

To explore the effects of 10% TFC on seedling growth, transcriptomic profiles of wheat tissues were investigated. Two biological replicates were set up for each treatment. For each treatment, 15-day-old seedlings were collected separately and used to prepare cDNA libraries, which were sequenced using the BGISEQ-500 sequencing platform. After joining overlapping reads and removing low-quality sequences from the raw reads, a total of 447,599,627 and 498,970,193 clean reads were obtained from the CK and treatment groups of seedling samples after quality filtering, respectively (Table S4). The cluster analysis results showed that the correlation between the biological replicates was high, indicating that the sequencing data were repeatable and reliable (Figure 6a). A total of 8286 differentially expressed unigenes (DEGs) were identified, including 2290 upregulated and 5996 downregulated genes, between the seed-coating-treated and the control samples (Figure 6b,c).

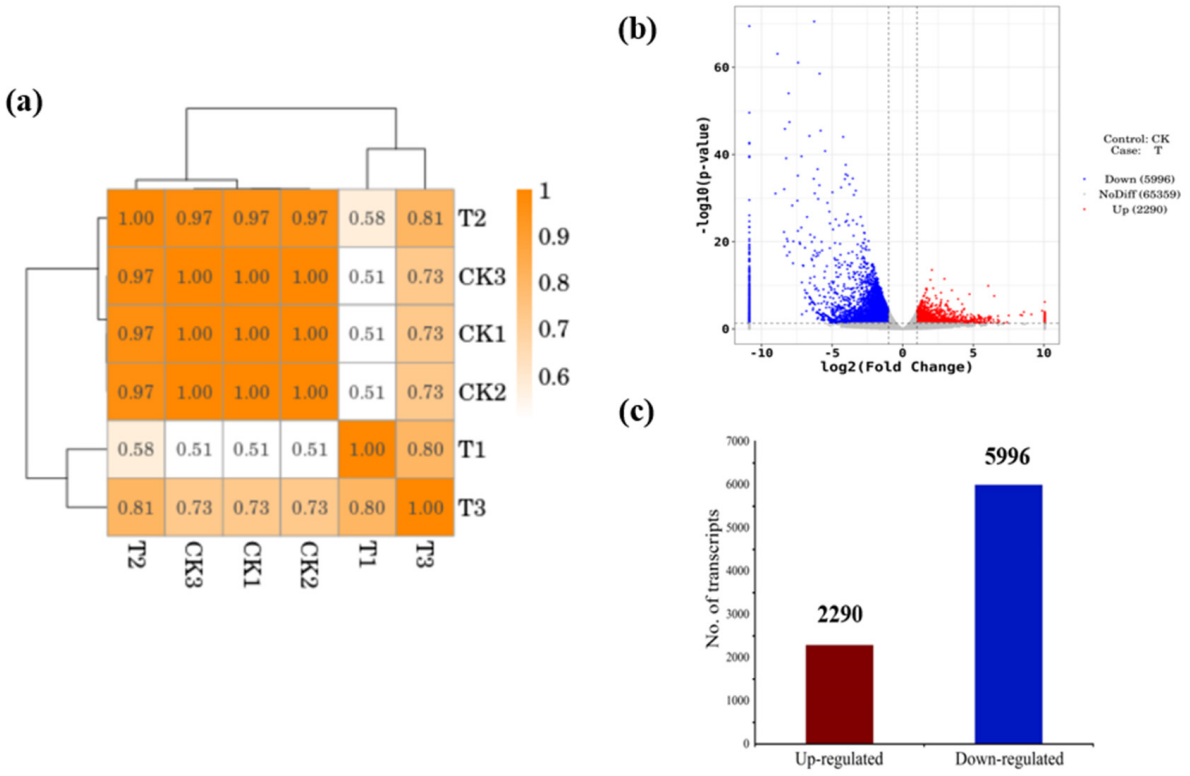

Figure 6. Differential gene expression analysis of wheat seedlings in response to 10% TFC. (**a**) Correlation analysis of the samples used for sequencing. The sample numbers are indicated, and the values in the squares are the Pearson correlation coefficients, calculated using R Studio. Dark colors indicate high expression, while lighter colors indicate lower expression. (**b**) Volcano plot of DEGs. *X*-axis: $\log_2$-fold change (10% TFC/control). *Y*-axis: the negative $\log_{10}$- adjusted *p*-value (FDR). Red

data points indicate upregulated transcripts and blue data points indicate downregulated transcripts. (**c**) Number of up- and downregulated DEGs. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

