The Positive Effects of Arbuscular Mycorrhizal Fungi Inoculation And/Or Additional Aeration On The Purification Efficiency of Combined Heavy Metals In Vertical Flow Constructed Wetlands

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Abstract

The extent to which, and mechanisms by which, arbuscular mycorrhizal fungi (AMF) and additional aeration (AA) purify wetlands polluted by combined heavy metals (HMs) are not well understood. In this study, vertical flow constructed wetlands (VFCWs) with the *Phragmites australis* (reeds)-AMF/AA were used to treat combined HMs-polluted wastewater. The results showed that (1) AA improved the AMF colonization in VFCWs, and AMF accumulated the combined HMs in their structures; (2) AMF inoculation and/or AA significantly promoted the reeds growth and antioxidant enzymes activities, thereby alleviating oxidative stress; (3) AMF inoculation and AA significantly enhanced the removal rates of Pb, Zn, Cu and Cd under the stress of high combined HMs concentrations comparing to the CK treatment (autoclaved AMF inoculation and no aeration), which increased by 22.72%, 30.31%, 12.64%, and 50.22%, respectively; (4) AMF inoculation and/or AA significantly promoted the combined HMs accumulation in plant roots and substrates, and altered the distribution of HMs at the subcellular level. We therefore conclude that AMF inoculation and/or AA in VFCWs improves the purification of combined HMs-polluted wastewater, and the VFCWs-reeds-AMF/AA associations exhibit great potential for application in remediation of combined HMs-polluted wastewater.

1. Introduction

Nowadays, heavy metals (HMs) pollution mainly caused by human activities, due to its high toxicity, non-biodegradability, and persistence nature, has posed an increasingly serious threat to human health and the stability of aquatic ecosystems even at trace levels (Jia et al. 2020, Yin et al. 2019, Yu et al. 2020). Actually, compared with single HM pollution, combined HMs pollution (e.g. Pb, Zn, Cu, and Cd) can be seen almost everywhere in wastewater (Zhao et al. 2017). What's worse, due to the cumulative effect and complexity of multi-metals contamination, it shows higher toxicity and may lead to a more serious environmental challenge (Shi et al. 2020, Wang et al. 2018). Therefore, there is an urgent need to develop effective techniques to relieve combined HMs negative influences. In general, the remediation of combined HMs by conventional technologies not only needs high energy consumption, but also generates a large amount of secondary wastes (Jacob et al. 2018). Conversely, natural restoration system, such as constructed wetlands (CWs), provides a high-efficiency, economical and eco-friendly mean for treating combined HMs wastewater (Ayaz et al. 2020, Papaevangelou et al. 2017, Zhang et al. 2020). The removal of combined HMs in CWs mainly relies on the uptake capacity of plants, physicochemical properties of substrates, and microbial functions, which are the main removal pathways (Chen et al. 2021, Vymazal &Březinová 2016, Yu et al. 2020). However, previous studies also reported that the removal rate of multi-metals in CWs was greatly affected by a series of abiotic factors (e.g. pH, temperature, oxygen content, etc.), and the tolerance of wetland plants to multi-metals toxicity was also limited (Fakhar et al. 2020, Zhang et al. 2020). Therefore, the issue that how to enhance the ecological function of wetlands has aroused significant concern from all over the world.

Arbuscular mycorrhizal fungi (AMF), a key group of soil microorganisms, can be symbiotic with more than 80% vascular plants in terrestrial (Trappe 1987). The significant roles of AMF in enhancing plants nutrition uptake, increasing plants resistance, and stimulating rhizospheric microbial activity under biotic and abiotic stresses, have been thoroughly reported (Camenzind et al. 2016, Huang et al. 2018, Riaz et al. 2021). Over the last two decades, it has been gradually reported that AMF could form mycorrhiza with wetland plants, which made it possible for them to perform their functions in aquatic habitats (Hu et al. 2020b, Xu et al. 2016). However, unlike the terrestrial environment, wetlands had a low AMF colonization due to inadequate dissolved oxygen content (Calheiros et al. 2019, Xu et al. 2016). With the in-depth research on mycorrhizal fungi by numerous researchers, AMF, as an effective strategy of bioaugmentation, could become a promising tool to improve the functions of treatment wetlands (Tondera et al. 2021). Surprisingly, a number of studies have proved that plants assisted by AMF have better performance than non-inoculated plants in CWs, for example in the purification of HMs wastewater (Hu et al. 2020c, 2021, Li et al. 2016). However, the role of AMF on the treatment of combined HMs pollution in aquatic environment remained poorly understood. At the same time, aeration is usually used to increase the amount of dissolved oxygen in water bodies, which is also well-known as a common physical method to improve the ability of wetlands to remove pollutants (Feng et al. 2021). Nevertheless, there is no information so far about the extent to which AMF or combined with aeration plays an ecological role in removing combined HMs in CWs and whether the combination improves the treatment efficiency. As a result, the applications of AMF inoculation and additional aeration (AA) in treating combined HMs require further study.

The main aims of this study were to (1) assess the effects of AMF inoculation and/or AA on the growth and physiological indexes of wetland plants under the stress of combined HMs; (2) evaluate the removal efficiencies and distribution of combined HMs in CWs with AMF inoculation and/or AA; (3) explore the role of AMF inoculation and/or AA on the removal of combined HMs in CWs. The results of this study can provide an understanding of the combination with AMF inoculation and/or AA in CWs for combined HMs removal.

2. Materials And Methods

2.1. System Construction

The experiment was carried out on an open-air balcony with a transparent shelter at Wuhan University of Technology in Wuhan, China (30°31'9" N, 114°20'48" E). Twelve parallel microcosm vertical flow CWs (VFCWs) **(Fig. S1)**, as described in detail by Xu et al. (2021), were constructed for treating simulated combined HMs polluted wastewater in this study.

2.2. AMF inoculum, wetland plants and synthetic wastewater

The AMF *Funneliformis mosseae* (BGC XJ01A) were purchased from the Institute of Plant Nutrition and Resource, Beijing Academy of Agriculture and Forestry Sciences, China. The *F. mosseae* inoculum (ca. 70 spores/g), consisting of roots, spores, and hyphae, was obtained by trap culture with *Zea mays* L. The *F. mosseae* inoculum was obtained according to the method described in Xu et al. (2021).

