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The Effect of Curcin Protein and Jatropha Plantation on Soil Fungi

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Abstract: *Jatropha curcas* is widely planted as a highly drought-resistant biodiesel feedstock. Curcin protein is one of the Jatropha ribosomal inactivation proteins with broad-spectrum antifungal activity that may enter the soil ecosystem as a result of large-scale Jatropha cultivation and affect fungi and various enzymatic activities in the soil. In this research, the influence of curcin protein and Jatropha planting on soil fungi was investigated, and the levels of curcin in various tissues and organs of Jatropha were measured with an enzyme-linked immunosorbent assay. It was found that the content of curcin in seed kernels reaches 2 mg/g, which is much higher than that in other tissues. After the seeds have fallen into the soil, the level of curcin in the soil rises rapidly, reaching 59.22 μg/g soil and 67.49 μg/g soil in different soil samples, respectively. It then falls by more than 99% within six days. High-throughput sequencing technology was used to study the soils treated with different concentrations of curcin, and the results of the soil fungal alpha diversity index analysis showed that the fungal communities did not change significantly, but the abundance of each fungal community changed significantly. The degree of influence of different concentrations of curcin treatment on the abundance of the soil dominant fungal community were investigated for concentrations of 0.5 μg/g, 50 μg/g and 5 μg/g, and showed that concentrations of 0.5 μg/g and 50 μg/g are more likely to change fungal community structure in soil, and with the increasing extension of the treatment time, they may be detrimental to the conservation of soil ecosystems. Internal transcribed spacer (ITS) sequencing of soil fungi from Jatropha planted and unplanted areas in four regions with different climate types showed that Jatropha planting significantly altered the soil fungal communities in each region. There was a negative impact on soil fungal communities in tropical maritime monsoon and subtropical dry and hot monsoon climates, while a positive impact was observed in subtropical monsoon and tropical highland monsoon climates due to Jatropha cultivation. In conclusion, Jatropha plantations and curcin protein have an impact on soil fungi and thereby affect the ecological system of the soil.

Keywords: *Jatropha curcas*; ribosome-inactivating proteins; curcin; soil enzyme activities; soil fungi

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1. Introduction

Soil is the most biologically diverse and the most active energy-exchanging and material-cycling layer of life in the surface system of the Earth, with the capacity to sustain plants and animals, maintain and improve water and air quality and support healthy human life. Soil fungi convert much of the decaying organic matter into plant-available nutrients through humification and mineralization. Fungi make up the major part of the microbial biomass and are most versatile in decomposing organic residues. They transform a larger portion of decaying plant residue into available nutrients. For instance, mycorrhizal fungi accelerate leaf litter decomposition in a northern hardwood forest [1]. They also increased plant uptake of nutrients such as nitrogen, phosphorus and potassium by influencing plant's root morphology and physiological characteristics, such as mycorrhizal fungi [2,3]. However, there are also many fungi that are causal agents of plant diseases.

Fungi pose a significant threat to both plant and animal biodiversity [4]. The richness of fungal decomposers was consistently and positively associated with ecosystem stability worldwide, while the opposite pattern was found for the richness of fungal plant pathogens, particularly in grasslands [5]. The relative stability of the structure of the soil fungal community therefore largely determines the balance of the soil ecosystem.

Plant growth had an important influence on changes in the populations of inter-rooted microorganisms, and any change in the composition or quantity of root secretions had the possibility to affect the soil microbiota [6–8]. For example, root exudates from slow- and fast-growing *Arabidopsis thaliana* plants have distinct effects on both microbial community structure and soil nutrients available to plants [9]. Even after harvest, the decomposition of plant litter continued to release new gene products into the environment [10], potentially changing the inter-root environment for long periods of time and thus having many effects on the soil environment. For example, Araujo et al. have showed a significant reduction in soil microbial biomass following the conversion of native savannah to eucalyptus forest [11]. The transgenic cotton line (25C-1) was significantly enriched in two beneficial bacteria, *Arthrobacter* and *Sphingomonas*, after continuous cultivation, which had a positive impact on the soil ecology [12]. The study by Kumar et al. investigated that changing land use (natural woodland, rotational cropland, and cropland) had significant effects on soil microbial biomass, with natural woodland having more biomass, cropland having medium biomass, and rotational cropland having less biomass [13]. This suggests that land use change can have a considerable impact on the soil microbial community.

Ribosome inactivating proteins (RIPs) are a group of active proteins widely found in plants and fungi that can disrupt ribosome structure, leading to ribosome inactivation and inhibition of protein biosynthesis [14–16]. Many studies have shown that RIPs have a variety of biological activities, such as antiviral, insecticidal, immunological and antifungal activities [17–20].

Jatropha curcas, a small, semi-fleshy tree or large shrub of the family *Euphorbia*, has been widely planted in tropical and subtropical regions for its high drought and barren tolerance as a biodiesel feedstock [21]. By 2012, China had approximately 200,000 hectares of Jatropha resources (including natural forests) [22]. There is a type I ribosome-inactivating protein curcin in Jatropha [23], which not only strongly inhibits protein synthesis in cell-free systems, but also has an inhibitory effect on plant pathogenic fungi at certain concentrations [24], such as *Saccharomyces cerevisiae*, *Rhizoctonia solani*, *Pyricularia oryzae*, *Gibberella zeae* and *Sclerotinia sclerotiorum* [24].

The Jatropha ribosomal inactivation protein curcin may enter the soil ecosystem through plant debris, seeds, pollen and root secretions because of large-scale cultivation of Jatropha and affect non-target organisms, fungi and various enzymatic activities in the soil, thus affecting soil ecological diversity and stability. The study aimed to examine the diverse impacts of Jatropha plantations and curcin on soil ecosystems.

2. Materials and Methods

2.1. Soil for Curcin Protein In Vitro Treatment Experiments

The experimental soil was collected from an oilseed rape field in Jinjiang District, Chengdu, Sichuan Province (longitude: 30°34′44″ E, latitude: 104°9′25″ N, altitude: 503 m), with a sample area of 23 m^2 and a maximum temperature of 16 °C and a minimum temperature of 12 °C on a cloudy day. The local area belongs to the subtropical monsoon climate zone, with hot and rainy summers and warm and humid winters. The average annual temperature is 16.6 °C, sunshine 1156.7 h and rainfall 966.9 mm. A total of 2 kg soil samples were taken from the surface layer at a depth of 1–15 cm via isometric sampling. Surface vegetation was removed, fresh soil was sieved to 2 mm and the soil was mixed thoroughly.

2.2. Sample Collection of Jatropha Cultivation Areas

As Jatropha is a tropical plant, the soils from Jatropha plantations and non-plantation areas were collected in four different tropical or subtropical climate regions, Jinhexiang, Sichuan Province (longitude: 101°42′ E; latitude: 27°06′ N, elevation: 2500 m), Xichang, Sichuan Province (longitude: 101°57′ E, latitude: 27°42′ N, elevation: 2000 m), Yuanmou, Yunnan Province (longitude: 101°49′ E; latitude: 25°50′ N, elevation: 1312 m) and Haikou, Hainan Province (longitude: 109°75′ E; latitude: 19°88′ N, elevation: 126 m). Among them, Jinhexiang belongs to the subtropical monsoon climate; Xichang belongs to the tropical highland monsoon climate; Yuanmou belongs to the subtropical dry and hot monsoon climate and Haikou belongs to the tropical maritime monsoon climate. Approximately 1 kg of fresh soil was collected at a depth of 1–20 cm using the five-point sampling method, mixed and passed through a 2 mm sieve after removing dead branches and leaves on the surface, and soil samples were taken in triplicate for a total of 24 samples.

