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Autotoxicity Hinders the Natural Regeneration of *Cinnamomum migao* H. W. Li in Southwest China

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Abstract: Autotoxicity is a widespread phenomenon in nature and is considered to be the main factor affecting new natural recruitment of plant populations, which was proven in many natural populations. *Cinnamomum migao* H. W. Li is an endemic medicinal woody plant species mainly distributed in Southwestern China and is defined as an endangered species by the Red Paper of Endangered Plants in China. The lack of seedlings is considered a key reason for population degeneration; however, no studies were conducted to explain its causes. *C. migao* contains substances with high allelopathic potential, such as terpenoids, phenolics, and flavonoids, and has strong allelopathic effects on other species. Therefore, we speculate that one of the reasons for *C. migao* seedling scarcity in the wild is that it exhibits autotoxic allelopathy. In this study, which was performed from the perspective of autotoxicity, we collected leaves, pericarp, seeds, and branches of the same population; we simulated the effects of decomposition and release of litter from these different anatomical parts of *C. migao* in the field; and we conducted 210-day control experiments on seedling growth, with different concentration gradients, using associated aqueous extracts. The results showed that the leaf aqueous extract ($leaf_{AE}$) significantly inhibited growth indicators and increased damage of the lipid structure of the cell membrane of seedlings, suggesting that autotoxicity from *C. migao* is a factor restraining seedling growth. The results of the analyses of soil properties showed that, compared with the other treatments, $leaf_{AE}$ treatment inhibited soil enzyme activity and also had an impact on soil fungi. Although $leaf_{AE}$ could promote soil fertility to some extent, it did not change the effect of autotoxic substances on seedling growth. We conclude that autotoxicity is the main obstacle inhibiting seedling growth and the factor restraining the natural regeneration of *C. migao*.

Keywords: *Cinnamomum migao*; autotoxicity; seedling growth; soil substrate; soil enzyme; soil fungi

1. Introduction

Autotoxicity is a special type of intraspecific allelopathy of plants [1]. It is known to be widespread in nature, particularly in artificial agroforestry systems [2], leading to population deterioration and regeneration failure [3]. Autotoxicity refers to the phenomenon in which plants release their metabolites into the surrounding environment by volatilization, rainwater leaching, decomposition, and root excretion [4]; these metabolites then inhibit seed germination or other individuals' or their own seedlings' growth directly or indirectly. Canopy trees' autotoxicity to their seedlings may play an important role in forest species replacement [5], and the existence of autotoxicity was proved in many natural populations [4,6]. Current studies on plant autotoxicity have shown that allelochemicals mainly inhibit plant growth in the following ways: (1) Plant growth is directly inhibited by affecting photosynthesis and altering plant cell membrane structures and plant defense systems [7,8]. (2) Plant

growth is indirectly affected by inhibiting nutrient absorption or changing soil enzyme activity [9,10]. (3) The rhizosphere microecosystems are changed through the interaction between plant metabolites and fungi to ultimately affect seedling growth [11] (Figure 1a).

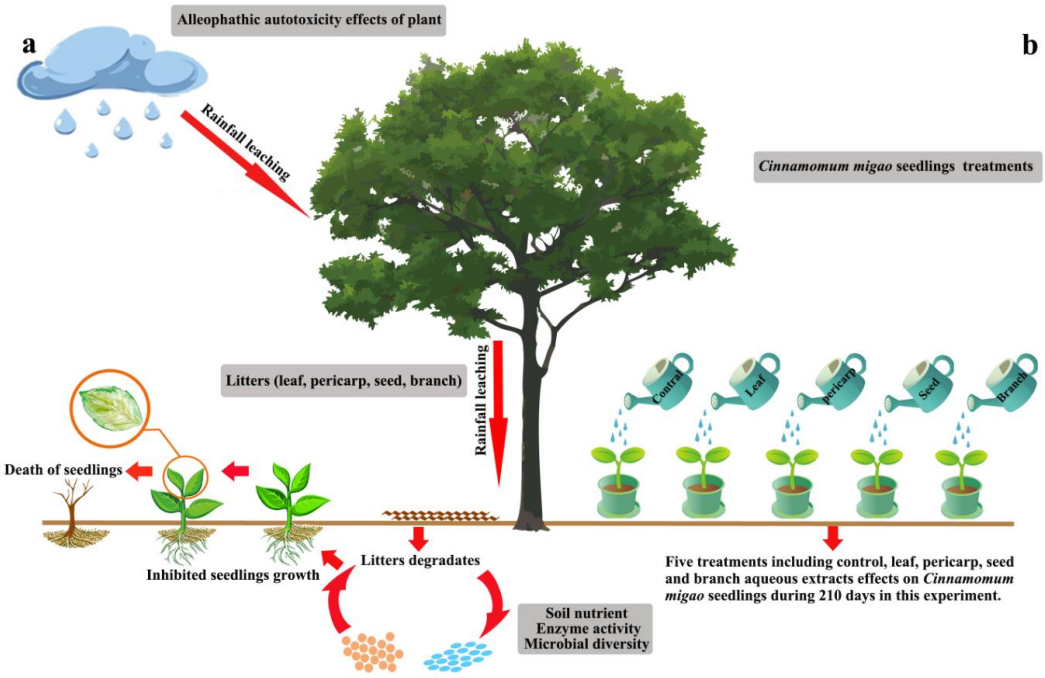

Figure 1. Process of plant autotoxicity (**a**) and our experimental design (**b**).

Autotoxicity is regarded as a negative effect on plant growth, and secondary metabolites produced by maternal plants could hinder the growth of their seedlings [12]. Phenolics and terpenoids released by woody plants play a key role in these interactions by influencing the structure and diversity of plant and soil communities [13–15]. Soil microorganisms can be directly affected by plant phenolics [16], but they can also use these phytochemicals as carbon sources, thereby modifying the chemical plant–plant interactions [17]. Phenolics from litter can inhibit the symbiosis between trees and fungi [18], which may have important consequences on the seedling development of trees. Similarly, phenolics released by plants may also play a key role in influencing the soil microbial community structure [19] and litter decomposition process [20]. For instance, autotoxicity of canopy trees on their own seedlings probably plays a role in forest species turnover along succession in Mediterranean forests [5]. Some autotoxic compounds released by asparagus (*Asparagus officinalis*) probably inhibit its own growth and can also be a reason for "asparagus decline" [21]. Phenolics released by the understory dwarf shrub *Empetrum hermaphroditum* impair the regeneration of the dominant tree *Pinus sylvestris* in boreal forests [22]. In addition, studies by Alías et al. [23] on the soil properties of the invasive rock-rose (*Cistus ladanifer*) population also showed that the compounds released by the plant itself were involved in autotoxicity and regeneration of the rock-rose population. *Cinnamomum migao* contains substances with high allelopathic potential, such as terpenoids, phenolics, and flavonoids [24]; generally, species with high allelopathic potential tend to have strong autotoxicity [25], and medicinal plants are more likely to have autotoxicity than other plants [14]. Therefore, we speculate that one of the reasons for seedling scarcity in the wild population of the species *C. migao* is that this species has autotoxic allelopathy [14].