A common wetland plant species, *Phragmites australis* (reeds), were chosen as the host plant in this study. The seeds, purchased from Fuliya Seed Industry Co., Ltd, China, were sterilized in 70% ethanol solution for 1 min, then buried in a tray filled with sterilized fine sand, and kept moist with sterilized water every day. Two weeks later, reed seedlings were transplanted into a sterile nursery cup with a diameter of 9 cm and a depth of 13 cm. The growth substrate was composed of river sand, vermiculite, and humus (5:1:1 in mass). Half of the seedlings were inoculated with *F. mosseae* inoculum, and the other half were inoculated with autoclaved *F. mosseae* inoculum. Tap water was added to keep moist before the seedlings were transplanted to the VFCWs. Three seedlings were transplanted into each VFCW system on October 26, 2019, and acclimated to the VFCWs under flooded conditions (2 cm depth of water above the surface of quartz sand) for 10 days.

The influents were synthetically prepared using $Pb(NO_3)_2$, $Zn(NO_3)_2$, $Cu(NO_3)_2$, $Cd(NO_3)_2$, and ultra-pure water in this study. Additionally, each VFCW was irrigated with 50ml modified Hoagland solution every week, in which KH_2PO_4 concentration was adjusted to 0.01 mM in order to prevent precipitation of Pb (Islam et al. 2008). Considering the limited tolerance of reeds to combined HMs, 2, 5, 2.5 and 0.5 mg/L of Pb, Zn, Cu and Cd were artificially prepared as the low concentrations of combined HMs, and 20, 50, 25 and 2.5 mg/L were set as the high concentrations of combined HMs, according to Cheng et al. (2002) and Fritioff andGreger (2006) with minor modifications.

2.3. Experimental setup

The experimental treatments were factorial combinations of three factors: (1) intermittent aeration at 4 h per day (0:00-1:00 h, 6:00-7:00 h, 12:00-13:00 h and 18:00-19:00 h) (IA) and no aeration (NA); (2) inoculation with *F. mosseae* (FM) and autoclaved *F. mosseae* (NM); (3) three concentrations of combined HMs (0, low and high). A total of 12 VFCWs were set up, and three reed seedlings with similar growth were planted into each VFCW. In the 6 VFCWs inoculated with *F. mosseae*, 50 g of AMF inoculum was first laid in the middle of the quartz sand layer, then mycorrhizal reed seedlings were placed on the inoculum, and finally covered with a layer of sterilized quartz sand. In the other 6 VFCWs, 50 g of sterilized AMF inoculum was first laid in the middle of the quartz sand layer, then the roots of non-mycorrhizal reed seedlings were directly transplanted into the middle of the quartz sand layer, and finally covered with a layer of sterilized quartz sand.

Once the plants were transplanted into the VFCWs on October 26, 2019, aeration was carried out in the wetland for 4 h (1 L/min). Tap water was added to a height of 2 cm above the quartz sand layer, and the outlet valve was kept closed for the next 10 days. Then, the VFCWs began to be operated without combined HMs loading. Tap water was fed in at 10:00 am, and discharged at 10:00 am in the next morning. The VFCWs were cyclically operated for 2 weeks to promote the coating of microorganisms on the substrate or plant roots surface, with a hydraulic retention time (HRT) of 24 h. During the operation of the VFCWs with combined HMs loading, the synthetic combined HMs wastewater were fed in and discharged with an HRT of 24 h. The influent of the next cycle was the effluent of the previous cycle, and the ultrapure water was replenished to the original height (62 height). The purification experiment of simulated combined HMs polluted-wastewater was operated for 15 cycles, e.g. 30 days. All water samples were collected and stored at 4°C.

2.4. Sample analysis

2.4.1. Sample handling and measurement

Plant shoots and roots were harvested separately at the end of the experiment, then washed with deionized water and dried the tissues. The root length, shoot height, fresh weight of the roots and shoots were measured directly with the tape measure and electronic balance. At the same time, the infection rates of inoculated plants were calculated according to the method of Phillips andHayman (1970) and Trouvelot et al. (1986). The superoxide dismutase (SOD) and peroxidase (POD) activities were determined using the nitro blue tetrazolium (NBT) method (Giannopolitis &Ries 1977) and the method by Chance andMaehly (1955), respectively. The reactive oxygen species (ROS) content and catalase (CAT) activity were determined by spectrophotometry, the root activity determined by the triphenyl tetrazole chloride (TTC) method, and the malondialdehyde (MDA) content was determined by the thiobarbituric acid colorimetric method (Gao 2006). Besides, chlorophyll content was determined by Portable chlorophyll meter (SPAD-502 Plus, KONICA MINOLTA, Japan). Dry weight of the shoots and roots was measured using an analytical balance after drying at 80 °C to constant weight.

The bioconcentration factor (*BCF*) and translocation factor (*TF*) were calculated using the following equation to evaluate the effect of AMF on the Pb, Zn, Cu, and Cd translocation-absorption capacity in wetland plants.

$$BCF=\frac{Pb, Zn, Cu, andCd \text{ concentrations in the whole plants}}{Pb, Zn, Cu, andCd \text{ concentrations in substrates}} \quad (1)$$

$$TF=\frac{Pb,\ Zn,\ Cu,\ \text{and}\ Cd \text{ concentrations in shoots}}{Pb,\ Zn,\ Cu,\ \text{and}\ Cd \text{ concentrations in roots}}$$

2.4.2. Detection of Pb, Zn, Cu, and Cd

The substrates were digested with the mixture of concentrated HF-HNO_3-$HClO_4$ mixed acid in a 5:2:1 volume ratio (Gao &Chen 2012). The harvested reeds were rinsed repeatedly with deionized water, and then exchanged with 20 mmol/L Na_2-EDTA for 20 min to remove the HMs ions adsorbed on the tissue surface, and finally digested on the electric heating plate with concentrated HNO_3-$HClO_4$ mixed acid by volume 4 :1 (Favas 2019). The distribution of Pb, Zn, Cu, and Cd in different components of reed cells was pretreated by centrifugation at 10000×g for 30 min, then determined by the method of Karmakar et al. (2019). The contents of Pb, Zn, Cu, and Cd in effluents, substrates, and plants were analyzed by ICP-MS (Prodigy 7, Teledyne Leeman Labs, USA).