2.3. Curcin Treatment Soil Experiment

Based on our previous studies [25] and research on the antifungal activity of curcin in vitro, three concentrations of 0.5 μg/g, 5 μg/g and 50 μg/g were prepared. 20 g of soil was weighed into 100 mL sterilized triangular flasks, 1 mL each of 10 μg/mL, 100 μg/mL and 1000 μg/mL protein solution was pipetted into the triangular flasks with the same volume of ddH20 as the blank control, a total of four groups of three replicates each, stirred well with a sterilized glass rod and the triangular flasks were sealed with a triangular flask sealing film with air holes. The sealed triangular flasks were incubated in the dark at 25 °C in an artificial climate incubator with humidity adjusted to 60% and samples were collected on the 1st, 3rd, 7th and 12th days after incubation.

2.4. DNA Extraction and Sequencing

A total of 48 samples were taken from soils treated with different concentrations of curcin protein, and a total of 18 samples were collected from soils in different climate regions with Jatropha plantation and non-plantation areas. The methods used to collect the samples are identical to those outlined in Section 2.2. The DNA of samples were extracted using the MP Biomedical Fast DNA soil sample extraction kit (Mobio Laboratories, Carlsbad, CA, USA) according to its instructions, and the samples were tested and then sent to BGI Genomics for ITS sequencing of the fungi. Raw data were spliced using FLASH (version 1.2.11) software, low quality data were filtered using Trimmomatic (version 0.33) software and chimeras were removed using UCHIME (version 8.1) software. Sequences were clustered using USEARCH (version 10.0). The OTU sequences were compared with the Unite Release 7.2 database using RDP classifier (version 2.2) software and species annotation was performed to cluster the samples.

2.5. Determination Method of Enzyme Activity

Soil sucrase enzyme activity was determined with a colorimetric method using 3,5-dinitrosalicylic acid [26]. Soil acid phosphatase activity was determined with a colorimetric method using disodium phenyl phosphate [27]. Soil urease activity was determined with the sodium phenol-sodium hypochlorite colorimetric method [28]. Soil catalase activity was determined with the potassium permanganate colorimetric method [27].

2.6. Determination of the Degradation Rate of the Jatropha Ribosome Inactivation Protein Curcin in Soil

Due to the high content of curcin in the seed kernel of Jatropha and its value for use in transgenic plants, the dynamics of its accumulation and degradation in the soil is an important indicator of its bioenvironmental risk. In order to simulate the degradation process of Jatropha seed kernels in the soil in a natural environment, soils from Jinhexiang, Sichuan Province (where Jatropha actually grows) and Lvyangcun, Chengdu, Sichuan

Province (where Jatropha does not grow, but with fertile soil and rich in microorganisms) were selected for comparison.

(1) Soil from Jinhexiang and Lvyangcun was taken and passed through a 20 mesh sieve.
(2) A total of 0.1 g of Jatropha seeds ground into powder with liquid nitrogen was added to 0.5 g of soil; 0.4 mL of distilled water was added, mixed with a pellet pestle (RS-Sigma) for 5 s, centrifuged at 3000 rpm for 30 s at room temperature, and the supernatant was aspirated to obtain a homogeneous mixture of soil, seeds and water. Twenty-one replicates were performed for the soil at each site (set of seven time points, three replicates per set).
(3) EP tubes were placed in a thermostat, maintained at 30 °C and 80% relative humidity and incubated in the dark. Samples were taken on days 0, 6, 12, 18, 24, 30 and 36 of degradation and three replicates were taken at each time point.
(4) Soil curcin protein was extracted and ELISA assays were performed.

2.7. Data Analysis and Processing

Diversity indices were mainly calculated using the R package Vegan (https://cran.r-project.org/package=vegan, accessed on 15 June 2023), and data processing, statistical analysis and graph plotting were performed in SPSS26, R (v4.2.0) and GraphPad Prism 8.0.1.

3. Results

3.1. Degradation of Curcin Protein in Soil and the Effect on Enzyme Activities

3.1.1. Possible Pathways of Curcin into the Soil and Its Degradation Dynamics in the Soil

The endosperm of Jatropha seeds is relatively rich in curcin protein [29], but other parts, such as the leaves, may also contain curcin. In order to investigate the possible pathways for curcin to enter the soil, five Jatropha trees were randomly selected from the Jatropha planting area and 100 g each of seeds, green leaves and roots were collected during the fruiting period, and 100 g each of dried and fallen Jatropha fruits and yellow leaves were collected from the plantation. The results showed that the content of curcin in seed kernels was up to 2 mg/g tissue, 13 μg/g in green leaves, 15 μg/g in yellow leaves, 2 μg/g in the fruit peel and 3 μg/g in roots, and the content of curcin in seed kernels was much higher than that in leaves, roots and pericarp (Figure 1A). It is therefore suggested that curcin enters the soil mainly through the seed kernel, in addition to being secreted by the root system, and may affect the balance of soil ecosystems.

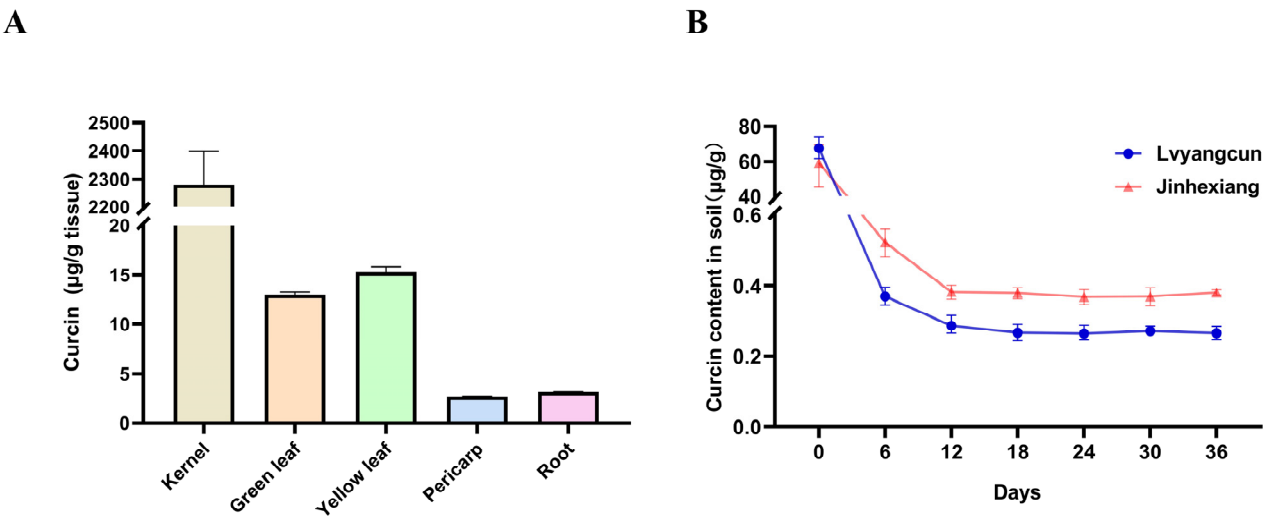

Figure 1. Possible sources of curcin in soils and its degradation dynamics. (**A**) Contents of curcin in different tissues of Jatropha and (**B**) Degradation trends of curcin in soil from Jinhexiang and Lvyangcun.

3.1.2. Degradation Dynamics of Curcin Protein in the Soil

In order to determine the amount of curcin in the soil around Jatropha in its natural growing state, the inter-root soil and the growing soil were collected at three different locations, three replicates of each location, ground to a fine powder, extracted curcin and assayed with monoclonal antibody indirect enzyme-linked immunosorbent assay (ELISA). Curcin was found to be below the minimum detection limit of the method in both inter-root soil and growing soil. The reason for this is thought to be that when seeds, leaves, fruits and other tissues enter the soil, curcin is rapidly degraded by biological factors (e.g., bacteria, fungi, etc.) or adsorbed by the soil.

