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Differences in Physiological Metabolism and Antioxidant System of Different Ecotypes of *Miscanthus floridulus* under Cu Stress

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Abstract: To reveal the similarities and differences in the resistance mechanisms of different ecotypes to Cu stress, a pot experiment was used to systematically compare the physiological responses of non-mining ecotype *Miscanthus floridulus* (collected from Boluo County, Huizhou City) and mining ecotype *Miscanthus floridulus* (collected from Dabaoshan mining area) under different Cu concentrations. The results showed that chlorophyll a, chlorophyll b and total chlorophyll in the leaves of the two ecotypes of *M. floridulus* were negatively correlated with Cu stress concentration ($p < 0.01$), but the extent of decrease for the ecotypes in the mining area was lower than that for the ecotypes in the non-mining area. The values of chlorophyll a/b for both ecotypes increased with increasing Cu treatment concentration, indicating that Cu is more harmful to chlorophyll b than to chlorophyll a for *M. floridulus*. Cu stress can lead to the accumulation of malondialdehyde (MDA) in the leaves of *M. floridulus* with the amount of MDA accumulation observed being greater in the non-mining ecotype than in the mining ecotype ($p < 0.05$). The content of antioxidant substances (ascorbic acid and reduced glutathione) in the mining ecotype *M. floridulus* was significantly higher than that in the non-mining ecotype. The activity of SOD in the leaves of non-mining ecotypes was inhibited by Cu stress and the activity of POD was increased by Cu stress. However, the increase in POD in the mining ecotypes was greater than that in the non-mining ecotypes and the activities of the two enzymes in the mining ecotypes were significantly higher than those in the non-mining ecotypes at the highest concentration of Cu. Cu had different effects on PPO activity in the leaves of the two ecotypes of *M. floridulus*. The plant leaves of the non-mining ecotype at 400 and 800 $mg \cdot kg^{-1}$ were significantly fewer than those of the control group ($p < 0.05$), which were 87.1% and 65.2% of the control group, respectively. The PPO activity in the plant leaves of the mining ecotype was higher than that in the leaves of the non-mining ecotype and was significantly higher at 400 and 800 $mg \cdot kg^{-1}$ than that of the control group ($p < 0.05$), at 226.5% and 268.1% of the control group, respectively. These results indicate that the mining ecotype *M. floridulus* is more resistant to copper stress, that resistant ecotypes have been formed, and that small-molecule antioxidant substances play an important role in increasing resistance levels.

Keywords: copper; *Miscanthus floridulus*; non-enzymatic substance; antioxidant system

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1. Introduction

With increasing growth in Cu-containing pollutants in industry, agriculture, transportation and other fields, especially in recent years, the frequent use of Cu-containing pesticides in agricultural production, the excessive mining of copper ores and the huge discharge of Cu-containing pollutants in industrial production have resulted in serious harm to animals, plants and the environment, and even endangered human health [1–3]. These harms to the ecosystem have attracted the attention of researchers [4,5]. The phytoremediation of mining wasteland with (super) enriched plants and tolerant plants is currently considered to be an economical and effective method [6,7]. Therefore, screening new repair species and exploring their mechanisms of tolerance to heavy metals has become a focus of academic research [8,9].

Previous studies have shown that, due to its long-term survival in a polluted environment, *Miscanthus floridulus* in metal mining areas may have undergone resistance evolution adapted to the polluted environment and formed resistant ecotypes [10–12]. Liao Bin et al. [13] found that there were significant differences in the critical concentration and symptoms of Cu poisoning between the two ecotypes of *Commelina communis*. The differences in the resistance mechanisms of different ecotypes to heavy metal stress may be caused by variation in genetic mechanisms, the functioning of the antioxidant enzyme system or the detoxification of heavy metal chelation, but there is no consensus at present [14–16]. *M. floridulus* is a perennial herb plant of the Miscanthus genus, which is widely distributed in southern China and is highly adaptable [17,18]. Sun Jian et al. [19] investigated heavy metal pollution in the soil and plants in a lead–zinc mining area in Chenzhou, Hunan Province, and found that *M. floridulus* had a large capacity for absorption and transport of lead and copper. Studying the physiological and ecological differences between resistant ecotypes and sensitive ecotypes is a valuable approach for revealing the mechanisms of plant resistance and underpins the widespread use of phytoremediation technology [20–22].

The Dabaoshan mine, located at the junction of Qujiang County and Wengyuan County, Shaoguan City, Guangdong Province, is a large iron polymetallic sulfide-associated deposit [23]. Zhao et al. [24] investigated the soil of Dabaoshan and found that heavy metal Cd pollution in the soil of Dabaoshan was the most severe, with more than 81.19% of the soil contaminated by Cd. Furthermore, more than 72.91% of the soil evidenced Cu pollution. According to a study of plants in the Dabaoshan mining area by Chen et al. [25], the rhizosphere of *M. floridulus* can activate Cu and other heavy metals significantly and may be considered a pioneer plant for local vegetation restoration. Qin et al. [26,27] conducted a number of studies on soil heavy metal content, soil enzyme activity and vegetation restoration in the Dabaoshan lead-zinc mining area. The results showed that *M. floridulus* was beneficial in accelerating the ecological restoration of abandoned mining areas. Previous studies have also shown that *M. floridulus* can grow normally in the heavily polluted tailings ponds in the Dabaoshan mining area and that its roots can absorb and fix heavy metal copper. Moreover, its biomass is large, so it represents good plant repair material [28,29].

To better apply *M. floridulus* to soil heavy metal pollution remediation practice, it is necessary to conduct in-depth research concerning the changes to its physiological and biochemical mechanisms. In this study, through soil culture experimentation, the physiological metabolism and antioxidant enzyme activity response of two ecotypes of *M. floridulus* under Cu stress were compared to provide a theoretical basis for the rational application of *M. floridulus* in the future phytoremediation of Cu-contaminated soil.

2. Materials and Methods

2.1. Materials

The experimental material was *M. floridulus* seedlings. New seedlings of the current year were collected in early March. The mining ecotype *M. floridulus* was collected from Dabaoshan Mining area, Shaoguan, Guangdong (24°33′36.6″ N, 113°43′14.0″ E), while the non-mining ecotype *M. floridulus* was collected from the hills and mountains of Boluo County, Huizhou, Guangdong (23°08′57.8″ N, 114°21′10.0″ E), in the same subtropical monsoon climate zone. The seedlings with soil were collected in the wild and immediately brought back to the school's greenhouse. The roots were rinsed with running water to prepare for planting experiments.