C. migao H.W. Li is a species of Lauraceae evergreen medicinal woody plant, which is defined as an endangered species by the Red Paper of Endangered Plants in China. It is endemic to China and mainly distributed in Southwestern China. The fruits of *C. migao*, which are effective in treating gastrointestinal and cerebrovascular diseases, are used as traditional medicine in Miao in China [24]. In the last 30 years, researchers have found that the natural population size of *C. migao* is very small, as

the majority of populations contain only two or three individuals, and the tree age is relatively high; the natural regeneration has some obstacles [26]. Moreover, recent investigations of this resource have found that many natural populations have disappeared, and the survival and reproduction of this species are greatly threatened [27]. However, to the best of our knowledge, no research was performed to explain the underlying cause of this phenomenon. Recent studies on the autotoxicity of medicinal plants mainly focused on medicinal herbs, such as *Codonopsis pilosula*, etc. [28]. Few studies have been performed on medicinal woody plants, and the mechanism underlying this autotoxicity has remained unclear [12].

Our previous studies confirmed that *C. migao* has a strong allelopathic effect [29]. To explain the difficulties associated with new recruitment of *C. migao*, the aim of this study was to understand the responses of the plants to autotoxicity and the possible mechanism of autotoxicity that inhibits plant growth. Accordingly, we proposed the following hypotheses: (1) autotoxicity is the main factor affecting seedling growth in *C. migao*, and (2) the main mechanism of autotoxicity is that decomposition and release of autotoxic substances from the litter of *C. migao* can be achieved by altering the soil environment (chemical property, soil enzyme activity, and fungi) to inhibit seedling growth and survival. To test these hypotheses, we adopted a method of irrigating the aqueous extract and simulated the effects of litter decomposition and release from different anatomical parts of *C. migao* on seedling growth under field conditions from the new perspective of plant autotoxicity [30]. The changes in morphology, physiological metabolism, soil substrate, and fungi, which are the four key factors affecting plant growth, were determined during seedling growth.

2. Material and Methods

2.1. Experimental Site

Experiments were conducted at the College of Forestry, Guizhou University, Huaxi District, Guiyang (106°42′E, 26°34′N, 1020 m a.s.l.). The climate in this region is subtropical monsoon, with a mean annual temperature of 15.3 °C and mean annual precipitation of 1129 mm.

2.2. Plant Materials

Fresh mature fruits of *C. migao* were collected from Luodian County, Guizhou Province (25°26′N, 106°31′E) from October to November 2017. They were then transported to the laboratory to remove their flesh immediately, followed by rinsing with water. Before germination, we cleaned the seeds, sterilized them by immersion in 0.5% $KMnO_4$ for 2 h, and then rinsed them in sterile water five times. Seed germination was performed in an artificial climate chamber (RXZ-1500, Ningbo Jiangnan Instrument Factory, Ningbo, China) with a germination box. After germination of the seeds, we transferred them to nutrient soil for planting in January 2018, and seedlings with identical growth were transplanted into nutrient bags for slow seedling treatment in mid-March. The pot-culture experiment began in April after the seedlings (16.25 ± 1.41 cmin height; number of leaves 5 ± 0.58) had been transplanted into plastic plots. The soil in our experiments was selected from the section loess of nongrowing plants and uniformly mixed with humus and perlite (loess: humus: perlite = 7:2:1); each pot contained approximately 2.5 kg of soil. All tested soils were sterilized under high pressure at 121°C twice, with each cycle lasting for 1 h. The litter of *C. migao* used in this experiment was directly collected from the forest floor. In addition, surface soil (0–5 cm) from under the canopy of nine natural populations of *C. migao* in Guizhou, Yunnan, and Guangxi provinces was sampled for sequence analysis to determine fungal diversity (Figure 2).

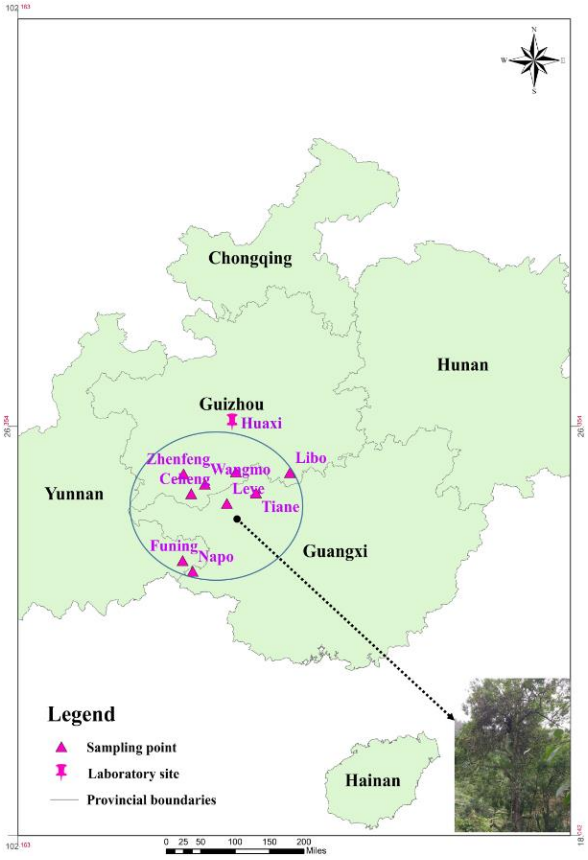

Figure 2. Experimental sites and soil sample collection sites for *C. migao* in Southwest China.

2.3. Experimental Design

Initially, we investigated and statistically analyzed the litter under the canopy of *C. migao* and found that the main components of the litter were leaves, fruits, and branches. Therefore, the materials were divided into four parts: leaf, pericarp, seed, and branch. To simulate natural conditions as much as possible, the litter was collected from under a canopy from November to December 2017 in Luodian County and classified. To prepare aqueous extracts, we cleaned all of the test materials and cut them into pieces (1 cm^2); gathered them into weights of 0, 1, 2, 3, and 4 g; and immersed them in 100 ml of deionized water. Solutions with concentrations of 0.00, 0.01, 0.02, 0.03, and 0.04 $g{\cdot}mL^{-1}$ were prepared in the dark. In accordance with the above method of aqueous extraction, five types of aqueous extract treatments were set up, namely, the control and treatment with the leaf aqueous extract ($leaf_{AE}$), pericarp aqueous extract ($pericarp_{AE}$), seed aqueous extract ($seed_{AE}$), and branch aqueous extract ($branch_{AE}$). Each treatment had nine replicates, giving a total of $4 \times 5 \times 9 = 180$ pots in this experiment. During the experiment, the aqueous extract was co-irrigated with litter (leaf, pericarp, seed, and branch) every 10 days, to better simulate the decomposition and release the effects of litter, and the positions of the pots were randomly moved. To maintain soil moisture content, quantitative deionized water was added to each pot at different times to supplement water. Through field monitoring, we found that seedlings usually withered or died within 8 months. Therefore, the experiment was designed to determine the growth indices after 210 days of treatment (Figure 1b).