Protein can combine with soil particles, increasing retention time. Some research has shown that clay minerals, humic acid and organic mineral aggregates in soil can adsorb Bt toxin and retain it, and the bound toxin still retains insecticidal activity, which has varying degrees of influence on soil organisms and soil enzymes [30–32]. Different masses of curcin were added to the soil, and the carrying capacity of the soil for curcin was determined with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot. When the addition of curcin was less than 40 mg curcin/g soil, there was no curcin band in the supernatant, which indicated that curcin was completely adsorbed by the soil; when the addition of curcin reached 40 mg curcin/g soil, the curcin band appeared in the supernatant, i.e., the adsorption of curcin by the soil reached saturation, and the non-adsorbed part was free in the supernatant, which indicated that the carrying capacity of the soil for curcin is about 40 mg/g soil. Therefore, there is a high likelihood of curcin retention in areas where *Jatropha curcas* is cultivated and in the inter-root soils where curcin was not detected.

In order to simulate the degradation process of seed kernels in the soil in a natural environment, the initial results of curcin were 59.22 μg/g soil and 67.49 μg/g soil after adding a mixture of Jatropha seed kernels to soils sampled from Jinhexiang and Lvyangcun, respectively, and after entering the soil, the content of curcin would decrease by more than 99% to less than 0.6 μg/g soil within six days and stabilize after 12 days, with curcin levels remaining at 0.4 μg/g soil in the Jinhexiang soil and around 0.3 μg/g soil in the Lvyangcun soil at a much faster degradation rate (Figure 1B). Therefore, the litter of the Jatropha plant, such as its seeds, can release curcin protein into the soil when it enters the soil and remain in the soil for a period of time.

3.1.3. Effect of Curcin on Soil Enzyme Activities

Soil enzymes were involved in almost all biochemical reactions in the soil, and their activity was closely related to soil properties, soil type and environmental conditions, characterizing the vigor of material metabolism in the soil. At the same time, soil enzymes influenced soil microbial populations and community structure, and were widely used as important indicators for assessing soil quality and soil biological activity [33,34].

To investigate the effect of curcin protein on soil enzyme activities after soil incorporation, the effect of curcin on soil sucrase, acid phosphatase, urease and catalase activities, respectively, was measured. In general, from the experimental results, curcin had a greater effect on soil sucrase and urease activities, showing a trend of promotion, then inhibition and finally promotion on sucrase and total inhibition on urease, while the effects on acid phosphatase and catalase were not obvious (Figure 2).

We hypothesize that curcin proteins are able to enter the soil through the Jatropha litter and remain there for a certain period of time, potentially resulting in a modified soil environment through the impact on soil fungi and enzyme activity.

3.2. Effect of Curcin Protein on Soil Fungi

3.2.1. Soil Fungi ITS Sequencing Quality Assessment Results

The soils treated with different concentrations of curcin protein and DNA in soils from different regions of Jatropha plantation and non-plantation areas were extracted for ITS sequencing of the fungus, and the OTU coverage ranged from 99.89% to 100%, and the

values indicated the probability of species detection. The OTU coverage of 24 samples from soils of Jatropha planting and non-planting areas in different regions was greater than 99.5%, and the rest of the indicators also met the criteria. The dilution curves of the two groups of samples tended to be flat, indicating that the sample sequences were adequate. In conclusion, the quality of the sequencing data is satisfactory and can be used for the next data analysis.

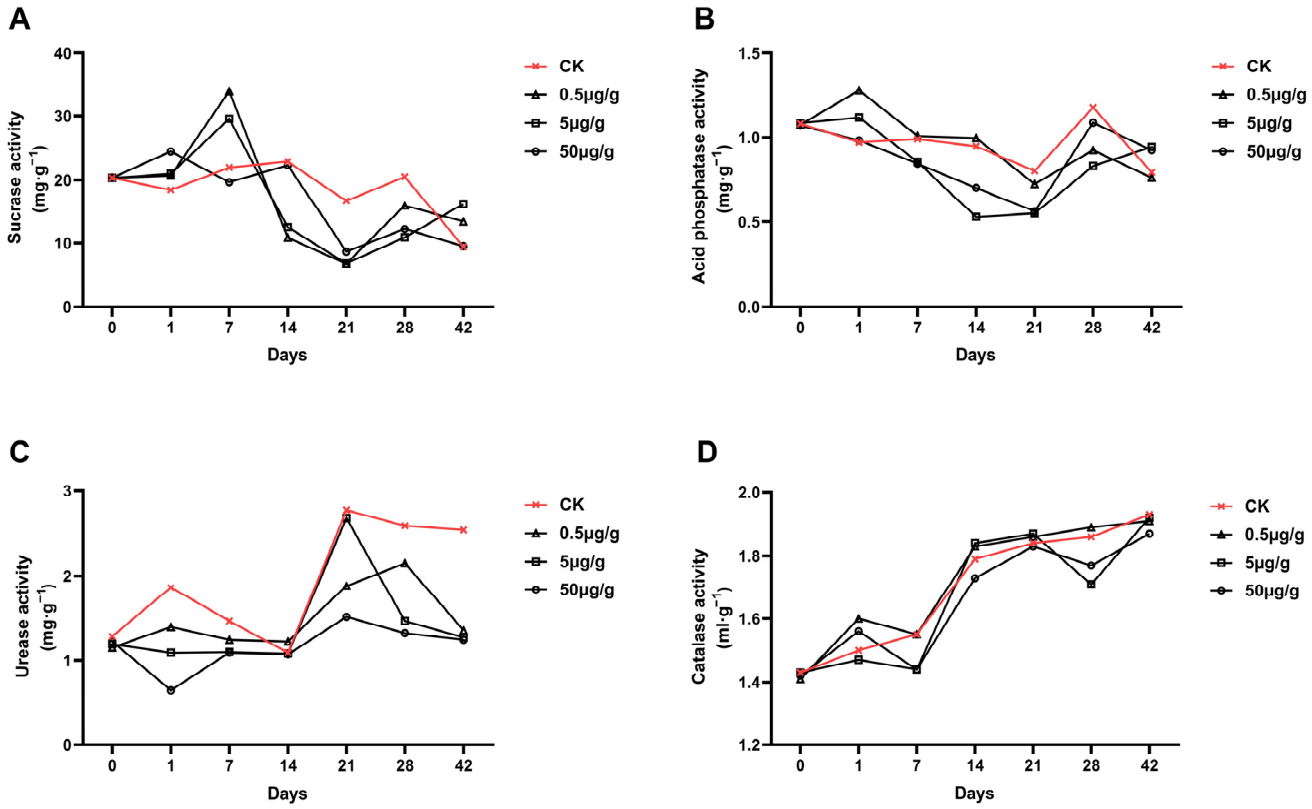

Figure 2. Effect of different concentrations of curcin on activities of enzymes in soil. (**A**) sucrase, (**B**) acid phosphatase, (**C**) urease and (**D**) catalase. CK, untreated soil sample that was used as the control.

3.2.2. Effect of Different Concentrations of Curcin Treatment on Soil Fungal Diversity

To explore the effect of curcin protein on soil fungi, soils were treated with different concentrations of curcin in vitro and fungal diversity indices were examined. The soil samples with 0.5 μg/g of curcin showed a significant increase in fungal abundance in the soil and no significant change in diversity at the early stage of treatment, and a significant decrease in fungal abundance and a significant increase in diversity at the late stage of treatment. A concentration of 5 μg/g of curcin in soil samples resulted in a significant increase in soil fungal abundance at the early stage and no significant change in the late stage. A concentration of 50 μg/g of curcin in soil samples showed an overall trend of increase in the OUT number of soil fungi after treatment; the Chao1 index increased in the early stage of treatment and showed no significant change in the late stage; the Shannon index showed an overall decreasing trend (Table 1). In general, different concentrations of curcin protein treatment caused different changes in the abundance and diversity of soil fungi, with 50 μg/g soil curcin having a more significant effect on soil fungi in the early stage, 0.5 μg/g soil curcin having a significant effect in the later stage, and 5 μg/g soil curcin having a relatively small effect on soil fungi. Concentrations of 0.5 μg/g and 50 μg/g are more likely to change fungal community structure in the soil, and with an increasing extension of the treatment time, they may be detrimental to the conservation of soil ecosystems.

Table 1. Alpha diversity index of soil fungi treated with curcin at different concentrations.