2.2. The Experimental Soil

The soil for this test was taken from the teaching vegetable garden of South China Agricultural University. The air-dried soil, screened to 2 mm, was put into plastic pots, with 1.2 kg per pot. The base fertilizer standard was 100 mg N kg^{-1} dry soil, added as H_2NCONH_2, and 80 mg P kg^{-1} and 100 mg K kg^{-1}, added as KH_2PO_4. This was mixed well and set aside.

The total amounts of Zn, Pb, Cu, and Cd in the soil were determined by digestion using HCl, HF, and perchloric acid, and then by ICP-OES (Optima5300DV, Perkin-Elmer, Sheldon, CT, USA) [30]. The basic chemical properties of the soil were determined using soil agrochemical analysis methods [31].

The basic chemical properties and heavy metal contents of the soil at the plant sample collection site and the test soil are shown in Table 1.

Table 1. The basic chemical properties of the soil at the sampling points of two ecotypes of *M. floridulus* and the soil for the pot experiment [27].

Soil Sample Point	pH	Organic C ($g \cdot kg^{-1}$)	Available P ($mg \cdot kg^{-1}$)	Available N ($mg \cdot kg^{-1}$)	Heavy Metal Content ($mg \cdot kg^{-1}$)			
					Zn	Pb	Cu	Cd
Dabaoshan Mining Area	6.2	14.7 ± 0.9 b	32.2 ± 2.0 b	30.2 ± 1.9 b	1768.7 ± 91.1 a	1253.3 ± 71.3 a	1701.3 ± 77.5 a	9.1 ± 0.9 a
Boluo County	6.6	13.8 ± 0.9 b	26.6 ± 1.8 b	28.4 ± 2.7 b	135.2 ± 13.1 b	242.6 ± 44.1 b	48.4 ± 9.5 b	1.1 ± 0.2 b
Soil samples tested	6.5	28.4 ± 1.8 a	61.5 ± 9.9 a	55.5 ± 9.1 a	80.2 ± 8.9 b	8.2 ± 7.2 c	1.5 ± 0.7 c	0.30 ± 0.1 b

Note: Data in the table are means ± SD (n = 3); different letters in the same vertical column indicate significant difference according to SSR test ($p < 0.05$), the same below.

2.3. The Experimental Design

The levels of Cu stress treatment were: CK (control), 50 $mg \cdot kg^{-1}$, 100 $mg \cdot kg^{-1}$, 200 $mg \cdot kg^{-1}$, 400 $mg \cdot kg^{-1}$, and 800 $mg \cdot kg^{-1}$. Cu was added in the form of $CuSO_4 \cdot 5H_2O$. After the soil was thoroughly mixed, deionized water was added to the soil pre-culture. After the soil was stable for 14 days, the *M. floridulus* seedlings were transplanted. Plants of the same weight and height were selected, separated according to the mining ecotypes and non-mining ecotypes, and assigned to each treatment concentration. Each ecotype was planted with 3 pots per treatment and 3 plants per pot. After transplantation, the soil in the basin was kept at 65% of the field water capacity. The physiological indexes were determined after 60 days of Cu stress.

2.4. Test Index and Method

The photosynthetic pigment of leaves was determined by an extraction method [32] using a 752 N ultraviolet spectrophotometer.

Preparation of supernatant extract: take 0.2 g of the blade to be tested (the last but one blade at the top), grind it quickly in an ice bath to homogenate, add 20 mL of precooled 0.05 $mmol \cdot L^{-1}$, pH 7.8 phosphoric acid buffer solution in batches, and centrifuge it at 4 °C for 10 min at 7000 $r \cdot min^{-1}$. The supernatant extract was used for the determination of protein content, superoxide dismutase (SOD), peroxidase (POD), polyphenol oxidase (PPO) activity and malondialdehyde (MDA) content.

The activity of SOD, POD and PPO was determined by the method introduced by Li Hesheng et al. [32]; the activity of SOD was determined by the nitrogen blue tetrazole (NBT) method; the activity of POD was determined by the guaiacol method; and the activity of PPO was determined by the colorimetric method.

The method of Zhang and Qu [33] was used for the determination of MDA. An amount of 2 mL of enzyme solution was taken and 2 mL of 0.67% TBA (thiobarbituric acid) was added. After mixing, the solution was boiled in a 100 °C water bath for 30 min, cooled down, and centrifuged again. The absorbance values of the supernatant at 450 nm, 532 nm and 600 nm were measured.

The permeability of the cell membrane was measured using a conductance meter [33], with slight changes in the procedure as follows: take 0.2 g of fresh leaves; wash and cut them with high-purity water; soak them in 20 mL of high-purity water; vacuum them with a vacuum extractor for 20 min; take them out and leave them to stand for 20 min; shake the leaves gently during this process, then measure the conductivity S1 with a conductivity meter (DDS-12, Shanghai, China) at a constant temperature of 20~25 °C; place them in a boiling water bath for 15 min after measurement; after cooling to room temperature, measure the conductivity S2; S1/S2 × 100% is the relative injury rate.

For the determination of ascorbic acid (AsA) content, the chemical colorimetric method introduced by Chen and Wang [34] was used. For determination of reduced glutathione (GSH) content, GSH extraction involved the same sample extraction method as for MDA. The method of Chen Jianxun et al. [34] was used for determining the GSH content and the mercapto reagent DTNB was used for determination.

Three strains were selected and tested for all indexes; all tests were repeated more than 3 times.

2.5. Data Processing

The statistical analysis of data was performed using a combination of Microsoft Excel 2007 and IBM SPSS Statistics 20.0 software. The significance of differences between means was analyzed using Duncan's multiple comparisons test (SSR test, $p < 0.05$).

3. Results

3.1. Effect of Cu Stress on Chlorophyll Content in Leaves of *M. floridulus*

Table 2 of the experimental results shows that, with increase in Cu treatment concentration, chlorophyll a, chlorophyll b, carotenoids and the total amount of chlorophyll in the leaves of the two ecotypes of *M. floridulus* decreased.

