Investigating *Phragmites Australis* Response to Copper Exposure Using Physiologic FTIR and Metabolomic Approaches

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Abstract

Background and aim *Phragmites australis* is a landscape plant with phytoremediation functions that is widely planted worldwide. However, little is known about the metabolomic background of the resistance mechanisms of *Phragmites* to heavy metals during its growth and development.

Methods Here, we performed copper stress studies on *Phragmites* and monitored physiological indicators such as malondialdehyde (MDA) and electrolyte leakage (EL). In addition, FTIR was used to study chemical composition changes in the roots, stems and leaves of *Phragmites* seedlings under excessive copper stress. Furthermore, LC-MS technology combined with metabolomics data processing software was used to analyze the metabolic profile of samples.

Result Copper contributed to the accumulation of MDA and EL. And the results of FTIR showed that the antioxidant effects of flavonoids and amino acids can be used by *Phragmites* leaf tissue to improve the tolerance of copper under 5 mg/L concentration. Further, the results of metabolomics reflected that *Phragmites* can improve its resistance to copper by increasing the accumulation of arginine and ayarin in the body. The former is accumulated through two pathways: the citrulline decomposition and conversion pathway and the circular pathway composed of ornithine, citrulline, L-Argininosuccinate and arginine. The latter is synthesized through the quercetin methylation pathway.

Conclusion This study provides insights into the resistance mechanism and repair performance of *Phragmites* and other plant accumulators in response to copper stress.

Introduction

Although copper is an essential trace element in plants, excessive copper is toxic to plants (Ugulu et al. 2012, Sahin 2016). Recently, copper pollution has attracted increasing attention due copper excessive emissions which are caused by different human activities such as mining, metal processing, fertilizer industry, fungicides, industrial and domestic wastewater and traffic discharges (Dogan,Baslar and Ugulu 2014, Unver et al. 2015, Ugulu,Unver and Dogan 2016). Excessive copper affects various physiological processes of plants, leading to abnormal growth and development (Ugulu 2015, Khan et al. 2019), and causes oxidative damage to cells by stimulating oxidative stress (Pisoschi and Pop 2015, Kapoor et al. 2019). Copper stress can stimulate a variety of defense mechanisms in plants such as antioxidant systems and antioxidants to control copper homeostasis (Yadav et al. 2018, Liu et al. 2019, Nazir,Hussain and Fariduddin 2019). Some metabolites (such as amino acids and flavonoids) have antioxidant effects in plants (Kovacik et al. 2010, Agati et al. 2020). Studying the changes of these metabolites and related metabolic pathways will help to reveal the intrinsic resistance mechanism of plants under heavy metal stress.

Generally, plant tolerance is a complex process that involves physiological, biochemical, and molecular changes, all of which being metabolic adjustments needed to adapt to environmental stresses to maintain plant growth (Lin and Aarts 2012, Farooq et al. 2016). By analyzing changes in metabolites, we

can assess the organism's reaction to environmental stresses (Hong et al. 2016). Studies show that metabolites themselves can act as modulators, which can enhance the signal transduction necessary for plants to quickly trigger tolerance mechanisms under stressful conditions (Hou, Ufer and Bartels 2016). In addition, metabolites can also play a direct role as final products to alleviate the harmful effects of various environmental pressures (Villiers et al. 2011). For example, metabolites can play an antioxidant role to respond to stress conditions (Arbona et al. 2013). Plants can also generate primary and secondary metabolites through a variety of complex metabolic pathways (Dixon and Strack 2003). Among them, primary metabolites such as sugars, amino acids, and lipids can provide necessary energy and molecules that are essential for growth and development. Therefore, the metabolic status of plants during growth can be known by monitoring the change of primary metabolites (Zhao et al. 2018). In addition, plants can produce a large number of secondary metabolites, such as flavonoids, terpenoids and glucosinolates, which can provide resistance to biotic and abiotic stressors (Keurentjes 2009). For instance, rice can produce many kinds of metabolites such as phenols, flavonols, carotenoids, and alkaloids under abiotic stresses (Kusano et al. 2015). Meanwhile, high Ni concentration has been shown to cause the roots of *Matricaria chamomilla* to produce oxidative stress responses, which stimulates the gradual accumulation of soluble phenols and flavonoids to resist oxidative damage (Kovacik, Klejdus and Backor 2009).

