CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

FACULTY OF ENVIRONMENTAL SCIENCES

LEAD TRANSFORMATION IN ARBUSCULAR MYCORRHIZAL ASSISTANT CONSTRUCTED WETLANDS

DIPLOMA THESIS

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Faculty of Environmental Sciences

DIPLOMA THESIS ASSIGNMENT

Meijun Chen

Geology

Environmental Geosciences

Thesis title

Lead transformation in arbuscular mycorrhizal assistant constructed wetlands

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Objectives of thesis

Investigate the transformation process of lead in constructed wetlands system with the addtion of arbuscular mycorrhizal.

Methodology

Setup several pot experiment to explore the transformation process of lead.

1.Set up two different types of wetland plant and two different water conditions(one water table is 3cm and other is 7cm)

And found out the best plant and water conditions for wetland plant growth.

2.Add different concentration of lead(5mg/l and 10mg/l)in the plants to study how wetland plants help decreasing lead containment and transformation of lead in wetlands.

The proposed extent of the thesis

50

Keywords

Lead, constructed wetland, arbuscular mycorrhizal

Recommended information sources

Acton, Q. A., ed. (2013). Issues in Global Environment—Pollution and Waste Management: 2012 Edition. Scholarly Editions. ISBN 978-1-4816-4665-9.

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Zhongbin Chen, Yi Chen, Jan Vymazal, Lumir Kule, Milan Kozeluh(2017), Dynamics of chloroacetanilide herbicides in various types of mesocosm wetlands,Science of the Total Environment 577 386-394.

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Dean

Prague on 21. 03. 2020

DECLARATION

I hereby declare that I have written this diploma thesis titled “Lead transformation in arbuscular mycorrhizal assistant constructed wetlands” independently under the direction of Zhongbing Chen. I have listed all literature and publications from which I have acquired information in the reference section.

In Prague, Czech Republic of 25.06.2020

Meijun Chen

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Thanks,

Meijun Chen

Abstract

Heavy metal pollution in water has gained many focuses in recent years. But arbuscular mycorrhizal fungi (AMF) assist plants to remove heavy metals from water in constructed wetlands (CWs) has been rarely studied, and the heavy metal transformation in wetlands with AMF has been poorly reported. This study investigated how lead (Pb) transformed in wetlands with the assistance of AMF. Eighteen pots experiment were settled at the campus of the Czech University of Life Science (CULS). *Iris wilsonii* were planted in pots in May 2019. Plant samples were collected in November.

The results showed AMF colonization decreased by 7.11% – 50.9% under Pb stress, but still sufficient enough to be functional for plants. AMF significantly increased plant growth, with root length, shoot height, root weight, shoot weight, phosphorus concentration, and potassium concentration increased by 19% – 39%, 3% – 16%, 44% – 67%, 20% – 86%, 11% – 23%, and 11% – 19%, respectively. AMF increased photosynthesis properties of plants such as photochemical efficiency and leaf chlorophyll contents, by 4% – 65% and by 6% – 39% under Pb stress respectively. The enzyme activities of POD, SOD and soluble protein contents were enhanced by 0.5% – 457%, 27% – 89%, and 36% – 103% respectively, and MDA and $O_2^{\cdot-}$ were decreased by 8% – 44% and 19% – 42% respectively. These findings suggested AMF could protect plants from oxidative stress through increasing activities of antioxidative enzymes to remove reactive oxygen species (ROS). Furthermore, AMF increased Pb accumulation in roots by 1% – 19%, but decreased in shoots and substrates (by 45% – 53% and 11% – 26% respectively). AMF bound most of Pb in roots, declined the transformation from roots to shoots and decreased the accumulation in substrates, which decreased the heavy metal contamination in wetlands. Therefore, AMF played an important role in assisting the Pb transformation of plants in CWs.