Table 2. Effect of different Cu treatment concentrations on chlorophyll content of two ecotypes.

Cu Treatment Concentration ($mg \cdot kg^{-1}$)		0	50	100	200	400	800
Chl a ($mg \cdot g^{-1}$FW)	Non-mining ecotype	1.30 ± 0.02	1.32 ± 0.01	1.21 ± 0.07	1.19 ± 0.03	1.01 ± 0.14	1.12 ± 0.11
	Mining ecotype	1.27 ± 0.02	1.27 ± 0.01	1.28 ± 0.01	1.21 ± 0.04	1.17 ± 0.12	1.14 ± 0.02
Chl b ($mg \cdot g^{-1}$FW)	Non-mining ecotype	0.77 ± 0.06	0.91 ± 0.07	0.50 ± 0.13	0.43 ± 0.04	0.30 ± 0.08	0.35 ± 0.10
	Mining ecotype	0.52 ± 0.08	0.47 ± 0.01	0.56 ± 0.01	0.48 ± 0.05	0.41 ± 0.12	0.38 ± 0.01
Carotenoid ($mg \cdot g^{-1}$FW)	Non-mining ecotype	0.42 ± 0.01	0.43 ± 0.01	0.37 ± 0.03	0.38 ± 0.02	0.33 ± 0.02	0.37 ± 0.04
	Mining ecotype	0.38 ± 0.01	0.37 ± 0.01	0.39 ± 0.01	0.37 ± 0.01	0.35 ± 0.05	0.34 ± 0.01
Total chlorophyll ($mg \cdot g^{-1}$FW)	Non-mining ecotype	2.07 ± 0.05	2.23 ± 0.07	1.71 ± 0.11	1.62 ± 0.05	1.31 ± 0.12	1.47 ± 0.20
	Mining ecotype	1.80 ± 0.09	1.74 ± 0.02	1.84 ± 0.02	1.70 ± 0.10	1.59 ± 0.21	1.52 ± 0.02
Chl a/b	Non-mining ecotype	1.70 ± 0.16	1.44 ± 0.09	2.49 ± 0.47	2.74 ± 0.18	3.44 ± 0.52	3.25 ± 0.66
	Mining ecotype	2.44 ± 0.32	2.61 ± 0.06	2.25 ± 0.06	2.50 ± 0.18	2.90 ± 0.60	3.00 ± 0.07

Under low concentration Cu stress (50 $mg \cdot kg^{-1}$), the various chlorophyll components and the total amount of chlorophyll of the non-mining ecotype plants increased slightly, but the difference did not reach a significant level ($p > 0.05$), indicating that the low concentration Cu stress slightly stimulated the chlorophyll synthesis of *M. floridulus*. When the concentration of Cu was greater than 100 $mg \cdot kg^{-1}$, the various components of chlorophyll, and the total amount of chlorophyll, began to decline significantly; the difference between the two ecotypes was significant ($p < 0.05$). The content of chlorophyll a, chlorophyll b, carotenoids and total chlorophyll in the 400 mg·kg^{-1} treatment group were 83.5%, 38.5%, 78.1% and 62.2% of the control group, respectively.

The contents of chlorophyll and the total amount of chlorophyll in the leaves of the mining ecotype *M. floridulus* showed a downward trend with increase in Cu concentration, but the degree of decline was not as large as that for the non-mining ecotype *M. floridulus*. Chlorophyll a and chlorophyll b concentrations were statistically different from the control group for the 400 mg·kg^{-1} and 800 mg·kg^{-1} treatment groups ($p < 0.05$). The total amount of chlorophyll was statistically different from the control for the 800 mg·kg^{-1} treatment group ($p < 0.05$), being 84.4% of the total chlorophyll in the control group.

As shown in Figure 1, with increase in Cu treatment concentration, the chlorophyll a/b value in the leaves of the two ecotypes of *M. floridulus* showed a trend of first decreasing and then increasing. The plant growth of the non-mining ecotype was more obvious. However, while the chlorophyll a/b values were higher than the control for most treatment groups, the chlorophyll a/b value for the 50 mg·kg^{-1} treatment group was slightly lower than that of the control group (85.5% of the control group). For the 400 mg·kg^{-1} treatment, the chlorophyll a/b value was 2.04 times that of the control group. The change in the chlorophyll a/b value of ecotype plants in the mining area was gentle, but also showed a trend of first decreasing and then increasing, being 1.25 times that of the control at 800 mg·kg^{-1}. These results suggest that Cu has a more harmful effect on chlorophyll b than chlorophyll a in the leaves of *M. floridulus*.

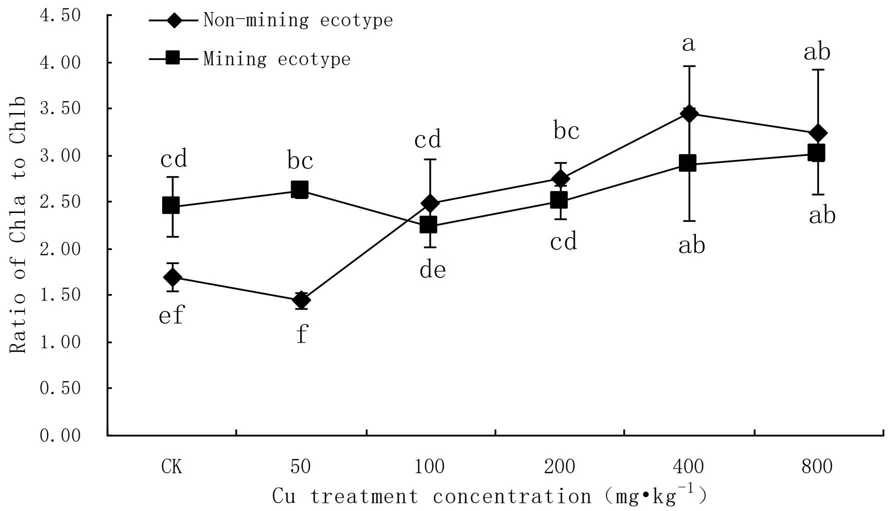

Figure 1. Chlorophyll a/b of two ecotypes of *M. floridulus* under different Cu treatment. Note: Error bars indicate standard deviation; different letters in the same group indicate significant difference at $p < 0.05$ according to Duncan's multiple range tests; the same below.