Day	Treatment	OTU	Chao1	ACE	Shannon	Simpson
1	CK	183.5 ± 0.5 f	221.1 ± 15.4 d	284.8 ± 2.6 c	4.21 ± 0.01 bc	0.031 ± 0.000 b
	C0.5	277.0 ± 3.07 bc	303.7 ± 11.3 b	298.0 ± 13.7 b	4.28 ± 0.05 bc	0.030 ± 0.004 b
	C5	300.0 ± 0.1 b	326.2 ± 4.2 b	321.1 ± 6.4 b	4.47 ± 0.01 ab	0.028 ± 0.00 b
	C50	275.5 ± 3.5 bc	302.7 ± 0.3 b	289.8 ± 4.0 bc	3.57 ± 0.01 d	0.08 ± 0.01 a
3	CK	229.5 ± 3.5 de	268.5 ± 2.5 c	284.4 ± 10.0 c	4.32 ± 0.06 b	0.021 ± 0.003 b
	C0.5	211.5 ± 0.5 e	313.8 ± 2.3 b	479.7 ± 9.7 a	4.44 ± 0.02 ab	0.021 ± 0.001 b
	C5	263.0 ± 4.0 cd	261.4 ± 12.6 c	265.4 ± 14.5 c	4.47 ± 0.01 ab	0.023 ± 0.00 b
	C50	235.5 ± 4.5 de	302.7 ± 0.3 b	275.6 ± 8.3 c	4.06 ± 0.08 c	0.06 ± 0.02 ab
7	CK	236.0 ± 12 de	248.1 ± 15.1 cd	257.6 ± 18.8 c	3.96 ± 0.08 c	0.034 ± 0.001 b
	C0.5	274.0 ± 10.0 c	268.5 ± 1.5 c	292.2 ± 13.2 bc	4.37 ± 0.08 ab	0.024 ± 0.001 b
	C5	293.5 ± 20.5 bc	297.6 ± 23.6 bc	254.8 ± 38.7 c	4.56 ± 0.08 a	0.024 ± 0.00 b
	C50	240.5 ± 4.5 d	244.0 ± 6.0 cd	241.4 ± 4.1 c	4.07 ± 0.00 c	0.039 ± 0.01 ab
12	CK	463.0 ± 1.0 a	489.8 ± 1.9 a	488.8 ± 1.9 a	3.56 ± 0.005 de	0.08 ± 0.002 a
	C0.5	236.5 ± 3.5 de	224.3 ± 13.8 d	244.1 ± 7.9 c	4.08 ± 0.005 c	0.03 ± 0.001 b
	C5	475.5 ± 18.5 a	508.0 ± 20.1 a	501.8 ± 22.8 a	3.60 ± 0.25 d	0.077 ± 0.05 a
	C50	469.0 ± 3.0 a	508.0 ± 5.1 a	501.3 ± 0.9 a	3.33 ± 0.01 e	0.08 ± 0.00 a

Note: Any difference between groups with the same marker letter is not significant, any difference with different marker letters is significant; C0.5: 0.5 μg/g curcin treatment, C5: 5 μg/g curcin treatment, C50: 50 μg/g curcin treatment and CK: control treatment.

3.2.3. Effect of Different Concentrations of Curcin Treatment on the Composition of Soil Fungal Communities

The sequencing results showed that Ascomycota was the dominant fungal phylum in the soils collected for this study, accounting for about 70% of the abundance. *Penicillium* and *Kazachstania* were the most abundant dominant fungal genera, *Mortierella* and *Purpureocillium* were the dominant fungal genera, *Aspergillus*, *Thermoascus*, *Metacordyceps*, *Rhizopus* and *Solicoccozyma* were the next dominant fungal genera (Figure 3).

After treating the soil with 0.5 μg/g of curcin, the overall abundance of the dominant fungal genus decreased in the first day treatment group and increased in the remaining three treatment groups. Soil dominant fungal genera showed similar trends in the first and third day treatment groups: the abundance of *Kazachstania* increased and the abundance of *Penicillium* and *Mortierella* decreased significantly; the abundance of soil dominant fungal genera showed the opposite trend in the seventh day treatment group; on the 12th day, a new dominant fungal genus *Saccharomyces* appeared and the abundance of *Kazachstania* increased, while the abundance of the remaining dominant fungal genera decreased (Figure 3).

After treatment of the soil with 5 μg/g of curcin, the total abundance of the dominant fungal phylum decreased only in the treatment group on the seventh day, but the change in the dominant fungal phylum was not significant in the treatment group in the remaining time periods; the overall abundance of the dominant fungal genus decreased in the treatment groups in different time periods, and the abundance of the genus *Kazachstania* increased and the genus *Penicillium* decreased in the treatment groups on the 1st and 12th day; there was no significant change in the abundance of the dominant fungal genus in the third day treatment group and in the seventh day treatment group, the abundance of the genus *Kazachstania* decreased and the abundance of the genera *Penicillium* and *Mortierella* increased (Figure 3).

After treatment of the soil with 50 μg/g of curcin, the overall abundance of both the dominant fungal phylum and the dominant fungal genus in the soil showed an increasing trend. The abundance of Ascomycota in the dominant fungal phylum increased significantly, but the abundance of Basidiomycota decreased significantly; the abundance of *Penicillium* in the dominant fungal genus showed a significant increase (Figure 3).