2.5. Statistical analysis

A three-way analysis of variance (ANOVA) was carried out to test the effects of main factors (inoculation of *F. mosseae*, aeration, and combined HMs) and their interactions on the growth, antioxidant enzyme activities, MDA and ROS of reeds. The Ducan's testing was used to compare treatment differences with P<0.05 set as a significant difference. All statistical analyses were performed using the IBM SPSS statistics version 26.0 software package for windows (IBM Corp., Armonk, NY, USA). The ‘Pheatmap’ and ‘Factoextra’ packages were used by R Software (version 4.1.0) to visualize the experimental data among the plant growth and physiological indexes in plant roots in all treatments. Origin 2021 (OriginLab, USA) was used to plot the figures.

3. Results And Discussions

3.1. AMF colonization under different treatments

As shown in Table 1, the frequency of mycorrhiza (F%) of reeds inoculated with *F. mosseae* were determined before those were transplanted into the VFCWs, being 50.00%, 53.30%, 50.00%, 53.30%, 50.00%, 46.70%, respectively. When the experiment ended at the 60th day, the F% values of the corresponding treatment increased by 40.00%, 25.14%, 20.00%, 18.76%, 13.40%, 7.07%, respectively, indicating that AMF could continue to infect the root system under flooded conditions. In particular, the F% values of the "IA+FM" treatment under the three concentrations of combined HMs (0, low, and high) were significantly higher than those of the "NA+FM" treatment (P<0.05), which increased by 10.58%, 17.64%, and 20.00%, respectively. Meanwhile, the intensity of mycorrhizal colonization (M%) and arbuscular abundance (A%) also showed a similar trend with the F%. These results suggested that AA had a positive effect on improving AMF colonization in VFCWs, especially under the high concentrations of combined HMs, which was consistent with the results of Xu et al. (2021).

Table 1
Root colonization of reeds in the six VFCWs inoculated with *F. mosseae*.

Treatment	HMs level	Days	F%	M%	A%
IA+FM	0	0	50.00±2.00fg	13.10±0.40d	0.80±0.10de
		60	70.00±2.00a	24.30±0.80a	2.80±0.30a
	low	0	53.30±1.60ef	14.80±0.30c	0.80±0.10de
		60	66.70±2.60ab	23.70±0.60a	2.10±0.20b
	high	0	50.00±1.70fg	8.50±0.50g	0.50±0.10f
		60	60.00±2.00cd	15.00±0.50c	1.10±0.20c
NA+FM	0	0	53.30±2.30ef	9.30±0.30g	0.40±0.10f
		60	63.30±2.60bc	17.40±0.40b	1.00±0.10cd
	low	0	50.00±1.70fg	11.70±0.50e	0.60±0.10ef
		60	56.70±2.20de	17.00±0.40b	0.90±0.10cd
	high	0	46.70±1.90g	10.50±0.50f	0.50±0.10f
		60	50.00±2.60fg	13.00±0.40d	0.60±0.10ef

F%: the frequency of mycorrhiza; M%: the intensity of mycorrhizal colonization; A%: the arbuscule abundance; IA: intermittent aeration; NA: no aeration; FM: inoculation with *F. mosseae*; 0: the day of the reeds were transplanted; 60: the day of the reeds were harvested.

Data are presented as Means ± SD. Significant differences between different treatments are presented by different letters (Duncan’s test, P<0.05).

Lower oxygen content in wetland habitats may be one of the most important factors leading to AMF not to colonize roots at a high level like in the terrestrial ecosystems (Huang et al. 2021, Wang et al. 2015). Similarly, Hu et al. (2020b) reported that AMF colonization under fluctuating water depth was higher than high water regimes, which was ascribed to the increasing oxygen content of the rhizosphere by constantly changing water regime. In addition to improving the availability of oxygen, the addition of aeration also promoted the growth of wetland plants, thus providing more surface areas for microorganisms growth, and also altered the diversity of microbial communities distributed in the rhizosphere (Feng et al. 2021). Ferreira et al. (2021) and Viollet et al. (2017) revealed that the diverse microbial communities (e.g. Plant growth-promoting rhizobacteria, nitrogen-fixing bacteria) had a significant influence on helping AMF acquire more nutrient and facilitating hyphal growth to produce spores, which played a positive role in promoting AMF colonization. We inferred here that it happened in wetlands environment as well. As the concentration of combined HMs increased, the F%, M%, and A% of the two inoculation treatments all showed a decreasing trend. A similar study by Hu et al. (2021) showed that the F% were 49.70% at 0 mg/ kg Cr concentration, but decreased to 40.90% and 23.90% at 5 and 25 mg/kg Cr concentration, respectively. This may be attributed to the negative influence of HMs on the germination and growth of hyphal (Göhre &Paszkowski 2006). What’s more, HMs had a great negative impact on the establishment of symbiotic relationship between AMF and plant roots, thereby decreasing root infectivity and colonization (Wu et al. 2019). Therefore, the AA provided progress for the lower AMF colonization in wetland plant roots.

3.2. Plant growth and physiological indexes under different treatments

3.2.1. Growth indexes, chlorophyll content and root vitality

Under the three concentrations of combined HMs, compared with the CK treatment ("NA+NM"), the reeds shoot height, root length, aboveground fresh weight, underground fresh weight, aboveground dry weight, underground dry weight, and chlorophyll content of AMF inoculation and/or AA treatments increased by 9.40-56.17%, 0.14-76.47%, 12.22-169.32%, 2.07-126.62%, 11.84-58.33%, 28.62-238.77%, and 36.52-519.67%, respectively (**Table S1**). Among them, those growth indexes and chlorophyll content of the "NA+FM" and "IA+FM" treatments were significantly higher than those in the CK treatment (P<0.05), and the growth promotion of the "IA+FM" treatment was the most significant. The root vitality of the AMF inoculation and/or AA treatments was significantly higher than the non-inoculated or non-aerated treatments (P<0.05). Compared with the reeds grown without combined HMs, the root vitality of reeds grown at low concentrations increased slightly, while that dropped significantly at high concentrations (P<0.05). Principal Component Analysis (PCA) results showed that these parameters were largely determined by Dim1 (67.90%), implying there was a strong correlation among them. Meanwhile, the results of scores of 12 treatments showed that AMF inoculation and AA had different effects on wetland plant physiological functions under the three concentrations of combined HMs (**Fig. S2**). The results of hierarchical clustering analysis indicated that AMF inoculation and AA showed obvious synergistic effects on the growth characteristics of wetland plants (Fig. 1).