To identify the functional pathways the DEGs are involved in, both GO and KEGG functional analysis were performed for up- and downregulated DEGs separately. In the biological process category, up- and downregulated DEGs were enriched for photosynthesis, plastid organization, and photorespiration. In the cellular component category, up- and downregulated DEGs were enriched for plastid, chloroplast, and thylakoid. In the molecular function category, up- and downregulated DEGs were enriched in catalytic activity and ribulose bisphosphate carboxylase activity (Figure 7a). Additionally, KEGG analysis showed that many DEGs were involved in genetic information processing and metabolism, including ribosome biogenesis in eukaryotes, plant hormone signal transduction, the biosynthesis of amino acids, and phenylpropanoid biosynthesis in photosynthetic organisms (Figure 7b). By comparison, we found that "phenylpropanoid biosynthesis", "plant hormone signal transduction", "biosynthesis of amino acid", and other metabolic pathways were the main gene-enriched pathways in wheat seedlings. In these pathways, there were 70 DEGs upregulated, including genes related to cell differentiation, seedling growth, and disease resistance, and eight DEGs that were downregulated and mainly related to the inhibition of plant growth.

(**a**)

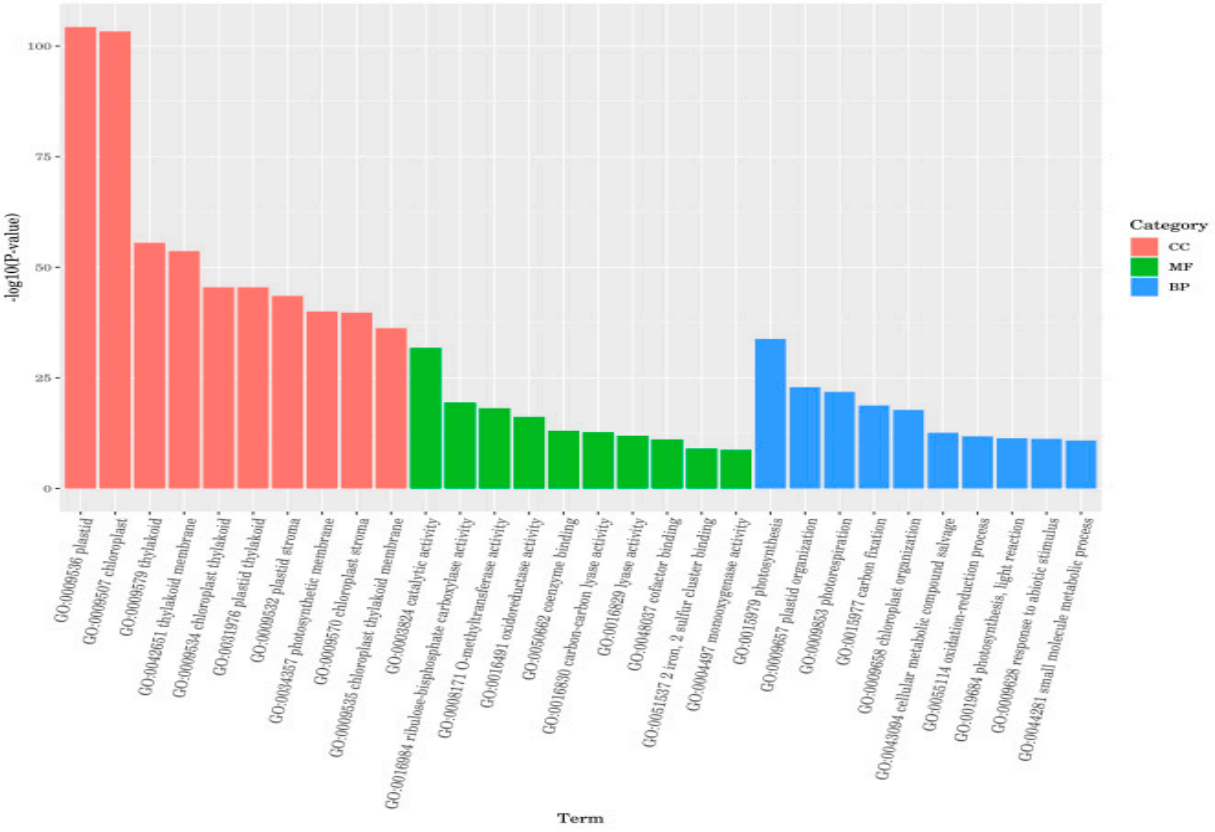

Figure 7. *Cont.*

(**b**)