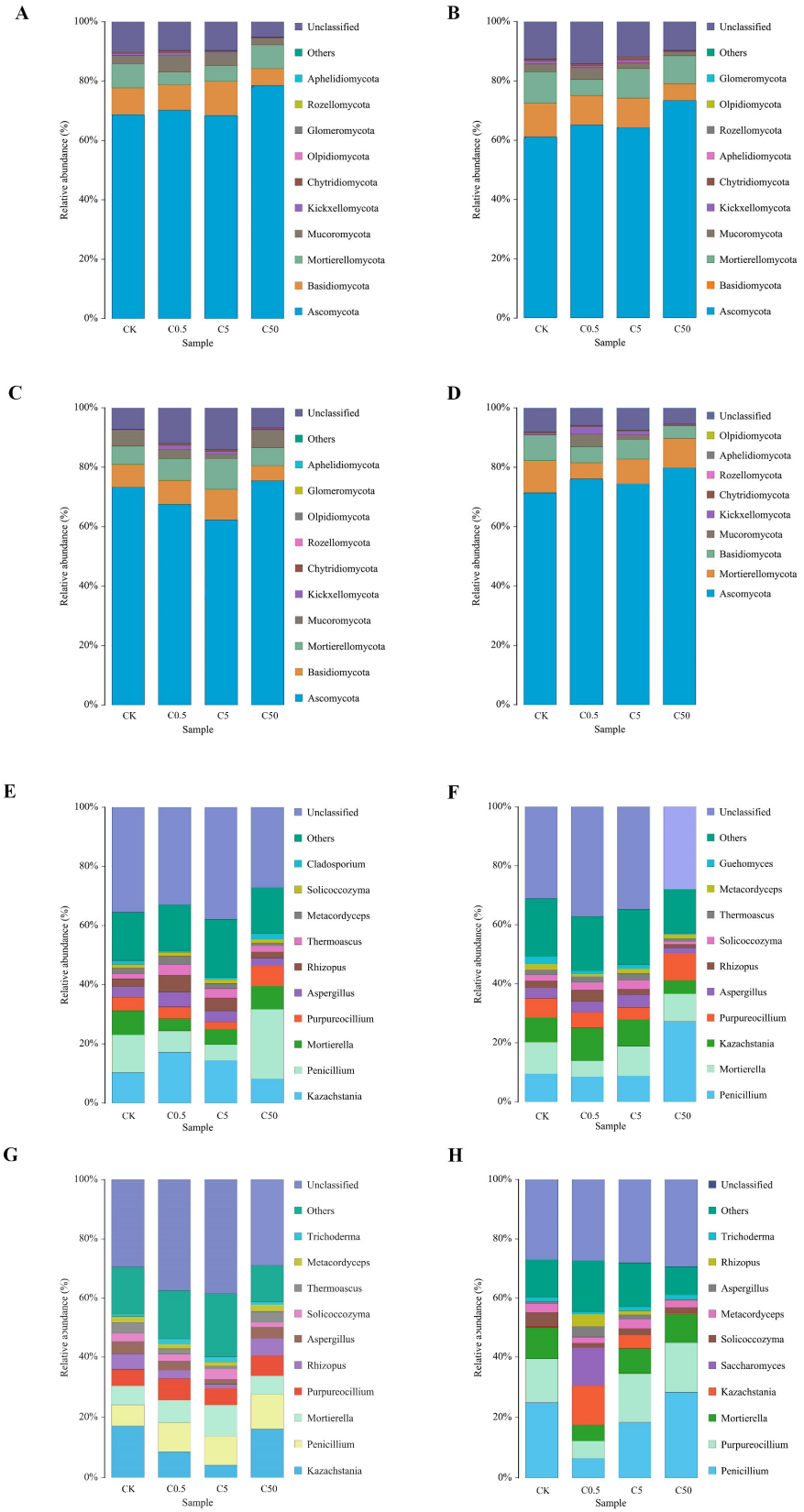

Figure 3. Composition of fungal communities treated with different concentrations of curcin. (**A**–**D**): 1 day, 3 days, 7 days and 12 days (phylum level); (**E**–**H**): 1 day, 3 days, 7 days and 12 days (genus level). C0.5: 0.5 μg/g curcin treatment, C5: 5 μg/g curcin treatment, C50: 50 μg/g curcin treatment and CK: control treatment.

3.2.4. Indicator Groups of Soil Fungal Communities Treated with Different Concentrations of Curcin

The different samples at each concentration were divided into four groups according to treatment duration and the fungal communities from phylum to genus in the samples were identified with LEfSe (Line Discriminant Analysis (LDA) Effect Size) analysis and the indicator groups detected in the CK and different concentrations of curcin treatment groups were presented with branching plots (Figure 4A,C,E,G); at the same time, histograms (Figure 4B,D,F,H) showed the communities that play an important role in fungal diversity (LDA score > 3). After one day of treatment, only the 0.5 μg/g soil curcin and 5 μg/g soil curcin treatment groups had significantly different fungal communities, whereas after 7 and 12 days, significantly changed fungal communities, i.e., indicator groups, were present in all four treatment groups at all concentrations.

Fungal indicator groups were more abundant in the 12th day's samples than in the other three groups with significant changes in the 0.5 μg/g soil curcin treatment group for the genera *Kazachstania*, *Aspergillus*, *Thermoascus* and *Saccharomyces* as indicator groups; in the 5 μg/g soil curcin treatment group, the abundance of fungi varied less, with only two indicator groups: *Fusarium* and *Papiliotrema*; in the 50 μg/g soil curcin treatment group, the indicator groups were *Penicillium*, *Gymnopilus* and *Beauveria*. Among them, *Kazachstania*, *Aspergillus*, *Saccharomyces* and *Penicillium* were the dominant fungal genera. It was found that the effect of 50 μg/g soil curcin on the dominant soil fungal genera increased with increasing treatment duration. After 12 days of treatment, the 5 μg/g soil curcin treatment had less effect on the soil fungal community than the 0.5 μg/g soil and 50 μg/g soil curcin treatments.

The effects of different concentrations of curcin treatments on soil fungal communities were all different, with the 0.5 μg/g and 5 μg/g soil curcin treatment groups having significantly different fungal communities, while the 50 μg/g soil curcin treatment group had the most significant change in fungal communities. It was also found that the effect of 50 μg/g soil curcin on dominant soil fungi increased with increasing treatment time. These results can contribute to a better understanding of the effects of curcin on soil microbial diversity and provide theoretical support for biological control in agricultural production.

3.3. Analysis of Soil Fungal Communities in Jatropha Planting and Non-Planting Areas in Different Climatic Regions

3.3.1. Comparison of Soil Fungal Diversity in Jatropha Planting and Non-Planting Areas in Different Climatic Regions

The ITS sequences of fungi from 24 soil samples collected from different regions were sequenced, and the results showed that the OTU coverage of soil samples from all regions was greater than 99.5%. The abundance and diversity of soil fungi in the soil samples from the planting areas of the tropical maritime monsoon climate (Haikou) and the subtropical dry and hot monsoon climate (Yuanmou) were lower than those from the non-planting areas, but the differences were not obvious. Unlike other localities where the abundance and diversity of fungi in soil samples from planting area and non-planting areas were consistent, the abundance of fungi in soil samples from the tropical highland monsoon climate (Xichang) showed that the non-planting area was greater than the planting area, and the diversity of fungi showed that the planting area was greater than the non-planting area. Noteworthy, the abundance and diversity of soil fungi in the soil samples from the subtropical monsoon climate plantation area (Jinhexiang) were significantly higher than those from the non-plantation area (Figure 5). Jatropha cultivation affects soil fungal communities, with varying trends observed in areas with different climate types.

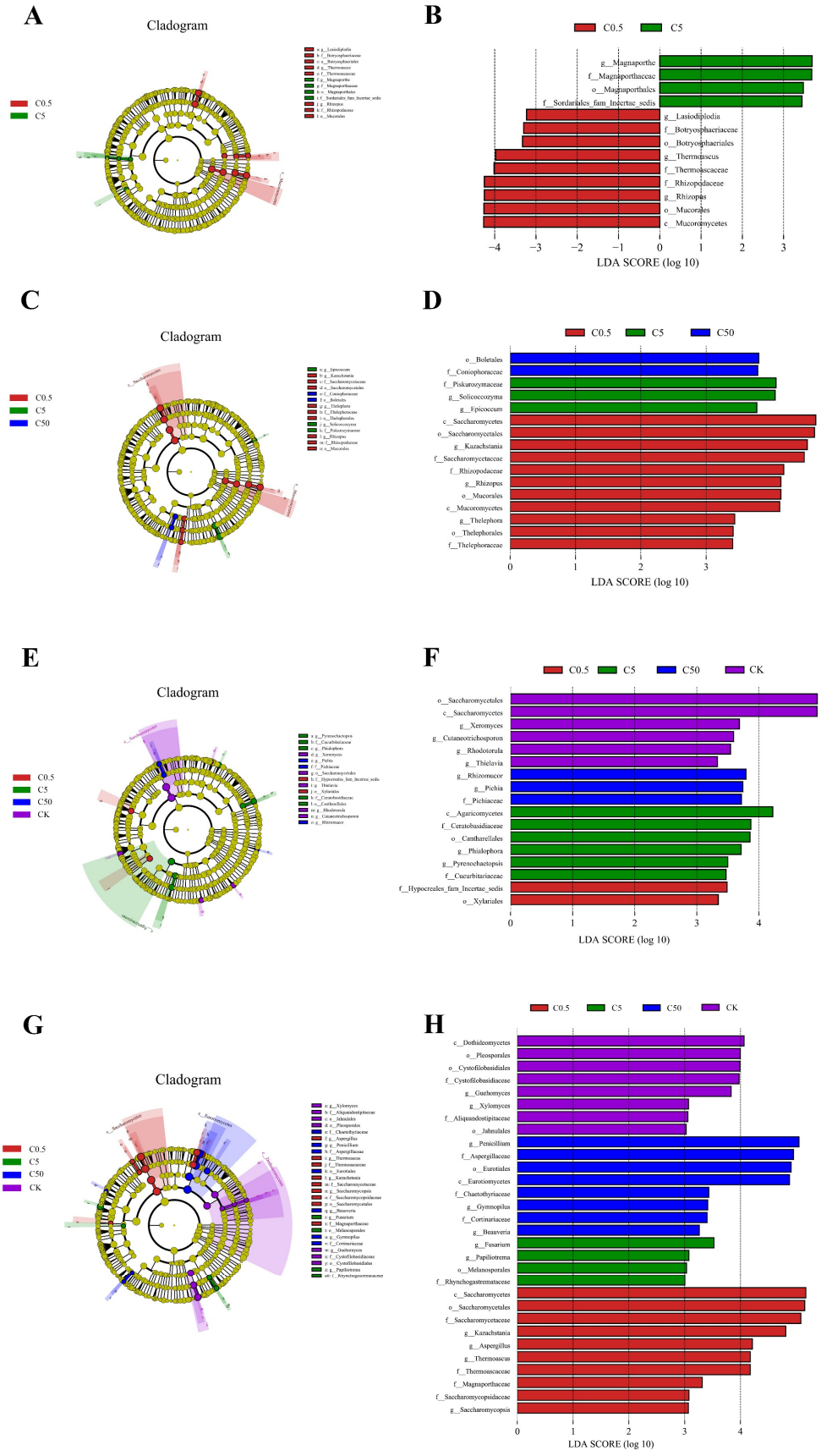

Figure 4. Phylogenetic distribution of soil fungal communities and linear discriminant analysis. Phylogenetic distribution of soil fungal communities at 1 day (**A**), 3 days (**C**), 7 days (**E**) and 12 days (**G**)