Keywords: Arbuscular mycorrhizal fungi; Pb transformation; Constructed wetlands; Heavy metals pollution; Reactive oxygen species; Antioxidative enzymes

CONTENTS

1. Introduction

1.1 Background

Heavy metal pollution became an important issue due to the damage to the ecosystem. Increased heavy metal pollution may because of human activities, for example, mining and smelting, battery manufacturing, disposal or recycling and burning fossil fuels (Tiwari et al. 2013). Due to the lack of advanced mining and environmental protection technologies, lead (Pb) has caused serious environmental pollution. As one of the abundant and pure toxic heavy metals, a large number of Pb has a high potential to contaminate water and inhibit plant growth (Tiwari et al. 2013).

Pb may inhabit photosynthesis progress of plants. It may decrease chlorophyll content and damage photosystem II (Kushwaha et al. 2018 ex. Qufei and Fashui, 2009). It can increase the production of reactive oxygen species (ROS), increase oxidative stress and affecting antioxidant protection. It can directly connect with protein lead to interrupt physiological pathways. It occupies the same pathway of important elements that caused related function loss (Sharma and Dietz, 2009). But plants improved many ways to decrease heavy metal toxicity. One way is to remove ROS by increasing antioxidant enzymes, for example, superoxide dismutase (SOD), peroxidase (POD). Another way is to enhance heavy metal tolerance with the assist of Arbuscular mycorrhizal fungi (AMF) (Yang et al. 2015 ex. Joner and Leyval, 2001).

AMF are common rhizosphere microbes that can form a beneficial symbiotic association with plants by inner root colonization. AMF may enhance the photosynthesis process of plants (Ramírez-Viga et al. 2018). AMF take carbohydrates from host plants and in return provide them with nutrient such as nitrogen (N), phosphorus (P) (Wu et al. 2016a). AMF may have the ability to be a bio-fertilizer to stimulate growth, gain water and uptake nutrients in plants thus increase the tolerance to heavy metals (Wang et al. 2018). AMF could extend their

mycelia which increases the effective absorbing zone of root. Therefore, use an appropriate plant that can absorb a high amount of heavy metals and with the assist of AMF can be a way to remove heavy metals of water in wetlands (Xu et al. 2018; Gunathilaka et al. 2018). However, the exact role of AMF in improving tolerance to heavy metals is unknown.

Wetland is a unique ecosystem between land and water, it can provide ecology functions. Wetland sediments can act as a natural sink to absorb the contaminants such as heavy metals from the industrial areas or agriculture areas (Zheng et al. 2015). Constructed wetland (CW) is an engineered system to moderate natural wetland to enhance the wastewater treatment process using plants, substrate and microbes in wetlands (Chen et al. 2017). Wetland plants are important for heavy mental removal because they can uptake and absorb heavy metals. But the aboveground of plants is the transportation of heavy metal to the water column of wetland thus can cause contamination. Therefore, heavy mental transformation in the wetland is becoming a popular topic in order to study how to decrease contamination and heavy mental removing mechanism in wetlands. In addition, the potential of AMF of being a barrier to decline transformation heavy mental from roots to shoots in contained soil has been poorly studied (Zheng et al. 2015), and the potential in wetlands is largely unknown.

1.2 Aim

The aim of this thesis is to study the transformation of Pb in wetlands with the assistance of AMF, and the influence of AMF on plant growth and physiological functions in wetland plant under Pb stress.

2. Literature review

2.1 Heavy metals pollution

Environmental pollution has gained many concerns for many years. Environmental pollution was defined as the physical and biological components of the earth or atmosphere system that are contaminated affecting regular environmental processes (Muralikrishna et al. 2017). Environmental pollution included air pollution, water pollution, land pollution and so on (Muralikrishna et al. 2017). Heavy metal pollution is also one kind of environmental pollution which gained much attention for some years. Heavy metal pollution is an anthropogenic problem that humans strive to solve. It is the main problem in European soil and groundwater (Panagos et al. 2013). Human activities like mining and some industries released mobile and bioavailable forms of heavy metal (Vareda et al. 2019 ex. Adriano, 2001). Heavy metal is a group of elements that density higher than 7g cm^{-3}. Heavy metal includes lead (Pb), zinc (Zn), copper (Cu), mercury (Hg), cadmium (Cd), chromium (Cr), selenium (Se), silver (Ag), and nickel (Ni) and so on (Kushwaha et al. 2018).