As one of the most common and widely distributed underground stem plants in the world, *Phragmites* has a strong viability and can survive and reproduce in the form of expanded rhizomes with buds (Wu et al. 2019). *Phragmites* has a strong environmental tolerance to resist heavy metals and other pollutants and has been widely used in the pollution remediation of wetlands (Wu et al. 2013, Ye et al. 2003). In addition, it is believed that *Phragmites* has strong heavy metal removal capabilities due to features such as rapid growth to form dense vegetation, higher above-ground biomass, typical tissue systems and defense mechanisms in the body, and higher bioaccumulation and heavy metal removal efficiency than other repair plants (Carricondo et al. 2020, Samaras, Sicilia and Garcia-Barriocanal 2021, Huang et al. 2018, Bonanno 2013).

In this study, we preliminarily assess the degree of plant damage in *Phragmites* under copper stress during growth and development by measuring the growth index, malondialdehyde (MDA), electrolyte leakage (EL), and copper concentration. Then, we further characterize the functional groups in *Phragmites* that have changed under copper stress by analyzing infrared data. Finally, we explore the mechanism of the molecular biological changes of internal metabolites of *Phragmites* leaves under copper stress using the results of LC-MS non-targeted metabolome analysis. The main goal of this study is to analyze the response mechanism of *Phragmites* to copper stress through metabolomics and further understand the tolerance and resistance mechanism of *Phragmites*.

Materials And Methods

Experimental Design and Growth Parameters

Paustralis seedlings used in the experiment were first hydrocultured for 20 days in pots containing 2 L of Hoagland nutrient solution, before being subjected to copper stress. $0.02 \text{ mg} \cdot \text{l}^{-1}$ nutrient solution acted as the control group and the copper concentration gradient was set to 5, 10, and $20 \text{ mg} \cdot \text{l}^{-1}$. Each treatment was repeated three times, and all solutions replaced every 2 days. The experiment lasted for 21 days. Three seedlings washed with deionized water were randomly selected at each treatment concentration to measure the shoot height, root length, shoot dry weight, and root dry weight.

Determination of Cu Concentrations

The method of analyzing the copper content in *Phragmites* seedlings is shown as follows. Firstly, the seedlings were washed then dried via baking, and then the dried samples were homogenized. Next, they were transferred to a Microwave Digestion System (MARS-5; CEM, Matthews, North Carolina) for mineralization with HNO_3 (67%)- HCl (30%)- HF (49%) acids (5 : 2 : 2, V/V/V). Lastly, analysis of the mineralized samples was conducted using Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) (Perkin Elmer Optima 5300DV, Waltham, Massachusetts).

Physiological Measurements of *Phragmites* Seedlings under Copper Stress

According to Heath et al. (Heath and Packer 1968), lipid peroxidation was assayed by determining the content of MDA in the functional leaf. A *Phragmites* leaf sample (0.1g) was homogenized with 5 mL of 10% trichloroacetic acid (TCA) solution and a small amount of quartz sand in the mortar. The homogenate was poured into a glass tube and centrifuged at 4000r/min for 15 min. 2ml of 0.6% Thiobarbituric acid (TBA) replaced 2ml of the supernatant in the centrifuge tube, and the new solution was mixed well and kept in a boiling water bath for 15. The reaction was stopped by cooling and the mixture was centrifuged again. The supernatant was used to measure the absorbance (OD) of the sample at 450nm, 532nm and 600nm wavelengths in a cuvette.

The EL induced by copper was estimated by measuring the electric conductivity (Pang et al. 2003). Plant samples of *Phragmites* leaves (0.1g) were placed into syringe containing 10 mL of deionized water. Then, the air between the leaves was exhausted by pumping repeatedly for several minutes. Then pour the samples and water into a beaker and wait for about 10 minutes. The electric conductivity was measured at room temperature (EC1). Thereafter, the beakers containing the fronds were placed in a boiling water (100°C) bath for 20 min and then cooled to room temperature, and the electric conductivity was measured again (EC2). The relative electrolyte leakage was calculated using the following formula: $\text{EL} = (\text{EC1} / \text{EC2}) \times 100$.