for four different treatments. Linear discriminant analysis was used to derive the associated fungal communities scoring ≥ 3 in the four different treatment groups on 1 day (**B**), 3 days (**D**), 7 days (**F**) and 12 days (**H**). Nodes of different colors represent each fungal community that had a significant effect on inter-taxon variation, yellow nodes had a non-significant effect, and node diameters represent the abundance of the community. c, o, f and g indicate class, order, family and genus, respectively; C0.5: 0.5 μg/g soil curcin treatment, C5: 5 μg/g soil curcin treatment, C50: 50 μg/g soil curcin treatment and CK: control treatment.

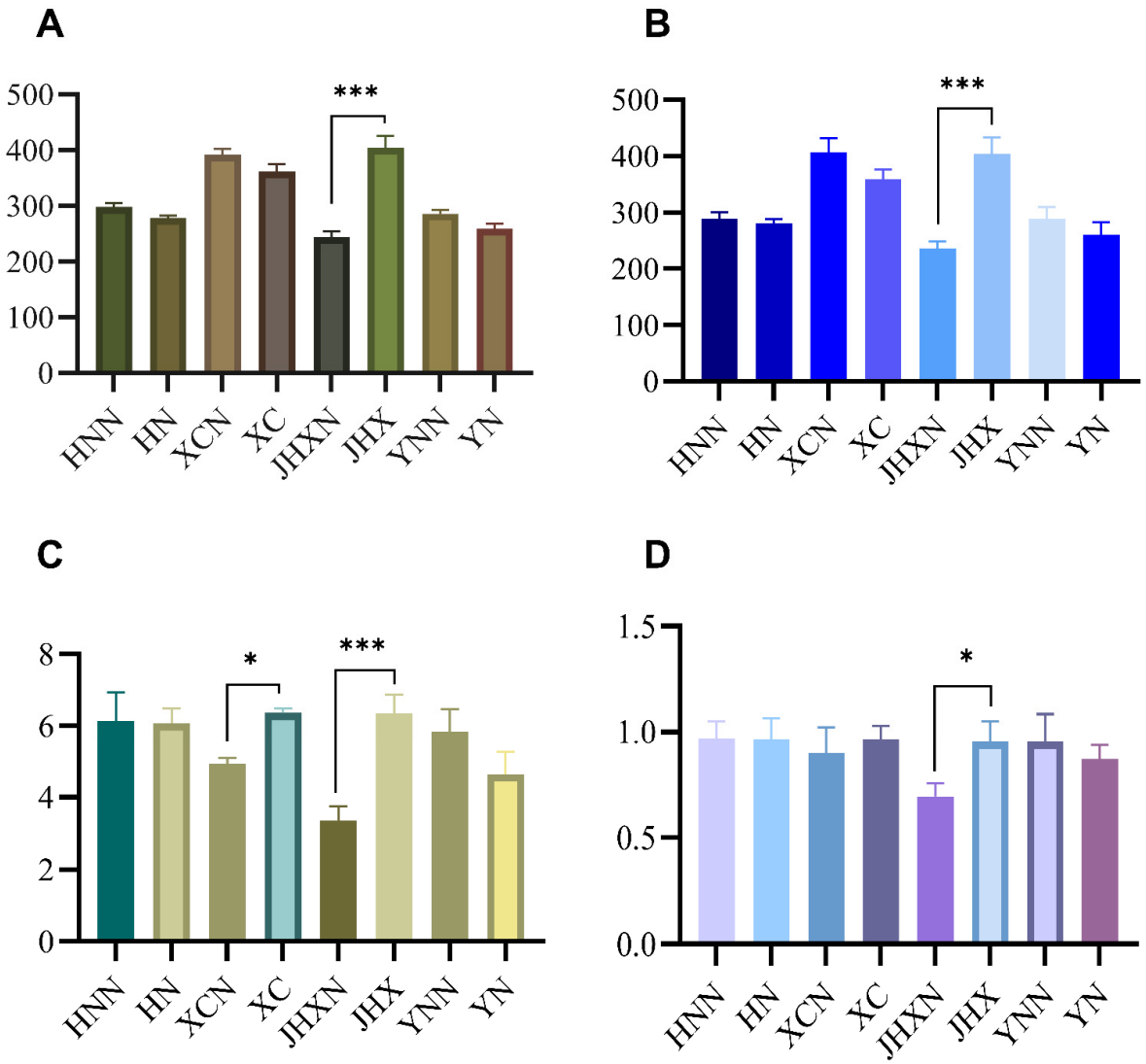

Figure 5. Alpha diversity of soil fungal in different areas. (**A**) Chao1, (**B**) ACE, (**C**) Shannon and (**D**) Simpson. HN: Haikou, Hainan planting area; HNN: Haikou, Hainan non-planting area; XC: Xichang planting area; XCN: Xichang non-planting area; JHX: Jinhexiang planting area; JHXN: Jinhexiang non-planting area; YN: Yuanmou, Yunnan planting area and YNN: Yuanmou, Yunnan non-planting area. Note: ***: $p < 0.001$. *: $p < 0.05$.

3.3.2. Soil Fungal Community Composition in Jatropha Planting and Non-Planting Areas in Different Climatic Regions

Soil fungal sequencing results from a total of 24 samples from different locations showed that at the phylum level, the abundance of Ascomycota (51.76%–94.20%) was absolutely dominant in different soil samples, and it was more abundant in non-plantation areas than in plantation areas in the tropical maritime monsoon climate (Hainan), tropical highland monsoon climate (Xichang) and subtropical monsoon climate (Jinhexiang), while in the subtropical dry and hot monsoon climate (Yunnan) soil samples it showed higher

abundance in planting areas than that in non-planting areas. The abundance of Ascomycota, Basidiomycota and Chytridiomycota found among in the tropical maritime monsoon climate (Hainan) planting area (59.21%, 14.27% and 0.16%) versus the non-planting area (81.60%, 3.25% and 1.75%), in the tropical highland monsoon climate (Xichang) planting area (51.76%, 6.67% and 0.74%) versus the non-planting area (72.19%, 23.27% and 0.25%), in the subtropical monsoon climate (Jinhexiang) planting area (80.52%, 7.19% and 0.56%) versus non-planting area (94.20%, 2.30% and 0.10%), and in the subtropical dry and hot monsoon climate (Yunnan) planting area (75.98%, 9.34% and 0.25%) versus non-planting area (60.33%, 26.56% and 4.59%) varied considerably (Figure 6A). At the genus level, the dominant fungal genera with higher abundance in the different soil samples differed and the composition of the fungal communities varied considerably. The fungal genera with the highest abundance in the planting and non-planting areas differed in soil samples from all areas except Jinhexiang (Figure 6B). The large differences in the dominant fungal phylum and dominant fungal genus between planting and non-planting areas suggested that Jatropha planting had a large impact on the dominant fungal community in the soil.