As one of the most abundant heavy metals, Pb has become the most widely distributed toxic heavy metal in the world due to human action (Cheng and Hu, 2010). Pb could contaminate water from mining and smelting, and corrosion of plumbing (Kushwaha et al. 2018). After getting into the water, Pb would stay in the water system and accumulate in sediment, which may become the second source of environmental pollution (Zhang et al. 2012). Pb could get into the body of human beings through contaminated air, soil, water and food (Needleman, 2004). It could affect human blood pressure, lungs, liver, kidneys, bone marrow and central nervous systems (Needleman, 2004).

Pb is toxic to plants. Pb may affect photosynthesis by decreasing chlorophyll and carotenoid content and damaging photosystem II. It may decrease the activities of enzymes like phosphatase, ATPase. Pb could decrease the water content in plants. And it may disturb replication and repair mechanisms (Kushwaha et al. 2018).

The toxicity of Pb to plants can be summarized by three reasons: 1) increase production of reactive oxygen species (ROS), increase oxidative stress and affecting antioxidant protection; 2) direct connection with protein lead to interrupt

physiological pathways; 3) occupation the same pathway of important elements that caused related function loss (Sharma and Dietz, 2009). ROS are essential signal molecules in stress response and remaining basic physiological functions in plants (Mittler, 2017). ROS are very active, they can damage regular metabolism by oxidative damage to protein, lipids and nucleic acids if there is no protection mechanism (Rout and Shaw, 2001).

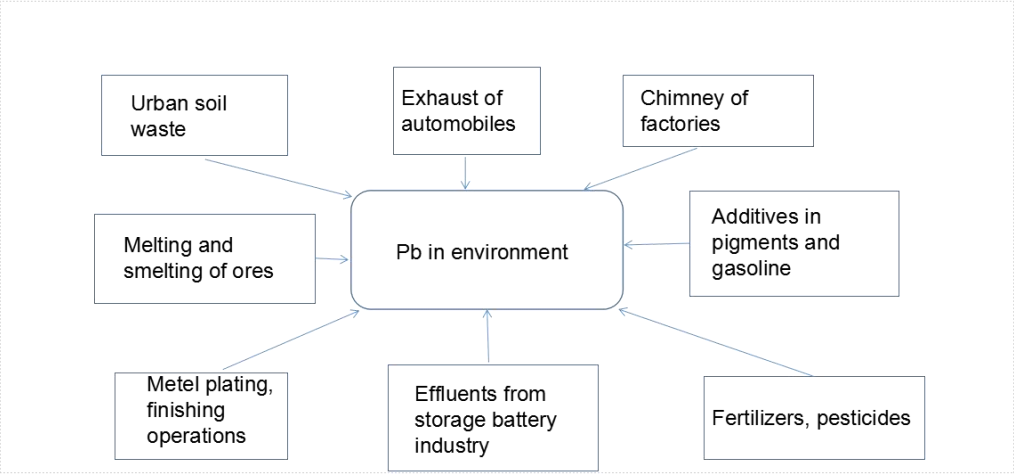

Fig. 1 Various sources of Pb pollution in the environment (modified from Sharma and Dubey, 2005)

2.2 Pb transformation in plants

Pb is barely soluble, therefore could easily uptake by the plant (Kushwaha et al. 2018). Plant factors such as root surface area, root exudates, mycorrhization and transpiration rate influence the utilization and absorption of Pb. Microbes and ectomycorrhiza may also affect the availability of transport and toxicity of Pb through biosorption, bioaccumulation and dissolution processes (Sharma and Dubey, 2005).