3.2. The Effect of Cu Stress on the Membrane Protection System of M. floridulus

(1) Effects of Cu stress on MDA content in leaves of *M. floridulus*

Malondialdehyde (MDA) is the product of membrane lipid peroxidation in plants, and its content reflects the degree of damage to plant cells caused by membrane lipid peroxidation generated by reactive oxygen species [35]. As can be seen from Figure 2, with increase in Cu treatment concentration, the MDA content in the leaves of the non-mining ecotype *M. floridulus* increased significantly and was significantly increased compared with the control ($p < 0.05$). The MDA levels were 108.5%, 122.1%, 135.1%, 151.3% and 213.2% of the control for each treatment concentration group, respectively. The amount of MDA in the leaves of the mining ecotype *M. floridulus* gradually increased with Cu treatment concentration, but the increase was not significant. The content of MDA in the treatment concentration groups was 123.1%, 135.2%, 152.3%, 159.5% and 189.5% of the control group, respectively. Under each treatment concentration, the MDA content of the non-mining ecotype plants was significantly higher than that of the mining ecotype plants ($p < 0.05$). The gradual increase in MDA content may have been due to the increased Cu treatment concentration, which gradually accumulated free radical content in the plant that could not be removed in time, resulting in severe oxidation of the cell membrane lipids and cell damage. Through regression analysis, for the non-mining ecotype b(MDA) = 0.0084x + 12.61 (x is the concentration of Cu, $R^2 = 0.95$, $p < 0.05$), and for the mining ecotype b(MDA) = 0.0032x + 9.85 (x is the concentration of Cu, $R^2 = 0.83$, $p < 0.05$). The results indicate that Cu had a substantial influence on the content of MDA in the leaves of the non-mining ecotype *M. floridulus*. MDA levels were significantly higher in non-mining ecotype plants than in mining ecotype plants, indicating that non-mining ecotype plants experienced more severe lipid oxidation in their cell membranes.

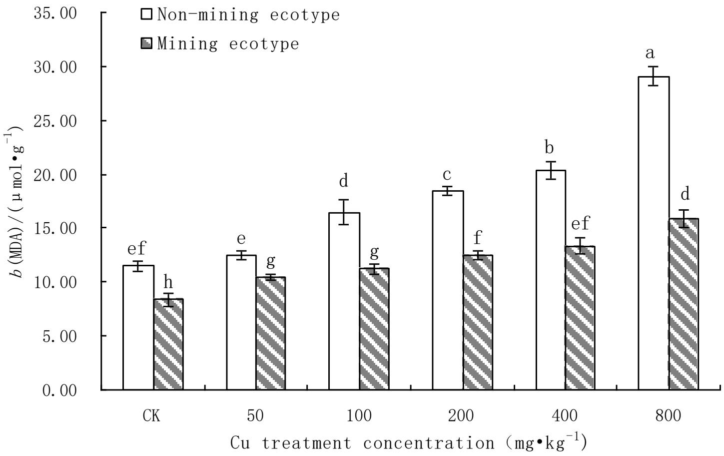

Figure 2. MDA concentrations of two ecotypes of *M. floridulus* under different Cu treatment. Note: Error bars indicate standard deviation; Different letters in the same group indicate significant difference at $p < 0.05$ according to Duncan's multiple range tests; the same below.

(2) Effect of Cu Stress on Cell Membrane Permeability in Leaves of *M. floridulus*

The permeability of the cell membrane is one of the indicators to evaluate the response of plants to pollution; an increase in cell membrane permeability is mainly manifested in an increase in relative electrical conductivity [36]. As can be seen from Figure 3, in the case of CK, there was no significant difference in the membrane permeability of the two ecotypes ($p > 0.05$) and the relative electrical conductivity of the leaf membrane showed an increasing trend with increase in Cu treatment concentration. Under a Cu treatment concentration of 400–800 mg·kg^{-1}, the relative electrical conductivity of plant leaves of the non-mining ecotype was 131.5%, 135.1%, 151.7% and 156.8% of the control, and that of the mining ecotype was 118.9%, 124.4% and 133.1% of the control, respectively. There were significant differences between the two ecotypes and the control ($p < 0.05$). The cell membrane permeability of the non-mining ecotype plants was higher than that of the mining ecotype plants; the difference was significant at the 400–800 mg·kg^{-1} treatment concentrations ($p < 0.05$).

3.3. Effect of Cu Stress on AsA Content of M. floridulus

As can be seen in Figure 4, the AsA content of the non-mining ecotype *M. floridulus* gradually increased with Cu concentration, with a similar pattern of change to that of the mining ecotype *M. floridulus*. However, in general, the ascorbic acid content of the mining ecotype *M. floridulus* was significantly higher than that in the non-mining ecotype ($p < 0.05$). At higher Cu concentrations (400 mg·kg^{-1} and 800 mg·kg^{-1}), the AsA content of both ecotypes of *M. floridulus* was significantly different from that of the corresponding control group (p <0.05). The AsA content of the mining ecotype *M. floridulus* was 2.74 times

(400 mg·kg^{-1} Cu treatment) and 1.90 times (800 mg·kg^{-1} Cu treatment) that of the non-mining ecotype *M. floridulus*, respectively.

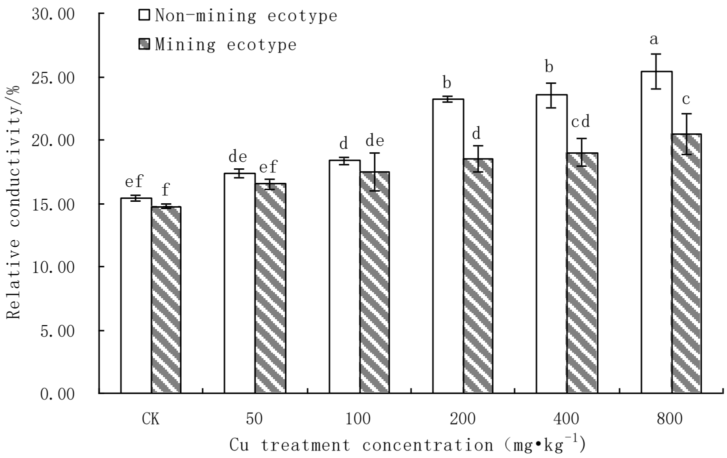

Figure 3. Change in relative conductivity of two ecotypes of *M. floridulus* under different Cu treatment. Note: Error bars indicate standard deviation; Different letters in the same group indicate significant difference at $p < 0.05$ according to Duncan's multiple range tests; the same below.