For aquatic plants, HMs stress could inhibit the root vitality, decrease the content of chlorophyll and plant biomass by reducing the uptake of essential macro and micronutrients (Wu et al. 2019, Zhao et al. 2019). The plant biomass and chlorophyll content of wetland plant (*Iris wilsonii*) inoculated with AMF under the stress of Cr contaminated-water were higher than those of non-inoculated control, which was consistent with our results (Hu et al. 2020c). Schück andGreger (2020) conducted a 5-day HMs exposure experiment on 34 types of wetland plants and measured 20 traits of each plant, and finally found that high biomass was one of the most important characteristics of the plant's ability to remove HMs. Therefore, wetland plants inoculated with AMF could strengthen their ability to remove HMs by increasing plant biomass. Meanwhile, AMF-inoculated plants can also alleviate the damage of combined HMs to chloroplast in leaves by restricting the transport to the aboveground parts (Janeeshma &Puthur 2020).

Similar with our result, Ban et al. (2021) revealed that the root vitality of reeds increased significantly under the low concentrations of CuO-NPs-polluted wastewater (50 mg/L), but decreased significantly at high concentrations (500 mg/L). A likely explanation was that plants themselves had the potential to improve their physiological metabolism level by increasing the root vitality to resist combined HMs stress at low concentrations. The widespread underground network between the mycelium and plant roots was beneficial for facilitating nutrient flow between roots, and preventing combined HMs from entering root cells (Alam et al. 2019). However, excessive HMs could destroy the root cell structure, significantly reduce root vitality, and cause obvious physiological and metabolic disorders (Liu et al. 2014).

Meanwhile, the AA not only increased the dissolved oxygen in the rhizosphere microenvironment and improved the vent ability in reed tissues, but also promoted the absorption of nutrients by roots, thereby promoting the growth of the reeds. There was a very marked synergistic effect of AMF inoculation and AA on growth indexes, chlorophyll, and root vitality of wetland plants for treating combined HMs polluted wastewater. Three-way ANOVA analysis also showed that AMF, aeration, combined HMs pollution, and the interaction of AMF with aeration had significant effects on the growth indexes, chlorophyll content, and root vitality (P<0.05) (Table 2).

Table 2
Three-way analysis of variance of AMF inoculation, aeration and combined HMs stress on reeds.

Index	AMF (F Value)	Aeration (F Value)	C (F Value)	AMF×Aeration (F Value)	AMF×C (F Value)	Aeration× C (F Value)	AMF×Aeration× C (F Value)
Shoot height	2928.66***	632.59***	1310.79***	10.35**	0.07 NS	27.83***	31.04*
Root length	2622.60***	126.29***	415.67***	40.96***	103.98***	18.86***	0.77NS
Aboveground fresh weight	300.55***	67.99***	153.61***	7.28**	1.15 NS	2.86 NS	2.81NS
Underground fresh weight	4509.45***	130.50***	337.32***	33.87***	61.31**	5.52*	2.17NS
Aboveground dry weight	601.27***	500.25***	1849.43***	89.10***	196.20***	94.55***	22.85***
Underground dry weight	57081.50***	8096.28***	920.36***	482.69***	256.66***	334.20***	2509.49***
Root vitality	39134704.50***	3325620.50***	8585079.50***	243602.00***	398605.50***	36639.50***	166784.00***
Chlorophyll	12745.40***	1674.39***	5347.21***	26.48***	236.40***	16.98***	102.30***
SOD	6316.69***	1271.17***	22993.76***	79.45***	626.93***	50.59***	106.24***
POD	62721.01***	2289.32***	175562.61***	12.90***	1574.04***	879.96***	124.05***
CAT	3254.36***	43.41***	21422.46***	241.29***	697.20***	41.62***	60.19***
MDA	6212.11***	239.08***	19795.75***	8.87**	1059.52***	74.87***	50.77***
ROS	352.80***	57.800***	491.79***	6.96**	59.89***	4.96*	1.36 NS

* means P<0.05, ** means P<0.01, *** means P<0.001, and NS means no significant difference. AMF represent inoculation of *F. mosseae*, C represent combined HMs stress.

3.2.2. ROS levels, lipid peroxidation and antioxidant response

It can be seen from **Table S2** that compared with the CK treatment ("NA+NM"), AMF inoculation and/or AA reduced the contents of MDA and ROS, and the "IA+FM" treatment showed the lowest values at different combined HMs concentrations. Conversely, the reeds SOD, CAT and POD activities of AMF inoculation and/or AA treatments were significantly higher than those of the "NA+NM" treatment under the three concentrations (P<0.05), with an increase of 7.32-176.78%, 3.70-74.88%, and 3.74-40.89%, respectively. Among them, the SOD, CAT and POD activities of reeds in the "IA+FM" treatment were the highest. PCA results showed the plant growth indexes had a strong negative correlation with ROS and MDA, which was consistent with Hu et al. (2020a) (**Fig. S2**). Those results of hierarchical clustering analysis indicated that inoculation with AMF and/or AA had a positive effect on alleviating oxidative stress and promoting the antioxidant enzyme activities of wetland plants under the stress of combined HMs (Fig. 1).

HMs pollution was one of the abiotic factors leading to oxidative stress in plants, which could induce excessive ROS and lipid peroxidation, resulting in cellular damage and disturbing cellular ionic homeostasis (Dubey et al. 2018). SOD, CAT and POD were the main antioxidant enzymes to remove ROS in plants, which could protect the structure and function of the cell membrane system and maintain the redox state of plant cells. Nafady andElgharably (2018) found that AMF inoculation significantly reduced MDA content when studying the effect of mycorrhizal symbiosis on *Zea mays* growth under combined HMs (Fe, Mn, Zn, Cu, Cd, and Pb) stress, which was consistent with our results. Similarly, whether in aquatic habitat or terrestrial habitat, mycorrhizal plants had better ROS scavenging ability and antioxidant ability than non-inoculated plants under HMs stress (Hu et al. 2020c, Zhan et al. 2018). In addition, AA had the potential to alleviate oxidative damage by enhancing plant antioxidant capacity and leaf photosynthesis, and upregulate the expression of the genes maintaining cell redox balance (Xiaochuang et al. 2020). Therefore, AMF inoculation and AA synergistically improved the physiological indexes of reeds and increased antioxidant enzyme activities (SOD, CAT, and POD) under the stress of combined HMs. Three-way ANOVA analysis also showed that the interaction of AMF with aeration had significant effects on antioxidant enzyme contents (P<0.05) (Table 2).