FTIR Spectroscopic Analysis

The root, stem, and leaf of *Phragmites* stressed by copper were separated into different beakers, numbered, and frozen. 4g of frozen plant samples was weighed accurately and grinded into powder. Next, the powdered plant sample was transferred to a sealed centrifuge tube, treated in an ice water bath for 20 minutes, and centrifuged for 10 minutes in a 4000 rpm high-speed centrifuge. The precipitate was

washed once each with iced acetone, iced methanol chloroform, and methanol, and centrifuged again at 4000 rpm for 10 min. After the second centrifugation, the precipitate was frozen, vacuum dried, weighed, and numbered. To determine the spectral information, 2 mg of the dried sample prepared above was thoroughly mixed with KBr. The absorbance of each component of *Phragmites* was then recorded with a FTIR (as per operator procedure).

Metabolomic Analysis

The extraction of metabolites was carried out as follows: Frozen leaf tissues (100 mg) from each sample group were grinded in 1 mL solution of methanol/water (1:1) at a frequency of 60Hz for 2 minutes. The samples were sonicated for 30 min and let stood at -20°C for 20 min. Next, the samples were centrifuged for 10 min (13000 rpm, 4°C) and 200 µL of the supernatant was drawn with a syringe. Finally, the samples were transferred to the LC injection vial and stored at -80°C until LC-MS analysis. Quality control samples (QC) were prepared by mixing extracts of all samples. All extraction reagents in this experiment were pre-cooled at -20°C before use.

The metabolite data in this experiment were acquired using a liquid-mass spectrometry system composed of Thermo Scientific UltiMate 3000 HPLC in tandem with Thermo Scientific Q-Exactive high-resolution mass spectrometer. Analytes were separated using an ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 µm). The mobile phases were (A) water with 0.1 % acetic acid and (B) acetonitrile with 0.1 % acetic acid. Gradient elution was performed at a flow rate of 0.35 mL/min under the following program: 0-0.5min, 5% B; 0.5-7 min, 5–100% B; 7–8 min, 100% B; 8-8.1 min, 100–5% B; 8.1–10 min, 5%B. The column temperature was kept at 50°C and the injection volume was 10 µL.

Statistical Analysis

All values in this study were the average of three replicate samples. The MDA concentration was calculated according to the following formula:

$$\text{MDA concentration } (\mu\text{mol} \cdot \text{L}^{-1}) = 6.45 \times (D_{532} - D_{600}) - 0.56 \times D_{450}.$$

where D_{532} , D_{600} and D_{450} represent the optical density values at the wavelengths of 532, 600, and 450 nm, respectively.

SPSS (Ver.25.0, US) software was used to perform statistical analyses. The data was analyzed using one-way ANOVAs and least significant difference (LSD) test was used to determine the significant differences between treatments ($P < 0.05$).

Results

Distribution of Copper and Effect on *Paustalis* Growth

In this study, the results showed that the copper content in the roots, stems, and leaves of *Paustralis* was constantly increasing as the copper concentration increased (Fig. 1). When the copper concentration changed from 5 mg·l⁻¹ to 10mg·l⁻¹, the copper content in the roots, stems, and leaves of *Paustralis* increased by 44.53%, 113.67%, and 105.32% respectively. Moreover, copper content in roots, stems and leaves increased by 16.57%, 18.7%, and 28.6% respectively when the copper concentration increased from 10 mg·l⁻¹ to 20 mg·l⁻¹. It can be seen that the copper content significantly increased in all parts of *Paustralis* as the copper concentration changed from 5 mg·l⁻¹ to 10 mg·l⁻¹, however the accumulation rate slowed down as the copper concentration changed from 10 mg·l⁻¹ to 20 mg·l⁻¹.

As shown in Table 1, in response to copper stress, the shoot height and root length of *Paustralis* had a tendency to decrease gradually from 5mg·l⁻¹ to 20 mg·l⁻¹ compared to the control group. Additionally, Table 1 shows that shoot dry weight and root dry weight decreases with increasing copper concentration.

Table 1
Effect of copper stress on shoot height, root length, shoot dry weight and root dry weight of *Paustralis* seedlings.