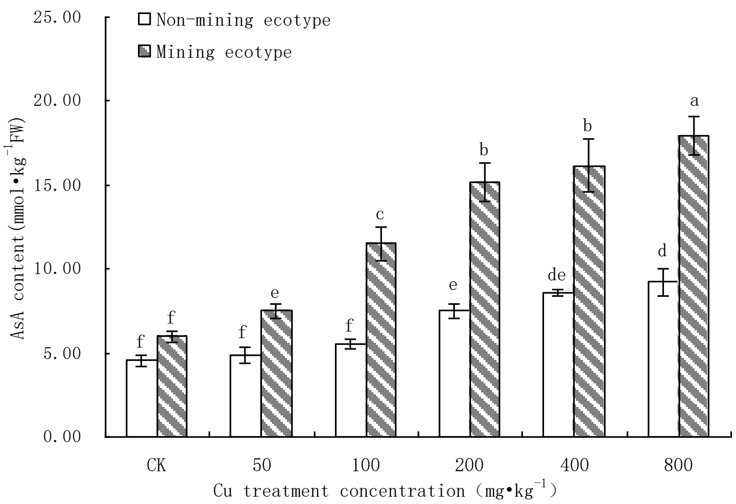

Figure 4. AsA concentrations of two ecotypes of *M. floridulus* under different Cu treatment. Note: Error bars indicate standard deviation; Different letters in the same group indicate significant difference at $p < 0.05$ according to Duncan's multiple range tests; the same below.

3.4. Effect of Cu Stress on the Content of GSH in M. floridulus

As can be seen from Figure 5, when the Cu concentration was 50 mg·kg^{-1}, the GSH content of the two ecotypes of *M. floridulus* increased compared with that of the control group. However, with continuous increase in Cu concentration, the content changes were different: the GSH content of the non-mining ecotype *M. floridulus* decreased rapidly, and the GSH content of the non-mining ecotype *M. floridulus* was significantly different from that of the control group when the Cu concentration was 400 mg·kg^{-1} and 800 mg·kg^{-1} ($p < 0.01$). The GSH content of the ecotype *M. floridulus* also decreased but did not reach a highly significant level compared with the control group ($p > 0.01$). In general, under the higher Cu treatment concentration, the GSH content of ecotype *M. floridulus* in the mining area was significantly higher than that in the non-mining area: The GSH content of the mining ecotype *M. floridulus* was 2.54 times (400 mg·kg^{-1} Cu treatment) and 3.81 times (800 mg·kg^{-1} Cu treatment) that of the non-mining ecotype *M. floridulus*.

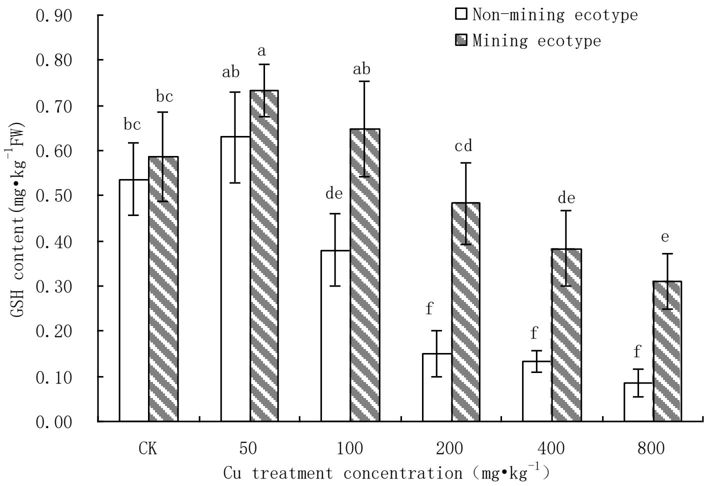

Figure 5. GSH concentrations of two ecotypes of *M. floridulus* under different Cu treatment. Note: Error bars indicate standard deviation; Different letters in the same group indicate significant difference at $p < 0.05$ according to Duncan's multiple range tests; the same below.

3.5. Effect of Cu Stress on SOD Activity of *M. floridulus*

Many studies have shown that the SOD activity of plant leaves is enhanced under heavy metal stress [37]. Figure 6 shows that Cu had a different effect on the SOD activity in the leaves of the two ecotypes of *M. floridulus*. The SOD activity in the leaves of the non-mining ecotype plants exposed to Cu stress first increased and then decreased and reached the highest level at 100 mg·kg^{-1}, which was 143.5% that of the control. The levels for concentrations of 400 mg·kg^{-1} and 800 mg·kg^{-1} were significantly lower than those of the control group ($p < 0.05$), being 85.2% and 61.5% of the control group, respectively.

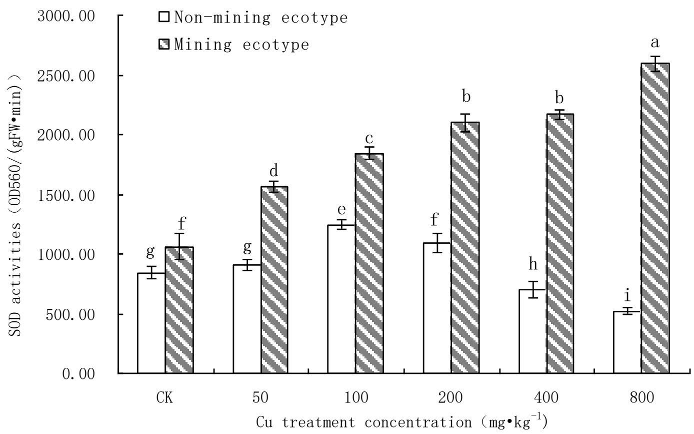

Figure 6. Activity of SOD of two ecotypes of *M. floridulus* under different Cu treatment. Note: Error bars indicate standard deviation; Different letters in the same group indicate significant difference at $p < 0.05$ according to Duncan's multiple range tests; the same below.