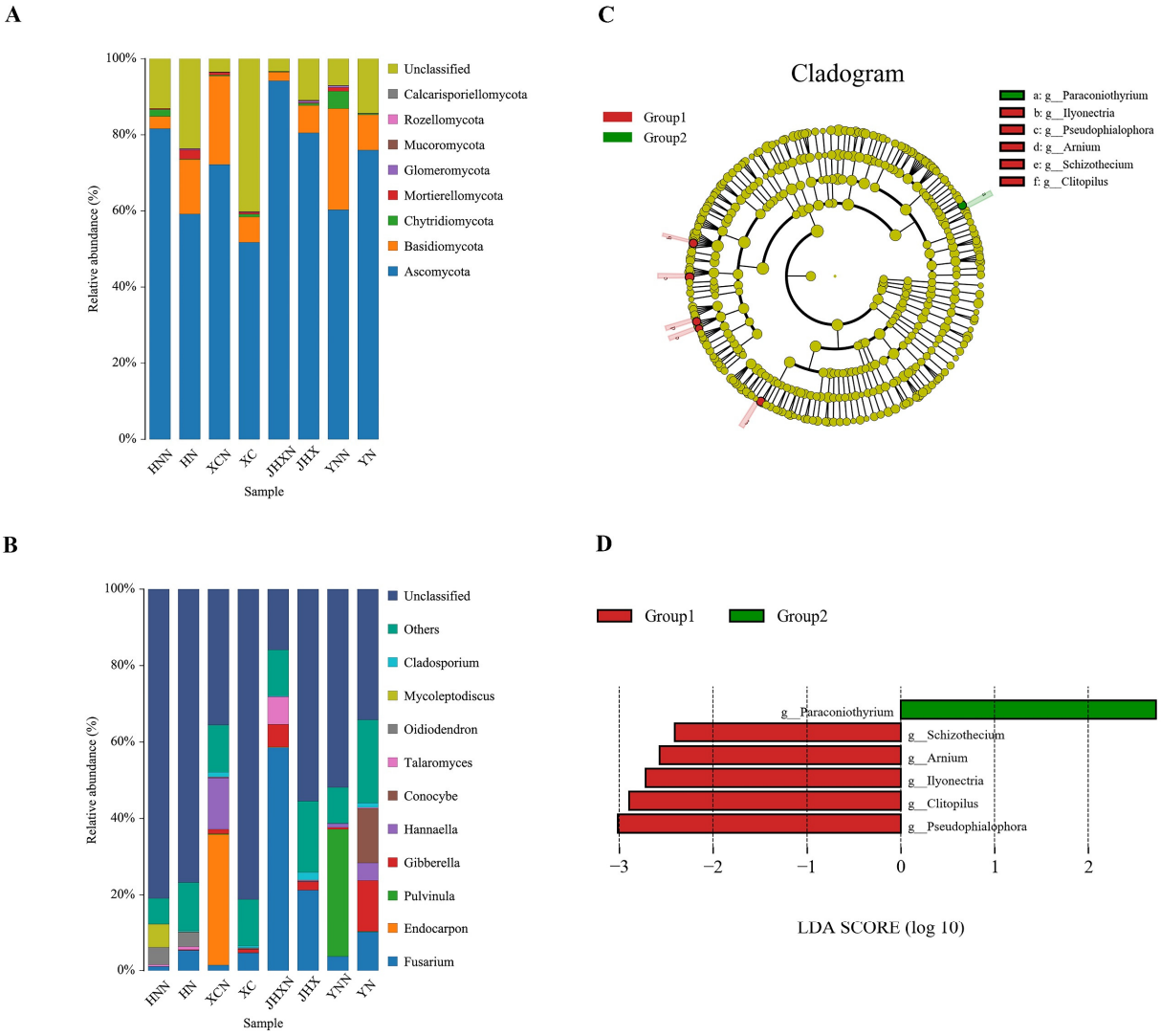

Figure 6. Composition, phylogenetic distribution and linear discriminant analysis of soil fungal communities in Jatropha planting and non-planting areas. Composition of soil fungal communities at different locations on (**A**) phylum level and (**B**) genus level. (**C**) Phylogenetic distribution of soil fungal communities in the two different treatments. (**D**) Associated fungal communities scoring ≥2 in the two treatment groups were derived using linear discriminant analysis.

Analysis of the significance of differences between groups revealed species that were only significantly different at the genus level when the LDA value = 2, and were mainly found in samples from all planting areas, including *Schizothecium*, *Arnium*, *Ilyonectria*, *Clitopilus* and *Pseudophialophora*, while only one species, *Paraconiothyruim*, was significantly different between the different non-planting area samples (Figure 6C,D). More species changed significantly in abundance in planting areas than that in non-planting areas, suggesting that Jatropha planting had a great impact on soil fungal composition.

4. Discussion

4.1. Retention and Degradation of Curcin Protein in Soil

Ribosomal inactivating proteins act as antifungal proteins; their broad-spectrum antifungal properties have great potential for agricultural pest and disease control [35]. Curcin is valuable for applying in transgenic plants because of its fungal resistance, and curcin has been demonstrated to be biologically active in both cell-free and whole animal systems [29]. There are no relevant studies on the retention and degradation dynamics of curcin proteins in soil. Saxena [36] and Stotzky [32,37] found that Bt toxins released into the soil by transgenic Bt plants and soil Bt bacteria are rapidly adsorbed by soil particles and humic acid to form bound Bt toxins that are difficult for microorganisms to degrade and thus remain biologically active for a longer period of time, and compete with other substances (i.e., heavy metal) in the environment for adsorption sites on mineral surfaces [38,39]. We hypothesized that curcin proteins and BT toxins may share similar degradation mechanisms.

Experimental results have shown that after Jatropha seeds are incorporated into the soil, the level of curcin is reduced by more than 99% within a short period of time. Curcin levels in soil are not always at extremely low levels and it is possible that after seeds, leaves, fruits and other tissues are introduced into the soil, due to biological factors (e.g., microorganisms) or some physical factors (e.g., ultraviolet light, temperature, humidity, etc.), the plant tissues decompose rapidly, the cells break down and curcin enters the soil, causing a rapid increase in the local soil curcin levels. Perhaps the adsorption of soil particles makes it difficult to extract curcin, leading to a rapid reduction in its content in a short period of time, while the degradation of curcin by soil microorganisms is the main reason for its continued reduction over time. Studies on the degradation of BT proteins in soil by Helassa et al. showed that a rapid decrease in the amount of extractable toxin was observed during the first 14 days, with a decrease of 86 $\pm$ 7.5% of the initial value, followed by a slower decrease [40]. The degradation trends of curcin protein and BT toxin in soil are similar, but the degradation rate of curcin is faster and reaches a steady state earlier.

After six days of degradation, the curcin levels stabilized, probably due to the gradual loss of moisture in the soil and the slowing of microbial activity. Although the initial addition was the same, differences were observed in the amount of curcin detected in the Jinhexiang and Lvyangcun soils. It is speculated that the reason for this may be firstly, the different extraction efficiency of the method for curcin in different soils, and secondly, it may be that curcin degrades at different rates in different soils and that soil properties (e.g., pH, mineral content composition, microbial community, etc.) affect the rate of protein degradation [41]. Jatropha seeds are mostly harvested at maturity as a feedstock for biodiesel production, but residues from other parts of the plant have a greater chance of entering the soil. Although tissues such as leaves, roots and fruit bark do not contain as much curcin as seed kernels', their long-term accumulation in the soil cannot be ignored. Residues from these parts of the plant may still be an important factor in the accumulation of curcin in soil and their effects on soil ecology deserve further investigation.

Curcin is capable of persisting in soil at low concentrations over extended periods of time, and the long-term efficacy of pest control using curcin may be influenced by several factors, consisting of the extent of its degradation, the nature of the degradation products and their distribution in soil. In cases where the degradation products retain antifungal

and antipest properties, curcin may still confer resistance to fungi and pests to some degree. Further research is required to affirm this.