2.4. Chemical Analyses

2.4.1. Analysis of Seedling Growth and Physiology

At the end of the experiment, we determined seedling growth and physiology; the determination methods were as follows. (1) Seedling height: The seedling height for each treatment was measured

using Vernier calipers and a tape measure. (2) Leaf area: Three pots of seedlings were selected for each treatment, and the leaf area of the third mature leaf below the apex of the seedlings was measured using a portable leaf-area meter (LI-3000C; LI-COR, Lincoln, NE, USA). (3) Biomass: At least three seedlings from pots undergoing different treatments were carefully removed from the soil to maintain their integrity, after which residual soil and impurities were removed by flushing with running water. After cleaning, the seedlings were oven-dried twice at 65 °C to a constant weight and then weighed.

Fresh plant materials (0.2 g) were collected from each treatment and homogenized with 5 mL of buffer (with 1% PVP), by grinding on ice, and then centrifuged at 4 °C. After centrifugation, the supernatants were used for measuring the levels of malondialdehyde (MDA), soluble protein, peroxidase (POD), and superoxide dismutase (SOD). In brief, the MDA content was estimated using the thiobarbituric acid method reported by Hodges et al. [31], with minor modifications. The soluble protein content was measured using the Folin's phenol reagent method [32]. POD activity was measured following the guaiacol method, with minor modifications [33]. SOD activity was analyzed using the nitroblue tetrazolium chloride method by Lacan and Baccou [34], with minor modifications.

2.4.2. Analysis of Soil Physicochemical Properties and Soil Enzyme Activity

At the end of the experiment, soil samples were collected from each pot immediately after the different treatments and divided into two parts: One was used for soil nutrient analysis, and the other was stored at 4°C for soil enzyme and soil fungal analyses.

Soil samples from the different aqueous extract treatments were naturally air-dried; the samples were sieved using a 2 mm diameter mesh to determine their chemical properties. In brief, the soil total nitrogen (TN) content was analyzed using the Kjeldahl method with a Foss–Kjeltec analyzer [35]. The soil total phosphorus (TP) content was determined by the Mo–Sb colorimetric method after soil was digested with a mixed acid solution of H_2SO_4 and $HCLO_4$ [36]. The soil total potassium (TK) content was measured using an alkali melting-flame photometer. Soil available N (AN) content was determined by the method used by Dorich and Nelson [37]. Soil available P (AP) content was obtained by $NaHCO_3$ extraction and analyzed by the sodium bicarbonate–molybdenum resistance colorimetric method [38]. The soil available K (AK) content was measured by the method of ammonium acetate leaching–flame photometry [38]. Soil enzyme activity, including catalase, urease, phosphatase, and invertase activity, was estimated using a soil enzyme activity kit (Beijing Solebo Biotechnology Co., Ltd.), in accordance with the manufacturer's instructions. The initial soil chemical composition and enzyme activity are detailed in Table 1.

Table 1. Initial chemical composition and soil enzyme activity of the tested soil samples.

Soil Properties	pH	Total N ($g \cdot kg^{-1}$)	Total P ($g \cdot kg^{-1}$)	Total K ($g \cdot kg^{-1}$)	Available N ($g \cdot kg^{-1}$)	Available P ($mg \cdot kg^{-1}$)	Available K ($mg \cdot kg^{-1}$)	Catalase $mL \cdot g^{-1} \cdot 20$ min	Urease $mgNH_3^-N/g$	Phopatase $umol \cdot g^{-1} \cdot d^{-1}$	Invertase $mg \cdot g^{-1} \cdot d^{-1}$
	6.06	1.2	0.2	0.29	52.5	3.5	13.03	1.43	0.015	23.53	1.5

2.4.3. Analysis of Soil Fungi

Surface soils (0.04 $g \cdot mL^{-1}$) from the four aqueous extract treatments and the control from our experiment and surface soils of 0–5 cmfrom nine areas in which *C. migao* is distributed were used to analyze the diversity of soil fungi composition. Each treatment and field sample had three biological replicates. Soil genomic DNA was extracted using an extraction kit (FastDNA®tSPINKit for Soil, MP Biomedicals Co., Ltd., Shanghai, China). PCR amplification was performed using TaKaRa *rTaq* DNA polymerase in a 20 µL reaction system containing the following: 4 µL of 5× Buffer, 2 µL of 2.5 mmol/L dNTPs, 0.8 µL of forward primer ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (5 µmol/L), 0.8 µL of reverse primer ITS2R (5′-GCTGCGTTCTTCATCGATGC-3′) (5 µmol/L), 0.4 µL of rTaq polymerase, 0.2 µL of BSA, 10 ng of template DNA, and ddH_2O to 20 µL. ABI GeneAmp 9700 was used for PCR. The PCR reaction parameters were as follows: 95 °C for 3 min; 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s; 72 °C for 10 min; and, finally, 10 °C until stopping the reaction. The PCR products

were sequenced using an Illumina Hiseq high-throughput sequencing platform by Shanghai Ouyi Biomedical Information Co., Ltd.

2.5. Statistical Analysis

The homogeneity of variance was determined before performing ANOVA, and the data were logarithmically transformed when required. Duncan's test was used for assessing significant differences in all of the parameters using the SPSS 21.0 statistics package (Chicago, IL, USA). All presented data are indicated as the means and standard errors (SEs) of at least three replicates. The final results of fungal sequencing were collated, and SR statistics tools were used (http://www.mdts-erver.com/srst/) to analyze soil fungal data. Graphs were constructed with OriginPro 9.0 (Origin Lab, Northampton, MA, USA).

3. Results

3.1. Effects of C. migao Litter Aqueous Extract on Seedling Growth

The seedling height, biomass, and leaf area of *C. migao* were significantly decreased compared with those of the control when the concentration of $leaf_{AE}$ was increased. At 0.04 g·mL^{-1}, the seedling height, biomass, and leaf area decreased by 32.07%, 49.02%, and 42%, respectively. Within the experimental concentration range, $pericarp_{AE}$, $seed_{AE}$, and $branch_{AE}$ significantly promoted seedling growth. Among them, at 0.04 g·mL^{-1} of $pericarp_{AE}$, seedling height, biomass, and leaf area increased by 57.29%, 37.36%, and 50.88%, respectively. Similar increases with $seed_{AE}$ treatment of 44.89%, 25.62%, and 39.97% at 0.04 g·mL^{-1}, respectively, and with $branch_{AE}$ treatment of 44.89%, 30.21%, and 47.31% at 0.04 g·mL^{-1}, respectively, compared with those of the control were also identified (Table 2). The response of seedling growth to different litter aqueous extracts differed in the same concentration range.

Table 2. Effects of aqueous extract of litter from different anatomical parts of *C. migao* on seedling growth.