The SOD activity in the plant leaves of the mining ecotype was much higher than that of the non-mining ecotype. The SOD activity increased significantly after Cu stress ($p < 0.05$). The SOD activity in the treatment groups was 141.1%, 171.5%, 195.6%, 205.1% and 245.5% that of the control group, respectively.

3.6. Effect of Cu Stress on POD Activity of *M. floridulus*

Figure 7 shows that Cu has a different effect on the POD activity in the leaves of the two types of *M. floridulus*. The POD activity of non-mining ecotype plants exposed to Cu stress first increased and then decreased and reached its highest level at 200 mg·kg^{-1}, which was 185.2% of that of control. Following this, it decreased significantly, and for the 400 mg·kg^{-1} and 800 mg·kg^{-1} treatment groups was lower than that of the control group (93.5% and 75.5%, respectively). The POD activity in plant leaves of the mining ecotype was much higher than in those of the non-mining ecotype. The POD activity increased significantly after Cu stress ($p < 0.05$). The POD activity in the treatment groups was 155.9%, 245.1%, 315.6%, 351.8% and 430.5% that of the control group, respectively.

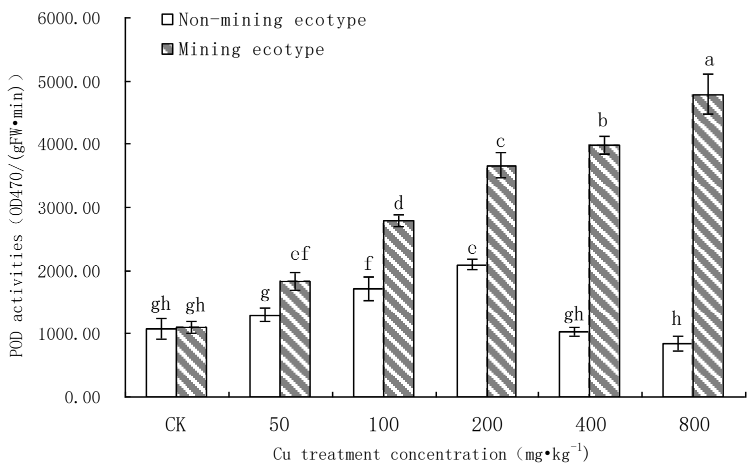

Figure 7. Activity of POD of two ecotypes of *M. floridulus* under different Cu treatment. Note: Error bars indicate standard deviation; Different letters in the same group indicate significant difference at $p < 0.05$ according to Duncan's multiple range tests; the same below.

3.7. Effect of Pb Stress on PPO Activity of M. floridulus

Figure 8 shows that Cu had a different effect on the PPO activity in the leaves of the two ecotypes of *M. floridulus*. The PPO activity in the leaves of the non-mining ecotype plants exposed to Cu stress first increased and then decreased, reaching its highest level at 100 mg·kg^{-1}, which was 138.7% of that of the control. Following this, the PPO activity of the 400 and 800 mg·kg^{-1} treatment groups was significantly lower than that of the control group ($p < 0.05$) at 87.1% and 65.2% of the control, respectively. The PPO activity in the plant leaves of the mining ecotype was higher than that in the leaves of the non-mining ecotype. The PPO activity significantly increased after Cu stress ($p < 0.05$). The PPO activity of each treatment concentration group was 151.1%, 190.8%, 207.5%, 226.5% and 268.1% of the control group, respectively.

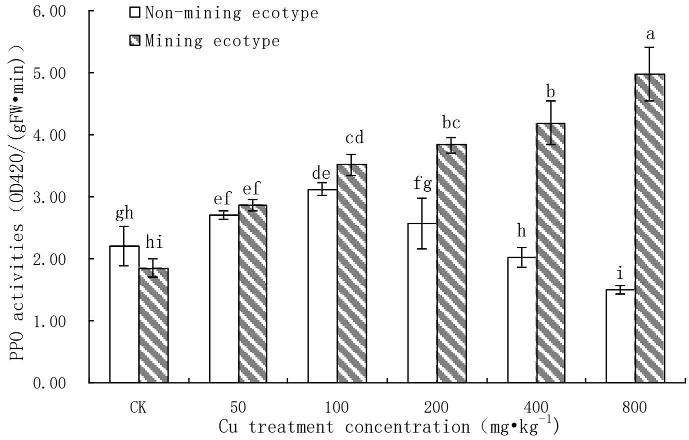

Figure 8. Activity of PPO of two ecotypes of *M. floridulus* under different Cu treatment. Note: Error bars indicate standard deviation; Different letters in the same group indicate significant difference at $p < 0.05$ according to Duncan's multiple range tests; the same below.

3.8. The Correlation between the Physiological Indexes and the Mass Fraction of Cu

As can be seen from Table 3, among the physiological indexes of the non-mining ecotype *M. floridulus*: MDA, RPP and AsA were significantly positively correlated with Cu treatment concentration ($p < 0.05$); GSH, SOD and PPO were negatively correlated with Cu concentration ($p < 0.05$); and there was no significant correlation between POD and Cu concentration. Among the other indexes, SOD, POD and PPO were significantly positively correlated ($p < 0.01$); MDA was negatively correlated with GSH, SOD and PPO ($p < 0.05$); and RPP was negatively correlated with GSH and PPO ($p < 0.05$).

Table 3. Pearson product-moment correlation matrix for MDA, Cu concentrations and *SOD, POD, PPO* of *Miscanthus floridulus*.