	Control	5 mg·l ⁻¹	10 mg·l ⁻¹	20 mg·l ⁻¹
Shoot height (cm)	36.0 ± 1.80 ^a	26.0 ± 1.45 ^b	23.2 ± 1.23 ^c	19.8 ± 0.33 ^d
Root length(cm)	13.7 ± 0.75 ^a	12.6 ± 0.72 ^a	12.2 ± 0.68 ^{ab}	11.0 ± 0.81 ^c
Shoot dry weight (mg)	110.8 ± 6.30 ^a	84.3 ± 5.16 ^b	66.0 ± 3.12 ^c	52.4 ± 4.20 ^d
Root dry weight (mg)	39.5 ± 6.20 ^a	28.0 ± 2.41 ^b	25.1 ± 3.94 ^{bc}	18.2 ± 3.20 ^c

Different lowercase letters within a column indicate a significant difference between the treatments (p < 0.05). Data shown are the average of three replications (n = 3).

Effect of Copper on EL and MDA in *Paustralis* Leaves

In this study, the MDA content of *Paustralis* leaves increased with an increase in copper concentration (Fig. 2a). As shown in Fig. 2b, a continuous increase in copper concentration led to the increase of EL. The results above reflect that excess copper can exacerbate the damage of cell tissues.

FTIR Analysis of *Paustralis* Seedlings

As shown in Fig. 3a, the absorption peaks of *Paustralis* leaves mainly changed in two places under copper stress. The hydroxyl (O-H) absorption peak of the control group was 3429 cm⁻¹, and the absorption peak moved to 3417 cm⁻¹ under a copper stress of 5 mg/L. Moreover, the absorption peaks of the control group was 1644 cm⁻¹, and the absorption peak moved to 1655cm⁻¹ under a copper stress of 5 mg/L.

In addition, Fig. 3b shows that the main FTIR changes in *Paustralis* stems were the O-H absorption peak at 3414 cm^{-1} - 3401 cm^{-1} and the N-H amide absorption peak at 1639 cm^{-1} - 1633 cm^{-1} . Figure 3c also shows that the O-H absorption peak of *Paustralis* roots was at 3388 cm^{-1} - 3403 cm^{-1} , and the C-C or C-O group absorption peak was at 1124 cm^{-1} - 1140 cm^{-1} .

Metabolomic Analysis of *Paustralis* under Copper Stress

In the metabolomic results, univariate analysis of fold-change and T statistical were used to test p-value to screen the differentially expressed metabolites. The screening criteria are ratio ≥ 2 or ratio $\leq 1/2$, p value < 0.05 and the fold change is the ratio of average content of metabolites in the two groups. The results reflected that there were a total of 228 differential metabolites in the positive ion sample group, which can be divided into 64 up-regulated differential metabolites and 164 down-regulated differential metabolites. Meanwhile, a total of 217 differential metabolites were in the negative ion sample group, of which 80 were up-regulated differential metabolites and 137 were down-regulated differential metabolites. In general, pathway analysis is based on the metabolic pathways of the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. We mapped the differential metabolites to the KEGG database to obtain the enrichment results of their metabolic pathways. By estimating pathway enrichment analysis of differential metabolites, we obtained 73 metabolic pathways and 1609 metabolites in the positive ion group, of which 215 are differential metabolites. At the same time, there were 69 metabolic pathways and 1044 metabolites in the negative ion group, which included 171 differential metabolites. For the screening of significant difference metabolic pathways, the significant difference metabolic pathway was selected as the pathway with a larger p-value ($p < 0.05$). Significantly different metabolic pathways in the positive ion group are: Starch and sucrose metabolism, Arginine and proline metabolism, Tryptophan metabolism, ABC transporters, Galactose metabolism, Tyrosine metabolism, Isoquinoline alkaloid biosynthesis, alpha-Linolenic acid metabolism, Monobactam biosynthesis, Lysine degradation, Aminoacyl-tRNA biosynthesis, and Photosynthesis. Significantly different metabolic pathways in the negative ion group are: Lysine degradation, Flavone and flavonol biosynthesis and Arginine biosynthesis.

The Analysis of Arginine Biosynthesis of *Paustralis* under Copper Stress

As shown in Fig. 5a, there are three differential metabolites in the arginine biosynthesis pathway. Glutamine and N-Acetyl-L-glutamate 5-semialdehyde are down-regulated metabolites, which are involved in citrulline synthesis and ornithine synthesis respectively. Arginine is an up-regulated metabolite, which is involved in both synthesis processes mentioned. Citrulline and ornithine also participate in the synthesis of arginine. In this study, the synthesis of arginine was divided into two pathways: firstly the direct catabolization of citrulline into arginine, and secondly the circular pathway composed of ornithine, citrulline, L-Argininosuccinate, and arginine (Fig. 5a).