Pb uptake by plants is mainly through the root (Sharma and Dubey, 2005). Pb is absorbed by the root surface first and then it will enter the root (Kushwaha et al. 2018). After it getting in the root system, it may stay in the root or goes to the upper part of the plant (Kushwaha et al. 2018). Most portion of Pb accumulate in the root

(around 98% or more), then the rest little portion of Pb translocated to other parts of plants (Kushwaha et al. 2018). The concentration of Pb in plant organs increases in the following order: seeds, inflorescence, stem, leaves, roots (Sharma and Dubey, 2005).

Pb remains in the roots is because Pb immobilizes on the ion exchangeable sites and extracellular precipitation on the cell wall (Sharma and Dubey, 2005). Pb mainly moves into the root apoplast and moves through the cortex in a radial manner and gathers close to the endodermis (Sharma and Dubey, 2005). The root endoderm could be a barrier to limit the transportation of Pb from root to other organs (Sharma and Dubey, 2005).

2.3 Constructed wetlands (CWs)

2.3.1 Definitions

Wetland is a transitional ecosystem between water and land (Cowardin, 1979), where the groundwater level is always at or close the surface, or the land is covered by shallow water. The only character of most wetlands is they are at least saturated with water or covered by water (Cowardin, 1979). Depend slightly on the geographic and topographic location of wetland, the wetland has different functions on the ecosystem. It has the following functions in general: water storage (flood control), groundwater refilling, reservoirs of biodiversity, climate change modification, trap sediments and heavy metals (United Nations Millennium Ecosystem Assessment and Ramsar Convention).

CW is an engineered system designed for wastewater treatment with moderated soil, plants, microbial as natural wetlands (Vymazal, 2005). They are designed to take advantage of natural wetland and make it easier to control (Vymazal, 2014). Some of the CW has been built only to treat wastewater, some others have multiple functions, for example, also for being a habitat for animals and for recycling in agriculture. A constructed wetland can be also called artificial wetland, man-made wetland or

engineered wetland (Vymazal, 2014).

2.3.2 Types of CWs

CW can be sorted by different criteria, but there are three main criterions. They are macrophyte growing type, hydrology (surface flow and sub-surface flow), and the flow way in sub-surface wetlands (horizontal and vertical). Different types of CWs can be combined to gain a specific function (Vymazal, 2005; 2008).

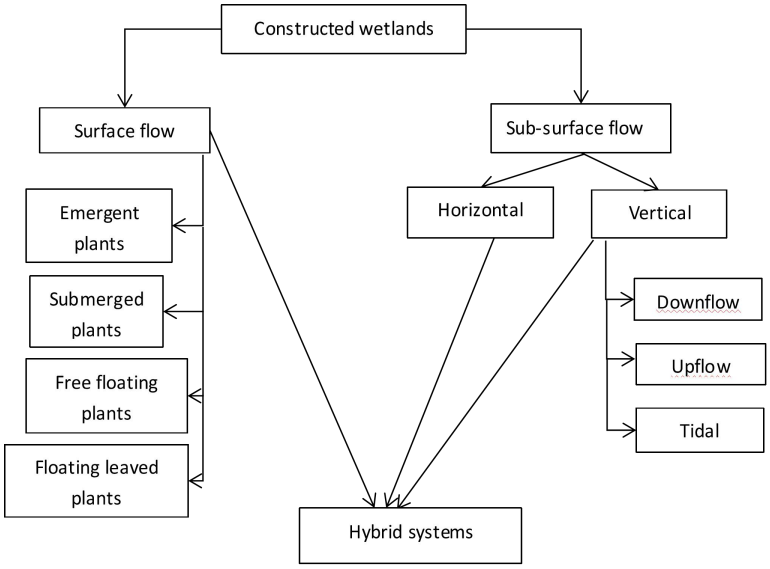

Fig. 2 Classification of constructed wetlands for wastewater treatment (Modified from Vymazal et al. 2008)