Group	Concentration (g·mL^{-1})	Height (cm)	Biomass (g)	Leaf Area (cm^2)
$Leaf_{AE}$	0	162.53 ± 3.75 a	148.30 ± 7.10 a	17.69 ± 4.30 a
	0.01	143.07 ± 7.30 b	110.80 ± 8.70 b	15.57 ± 2.88 bc
	0.02	131.23 ± 8.60 bc	99.80 ± 9.10 bc	14.35 ± 1.24 bc
	0.03	129.30 ± 6.80 c	95.30 ± 4.40 c	12.41 ± 2.65 bc
	0.04	110.40 ± 4.96 d	75.60 ± 5.70 d	10.26 ± 3.68 c
$Pericarp_{AE}$	0	162.53 ± 3.75 d	148.30 ± 7.10 c	17.69 ± 4.30 b
	0.01	220.60 ± 6.10 c	176.30 ± 6.40 b	23.46 ± 3.41 ab
	0.02	225.50 ± 7.50 bc	182.40 ± 6.30 b	22.33 ± 3.62 ab
	0.03	235.80 ± 5.20 b	183.80 ± 5.50 b	22.89 ± 3.05 ab
	0.04	255.60 ± 5.70 a	203.70 ± 7.70 a	26.69 ± 3.67 a
$Seed_{AE}$	0	162.53 ± 3.75 d	148.30 ± 7.10 b	17.69 ± 4.30
	0.01	168.80 ± 5.30 cd	152.50 ± 7.30 b	19.43 ± 2.78
	0.02	178.50 ± 6.00 c	157.00 ± 6.80 b	19.59 ± 3.88
	0.03	225.50 ± 6.70 b	176.60 ± 8.20 a	21.47 ± 4.26
	0.04	235.50 ± 4.80 a	186.30 ± 5.80 a	24.76 ± 3.86
$Branch_{AE}$	0	162.53 ± 3.75 c	148.30 ± 7.10 c	17.69 ± 4.30 c
	0.01	170.60 ± 5.90 c	150.30 ± 4.80 c	19.82 ± 4.18 ab
	0.02	192.50 ± 6.70 b	164.50 ± 6.20 b	23.52 ± 4.11 ab
	0.03	232.80 ± 7.50 a	187.50 ± 6.60 a	24.38 ± 3.65 ab
	0.04	235.50 ± 5.10 a	193.10 ± 5.70 a	26.06 ± 3.72 a

The indices of height, biomass, and leaf areas for *C. migao* seedlings after the application of $leaf_{AE}$, $pericarp_{AE}$, $seed_{AE}$, and $branch_{AE}$ treatments for 210 days. Values are expressed as mean ± SE. The values marked with different letters are significant at $p < 0.05$ (Duncan's test).

3.2. Effects of C. migao Litter Aqueous Extract on the Antioxidant Systems of Seedlings

Plants activate their own defense systems to resist toxicity caused by reactive oxygen species (ROS) when they are under stress, such as enhancing their antioxidant enzyme activities. Our results show that the MDA content in the leaves of *C. migao* seedlings increased as the concentration of $leaf_{AE}$ increased (Figure 3a). Among the treatments, the MDA content was the highest when the concentration of $leaf_{AE}$ was 0.04 g·mL^{-1}, which was 3.3 times that of the control. In $pericarp_{AE}$, $seed_{AE}$, and $branch_{AE}$ treatments, the MDA content changed linearly and was higher than that of the control. This illustrates that different aqueous extracts could cause different degrees of peroxidation of the cell membrane lipid structure. The soluble protein content decreased as the concentration of the extract increased under different treatments. It is noteworthy that the soluble protein content of $leaf_{AE}$ at 0.04 g·mL^{-1} was the lowest, which was reduced by 55% compared with that in the control (Figure 3b). Moreover, the antioxidant enzyme activities of the seedlings were also affected by the concentration of aqueous extract. The activities of POD and SOD in the seedlings treated with $pericarp_{AE}$, $seed_{AE}$, and $branch_{AE}$ were higher than those of the control. This indicates that, under the synergistic action of POD and SOD, excessive free radicals in the cells could be effectively removed, thereby maintaining the structural stability of the cell membrane. Conversely, under a low concentration of $leaf_{AE}$ (>0.01 g· mL^{-1}), *C. migao* seedlings were placed under stress, which resulted in membrane lipid peroxidation, a sharp decrease in the soluble protein content, reductions in POD and SOD activity (Figure 3c,d), and significant inhibition of the antioxidant capacity of the seedlings.

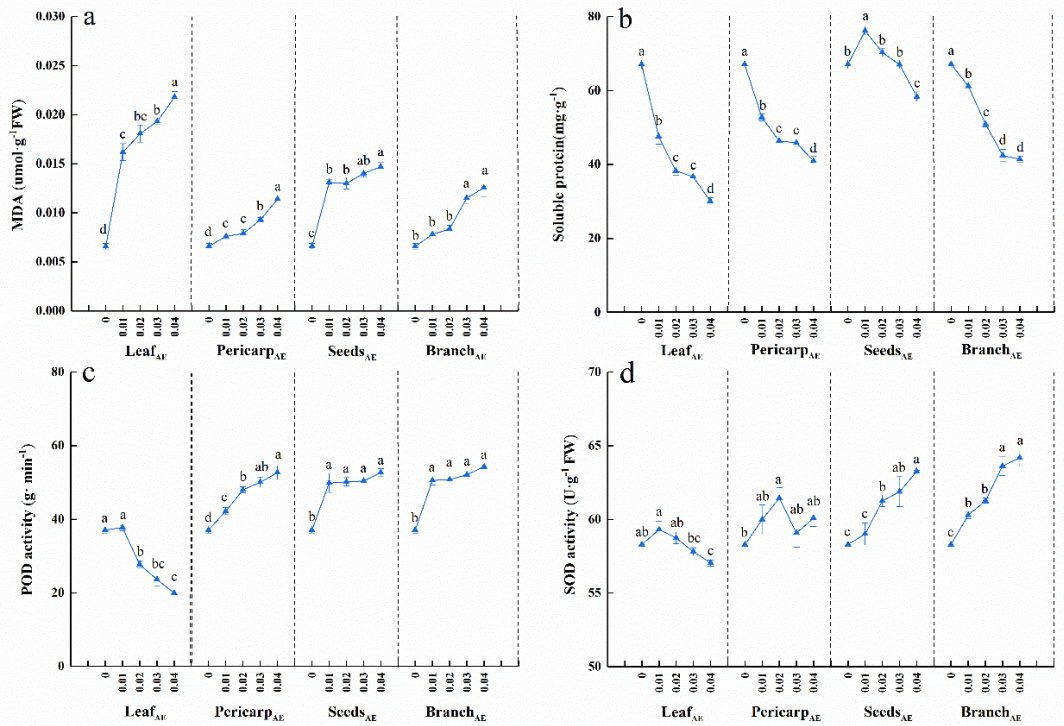

Figure 3. The MDA (**a**), soluble protein (**b**), POD (**c**), and SOD (**d**) contents in *C. migao* seedlings after the application of litter aqueous extract treatments for 210 days. The *x* axis denotes the concentration of the aqueous extract and the *y* axis denotes the content. Values are expressed as mean ± SE. The values marked with different letters are significant at $p < 0.05$ (Duncan's test).