4.2. Assessment of the Effect of Exogenous Curcin Protein on Soil Enzyme Activity and Fungal Communities

Soil enzymes are involved in almost all soil biochemical reactions and are now widely used as important indicators of soil quality and soil biological activity [33,34]. Sucrase is an important hydrolytic enzyme in soils, affecting the decomposition and conversion of soil organic carbon; acid phosphatase is the main enzyme in the phosphorus cycle and is an indicator of soil organic phosphorus mineralization and biological activity, converting organic phosphorus to inorganic phosphorus as an effective nutrient; urease promotes the hydrolysis of nitrogenous organic matter and is closely related to the formation and effectiveness of nitrogen in soils [42,43]. Soil catalase activity, which correlates with soil respiration intensity and soil microbial activity, is effective in preventing hydrogen peroxide toxicity and is an important indicator of soil microcosm environment [44]. The results of this study showed that the Jatropha ribosomal inactivation protein curcin had a greater effect on soil sucrase and urease activities under laboratory conditions, and it is speculated that Jatropha cultivation may also alter soil enzyme activities through curcin protein.

Among the dominant fungal communities in curcin-treated soils, *Penicillium* and *Aspergillus* were mostly soil saprophytic fungi with high reproductive capacity and high environmental adaptability. The same saprophytic fungi were also known as *Mortierella* and *Rhizopus*. *Rhizopus* was an important producer of lipase [45,46], and a common pathogenic genus, causing a variety of plant diseases such as soft rot [47]. *Purpureocillium* was an important biocontrol fungus and was very effective against root-knot nematodes that lead to serious damage to cash crops [48]. There is a certain antagonism between the different flora in the soil, and the beneficial flora and pathogenic flora restrain each other to bring the soil ecology into balance, increasing the defenses and resistance of the soil ecosystem, reducing the occurrence of pests and diseases and preventing soil pollution, which is conducive to the healthy functioning of the soil ecosystem.

Zhaolei et al. [49] employed T-RFLP fingerprinting to examine the impact of varied Cry1Ac toxin concentrations on the diversity of soil fungal communities. The findings indicate that soil fungal community structure remained unaltered over the incubation period of 100 days. These outcomes bear partial similarity to those of the current study. In this study, 0.5 μg/g soil curcin resulted in a significant reduction in soil fungal OUT after 12 days of treatment compared to control samples, but a significant increase in the Shannon index, indicating that application of exogenous curcin protein reduced the abundance of fungal communities but increased the number of fungal species at the taxonomic level, thereby increasing the diversity of fungal communities. It is noteworthy that the protein treatment did not significantly alter the dominant species composition of the soil fungal community, but rather the abundance of some of the dominant species, and the effect was more pronounced in the 0.5 μg/g soil curcin treatment group.

4.3. Analysis of Soil Fungal Communities in Jatropha Planting and Non-Planting Areas in Different Regions

Studies have shown that 20%–50% of the photosynthetic assimilation products of plants are transferred to the below-ground fraction, and most of them are released to the root zone in the form of organic and inorganic secretions, which affect the composition of the soil microbial community [50]. This conclusion was confirmed by Corey et al. [51] who added a plant root secretion to the test group for incubation and obtained a soil fungal community that was very similar to that of the soil in which the plant was grown, suggesting that inter-root secretions are a mechanism for regulating the composition of the soil fungal community. Because curcin protein can alter the composition of soil fungal communities and can enter the soil in a variety of ways, the differences in soil fungal communities between Jatropha planting areas and non-planting areas in different climatic regions were investigated. The results showed that Jatropha cultivation in four different

climatic regions had a major effect on the dominant fungal community in the soil. This indicates that Jatropha cultivation has an impact on the composition of soil fungi.

Kumar's study [52] showed that the diversity of soil fungi is generally correlated with the physicochemical properties of the soil, and the differences in the physicochemical properties and soil texture of the soil samples from the three sites may be one of the reasons for the differences in the effects of Jatropha cultivation on the fungal communities of each site. Additionally, the differences in the effects of Jatropha on soil fungal communities were also related to the age and species of Jatropha. It has been shown that plants release different amounts of material into the environment through root secretions, pollen and plant residues during different growth periods [53], and the growth status of Jatropha may vary from region to region, with different effects on soil fungal communities. This study did not sample the soil physicochemical properties of the non-planting areas of the site. Therefore, the study cannot reflect the effect of Jatropha planting on soil physicochemical properties, nor can it be ruled out that different physicochemical properties cause differences in soil fungal communities, which will be incorporated into the experimental design of the following study.

4.4. Shortcomings and Prospects

This study used fungal ITS sequencing to analyze changes in the structure of soil fungal communities. Compared to previous studies that used PLFA to analyze the effects of Jatropha on soil microorganisms [54], the target population is more refined (only soil fungi were studied) and the research methods are more advanced (PLFA can only analyze quantitatively dominant microbial communities, whereas fungal ITS sequencing can detect low abundance microorganisms and is more accurate). While most studies of fungal community composition have a long treatment period, this study focuses on changes in fungal community composition in the short term after treatment.

This study is only a preliminary exploration of the dynamics of curcin degradation in soil, and further research is needed to clarify the detailed process of curcin degradation. For example, the sampling time points between 0 and 6 days should be added to explore the process of curcin degradation from 100% to less than 1%, and the sampling time after 36 days should be extended to explore the maximum time that curcin can remain in soil within the detection limit. In addition, the degradation dynamics of curcin can be studied under specific microbial populations, different UV irradiation conditions and different temperatures and humidity.

The detection methods of high convenience and high sensitivity are urgently in need. For samples measurements, updated extraction methods (e.g., bioassay) and experimental analysis methods are needed to eliminate the bias caused by the determination of curcin protein concentration. In reality, our research on curcin protein and Jatropha cultivation is short-term, which may result in underestimating or overestimating the impact on non-target organisms. Hence, extended observations are imperative for comprehending the impact on soil. Overall, the use of curcin protein as a biopesticide may require additional considerations in terms of its effects on soil microbial communities and nutrient cycling. Further research is needed to fully understand the long-term impacts of curcin protein on soil ecosystems and to evaluate its use as a sustainable agricultural tool.

5. Conclusions

Curcin proteins can enter the soil through various means and persist for an extended period, resulting in certain impacts on soil enzyme activities and fungal communities. Furthermore, Jatropha farming elicits divergent effects on soil fungi under varied climatic conditions.

The results showed that the curcin content in Jatropha seed kernels is up to 2 mg/g tissue, which is much higher than in other tissues. The soil carrying capacity for curcin is approximately 40 mg/g. After Jatropha seeds were incorporated into the soil, the level of curcin in the soil increased rapidly and decreased by more than 99% within six days,

after which it stabilized, while the rate of curcin degradation in the soil varied from soil to soil. Curcin had a more significant effect on the fungal community at a later stage after entering the soil. Concentrations of 0.5 μg/g and 50 μg/g cause more structural changes in soil fungal communities than 5 μg/g curcin, which may disrupt the balance between soil microorganisms and be detrimental to the conservation of soil ecosystems.

The effects of Jatropha cultivation on the abundance and diversity of soil fungi were decreasing in the subtropical dry and hot monsoon climate and the tropical maritime monsoon climate and increasing in the subtropical monsoon climate, and effects on soil fungi in the tropical highland monsoon climate were decreasing in abundance and increasing in diversity; the dominant fungal phylum in each region remained unchanged, all being Ascomycota, but the dominant fungal genus with the highest abundance changed, except in Jinhexiang. Various soil properties vary in different climate types, resulting in contrasting impacts of Jatropha cultivation on soil fungi. There was a detrimental impact on soil fungal communities in tropical maritime monsoon and subtropical dry and hot monsoon climates. It is recommended that land managers in these regions prioritize the risk level of soil organisms in Jatropha cultivation areas. Conversely, there was a beneficent effect in subtropical monsoon and tropical highland monsoon climates, thus offering potential for soil utilization in Jatropha plantation areas within these regions.

Further research is required to investigate the impact of Jatropha cultivation and curcin proteins on soil microorganisms. To enhance utilization and preservation, fundamental research is necessary to examine the underlying mechanisms, such as investigating the enduring consequences of curcin on soil microorganisms.

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