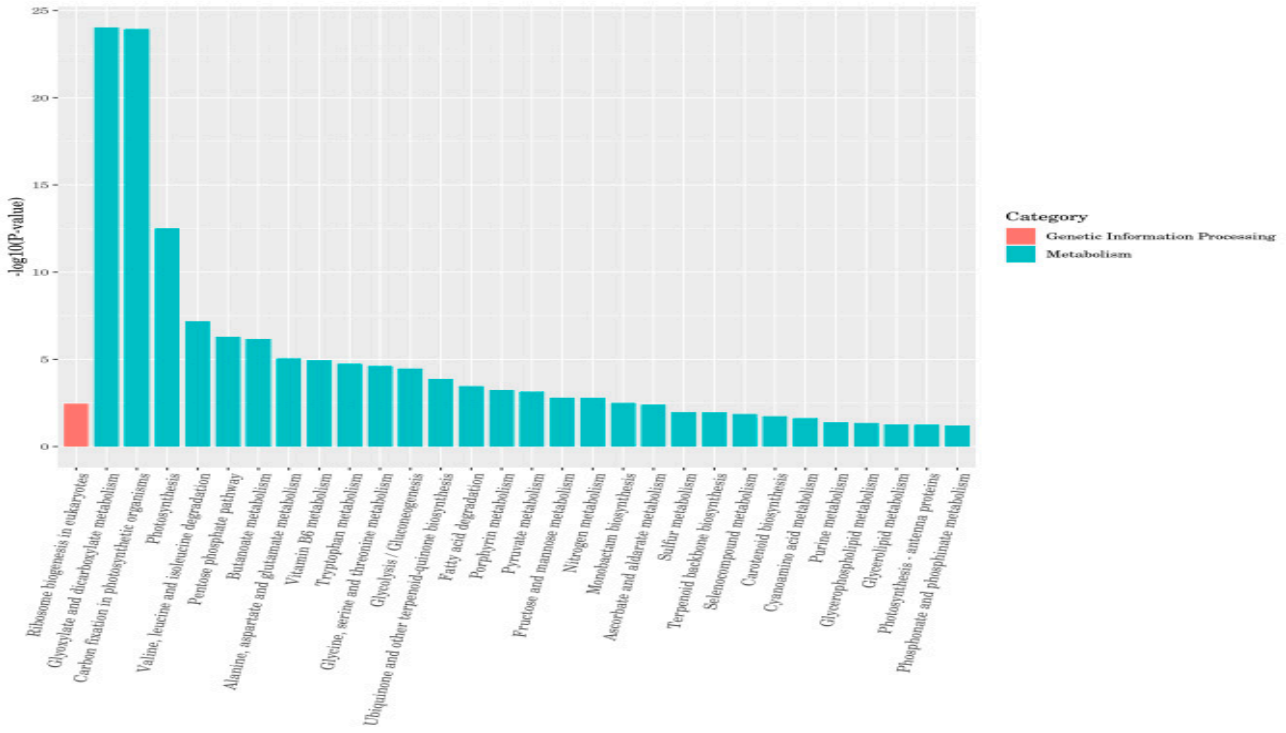

Figure 7. Major pathways differentially regulated by 10% TFC in wheat seedlings, as indicated by (**a**) GO and (**b**) KEGG enrichment analysis.

4. Discussion

Chemical seed-coating agents have been widely used in the field because of their plant protection and seedling growth promotion effects. In particular, so far, studies on their effects on rhizosphere microecology and the underlying molecular mechanism have been limited. This study investigated the responses of wheat rhizosphere soil microbial communities and transcriptome to seed-coating agents. These findings indicated that coating seeds with fungicides affected bacterial, fungal, and archaeal communities in rhizosphere soil, particularly for fungi, but did not lead to significant changes in the alpha diversity indices. This result is consistent with previous findings [36]. On the one hand, this may be due to bacterial, fungal, and archaeal communities sensitive to coating agents being replaced by insensitive ones. Figure 2a–c support this conclusion, with differences in the classification and proportion of dominant fungal phyla. On the other hand, this may be because the 10% TFC treatment increased the abundance of soil fungal communities, leading to changes in the qualitative composition of soil fungal microflora, and promoting the growth of some dominant fungi. In this case, these fungi increased in quantity.