Surface flow CWs

CWs with the surface flow (FWS CW) composed of basins (or channels) with soil (or other substrates) to assist the plants and water flow through CW with shallow depth (Vymazal, 2014 ex. Reed et al. 1988). FWS CW with emergent macrophytes can be used as a biological treatment system. FWS CWs could help to remove organic compounds, nitrogen and phosphorus. It can also remove suspended solids by filtrating, precipitation, aggregation and surface adhesion. Wetland vegetation can help the precipitation process by decreasing water mixture and resuspension of particles on the surface of the precipitation (Vymazal, 2014).

FWS CW can remove heavy metals. Heavy metals can be absorbed in plants and soils in wetlands. The study has proved FWS CW could remove Cd, Ni, Pb, Cu, Zn and Fe and they were mostly accumulated in roots compared with shoots. And there is no obvious heavy metals accumulation in the vertical soil profile (Lavrnic et al. 2018). In addition, FWS CWs are suitable to remove Fe, Mn, Hg and Ag in wastewater (Vymazal, 2005; Ghermandi et al. 2007).

Horizontal sub-surface flow CWs (HFCW)

In HFCWs, the wastewater flows horizontally via the porous medium under the surface. It has aerobic, anaerobic and anoxic zones. Aerobic zones appear surrounding the roots and rhizomes, releasing oxygen to the substrate (Vymazal, 2014 ex. Cooper et al. 1996).

HFCW can help to remove suspended solids, organic matter, nitrogen and phosphorus in wastewater. HF CWs could also remove heavy metals which various studies have proved. Vymazal (2005) studied HF CWS removed A1 (>98.9%), Zn (94.1%), and Cr (>92.8%), Cu (>75%), Pb (>73%), Ni (55.7%). Gill et al. (2014) studied runoff loads over 6 years period found 73% (Zn), 60% (Cu), 20% (Pb) and 7% (Cd) were removed. Lesage et al. (2007) assessed domestic wastewater treated in HF CWS for three years of operation, and found more than 84% of Al, Cu, and Zn were removed.

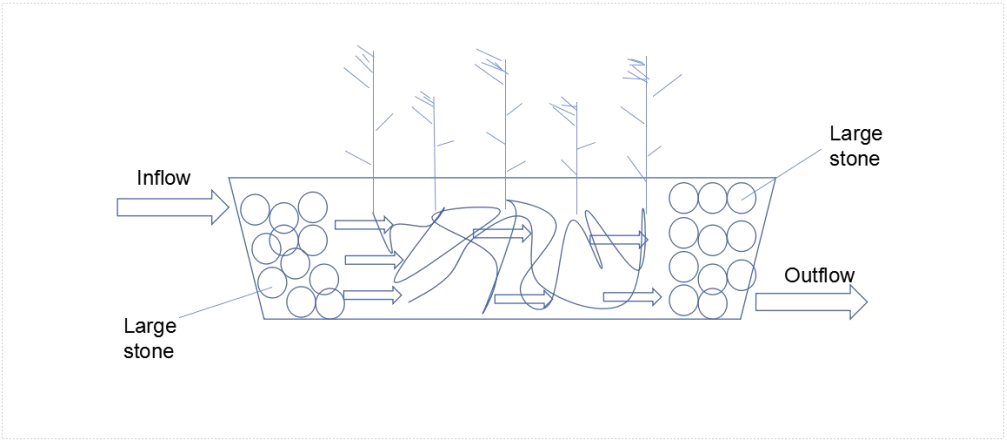

Fig. 3 Constructed wetland with horizontal subsurface flow (modified from Vymazal, 2001)

Vertical sub-surface flow CWs

Vertical flow (VF) CW is a type of CW that water flows vertically by filtrating between gravel or sand. It is with largely discontinuous inflow, thus it has a good oxygen transmission and the capability to nitrify (Vymazal, 2014 ex. Cooper et al. 1996). The oxygen transmission provides much more oxygen to the filtration bed than the plant stomata system (Vymazal, 2014). Most of VF systems are staged systems with several similar beds (Vymazal, 2014 ex. Cooper, 1999).