3.3. Effects of C. migao Litter Aqueous Extracts on N, P, and K in Soils of Potted Seedlings

The TN content of the soil was increased with increasing concentration of the aqueous extracts; the TN content increased by 69.68%, 42.26%, and 40.98% with $leaf_{AE}$, $pericarp_{AE}$, and $seed_{AE}$ at 0.04 g·mL^{-1}, respectively, compared with that of the control. The difference was the most significant when the

concentration of $leaf_{AE}$ was 0.04 g·mL^{-1} ($p < 0.05$) (Figure 4a). The TP content in soil was higher than that in the control after all treatments. The most significant difference was found when the concentration of $pericarp_{AE}$ was 0.04 g·mL^{-1} ($p < 0.05$) (Figure 4b). The TK content after all treatments significantly increased ($p < 0.05$) compared with that of the control, which increased with increased aqueous extract concentration (Figure 4c). The AN content showed an upward trend under $leaf_{AE}$ and $seed_{AE}$ treatments, and the AN content significantly increased under $seed_{AE}$ ($p < 0.05$). The $pericarp_{AE}$ and $branch_{AE}$ treatments showed downward trends for the AN content, and this variable in soil was the lowest when $branch_{AE}$ was 0.01 g·mL^{-1} (Figure 4d). The soil AP content showed an upward trend with the four kinds of aqueous extracts, with $pericarp_{AE}$ treatment causing the highest increase ($p < 0.05$) (Figure 4e). In addition, with increases of all aqueous extracts, the soil AK content significantly increased compared with that in the control group ($p < 0.05$) (Figure 4f).

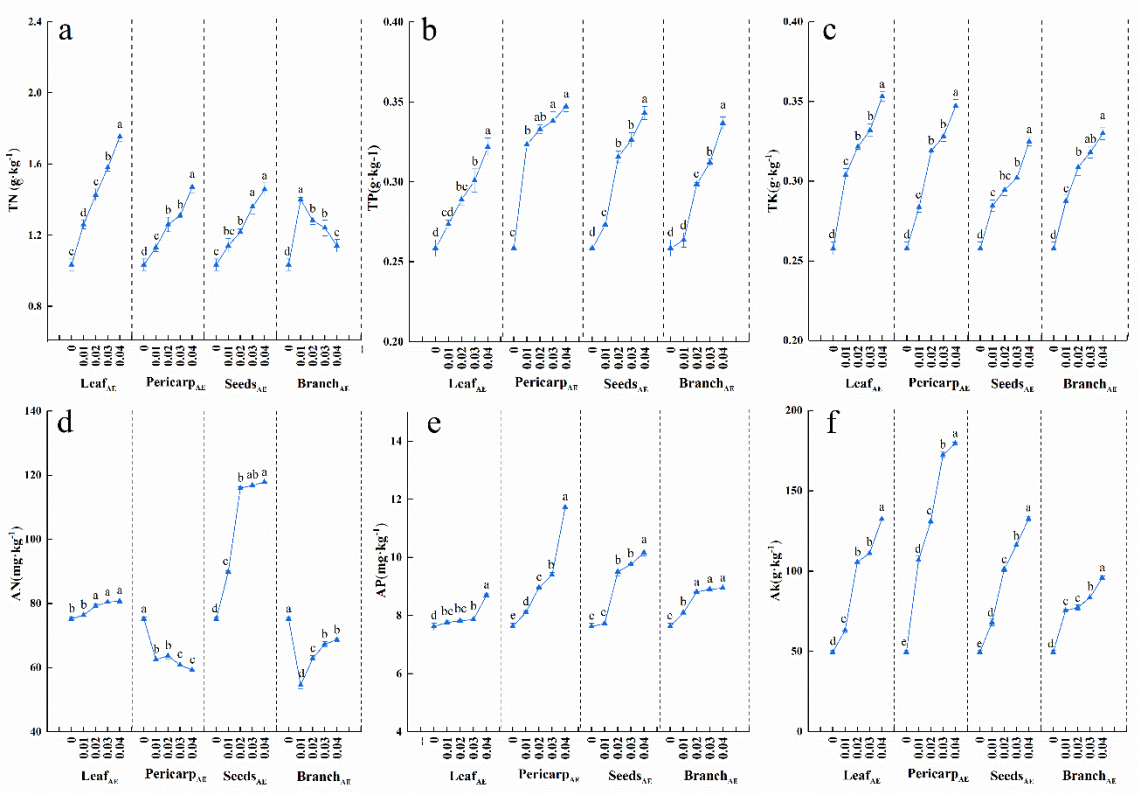

Figure 4. The TN (**a**), TP (**b**), TK (**c**), AN (**d**), AP (**e**), and AK (**f**) contents in soil planted with *C. migao* seedlings after litter aqueous extract treatments for 210 days. The *x* axis denotes the concentration of the aqueous extract, and the *y* axis denotes the content. Values are expressed as mean ± SE. The values marked with different letters are significant at $p < 0.05$ (Duncan's test).

3.4. Effects of C. Migao Litter Aqueous Extract on Soil Enzyme Activities of Potted Seedlings

In the $leaf_{AE}$ and $branch_{AE}$ treatments, the soil catalase activity was significantly decreased compared with that in the control ($p < 0.05$), with increasing extract concentration. The soil catalase activity in the $pericarp_{AE}$ and $seed_{AE}$ treatments first decreased and then increased (Figure 5a). This indicated that the $leaf_{AE}$ and $branch_{AE}$ treatments had significant negative regulatory effects on soil catalase, whereas the $pericarp_{AE}$ and $seed_{AE}$ treatments showed a promoting effect. The soil urease activity showed an increasing trend in all the treatments, being significantly higher than that in the control ($p < 0.05$). All treatments promoted soil urease activity (Figure 5b). The soil phosphatase activity decreased with the increases in the concentrations of $leaf_{AE}$ and $pericarp_{AE}$; the value after the $leaf_{AE}$ treatment was lower than that in the control, while that after the $pericarp_{AE}$ treatment was lower than that in the control at 0.02–0.04 g·mL^{-1}. The $seed_{AE}$ and $branch_{AE}$ treatments could also promote

soil phosphatase activity (Figure 5c). Moreover, under all the aqueous extract treatments, the soil sucrase activity was higher than that in the control, and the promoting effect of $seed_{AE}$ at 0.04 g·mL^{-1} was the most pronounced (Figure 5d).