3.3. Distribution of Pb, Zn, Cu and Cd in VFCWs

3.3.1. Distribution of Pb, Zn, Cu and Cd in wetland plants

As shown in Fig. 2, the concentrations of Pb, Zn, Cu and Cd in the roots of the reeds were higher than those in the shoots under the low and high concentrations of combined HMs, indicating that HMs were mainly accumulated in the roots of wetland plants. Meanwhile, the concentrations of Pb, Zn, Cu and Cd in the reeds were all increased greatly as the concentration increased. Under the two concentrations of combined HMs, the concentrations of Pb, Zn, Cu and Cd in the roots of AMF inoculation and/or AA treatments were higher than those of the CK treatment ("NA+NM"), particularly, the "IA+FM" treatment showed the highest values, which increased by 28.07-168.98% and 42.83-486.31%, respectively (P<0.05). Conversely, the concentrations of Pb, Zn, Cu and Cd in the shoots of the "IA+FM" treatment at low and high levels were significantly lower than those of the CK treatment, which decreased by 48.80-89.82% and 54.56-76.49%, respectively (P<0.05). Interestingly, the "IA+NM" treatment showed the highest concentrations of HMs in the shoots at high level (P<0.05), which may be related to the improvement of water and nutrients delivery to the shoots with the help of AA owing to the inhibition of transpiration by HMs (Rucińska-Sobkowiak 2016). Meanwhile, the ranking of HMs concentrations in reeds was Cu > Pb > Zn >> Cd. Moreover, it could also be seen from the **Table S3** that AMF inoculation and AA deeply increased the *BCF* and decreased the *TF* of the reeds.

Similar results were obtained by Han et al. (2021b) and de los Angeles Beltrán-Nambo et al. (2021), AMF played a positive role in promoting the accumulation of HMs in roots and inhibiting their transfer to the shoots. Moreover, Huang et al. (2017) reported that AMF symbiosis with higher *BCF* and lower *TF* promoted the tolerance of *P. australis* to Cd stress compared to the non-inoculated groups, which was consistent with our results. Meanwhile, previous studies also proved that the longer roots of mycorrhizal plants had the potential to increase the accumulation of combined HMs in roots, and combined HMs were preferentially accumulated in mycelium of fungi rather than root cells (Wu et al. 2016). This might be the underlying mechanism that AMF plants were able to increase HMs influx in roots. The differences in the ability to accumulate Pb, Zn, Cu and Cd between the results of the present study and Fritioff andGreger (2006) might be determined by the characteristics of plants and HMs. AA accelerated the absorption and transportation of water and nutrients, improving the growth of plants, which was helpful to strengthen the resistance to combined HMs stress and the transfer of HMs from roots to shoots. What's more, AA improved the colonization of AMF in plant roots, and AMF colonization led to higher combined HMs accumulation in roots and improved plant physiological status, which could strengthen the ecological function of VFCWs. In summary, AMF inoculation and AA were beneficial for wetland plants to resist combined HMs stress.

3.3.2. Distribution of Pb, Zn, Cu, and Cd in the substrates

As shown in Fig. 3A **and B**, AMF inoculation and/or AA significantly promoted the adsorption of Pb, Zn, Cu, and Cd in the substrates than those in the CK treatment ("NA+NM") under the low and high concentrations (P<0.05), particularly, the "IA+FM" treatment showed the highest Pb, Zn, Cu, and Cd accumulation in the substrates. Compared with the CK treatment, the concentrations of Pb, Zn, Cu, and Cd in the substrates of the "IA+FM" treatment at low concentrations were 44.31, 32.34, 42.28 and 1.58 mg/kg, respectively, with an increase of 45.40%, 6.52%, 47.20%, and 17.10%, respectively, and the concentrations at high concentrations were 85.47, 105.97, 159.44, and 3.89 mg/kg, respectively, with an increase of 50.86%, 61.30%, 59.49%, and 48.80%, respectively. What's more, the ranking of Pb, Zn, Cu, and Cd accumulation contents in the substrates was Cu > Pb ≥ Zn >> Cd.

In this study, it can be seen that inoculation with AMF enhanced the adsorption of combined HMs by the substrates. We speculated here that this was related to the fact that AMF could secrete some active matters (e.g. glomalin related soil protein (GRSP)) to chelate the combined HMs in substrates, absorb the HMs ions via their hyphae and change the rhizosphere microbial community (Ma et al. 2019, Wang &Feng 2021, Wang et al. 2020). GRSP released by AMF via mycelium and spore walls was water-insoluble glycoprotein, performing a remarkable role in chelating HMs (Wu et al. 2014). It was confirmed that AMF inoculation presented a noteworthy decrease of Cd, Pb, Zn, and Cu concentrations due to the release of GRSP by Nafady andElgharably (2018). Meanwhile, AA increased the oxygen content in the VFCWs, leading to the growth of microorganisms on the surface of the substrates and roots to form more biofilms, which can produce more extracellular polymeric substances (EPS) to remove combined HMs through chelation, surface precipitation, and ion exchange (Li &Yu 2014, More et al. 2014, Riaz et al. 2021). What's more, AA could also increase the frequencies of mycorrhizal symbiosis, thereby promoting the HMs adsorption of the substrates through the potential mechanisms mentioned above. Under the two concentrations of combined HMs, the adsorption difference of Cu, Pb, Zn and Cd might be attributed to the pH, redox potential, different chemical composition, and structure of the substrate material. For example, Hernández-Montoya et al. (2013) reported that zeolitic showed a selectivity in the adsorption process of Pb, Zn and Cd, and the ranking was Pb > Zn > Cd.

3.3.3. Removal rates of Pb, Zn, Cu, and Cd under different treatments

It can be seen from Fig. 4A that the removal rates of Pb, Zn, Cu and Cd of the four treatments all exceeded 80% at low concentrations, and those of the CK treatment ("NA+NM") were 95.17%, 80.36%, 91.66% and 80.68%, respectively. Among the four treatments, the removal rates of the "IA+FM" treatment were the highest, being 98.49%, 92.64%, 97.72%, 92.06%, respectively, increased by 3.48%, 15.28%, 6.61%, 14.11%, respectively, comparing with the CK treatment. At high concentrations, the removal rates of Pb, Zn, Cu, and Cd were lower than those at low concentrations. Comparing with the CK treatment, the removal rates of the AMF inoculation and/or AA treatments increased by 7.84-22.73%, 16.34-30.31%, 5.62-12.63%, 27.88-50.22%, respectively (Fig. 4B). At high concentrations, AMF inoculation and/or AA could significantly improve the removal efficiencies of Pb, Zn, Cu and Cd (P<0.05). However, AMF inoculation and/or AA could significantly improve the removal rates of only Zn and Cd at low concentrations (P<0.05).