Type	Item	Cu	Chl	MDA	RPP	AsA	GSH	SOD	POD	PPO
Non-mining ecotype	Cu	1								
	Chl	−0.671 **	1							
	MDA	0.972 **	−0.717 **	1						
	RPP	0.855 **	−0.757 **	0.902 **	1					
	AsA	0.884 **	−0.810 **	0.891 **	0.936 **	1				
	GSH	−0.763 **	0.842 **	−0.818 **	−0.903 **	−0.883 **	1			
	SOD	−0.713 **	0.310	−0.560 *	−0.437	−0.530 *	0.316	1		
	POD	−0.465	0.086	−0.296	−0.080	−0.203	−0.008	0.852 **	1	
	PPO	−0.747 **	0.354	−0.635 **	−0.548*	−0.649 **	0.478 *	0.853 **	0.650 **	1
Mining ecotype	Cu	1								
	Chl	−0.667 **	1							
	MDA	0.920 **	−0.698 **	1						
	RPP	0.797 **	−0.466	0.909 **	1					
	AsA	0.831 **	−0.622 **	0.932 **	0.852 **	1				
	GSH	−0.794 **	0.424	−0.718 **	−0.738 **	−0.762 **	1			
	SOD	0.868 **	−0.543 *	0.955 **	0.883 **	0.939 **	−0.686 **	1		
	POD	0.875 **	−0.583 *	0.952 **	0.873 **	0.963 **	−0.740 **	0.970 **	1	
	PPO	0.861 **	−0.501 *	0.922 **	0.855 **	0.909 **	−0.702 **	0.976 **	0.959 **	1

Note: Cu: Cu treatment concentration; Chl: total chlorophyll; RPP: cell membrane permeability; MDA: malondialdehyde; AsA: ascorbic acid; GSH: reduced glutathione; POD: peroxidase enzyme; SOD: superoxide dismutase; PPO: polyphenol oxidase; * and ** indicate significance under $p < 0.05$ and $p < 0.01$, respectively.

As can be seen from Table 3, among the physiological indexes of the mining ecotype *M. floridulus*: MDA, RPP, AsA, SOD, POD and PPO were significantly positively correlated with Cu treatment concentration ($p < 0.05$). Among the other indexes, SOD, POD and PPO were significantly positively correlated ($p < 0.01$); MDA was positively correlated with SOD, POD and PPO ($p < 0.05$); and RPP was significantly positively correlated with SOD, POD and PPO ($p < 0.05$).

4. Discussion

4.1. Analysis of the Effect of Cu Stress on Chlorophyll Content of M. floridulus

Cu is a trace mineral element necessary for the normal life activities of plants, and chlorophyll a and chlorophyll b are composed of a metal pigment protein [38]. Therefore, when the concentration of Cu is low, it may participate in and promote the synthesis of plant pigment protein, which is conducive to the various life activities of plants and will not cause obvious damage to them. When the content of Cu is higher than that required by plant life, a significant quantity of oxygen free radicals cannot be quickly removed, which destroys the structure and functioning of mitochondria and chloroplasts [39]. It also causes the oxidation of pigment proteins, inhibits the synthesis of chlorophyll, especially causing obvious damage to chlorophyll b, hindering the photosynthesis of plants and affecting their normal growth and development [40]. Many studies have confirmed that excessive copper can cause a decrease in chlorophyll content in plants [41,42]. Prasad et al. [41] suggested that a reduction in chlorophyll content caused by copper was mainly a result of the decomposition of chlorophyll caused by copper. Some studies have also indicated that copper can replace magnesium in the reaction center of chlorophyll, thus destroying the functioning and structure of chlorophyll [42].

The present study showed that the total amount of chlorophyll a, chlorophyll b, carotenoid, and chlorophyll in the leaves of the two types of *M. floridulus* first increased and then decreased as the Cu stress concentration increased. This may be because low Cu concentration affected the stimulation of chlorophyll synthesis, which would have a greater toxic effect as the Cu concentration increased. The total chlorophyll, chlorophyll a and chlorophyll b of the two ecotypes were negatively correlated with the Cu stress concentration ($p < 0.05$), suggesting that chlorophyll can be used as an indicator to show the degree of Cu stress.

Based on comparison between the two ecotypes, the chlorophyll content in the leaves of the non-mining ecotype decreased more rapidly; Cu stress caused more significant damage to chlorophyll b in the non-mining ecotype. In addition, in terms of appearance, the leaves of the non-mining species became more greenish-yellow than those of the mining species as the Cu concentration increased, presumably because the *M. floridulus* of the mining species has a resistance mechanism that reduces the damage caused by Cu to chlorophyll. This chlorophyll protection mechanism may enable the normal photosynthesis and substance synthesis of plants in polluted conditions.

4.2. Effects of Cd Stress on Physiological Metabolism of M. floridulus

Cell membranes are selective permeable membranes that control and regulate the transport and exchange of substances inside and outside the cell. Their permeability is one of the indicators used to assess the response of plants to pollution. Under adverse conditions, an increase in the permeability of the plant cell plasma membrane is mainly manifested as an obvious increase in relative conductivity [43]. Malondialdehyde (MDA) is a product of membrane lipid peroxidation. The intracellular MDA level indirectly indicates the level of reactive oxygen species and the degree of cellular damage in the plant. When plants are subjected to heavy metal stress, the balance between the production and removal of free radicals in the cell is impaired, leading to an increase in MDA content and the production of a large number of reactive oxygen species [44,45].

The results of this study showed that the membrane permeability of the leaves of the two ecotypes increased with Cu concentration and that the membrane permeability of the two ecotypes had a significant positive correlation with Cu stress concentration. With treatment at 800 $mg \cdot kg^{-1}$, the number of non-mining ecotype plants was 1.6 times that of the control, while the number of mining ecotype plants was 1.3 times that of the control. This may have been due to the protective mechanism of the mining ecotype *M. floridulus*, which reduced damage to the cell membrane of the plant under Cu stress. The MDA content of the leaves of both ecotypes of *M. floridulus* increased after Cu treatment compared to the control. Under 800 $mg \cdot kg^{-1}$ treatment, the non-mining ecotype plants were 2.2 times as many as the control, while the mining ecotype plants were 1.8 times as many as the control, indicating that the two ecotypes of M. floridulus had different responses to Cu stress. The mining ecotype plants showed significantly stronger resistance and less damage under Cu stress, which was similar to the experimental results of a study by Xie Mingji et al. [46] involving comparison of the MDA content of mining type and non-mining type *Elsholtzia chinensis* under copper stress.