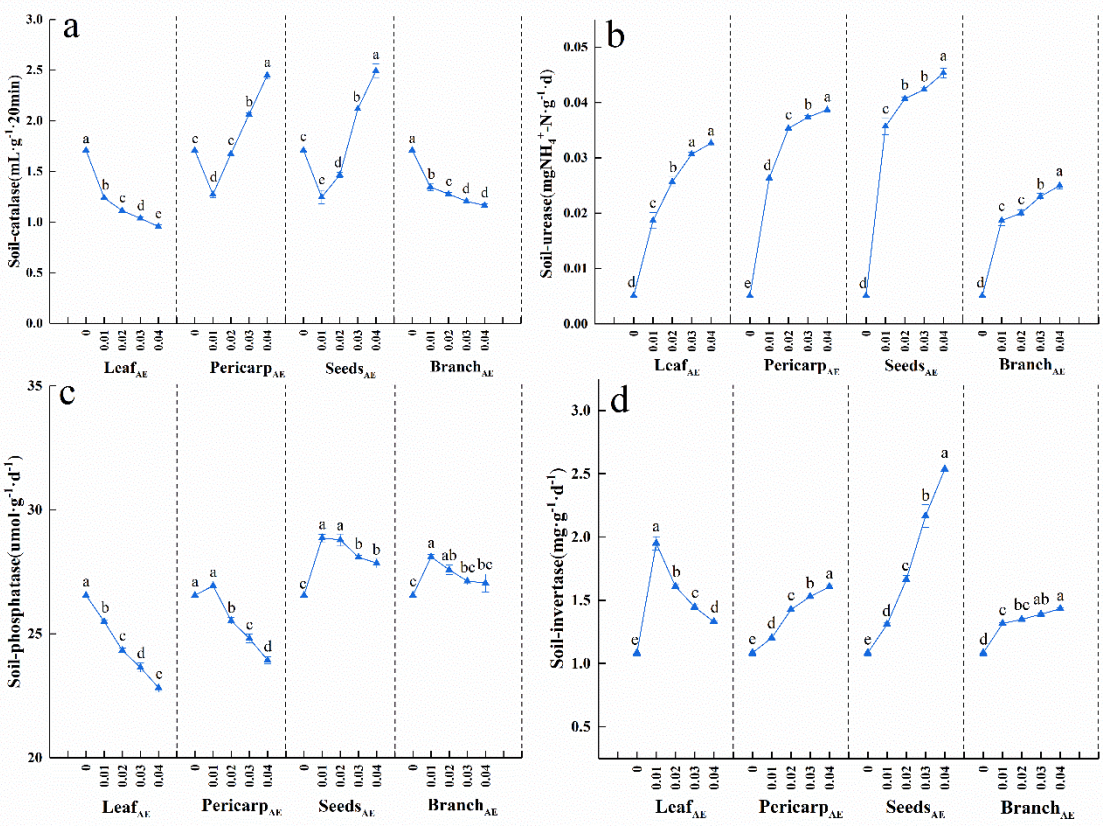

Figure 5. The catalase (**a**), urease (**b**), phosphatase (**c**), and invertase (**d**) contents in soil planted with *C. migao* seedlings after litter aqueous extract treatments for 210 days. The *x* axis denotes the concentration of the aqueous extract and the *y* axis denotes the content. Values are expressed as mean ± SE. The values marked with different letters are significant at $p < 0.05$ (Duncan's test).

3.5. Composition of Fungi in Different Plots and Experimental Treatments

Fungi in the surface soil of nine areas below *C. migao* canopy and upon the treatments with the highest concentration of the extracts were sequenced by high-throughput sequencing technology. As shown in Figure 6a, the common fungi (after the removal of unidentified fungi) in the surface soil below the canopy in the nine counties (Luodian, Ceheng, Libo, Zhenfeng, Wangmo, Napo, Funing, Tian'e, and Leye) were identified as *Archaeorhizomyces sp.*, *Chaetomium nigricolor*, *Chaetosphaeria vermicularioides*, *Chloridium sp.*, *Cladophialophora sp.*, *Cryptococcus podzolicus*, *Cylindrocladiella pseudoparva*, *Ilyonectria mors-panacis*, *Lophiostoma sp.*, *Microascaceae sp.*, *Mortierella biramosa*, *Myxocephala albida*, *Penicillifer martinii*, *Penicillium ochrochloron*, *Rozellomycota sp.*, *Trichoderma koningiopsis*, and *Trichosporon sporotrichoides*. Based on the relative abundance, the most abundant fungal species in Luodian, Ceheng, Libo, Zhenfeng, Wangmo, Funing, and Leye was *Cryptococcus podzolicus*, the most abundant fungal species in Napo was *Mortierella biramosa*, and the most abundant fungal species in Tian'e was *Penicillium ochrochloron*. Comparing the species' composition in terms of the top 30 species under the different treatments (Figure 6b), it could be found that the species overlapping between natural conditions and artificial controlled experimental conditions were *Cladophialophora* and *Lophiostoma*. The average abundance of *Cladophialophora* in each treatment group was in the order $pericarp_{AE}$ > $leaf_{AE}$ > $seed_{AE}$ > control > $branch_{AE}$, whereas that of *Lophiostoma* was $branch_{AE}$ > $leaf_{AE}$ > $pericarp_{AE}$ > $seed_{AE}$ > control.

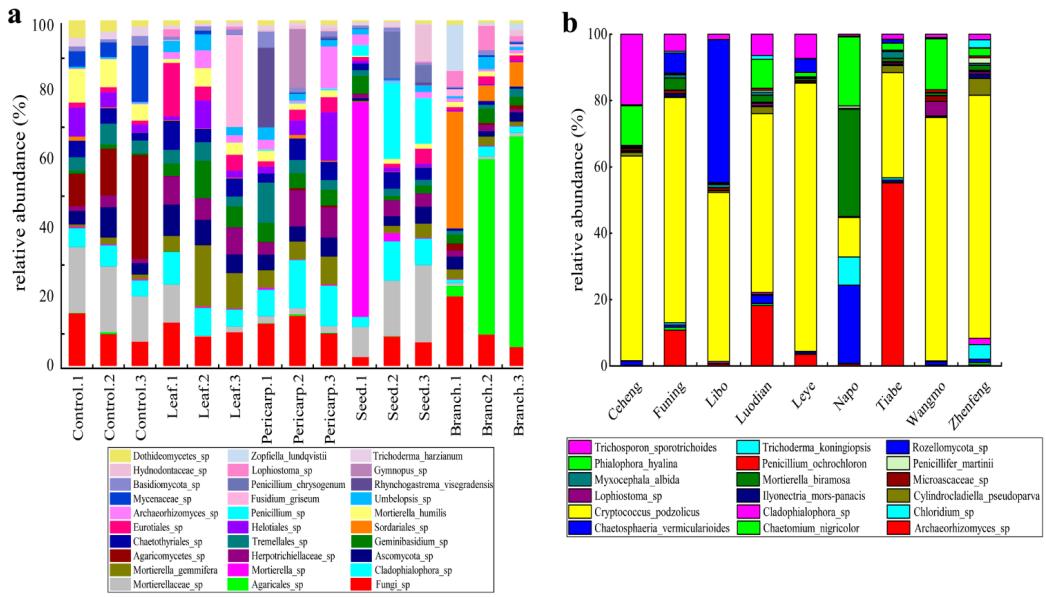

Figure 6. Relative abundance at the species level. Notes: (**a**) Relative abundance of the top 30 species under different treatments. (**b**) The common fungal species in nine areas.