The Zn and Cd removal rates of the inoculated treatments ("IA+FM", "NA+FM") were significantly higher than that of the CK treatment under the low concentrations (P<0.05), but not Pb and Cu. This may be due to the fact that the removal rates of Pb and Cu had been at a very high level (more than 90%), which made the role of AMF not obvious in promoting the performance. However, with the increase of combined HMs concentration, the adsorption efficiency of the substrates would be lower, and the toxicity of HMs to plants would be greater. Therefore, compared with the CK treatment, the addition of AMF had a more obvious effect on the removal of Pb, Zn, Cu and Cd at high concentrations than that at low concentrations, with an increase of 11.34%, 20.03%, 5.62%, 32.62%, respectively. Gunathilakae et al. (2018) found that AMF inoculation showed a more obvious effect on the removal of the high Cd level (50 ppm) than the low Cd levels (0, 5, 10, and 20 ppm) when evaluating the role of AMF on the phytoremediation potential of water hyacinth in Cd uptake, which was consistent with our result.

3.3.4. Distribution of Pb, Zn, Cu, and Cd in subcellular components

As shown in **Fig. S3**, Pb, Zn, Cu and Cd were mainly distributed in the cell wall, especially for the underground part of the reeds. The proportions of Pb, Zn, Cu, and Cd in the cell wall in the aboveground part of the reeds under the two concentrations were 30.39-73.93%, 46.74-58.43%, 43.90-72.73% and 35.23-44.71%, respectively, and those of the underground part of the reeds were 71.85-92.44%, 70.27-88.56%, 72.42-90.11% and 48.40-69.07%, respectively. As the concentration of combined HMs increased, there was a decline in the proportions of Pb, Zn, Cu and Cd in the cell wall in the aboveground part of the reeds, while a contrasting tendency was found in the underground part, except for "IA+FM" treatment. For the aboveground and underground parts, the Pb, Zn, Cu, and Cd proportions in the reeds cell wall of the "IA+FM" treatment were higher than those of the CK treatment ("NA+NM") at low concentrations. The cumulative concentration of single Pb, Zn, Cu, Cd in the aboveground and underground parts of the reeds all was cell wall > organelle ≥ soluble component.

The different types and concentrations of HMs induced a distinct subcellular combined HMs distribution. The components of cell wall (e.g. polysaccharides and proteins) provided a large number of retention sites to bind HMs and restricted their transmembrane transport, leading to a lower toxicity than the free state of that in soluble component and organelle (Han et al. 2021a, Mwamba et al. 2016). Gao et al. (2021) revealed that AMF inoculation improved the ability of the plant cell wall in fixing Cd by increasing the cell wall components, resulting in more Cd fixed in the cell walls, which was consistent with our study. More importantly, the fixation of HMs by cell wall was considered as the first barrier to protect protoplasts from the toxic effects of HMs in plants, while the mycorrhizal plants could promote the deposition of HMs on the plant cell walls and reduce the distribution in organelles, thereby alleviating the damage of HMs to the host plant organelles (Huang et al. 2018). Additionally, Fritz (2007) reported that the HMs ions affinity in pectin (a component of cell wall) showed a difference depending on the characteristics of them (Al^{3+} > Pb^{2+} > Cu^{2+} > Co^{2+} > Ni^{2+} > Zn^{2+} > Cd^{2+} > Ca^{2+}), which might account for the differences in the distribution of Pb, Zn, Cu and Cd in subcellular components in our study. According to the result of Considine andFoyer (2021), oxygen may be as a signal for plant growth, which affecting the root and shoot growth and structure, resulting in different distributions of combined HMs in plant subcellular. This study provides sufficient evidence on a subcellular level to illustrate how AMF inoculation and AA benefitted combined HMs tolerance.

3.3.5. Localization of Pb, Zn, Cu, Cd in ultrastructure of mycorrhiza

The normal root cells of reeds were presented in Fig. 5A (e.g. i: nucleolus, h: nucleus, g: cell membrane) applying transmission electron microscopy (TEM). However, under the stress of combined HMs, the root cell morphology of reeds not inoculated with AMF changed greatly, such as deformation of cell wall (k) and helical curl of cell membrane (l) (Fig. 5B). In addition, there were many thickened cell walls (j) and compartmentalized structures about 2-3 μm in diameter in reeds inoculated with AMF under the stress of high combined HMs concentrations (Fig. 5C, D, E, F). Meanwhile, there were many black deposits (a, b, c, d, e, and f) in cell walls and cell interiors, which were suspected to the Pb, Zn, Cu, and Cd. Interestingly, the mycelium of AMF penetrating the cell wall (red circle) of reed from one cell to another were observed **(**Fig. 5F**)**, which showed the process of AMF infecting plant cells. Additionally, Energy Dispersive Spectrometer (EDS) was conducted for those selected black deposits (a, b, c, d, e, and f), and the results were shown in (**Fig. S4**). The presence of Pb, Zn, Cu and Cd peaks indicated that the added HMs ions entered reed cells (a, b, c, d) and AMF structures (e, f). Since the samples were organic matter and the sample holder was made of aluminum material, there was no doubt that the C, O and Al elements were also identified.

TEM can be used to observe the injuries to different organs of plants in response to combined HMs stress. In this part, we can clearly observe how AMF-inoculated plants resist HMs damage at the cellular level. Huang et al. (2018) found that the organelle integrity of plant cells became worse with the increase of Cd concentration applying TEM, while the root cell structure of plants inoculated with AMF were more complete. In addition, Abdel-Fattah et al. (2011) and Xu et al. (2018) found that increasing cell wall thickness and altering subcellular compartmentalization by AMF was a significant mechanism for plants detoxification to HMs, which was consistent with our observations. Meanwhile, a similar result reported by González-Guerrero et al. (2008) that Zn, Cu and Cd also could be accumulated in the cell walls of AMF when these HMs were added to the extra-root mycelium.