The Analysis of Flavone and Flavonol Biosynthesis of *Paustralis* Copper Stress

In this study, as shown in Fig. 5b, there are 6 differential metabolites in the biosynthesis of flavonoids and flavonols under copper stress, of which ayarin was the only up-regulated compound. And ayarin was

involved in the flavonol resistance mechanism of *P. australis* under copper stress. The result showed that during the biosynthesis of ayanin, quercetin was firstly methylated to form 3-O-methylquercetin, then transformed into 3,7-O-Dimethylquercetagenin and finally obtain ayanin. (Fig. 5b). Moreover, apigenin and kaempferol produced in the process of flavonoids biosynthesis. The results also showed that apigenin can be transformed into Cosmosiin, Apin, Isovitexin and Vitexin and they were all down-regulated metabolites. And kaempferol was transformed into quercetin conducted by flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3'5'H), and quercetin continued to undergo methylation and a series of reactions to finally form ayanin. Meanwhile, kaempferol can also be converted to kaempferin by UDP-glycosyltransferase 78D1 (UGT78D1), kaempferin was down-regulated metabolite.

Discussion

The distribution of copper reflect that *P. australis* has a great potential to accumulate Cu in their body parts. This is reflective of the addition of copper inhibiting the growth of *P. australis* due to large amounts of copper accumulation in the cells (Ju et al. 2019, Saleem et al. 2020, Liu et al. 2018).

MDA is the oxidation product of lipid peroxidation, indicating the generation of free radicals and reflecting the degree of damaged membranes which are caused by abiotic stress (Riaz et al. 2021, Ohkawa H 1979). Lipid peroxidation is mediated by free radicals (Slater 1984a;b), which is the best measure (Halliwell 1991) to test the damage that is caused by increasing reactive oxygen species (ROS). Judging by the growth trend of MDA, it can be inferred that the increasing copper content will induce a large amount of ROS in *P. australis* leaves. And ROS can cause lipid peroxidation and cell membrane damage. Kumar et al. (Kumar et al. 2020) have reported that cell damage can increase the permeability of ions in cells and lead to the death of the cell. The degree of copper damage to cells of *P. australis* leaves can be assessed by measuring EL in the cells. In this study, MDA content and EL all increased with increasing copper concentration. Farid et al. (Farid et al. 2020) concluded that increasing chromium content in the soil can promote the production of EL and MDA in sunflower tissues. In this project, the continuous increase of MDA and EL can reflect the activation of the oxidative stress response of *P. australis* leaves, and the antioxidant defense system can be activated to resist oxidative stress. Saleem et al. (Saleem et al. 2020) found the EL and MDA contents of the *Hibiscus cannabinus* L. to increase under copper stress, and excess copper induced oxidative damage in *Hibiscus cannabinus* L. roots and leaves. However, they found that antioxidants (SOD, POD, CAT and APX) can eliminate the activity of ROS when their content was up to $120 \mu\text{mol}\cdot\text{L}^{-1}$, which shows that the active antioxidant systems can help *Hibiscus cannabinus* L. resist copper stress (Saleem et al. 2020). Zhu et al. (Zhu et al. 2020) reported that the Cd content in cotton roots and leaves is positively correlated with MDA and EL, while negatively correlated with SOD, CAT, chlorophyll, and photosynthetic parameters, and the antioxidant mechanism of cotton can be enhanced by adding biochar and biofertilizer. Therefore, in this study, the increase of MDA and EL in *P. australis* leaves under copper stress indicates that the cells have undergone lipid peroxidation reactions triggered by free radicals, leading to increasing cell damage, and it is speculated that the anti-oxidant defense system of *P. australis* leaves are activated at this time.