VF CWs could remove heavy metals in wastewater. For example, Mustapha et al. (2018) found *Cyperus alternifolius, Typha latifolia,* and *Cynodon dactylon* all have the ability to remove Cu, Zn, Pb, Fe, Cd and Cr from the refinery wastewater in VF CWs, but the one planted with *T. latifolia* had the best heavy metal removing results. Most metals were accumulated in roots, followed by leaves and stems. Lee and Scholz (2006) reported Cu and Ni had significant removal efficiency in VF CWs. Aslam et al. (2007) found high removal efficiency of Fe^{2+}, Cu^{2+}, Zn^{2+} in wastewater from an oil refinery in Pakistan. Plants took up 35-56% amounts of heavy metals.

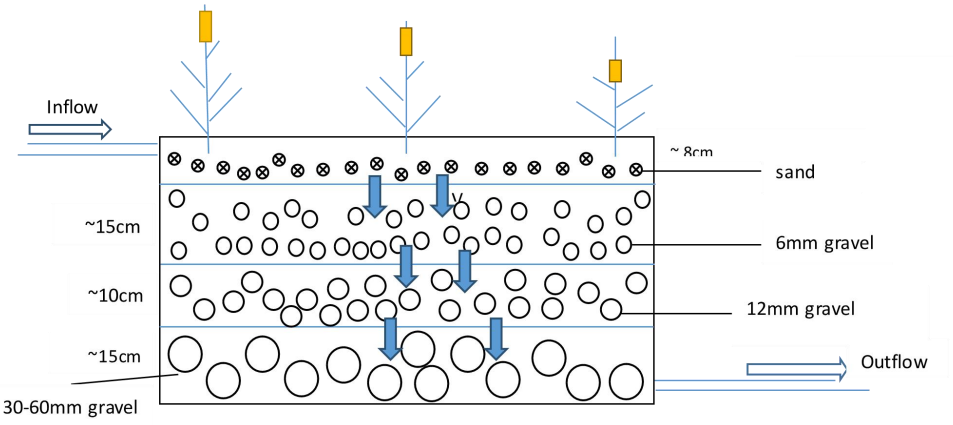

Fig. 4 Typical arrangement of a downflow vertical-flow constructed wetlands (Modified from Vymazal, 2014 ex. Cooper et al. 1996)

Hybrid CWs

It is a type of combined wetland system that always composed two-stage of VF beds (“filtrating beds”) and follow with two or three HF beds (“removing beds”) (Vymazal, 2014). The VF beds are always planted with *Phragmites australis*, however, the HF beds are with emergent macrophytes, such as *Iris*, *Typha* and *Sparganium* (Vymazal, 2014). Hybrid CW is always with HF and VF, but it can be also combined with other CWs (Vymazal, 2014). In hybrid systems, it can have various advantages of different CWs (Vymazal, 2014 ex. Cooper, 1999).

2.4 Arbuscular mycorrhizal fungi (AMF)

AMF is a tight, interconnected association that forms in the roots of plants and soil fungal members (Smith and Read, 2008). The association allows nutrient exchanging (carbohydrates given by the plant, mineral nutrients are given by the fungi) (Fester, 2012). AMF is a major part of soil microbe, occupied 30% of soil microbial biomass (Xu et al, 2016 ex. Olsson et al. 1999).

AMF may have a lot of positive effects on host plants, for example, it may improve plant growth by increasing nutrients such as nitrogen and phosphorus (Zhang et al. 2020 ex. Seshadri et al. 2017). AMF might improve the photosynthesis process (Zhang et al. 2020). It may improve the heavy metals, salinity, drought tolerance of plants (Wężowicz et al. 2015). AMF may decrease the damage caused by environmental stress through decreasing lipid peroxidation and increasing antioxidant enzymes (Zhang et al. 2010). It could affect the transportation and distribution of pollutants in plants (Schneider et al. 2013). In addition, it may improve the microbial activity of the host rhizosphere, the stability and diversity of the plant community (Xu et al, 2016 ex. Artursson et al. 2005).