AsA is a very important antioxidant in plants. Mediated by AsA, H_2O_2 accepts NADPH electrons and is reduced to H_2O, thus eliminating H_2O_2 toxicity. AsA can also react directly with superoxide free radicals and hydroxyl free radicals to remove these toxic molecules [47,48]. Studies have shown that AsA can inhibit membrane lipid peroxidation [49]. In this experiment, the content of AsA in the mining area was significantly higher than that in the non-mining area and the content of AsA increased significantly with increase in the treatment concentration; there was an obvious positive correlation between the content of AsA and treatment concentration. This result suggests that AsA can reduce the peroxidation damage caused by copper, which is consistent with the results of other studies [47]. GSH is a specific peptide ubiquitous in plants and is an important free radical scavenger that stabilizes the SH group in proteins and plays an important role in maintaining the structural integrity of membranes and preventing membrane lipid peroxidation. Under higher concentration copper treatment, the GSH content of the mining ecotype *M. floridulus* was significantly higher than that of the non-mining ecotype, indicating that GSH plays a positive role in alleviating the peroxidation damage caused by copper [47,50].

4.3. Effects of Cu Stress on the Antioxidant Enzyme System of M. floridulus

Studies have shown that excessive heavy metals produce reactive oxygen species in plants and break the dynamic balance of their scavenging mechanism, resulting in peroxide damage [51]. There are two types of protection mechanisms in plants against reactive oxygen species, namely, enzyme-induced and non-enzyme-induced mechanisms. The enzyme system includes SOD, POD, etc. Non-enzymatic systems mainly consist of minor molecules, such as AsA and reduced GSH [52,53].

Normally, the metabolism of ROS in plants is kept in equilibrium and cells are protected from damage. However, under stress, the production rate of ROS in the plant exceeds the plant's ability to remove ROS and excessive accumulation of ROS leads to peroxidation damage. In order to keep the ROS in plants at a certain equilibrium level, the antioxidant protection enzyme system can be quickly activated in plants under various stresses to reduce the damage of ROS [54]. The SOD can break down O_2^- into H_2O_2 and O_2, reducing the accumulation of O_2^- in the body. POD is an enzyme containing Fe, which can decompose H_2O_2, the product of SOD, into H_2O [55,56]. PPO is a stress-response enzyme system widely present in plants and its activity is closely related to the plant's metabolic strength and environmental stress response [56]. In plant respiration, the end of the respiratory chain oxidase, of which PPO is one, directly transfers electrons released during the oxidation of respiratory substrate intermediate products to O_2. PPO can catalyze the oxidation of phenolic compounds to quinones and decrease in activity of this enzyme affects the respiration of plants [56,57].

As can be seen from the results of this study, the response of antioxidant enzymes to Cu stress is very different for the two ecotypes. The SOD and POD activities of the non-mining ecotypes exposed to Cu first increased and then decreased in each treatment group, while the SOD and POD activities of the mining ecotypes exposed to Cu were significantly increased ($p < 0.05$). In addition, the SOD and POD activities in the leaves of the mining ecotype plants were much higher than those of non-mining ecotype plants and remained at a high level under high concentration Cu (800 $mg \cdot kg^{-1}$) stress. This indicates that the mining ecotype *M. floridulus* is more resistant to Cu stress than the non-mining ecotype.

PPO is barely mentioned in studies of heavy metal stress on plants. In this study, it was found that the response of PPO activity to Cu stress in the leaves of the two ecotypes was very different. The PPO activity in the non-mining ecotype plants first increased and then decreased with increase in Cu treatment concentration, while that in the mining ecotype plants increased significantly ($p < 0.05$). Correlation analysis showed that the activity of PPO in the leaves of non-mining ecotype *M. floridulus* was negatively correlated with the concentration of Cu ($p < 0.05$) and the activity of PPO in the leaves of mining ecotype *M. floridulus* was significantly positively correlated with Cu concentration ($p < 0.05$). The relationship between PPO activity and Cu concentration in the leaves of the two ecotypes of *M. floridulus* was very different and the internal relationships and specific mechanisms underpinning this need further study.

Stress also promotes the production of ROS in plants. Under normal circumstances, the production and removal of ROS in plant cells are in a balanced state, while increase in ROS in plants under stress can, on the one hand, induce an increase in the activities of related protective enzymes, such as SOD, POD and CAT, and, on the other hand, directly destroy biological macromolecules, resulting in the loss of enzyme activity [58]. In general, the antioxidant enzyme activity of mining ecotype plants was higher than that of non-mining ecotype plants. The most significant difference was that the antioxidant enzyme activity of the non-mining ecotype plants was not high under the stress of a high concentration of Cu at 800 $mg \cdot kg^{-1}$, while the enzyme activity of the mining ecotype plants was maintained at a high level under the stress of this concentration of Cu. This behavior of mining ecotype *M. floridulus* implies that the plants possess a tolerance mechanism enabling adaptation to heavy metals [59,60]. This may be because mining ecotype plants have previously been damaged to a certain extent by long-term adverse conditions and that the two ecotypes have become physiologically and ecologically different in different habitats. The results show that the mining ecotype *M. floridulus* is better able to protect itself under the stress of Cu.

5. Conclusions

(1) Chlorophyll a, chlorophyll b and total chlorophyll in the leaves of two ecotypes of *M. floridulus* were negatively correlated with Cu stress concentration ($p < 0.01$), but the extent of decrease for the ecotypes in the mining area was lower than that for ecotypes in the non-mining area. The values of chlorophyll a/b for both ecotypes increased with increasing Cu treatment concentration, indicating that Cu is more harmful to chlorophyll b than to chlorophyll a for *M. floridulus*.
(2) Under Cu stress, the content of antioxidant substances (GSH, AsA) in the mining ecotype was significantly higher than that in the non-mining ecotype. The membrane permeability increased for both ecotypes at high concentrations of copper treatment, and the MDA content of the non-mining ecotype was significantly higher than that of the mining ecotype. The experimental data obtained showed that, under copper stress, the non-mining ecotype *M. floridulus* suffered more severe peroxidation damage than the mining ecotype. The endogenous GSH and AsA of *M. floridulus* play an important role in scavenging free radical accumulation caused by excess copper.
(3) The SOD activity in the leaves of the non-mining ecotype was inhibited by Cu stress, and the POD activity was increased by Cu stress, but the increase for the mining ecotype was larger than that for the non-mining ecotype. At the highest Cu concentrations, both enzyme activities were significantly higher in the mining ecotype plants than in the non-mining ecotype plants. The results suggest that, in the long-term adaptation process, the mining ecotype *M. floridulus* becomes a resistant ecotype, and that the non-enzymatic system plays an important role in raising the level of resistance.

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