Infrared spectroscopy can identify the functional groups contained in a molecule (Zhang et al. 2020). Dhivya et al. (Dhivya 2017) have reported that the change of O-H may be related to the production of polyphenols and flavonoids, and another study shows that phenolic compounds and flavonoids are considered antioxidants and can be used as reducing agents and free radical scavengers (Gupta and Gupta 2011). At the same time, it is reported that the antioxidant activity of phenolic compounds and flavonoids is proportional to the presence of O-H in the sample, and the position of O-H can also affect free radical scavenging activity (Patle et al. 2020, S. Meenakshi 2009, Balasundram, Sundram and Samman 2006). Alica Bartošová et al. (Alica Bartošová 2015) showed that 1644 cm^{-1} is the characteristic band of protein spectrum. Yu et al. (Yu et al. 2020) analyzed infrared spectra and found that the absorbance of high concentration of Cd in the roots is greater than that of low concentration at the absorption band of 1631 cm^{-1} - 1637 cm^{-1} . High concentration of Cd can induce *C. Canadensis* (L.) Cronq seedlings to produce many proteins, amino acids, and other substances, and can also enhance stress resistance, provide nitrogen sources, reduce heavy metal toxicity and stabilize the internal environment by means of osmotic adjustment. Therefore, in this study, the FTIR results demonstrate that the O-H changes in *P. australis* leaves are related to the production of polyphenols and flavonoids, and the content of proteins and amino acids have an influence on the changes of absorption band near the 1644 cm^{-1} under copper stress. According to Yu et al. (Yu et al. 2017), FTIR analysis shows that under Cd stress, the O-H absorption peak of *V. zizanioides* roots is higher than that of the control group. The O-H of the root cells was complexed with C, which formed stable compounds to improve the plant's resistance to Cd. In this study, it is speculated that the increased O-H in the roots of *P. australis* are complexed with Cu ions to improve the tolerance of the roots under copper stress.

In this study, infrared spectroscopy detected the changes of the functional groups, some of which were related to amino acids and flavonoids in *P. australis* leaves. By analyzing the metabonomic results, we found that many amino acid-related pathways were significantly enriched, one of which being the arginine biosynthesis pathway. The metabolic activity of the compounds in the arginine biosynthesis pathway can not only maintain the balance of citrulline and ornithine in *P. australis* leaves, but also urge *P. australis* leaves to accumulate a large amount of arginine. These amino acids can not only chelate heavy metals but can also have antioxidant effects. In addition, the flavonoids and flavonols biosynthesis pathway in the metabolome revealed the production process of specific flavonoids and flavonols and their changes in response to copper stress. Interestingly, these compounds also have high antioxidant activities. Therefore, this project will mainly analyze the arginine biosynthesis pathway and the flavonoids and flavonols biosynthesis pathway to explain their resistance mechanisms in *P. australis* leaves under copper stress.

In the arginine biosynthesis pathway, several studies have shown that a large amount of arginine in plants can reduce toxicity by chelating heavy metal ions. At the same time, arginine can also synthesize antioxidant peptides with other substances to inhibit the destruction caused by ROS and the peroxidation of essential fatty acids (Rani, Pooja and Pal 2018, Nasibi et al. 2013, Koilraj, Kalusulingam and Sasaki 2019, Maestri, Marmiroli and Marmiroli 2016). In the first pathway, the citrulline can be converted into

arginine. Hartman et al. (Hartman et al. 2019) have reported that citrulline is a precursor of arginine in the pathway of citrulline catabolizing into arginine. In the second circular pathway, ornithine is firstly converted to citrulline by the catalysis of ornithine transcarbamoylase (OTC). Then, L-Argininosuccinate is formed by the connection of aspartic acid with citrulline conducted via argininosuccinate synthase (ASS1). Finally, the synthesis of arginine is catalyzed by argininosuccinate lyase (ASL), and arginine is sequentially catabolized into ornithine by arginase (Winter et al. 2015, Joshi and Fernie 2017, Monne et al. 2015). According to Fig. 5a, arginine can also be consumed to transformed into citrulline. Therefore, it is inferred that the second circular pathway dominates the process of arginine accumulation. And the accumulated arginine was used to resist copper stress.