Proteobacteria, Actinobacteria, Firmicutes, *Bacillus*, *Mortierella*, *Fusicolla*, and *Fusarium* were the main phyla or genera of the rhizosphere soil microorganism community whose relative abundances were increased by the coating agents. Proteobacteria may suppress plant diseases and promote plant growth [37,38], and Actinobacteria are dominant phyla that are beneficial to plants, as they maintain various normal functions and possibly control soil-borne pathogens [39–41]. Firmicutes is an important rhizosphere bacterium that can promote plant growth via nitrogen fixation, phosphate solubilization, and plant hormone production [42]. Proteobacteria, in particular, have been found in the rhizosphere of most plant species [43]. Actinobacteria found in wet rhizosphere soil can enhance seedling vigor by promoting the decomposition or formation of humus. The increases in the relative abundance of these potential beneficial microbial genera could affect subsequent seedling growth. *Bacillus* has the potential to antagonize plant pathogens [44]. *Mortierella* and *Fusicolla* are fungal genera that are beneficial for plant growth and development [45,46].

Additionally, some specific *Fusarium* make important contributions to plant defense. A recent study proved that *Fusarium* can promote the symbiotic effect of arbuscular mycorrhizal fungi and alleviate the deleterious effect of salinity on wheat growth [47]. Thus, 10% TFC increased the relative abundance of fungi at a genera level, and caused the inhibition of pathogenic microorganisms, contributing to wheat growth. These small changes in abundance makes it tempting to speculate that 10% TFC treatment may not only have a direct beneficial effect on plant health and root growth by interacting with plant signaling cascades, but that this could also have an indirect effect through the stimulation of rhizosphere bacteria by producing molecules of benefit to the plants. In addition, soil microbial diversity is an important indicator for the health of soil environments [43]. Korenblum et al. indicated that variations in rhizosphere soil microbiota are important factors regulating plant growth and development [48]. We therefore suggest that 10% TFC altered the relative abundance of some beneficial and harmful fungi in the rhizosphere soil of wheat, which increased the abundance of beneficial fungi in the rhizosphere soil, thereby improving the growth environment of wheat roots.

Further qPCR analysis revealed that the relative abundance of both rhizosphere bacterial 16S rRNA (Acidobacteria, Chloroflexi, Bacteroidetes, and Gemmatimonadetes) and fungal ITS (Mucoromycota) genes were reduced by coating agents. This is because fungicides and their degradation products may be toxic against specific components of the resident microbiome [49]. The fungal Shannon diversity index indicated that seed-coating agents had significantly promotional effects on fungal diversity, while they did not affect the bacterial alpha diversity indices, which is similar to the findings of Du et al. 2022 [50]. The results of PCoA showed that the seed-coating agents significantly changed the rhizosphere microbial community structure, possibly due to the influence of soil on the rhizosphere microbial communities. Previously reported that soil origin was an important factor affecting the changes in exogenous substance on the rhizosphere microbial community. It is worth noting that the soil's physiochemical properties were also an important factor affecting the changes in rhizosphere microbial communities, such as soil PH, structure, and organic matter content [51].

LEfSe results showed that seed-coating treatments significantly enriched fungal taxa such as *Septoria*, *Sordariales*, *Fusarium*, *Chaetomium*, *Mortierella*, *Mucor*, and *Syncephalis* in the seed-coating treatment groups of the soil. Studies have shown that Sordariales is a key microorganism for improving soil quality [52]. Among the above, *Chaetomium* and *Mortierella* were negatively correlated with disease incidence [53]. Bacterial taxa such as Nitrospirae, *Archangium*, Chloroflexales, *Massilia*, and *Tepidisphaerales*, were simultaneously enriched in both treatment groups of the soil (Figure 5h). According to Wu et al., Nitrospirae may be a potential microplastic-degrading bacteria [54]. Chloroflexales is reported to be an oligotrophic bacterium that can improve soil bacterial activity and nutrient cycling [55]. *Tepidisphaerales* is an aniline-degrading bacteria in an acidic environment [56]. We therefore speculate that the relative abundance of some beneficial microorganisms increased under the effect of seed coating, exerting a regulatory function in the soil ecology and plant health.