3.3.6. Combined HMs mass balance

As shown in Fig. 6, we found that substrate was the main component for HMs removal in these VFCWs, accounting for more than 50%, which was consistent with the results of Ban et al. (2021). Under the two concentrations, the proportions of combined HMs in the plants of the AMF inoculation and/or AA treatments were higher than those of the CK treatment ("NA+NM"), with the following ranking order: "IA+FM" > "NA+FM" > "IA+NM" > "NA+NM". Besides, the proportions of combined HMs in the roots of "NA+FM" treatment (more than 85%) were higher than those of the CK treatment at the low and high concentrations. Conversely, compared with the CK treatment, the proportions of combined HMs in the water of the AMF inoculation and/or AA treatments under the two concentrations decreased by 34.11-64.34% and 15.44-30.63%, respectively. The proportion of combined HMs in water of the "IA+FM" treatment under the two concentrations was the lowest among the four treatments at 4.60% and 27.40%, respectively.

The removal rates of combined HMs in VFCWs were closely related to the synergistic effects of the plants, microorganisms and substrates. Under the stress of combined HMs, AMF decreased the combined HMs concentration in water by improving the plants uptake, promoting that absorbed in substrates, which was consistent with Riaz et al. (2021). In this study, the combined HMs contents of the inoculated treatments in substrates were lower than that in the CK treatment at low concentrations, which was not consistent with Hu et al. (2021). The reason might be that the wetland plants adsorbed more HMs due to the low concentrations and toxicity of combined HMs, thus resulting in the reduction of combined HMs in the substrates. Besides, AMF accumulated HMs in their intra-radical and extra-radical mycelium, thus resulting in lower HMs contents in the water. Wu et al. (2016) showed that the extraradical mycelium of AMF could absorb and transport Cr to mycorrhizal roots from a distance, which could contribute to promoting the ability of the wetland system to purify HMs-polluted wastewater. In addition, the effect of AA on the mass balance of combined HMs in aquatic habits was mainly related to the enhanced microorganisms activities and improved HMs uptake of wetland plants. Therefore, we found that AMF inoculation and AA showed significant synergistic effect on the removal of combined HMs from VFCWs.

4. Conclusions

The utilization of AMF inoculation and additional intermittent aeration played a significant role in promoting the growth indexes, chlorophyll, SOD, POD, and CAT of the reeds under the stress of combined HMs. Meanwhile, the AA provided a better performance for AMF colonization in the roots of wetland plants. Moreover, compared with the CK treatment ("NA+NM"), the combination of AMF inoculation and aeration increased the removal rates of Pb, Zn, Cu, Cd, especially under the stress of high combined HMs concentrations, which increased by 22.72%, 30.31%, 12.64% and 50.22%, respectively. Besides, inoculation with AMF and AA changed the subcellular structures distribution of combined HMs in reeds, and that mainly distributed in the cell walls. TEM and EDS showed that AMF inoculation and AA could induce thicker cell walls of plant root and allow more combined HMs to be deposited in plant cell walls and fungal structures. Overall, the applications of AMF inoculation and AA exhibit significant synergistic effect in remediation of combined HMs polluted wastewater.

Statements And Declarations

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Author contribution

Zhouying Xu: Conducting most of the experiment. **Kaiguo Li**: Writing-review & editing. **Chen Wu**: Conducting part of the experiment. **Wenxuan Li**: Searching for references. **Xi Chen**: review & editing. **Jun Huang**: Searching for references. **Xiangling Zhang**: review & editing. **Yihui Ban**: review & editing.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information fles.

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Conflict of interest The authors declare no competing interests.

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Figures

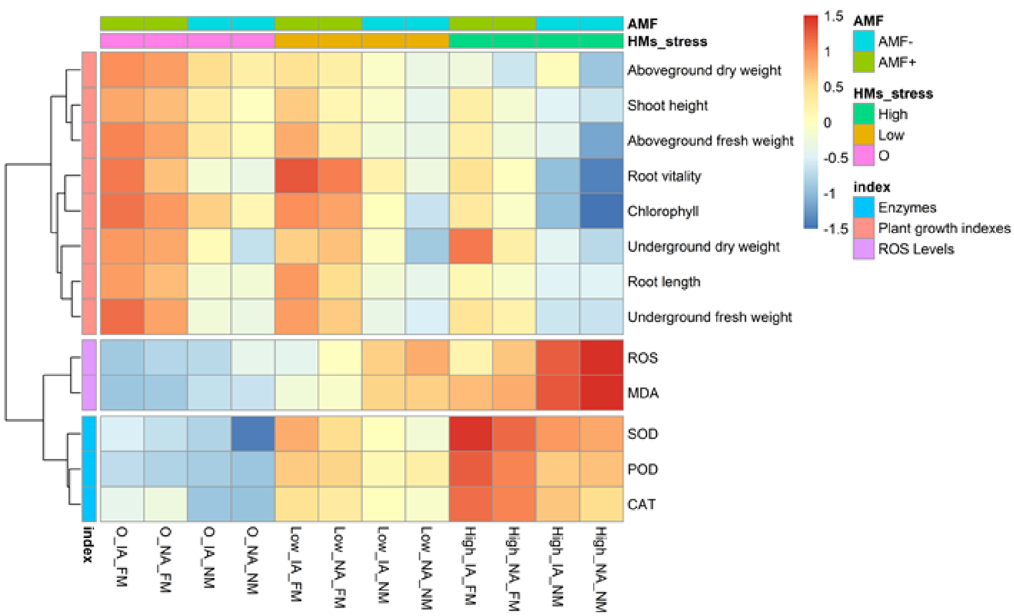

Figure 1

The cluster analysis among plant growth (shoot height, root length, aboveground fresh weight, underground fresh weight, aboveground dry weight, underground dry weight, chlorophyll, and root vitality) and physiological indexes (MDA, SOD, POD, CAT, and ROS) in plant roots, colors in the heatmap indicate the correlation between the different data sets. IA: intermittent aeration; NA: no aeration; FM: inoculation with *F. mosseae*; NM: inoculation with autoclaved *F. mosseae.*