The Adonis analytical method was used for analyzing the differences in the composition of fungi between the different treatment groups (Table 3). The results indicated that the composition of fungi significantly differed among the five treatments. It was also proven that the soil fungal community may have been changed by the extract treatment from different anatomical parts of *C. migao*.

Table 3. Composition of fungi among the four treatments by the Adonis analysis method.

Title	Df	Sum of Squares	Mean Squares	F. Model	R^2	P (>F)
Group_factor	4	1.7057	0.42642	3.4234	0.57795	0.001
Residuals	10	1.2456	0.12456		0.42205	
Total	14	2.9513			1	

$p < 0.05$ indicates that the feasibility of this test is high and that there are significant differences at an intergroup level.

Similarly, UPGMA sample similarity cluster analysis (BinaryJacCard distance) was performed on the three replicates of fungal communities (Figure 7). The results showed that the control, $pericarp_{AE}$, and $seed_{AE}$ (1,2) treatments were grouped into a single branch, whereas the $seed_{AE}$ (3) and $leaf_{AE}$ treatments were grouped together into another single branch. The clustering results showed that there was good similarity within the group, indicating that the change in the fungal composition among the different treatments was closely related to the type of litter.

4. Discussion

Obstacles to the natural regeneration of *C. migao* were always an important factor affecting its population continuity. Early studies also confirmed that *C. migao* has strong allelopathic effects on many plants [29]. Therefore, we hypothesized that *C. migao* is a medicinal plant with autotoxic allelopathy. One of the main methods to affect population regeneration is through the decomposition of litter and release of autotoxic compounds that alter the structure and properties of the ecosystems. Although our laboratory experiments have some limitations, the results indicate that the autotoxicity of *C. migao* is an important factor hindering its seedling growth and survival.

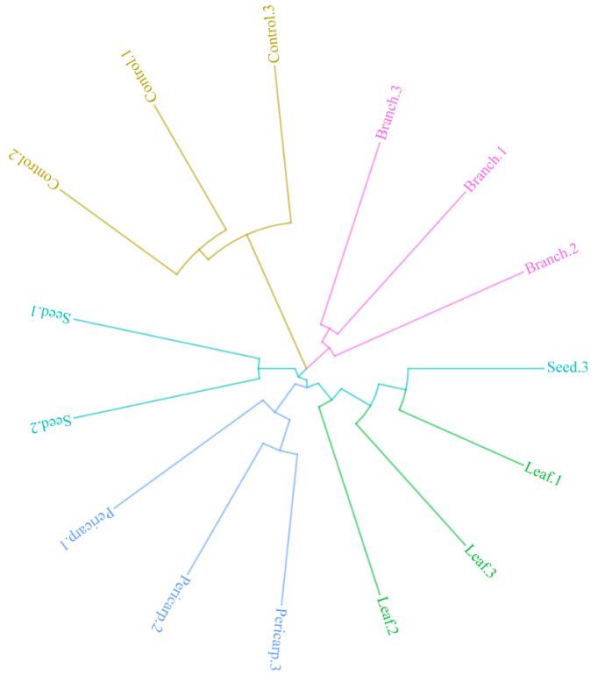

Figure 7. UPGMA sample similarity clustering results.

4.1. Seedling Growth and Antioxidant System Responses to Aqueous Extracts from Different Anatomical Parts of C. migao

Under autotoxicity, plants usually suffer a reduction in biomass, including inhibition of leaf, stem, and root growth [10]. When the concentration of autotoxic substances is low, it may promote plant growth, but if the concentration exceeds a threshold value, it will lead to unhealthy growth or other adverse effects [7]. Our study found that all growth indices of seedlings treated with $leaf_{AE}$ were inhibited (Table 2). The height, biomass, and leaf area of seedlings were significantly decreased with an increase in the concentration of $leaf_{AE}$ ($p < 0.05$), which may be the physio-morphological reaction to the autotoxic compounds contained in leaves on the seedlings [39]. This is similar to the findings in a study on biomass accumulation with the allelopathy of *Eucalyptus* [40]. Contrary to $leaf_{AE}$ treatment, $pericarp_{AE}$, $seed_{AE}$, and $branch_{AE}$ treatments showed different degrees of promotion of seedling growth. The reason for this may be that the types of compounds are different and the levels of autotoxic substances in pericarps, seeds, and branches aqueous extracts at the same concentration are lower than that in leaves, which does not reach the threshold of seedlings' tolerance [7]. For instance, the *Pinus harcus* leave aqueous extract inhibited wheat growth, whereas aqueous extracts from other parts promoted it [41]. Meanwhile, preliminary tests were undertaken to clarify the mechanism of autotoxins in *C. migao* seedlings.

Plants may undergo secondary stress under autotoxicity, for example, oxidative stress, which results in the production of excessive ROS; causes accumulation of osmolytes, such as peroxide anion free radical O_2^-, hydroxyl radical OH^-, and hydrogen peroxide (H_2O_2) [42]; and leads to peroxidation of the membrane lipid structure in plant cells and, finally, MDA formation. The MDA content can be used as an important index for judging the degree of peroxidation damage in plant cell lipid membranes [43]. Our results showed that the content of MDA in $leaf_{AE}$ was higher than that in other aqueous extracts (Figure 3a). Plants produce protective enzymes to cope with the damage caused by free radicals, including the cooperation of POD and SOD, which can effectively reduce and eliminate the damage caused by free radicals to cell membranes [44]. POD and SOD activities in seedlings were decreased with increasing applications of *C. migao* $leaf_{AE}$ at the end of the experiments (Figure 3c,d). This indicated that the $leaf_{AE}$ treatment had an inhibitory effect on the antioxidant system of seedlings; POD and SOD could not remove free radicals promptly, which made the seedlings

undergo membrane lipid peroxidation for a long time. However, the pericarp$_{AE}$, seed$_{AE}$, and branch$_{AE}$ treatments had no significant effect on seedling physiology (Table 2 and Figure 3), whereas the MDA content and antioxidant enzyme activity increased with increasing concentration, and the activity of antioxidant enzymes remained at a high level. This indicated that membrane lipid peroxidation occurs in seedlings after aqueous extract treatment [5] (Figure 3a). POD and SOD antioxidants can remove free radicals in seedlings over time, thereby preventing plant damage [9]. The increases in POD and SOD in our study also supported the notion that seedlings could cause biotic stress under autotoxicity. Similarly, soluble proteins are involved in the metabolism of various enzymes, the content of which could indirectly reflect the ability of plants to perform material synthesis and metabolism [45]. The decrease in the soluble protein content after leaf$_{AE}$ treatment was higher than that after other aqueous extract treatments, indicating that the structure and function of membrane lipids in seedling leaves were damaged for a long time, and this might have led to the decreased activities of various enzymes involved in synthesis and metabolism. Studies have indicated that 30%–50% of the leaf soluble protein is Rubisco, which plays a decisive role in determining the plants' rate of photosynthesis [46]. Therefore, we considered that leaf$_{AE}$ treatment affected the protective enzyme system and photosynthetic capacity of seedlings, which was consistent with the finding of inhibition of seedling growth after leaf$_{AE}$ treatment at the end of the experiment. Although the effects of aqueous extracts from different anatomical parts of *C. migao* on the seedlings differed, the experimental results of leaf$_{AE}$ treatment on the morphological and physiological inhibition of seedlings also validated our first hypothesis, i.e., autotoxicity is the main factor affecting seedling growth.