According to differential intermediate metabolites in plants, there are two pathways for the synthesis of citrulline in plants: the glutamine synthesis pathway and the arginine synthesis pathway (Domingos et al. 2015, Pandey 2018, Fragkos 2018). In the first pathway, Joshi et al. (Joshi and Fernie 2017) report that glutamine accumulation is a necessary prerequisite for the synthesis of citrulline through carbamoyl phosphate synthetase (CPS). In the second pathway, arginine can be oxidized to citrulline by the catalysis of nitric oxide synthase (NOS) according to Maurya et al. (Maurya and Rani 2017). In this study, glutamine was a down-regulated metabolite, and the biosynthesis of citrulline was reduced in the glutamine synthesis pathway. However, arginine was an up-regulated metabolite, which increased citrulline synthesis. The two pathways jointly maintained the stability of citrulline in *P.australis* leaves. Citrulline can maintain nitrogen homeostasis by playing a role in plant nitrogen transport under abiotic stress and maintaining cell osmotic pressure, and it is also an effective free radical scavenger (Joshi and Fernie 2017, Breuillard, Cynober and Moinard 2015).

In addition, the synthesis of ornithine is also divided into two pathways: the glutamate synthesis pathway and the arginine synthesis pathway (Monne et al. 2015, Chen et al. 2019). In the first pathway, Winter et al. (Winter et al. 2015) found that glutamate synthesizes ornithine in a cyclic fashion through several acetylation intermediates. In the second pathway, Singh et al. (Singh et al. 2020) showed that arginine synthesizes ornithine through arginase. In this study, N-Acetyl-L-glutamate 5-semialdehyde, a key acetylation intermediate in the glutamate pathway, was down-regulated, which indicates that the accumulation of ornithine was reduced in this pathway. In the arginine pathway, arginine was an up-regulated metabolite, which promoted the accumulation of ornithine. It can be speculated that the two pathways worked together to maintain the balance of ornithine. Studies show that excessive accumulation of ornithine can not only cause the toxicity of plants, but also limit the synthesis of polyamines. Therefore, it is necessary to maintain homeostasis of ornithine in plants. A proper amount of ornithine can be used as a precursor of polyamines, a signal molecule and a nitrogen carrier in plants, and the nitrogen carried can resist oxidative damage by enhancing the antioxidant defense system (Winter et al. 2015, Joshi and Fernie 2017, Pandey 2018, Jortzik et al. 2010).

In the flavonoids and flavonols biosynthesis pathway, flavonoids have an important function in many plants, such as pigmentation, preventing dormancy, improving fertility, protecting from ultraviolet rays,

defending against plant pathogens, and preventing biological and abiotic stress. Flavone and flavonol are flavonoids (Iwashina 2003, Jia et al. 2012).

Ayarin is a flavonol derived from the gradual methylation of quercetin (Vitalini et al. 2011). Flavonols can act as antioxidants and activate the antioxidant system when plants resist adverse environment and abiotic stresses, and can also eliminate oxidative stress induced by ROS (Zhang et al. 2020, Watkins, Hechler and Muday 2014). Several studies have shown that as a precursor of ayarin, quercetin can also inhibit lipid peroxidation by scavenging ROS and chelating metal ions which can cause the production of ROS (Ishige, Schubert and Sagara 2001, Kato et al. 2016, Jeffrey B. Harborne 2000). It is mentioned above that quercetin forms ayarin via the process of 3-Omethylation. Although the oxidation ability of ayarin is weaker than quercetin, the process of 3-Omethylation greatly improves the free radical scavenging ability of ayarin. This is because methylated quercetin is an effective metal chelating agent that will chelate Cu ions to form a complex (Vitalini et al. 2011, Kato et al. 2016, Pekal, Biesaga and Pyrzynska 2011, Bukhari et al. 2009). Therefore, in this study, it is speculated that *P.australis* resists oxidative stress by exerting a higher antioxidant capacity through the chelation of ayarin.

As a naturally occurring flavonoid in plants, apigenin has significant antioxidant activity, which can have an effect on scavenging free radicals to inhibit the oxidative stress response of plants (Dou et al. 2020). Studies show that apigenin can also combine with sugar to form Cosmosiin, Apin, Isovitexin and Vitexin and other compounds, and these compounds are glycosides naturally occurring in plants (Meyer et al. 2006, Ali et al. 2017, Peng et al. 2008). The researches also show that apigenin should have decreased when these glycosides were down-regulated. However, apigenin have no significant changes. And studies have found that chalcone synthase (CHS) and flavone synthase I (FSI) are the key enzymes involved in the production of apigenin in the process of flavonoid biosynthesis (Li, Feng, et al. 2020, Yan et al. 2014). Therefore, it is speculated that the apigenin content in *P.australis* leaves stayed stable because the process of flavonoids biosynthesis can produce apigenin when apigenin was consumed by other reactions.