To elucidate the molecular mechanisms underlying the TFC-induced effects on plant growth and disease resistance, 15-day-old seedlings were collected separately and used to prepare cDNA libraries, which were sequenced using the BGISEQ-500 sequencing platform. Seed-coating treatments caused most classes of genes to be upregulated in the grown seedlings, including *PAL*, *COMT*, and *CYP84A*. The phenylalanine ammonia-lyase (*PAL*) gene family was actively expressed. The phenylpropane metabolic pathway directly and indirectly generates intermediate products in the phenylpropane skeleton, such as trans-cinnamic acid, coumaric acid, and sinapic acid, and the contents of these substances are closely related to *PAL* activity. It is worth noting that one of the physiological roles of *PAL* is to promote cell differentiation and plant growth, which play an important role in plant growth and physiology [57]. The results showed that the application of seed coatings (i.e., 10% TFC) can accelerate seedling growth by promoting *PAL* expression. Additional studies

are necessary to confirm whether similar molecular mechanisms exist among different members in the regulation of phenylpropanoid metabolism and plant immunity.

In addition to the diverse physiological processes mentioned above, seed coatings also regulate plant responses to pathogens. Using KEGG enrichment analysis, the application of seed coatings increased the expression of genes involved in plant hormone signal transduction pathways (*JAZ*, *BZR1*, *PR1*, and *JAR1*), and reduced the regulation of plant growth inhibition (*DELLA* and *BKI1*). Very recently, we observed that *PR1* is a water-soluble protein that is produced by plants in response to infection by pathogens or stimulation by biotic factors [58]. DELLA proteins are transcription factors that negatively regulate gibberellin. These genes were beneficial for wheat growth in adverse environments. Collectively, these results indicate that the application of seed coatings (10% TFC) can promote resistance in wheat seedlings by stimulating the expression of plant hormone signal transduction pathways.

5. Conclusions

In the field, we assessed the ability of 10% TFC to affect wheat growth in early growth stages of wheat, with some variations among products. This study revealed that, after the application of 10% TFC, wheat seedling growth was significantly promoted. In a greenhouse, we analyzed 16S/ITS rDNA profiles of the rhizosphere soil microorganism communities of these pot-grown wheat seedlings treated with 10% TFC. The application of seed coatings significantly altered the community structure and composition of the bacterial and fungal communities, increasing beneficial microorganisms and reducing harmful microorganisms, thus making the soil more conducive to wheat growth and thereby increasing the yield and quality of wheat. The transcriptome data revealed it likely did so by stimulating the gene expression related to phenylpropanoid biosynthesis, in order to promote cell differentiation and seedling growth, and suppressed the expression of growth-inhibiting genes through the regulation of plant hormone signaling. Considering this, 10% TFC could improve the growth environment of wheat roots by regulating rhizosphere processes, as well as significantly promoting the molecular mechanisms underlying wheat seedling growth, which is potentially beneficial for increasing wheat growth. Moreover, this research provides fundamental insight into the effects of 10% TFC on wheat seedling growth and rhizosphere microecology, which will be helpful for the further elucidation of the molecular mechanisms underlying TFC-induced effects on plant growth and disease resistance. Further research is now required to validate the efficacy of TFC across different environmental conditions and crop varieties.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14061209/s1. Table S1. The fungi diversity indices (at 97% sequence similarity) in rhizosphere soils, and the rhizospheres of untreated control wheat plants (RhC) and of plants treated with 10% TFC. Figure S1. Rarefaction curves of fungi communities in all treatments. Figure S2. Fungi genera (relative abundance $\geq$ 0.5%) in the soil fungal communities under the two treatments. Figure S3. Differences in seed-coating treatment at fungal phylum, class, order, family, and genus levels. Table S2. The bacterial diversity indices (at 97% sequence similarity) in rhizosphere soils, and the rhizospheres of untreated control wheat plants (RhC) and of plants treated with TFC. Figure S4. Rarefaction curves of bacterial communities in all treatments. Figure S5. Effects of seed-coating treatment on bacterial PCoA of bacterial community structure (the points with different colors indicate different groups). Figure S6. Differences in seed-coating treatment at bacterial phylum, class, order, family, and genus levels. Table S3. The archaeal diversity indices (at 97% sequence similarity) in rhizosphere soils, and the rhizospheres of untreated control wheat plants (RhC) and of plants treated with TFC. Figure S7. Rarefaction curves of archaeal communities in all treatments. Figure S8. Archaea genera (relative abundance $\geq$ 0.5%) in the soil archaeal communities under the two treatments. Figure S9. Differences in seed-coating treatment at archaea phylum, class, order, family, and genus levels. Table S4. Correlation analysis of the samples used for sequencing.

Author Contributions: Conceptualization, C.C., S.H. and D.X.; investigation, C.C., S.L., S.Z. and W.W.; formal analysis and writing (original draft preparation), C.C. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: The authors declare no conflicts of interest.

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