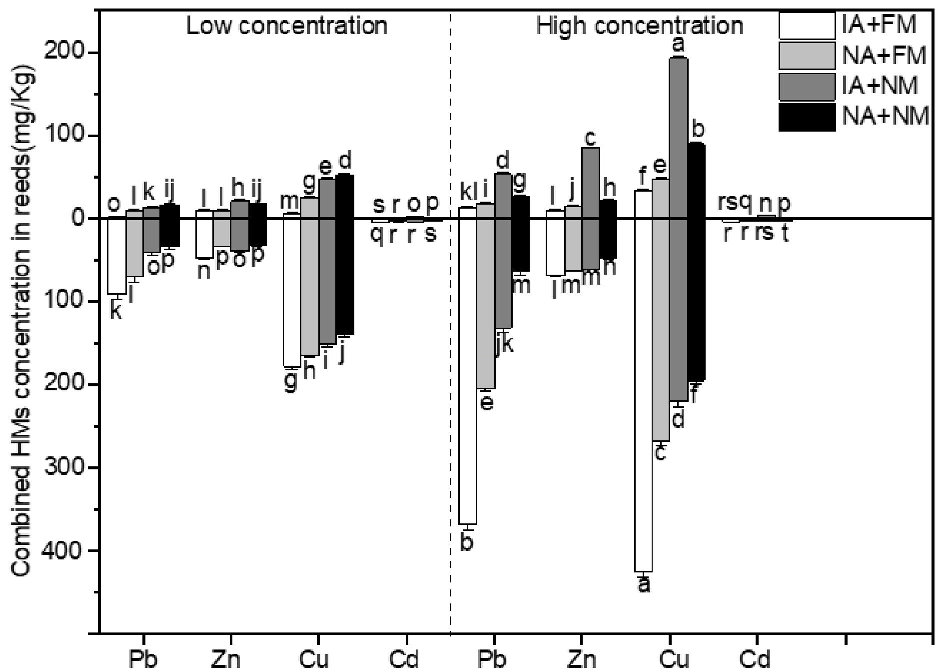

Figure 2

The distribution of combined HMs in the aboveground and underground parts of reeds at different concentrations. IA: intermittent aeration; NA: no aeration; FM: inoculation with *F. mosseae*; NM: inoculation with autoclaved *F. mosseae*. Different letters show the significant difference (P<0.05).

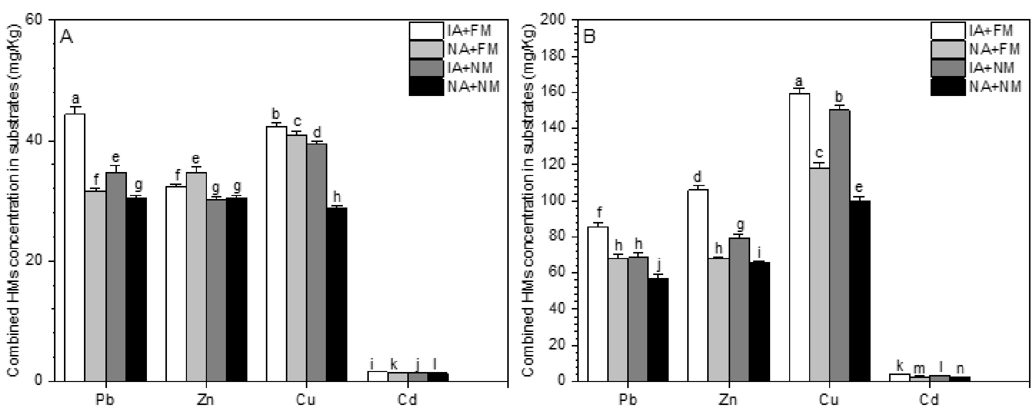

Figure 3

The accumulation of substrates on combined HMs in different VFCWs. A shows wastewater with low concentrations of combined HMs (Pb: 2 mg/L, Zn: 5 mg/L, Cu: 2.5 mg/L, Cd: 0.5 mg/L); B shows wastewater with high concentrations of combined HMs (Pb: 20 mg/L, Zn: 50 mg/L, Cu: 25 mg/L, Cd: 2.5 mg/L). IA: intermittent aeration; NA: no aeration; FM: inoculation with *F. mosseae*; NM: inoculation with autoclaved *F. mosseae*. Different letters show the significant difference (P<0.05).

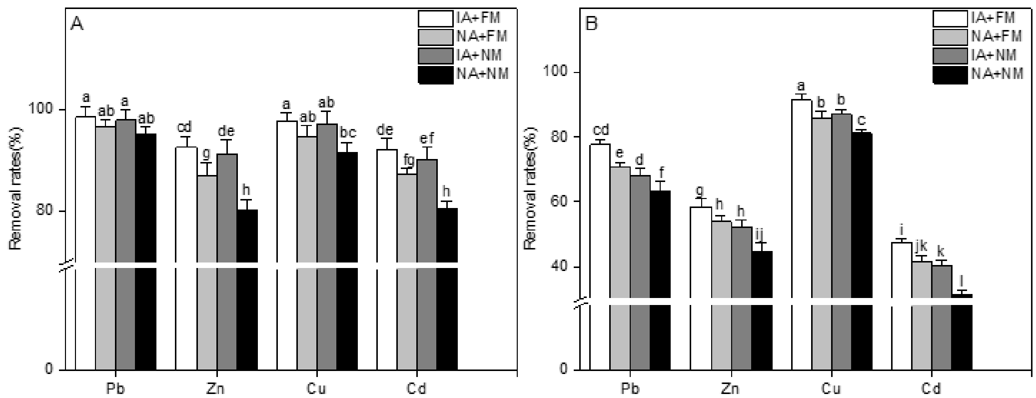

Figure 4

Purification effects of different VFCWs on combined HMs polluted wastewater. A shows the removal rates at low concentrations of combined HMs polluted wastewater (Pb: 2 mg/L, Zn: 5 mg/L, Cu: 2.5 mg/L, Cd: 0.5 mg/L); B shows the removal rates at high concentrations of combined HMs polluted wastewater (Pb: 20 mg/L, Zn: 50 mg/L, Cu: 25 mg/L, Cd: 2.5 mg/L); IA: intermittent aeration; NA: no aeration; FM: inoculation with *F. mosseae*; NM: inoculation with autoclaved *F. mosseae*. Different letters show the significant difference (*P*<0.05).

Figure 5

The transmission electron microscope images of reed roots of different treatments. A shows the normal root cell of reed; B shows the root cell morphology of reed not inoculated with AMF under the combined HMs stress of high concentrations; C, D, E, and F show the root cell morphology of reed inoculated with AMF under the combined HMs stress of high concentrations. i: nucleolus, h: nucleus, g: cell membrane, k: the deformation of cell wall, l: the helical curl of cell membrane, j: the thickened cell walls.

Figure 6

The combined HMs mass balance in different VFCWs. IA: intermittent aeration; NA: no aeration; FM: inoculation with *F. mosseae*; NM: inoculation with autoclaved *F. mosseae.*

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