Kaempferol is also a natural flavonoid with powerful antioxidant activity (Deng et al. 2019). Studies show that kaempferin is the derivative of kaempferol, and UGT78D1 is as the key enzyme to conduct kaempferol transform into kaempferin (Li, Hossain, et al. 2020, Lee et al. 2017), so kaempferol should have decreased when it was converted into ayarin and kaempferin. However, kaempferol have no significant changes in the present research. Dong et al. (Dong and Lin 2021) have studied that kaempferol is produced in the process of flavonoid biosynthesis and requires the participation of flavonol synthase (FLS), which acts on dihydroflavonols to produce flavonols such as kaempferol. Furthermore, Guo et al. (Guo et al. 2019) have reported that F3'H and F3'5'H can promote the accumulation of quercetin, and F3'H plays a leading role in this process. Therefore, it is speculated that kaempferol can be produced in the process of flavonoid biosynthesis when kaempferol was consumed by other reactions, which was greatly balance the content of kaempferol in *P.australis* leaves. By maintaining the content of kaempferol in *P.australis* leaves and avoiding the decrease of its content, the oxidative stress response caused by copper stress can be resisted.

Conclusions

The main goal of this report is to evaluate the resistance mechanism of *Phragmites* to copper stress during growth and development from the aspect of metabolomics analysis. Here, we studied the variation of the physiological indicators of *Phragmites* leaves and found that the content of MDA and EL gradually increased with the increase of copper concentration, which reflected that the degree of oxidative damage of *Phragmites* leaves under copper stress was increasing. Additionally, we used the FTIR to research the change of chemical composition in the roots, stems and leaves of *Phragmites* seedlings, the results showed that flavonoids and amino acids were the main increased substances in *Phragmites* leaves, which is helpful to explain how plants show tolerance to copper stress. Besides, by analyzing the metabolomics results to identify significant differential metabolic pathways and differential metabolites that were significantly up-regulated or down-regulated in the corresponding pathways, including Arginine Biosynthesis, Flavone and Flavonol Biosynthesis, arginine and ayarin and so on, which were all related to the resistance of *Phragmites* leaves. This project is devoted to improve the understanding of the physiological and molecular mechanisms involved the process of growth and development of *Phragmites* leaves under copper stress, and provided a theoretical basis for improving the resistance mechanism and repair performance of *Phragmites* to heavy metal.

Declarations

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Conflict of interest The authors declare that they have no conflict of interest.

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Figures

Figure 1

Distribution of copper in (a) roots, (b) stems and (c) leaves of *P. australis* seedlings. Vertical bars represent Cu concentration. Different lowercase letters within a column indicate a significant difference between the treatments ($p < 0.05$). Data shown are the average of three replications ($n=3$).

Figure 2

Effects of Cu treatment concentrations on the contents of malondialdehyde (MDA) (a) and electrolyte leakage (EL) (b) in leaves of *P. australis* seedlings. Different lowercase letters within a column indicate a significant difference between the treatments ($p < 0.05$). Data shown are the average of three replications ($n=3$).

Figure 3

Absorption FTIR spectra in the (a) leaves, (b) stems, and (c) roots of *P.australis* seedlings by copper treatment.

Figure 4

(a) The volcano graph of differential metabolites. The red origin represents the differential metabolites that are significantly up-regulated in the experimental group, and the green origin represents the significantly down-regulated differential metabolites. Gray dots represent insignificantly differential metabolites. (b) The heat map of differential metabolites. Perform Hierarchical Clustering on the expression of significantly differential metabolites. (c) General overview of significantly differential metabolites in positive ion group and negative ion group. (d) Statistical graph of differential metabolites (Flavone and flavonol biosynthesis and Arginine biosynthesis).

Figure 5

The variation of differently expressed metabolites in the Arginine biosynthesis pathway and Flavone and flavonol biosynthesis pathway of *P. australis* seedlings. The up-regulated were marked with red fonts, down-regulated were marked with blue fonts, insignificant were marked with black fonts and white box indicates enzyme.