4.2. Soil Nutrition and Enzyme Activity Responses to Aqueous Extracts of Different Anatomical Parts of C. migao

Soil nutrient elements and enzyme activities play an important role in plant growth. Generally, allelopathic (autotoxic) plant litter has a major impact on soil N, P, and K contents and soil enzymes [47]. Allelopathic substances could alter soil fertility and enzyme activity when they enter the soil, which indirectly leads to the occurrence of autotoxicity [48]. Soil enzymes are catalytically, biologically active substances secreted by fungi, plants, and living animals, which are released after the decomposition of animal and plant residues [49]. They are closely related to the transformation and utilization of soil N, P, and K [50]. Soil catalase mainly decomposes H_2O_2 produced by microorganisms to reduce toxicity; in the present study, the soil catalase content significantly decreased with an increase in the concentration of leaf$_{AE}$ compared with that in the control ($p < 0.05$), indicating that leaf$_{AE}$ treatment significantly inhibited the ability of soil catalase to scavenge H_2O_2, thereby indirectly increasing the toxicity to seedlings [51] (Figures 5a and 3a). Soil urease can catalyze the hydrolysis of urea in soil and improve the soil N supply capacity. Our study found that treatment with aqueous extracts from different anatomical parts of *C. migao* increased soil urease activity, which was similar to the changes in soil urease activity reported in studies of allelopathy on *Allium sativum* bulbs by *Astragalus mongholicus* root aqueous extracts [52]. Generally, invertase activity is positively correlated with soil urease and soil fertility. Compared with the control, the increase in soil fertility had positive effects on soil invertase and urease activities in our study, which illustrates this point [53]. In some autotoxic species, allelochemicals may cause deterioration of the soil substrate, thereby inhibiting the secretion and release of soil phosphatase by plant roots and soil microbial metabolic activities [11] and also affecting the conversion of soil organic P. Our results showed that the TP and AP contents upon different treatments were significantly higher than those in the control, whereas the soil phosphatase activity showed an opposite trend, with an increase in the concentration of the aqueous extract, and leaf$_{AE}$ concentration was always lower than in the control at 0.01–0.04 g·mL^{-1}. One potential reason for this is that the method of co-irrigation of extract and litter was adopted in this experiment, and the later decomposition of litter could supplement soil elements. Furthermore, it is possible that the aqueous extract has a negative effect on the metabolic activity of roots and the composition and activity of soil microorganisms, thus changing the intensity of secretion, release, and modification of

soil enzymes [11]. Although aqueous extract treatments supplemented the soil nutrients, they did not change the inhibitory effect of $leaf_{AE}$ on *C. migao* seedling growth, which also confirmed that an improvement in soil fertility could not alleviate the negative effects of autotoxicity on plants [8]. Allelochemicals could affect soil physicochemical properties, microbial composition, and soil enzyme activity after entering the soil, thereby changing the growth of plants [54]. Our study found that litter aqueous extract caused certain changes in soil conditions compared with those in the control, particularly the $leaf_{AE}$ treatment, which altered the soil conditions to indirectly inhibit seedling growth. Therefore, our second hypothesis was partially validated in this experiment. In addition, the inhibition of seedling growth by $leaf_{AE}$ treatment indicates that there are indeed autotoxic substances in *C. migao* leaves, and the types and contents of these substances still need further study.

4.3. Soil Fungal Component with Different Treatments and in Contrast to that in the Field

Soil fungi are essential components of forest ecosystems and are considered to be key factors affecting plant growth. Many autotoxic substances bind in the form of acyl/glycosyl groups in plants. These toxic substances do not directly act on plants and do not exhibit autotoxicity, because of the lack of soil fungi participated [55]. However, when autotoxic compounds enter the soil in the form of litter, they lead to the degradation of acyl/glycosyl groups with the participation of fungi and conversion of inactive to active forms, which finally cause autotoxicity [56]. The soil fungal community is the main part of its microbial population and a decomposer of soil organic compounds [57]. It plays an important role in regulating soil enzyme activities [58] and is directly related to invertase, urease, and phosphatase activities [44]. In this study, soil fungal composition after different aqueous extract treatments were compared with that in the nine areas to explore the differences in fungal diversity in potted soils after different treatments. This could verify the possible effects of autotoxic substances on the soil after entering it. Moreover, the study could determine whether there are common fungi involved in the interaction of litter catabolism and soil substrate change during the process of autotoxicity. Our results showed that there were significant differences in the fungal composition of surface soil between different anatomical parts of aqueous extract treatments. Next, we performed comparisons with 30 common fungi found in the nine areas and the two fungi found in the pot experiments; both of these groups had a high abundance under $leaf_{AE}$ treatment. This indicates that the different secondary metabolites of litter resulted in an interaction between fungi in soil, which resulted in the difference in the fungal composition after different treatments. It also indicates that allelochemicals could affect plant growth by changing soil fungal composition [59]. There are only two kinds of fungi in common between the pot experiment and the natural population. It may be that the pot experiment used high-temperature-sterilized soil, and the species and quantity of fungi carried by the litter itself are limited [13], which cannot fully reflect the fungal composition under natural conditions. In addition, the climate of the experimental site differs from the natural conditions; therefore, the main reason for these differences may be that the in situ environment is more suitable for related fungal growth [12]. From the perspective of revealing the relationship between fungi and autotoxicity, in situ experiments will be indispensable, which will be the focus of our work in the future.

5. Conclusions

In summary, our results demonstrated that the $leaf_{AE}$ treatment could inhibit seedling growth to different degrees, and the inhibitory effects mainly act via decreasing growth targets and damaging cell membrane lipid structures. Our results also revealed that soil enzyme activities were inhibited by $leaf_{AE}$, and the soil fungal composition after $leaf_{AE}$ treatment was significantly different from that after the other treatments (including the control). Although the soil fertility was somewhat improved by $leaf_{AE}$ treatment, it did not promote seedling growth, which indicated that the autotoxic substances contained in leaves are important factors affecting seedling growth. This is consistent with the long-term accumulation of leaf litter in the field and also supports the view that autotoxicity is an important obstacle to the natural regeneration of *C. migao*. From the perspective of species

protection, the litter under the canopy should be cleaned up in time to reduce accumulation of autotoxic substances in soils under canopy and conserve the natural population. Meanwhile, measures for ex situ conservation could be considered after artificial seedling cultivation in similar habitats.

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