2.4.1 Possibility of applying AMF in CWs

AMF can be considered as an ideal assistant for phytoremediation of contaminated water (Xu et al. 2016). For example, AMF could be support for phytoremediation of benzene and ammonia in contaminated groundwater in CW

(Fester, 2013). AMF could help the wetland plants to remove organic pollutants and nutrition in wastewater (Calheiros et al. 2019).

AMF has the potential to assist plants in CWs to remove heavy metals in wastewater. For example, Gunathilaka et al. (2018) found AMF can be used to assist *Eichhornia crassipes* to remove Cd in wastewater by making plants more effective. It was observed a high growth rate, high concentration of Cd in roots and shoots, high dry biomass of roots and shoots in AMF inoculated treatment. And Wężowicz et al. (2015) reported AMF could help *Iris pseudacorus* to remove Pb, Fe, Zn, and Cd from industrial water by increasing stress tolerance of plants. Xu et al. (2018) found VFCWs with AMF have significantly higher efficiency to remove Cd and Zn in wastewater than without AMF. The efficiency of removal Cd and Zn in controls were 78.56% and 73.01%, and the efficiency of removal Cd and Zn with AMF were 95.56% and 86.88% respectively.

2.4.2 Factors affecting the application of AMF in CWs

A lot of factors affect the application of AMF in CWs, for example, flooding (hydrologic condition), phosphorus, salinity, plant species and aerenchyma, CW types, the quality of wastewater and so on (Xu et al. 2016).

Flooding (hydrologic condition)

There are different saying about flooding effect on AMF colonization. Miller (2000) and Wang et al. (2010) (Xu et al. 2016 cited) found there is a decreasing AMF colonization with flooding, Wirsel (2004) even found continuing flooding could cause zero colonization. Colonization decreased because flooding conditions might affect root morphology and physiology (Xu et al. 2016). Controversially, Miller and Bever (1999) (Xu et al. 2016 cited) reported the wettest part of the hydrological gradient in the Gulf of Florida, USA, was found to have the largest total number of fungal spores. AMF might decrease the effect of flooding by enriching oxygen in the root or rhizosphere (Xu et al. 2016). Therefore, flooding condition is variable in different situations, but it is essential for AMF colonization. It is important to take

into account the flooding condition in CWs regarding the AMF colonization.

Phosphorus (P)

Phosphorus level in the rhizosphere is a major abiotic factor that influences the AMF colonization in roots (Xu et al. 2016). But AMF colonization and P level of the environment have a complicated relationship. *Typha angustifolia* is colonized in low-phosphorus treatment but does not exist in high-phosphorus treatment (Xu et al. 2016 ex. Tang et al. 2001). However, *Carex Lasiocarpa* and *Typha latifolia* have no mycorrhizal in a low P condition (Xu et al. 2016 ex. Cornwell et al. 2001). So Wang et al. (2010) (Xu et al. 2016 cited) suggested that there is a "bell-shaped" relationship between AMF colonization and soil P in wetland ecosystems, that is, AMF colonization is inhabited at high or low P levels. Therefore, attention should be paid to the content of phosphorus in CW to ensure AMF colonization.

Operation modes of CW

Intermittent operation of water flow, variation of wet and dry, and aeration would supply oxygen to CW, which brings benefit to the growth, richness and variety of AMF (Xu et al. 2016). For example, Miller (2000) (Xu et al. 2016 cited) found the AMF colonization is higher in intermittent flood conditions than continuous flood conditions. In addition, study found adjustment of operation modes in SFCW could improve the oxygen transfer capacity to provide enough oxygen for microorganisms (including bacteria and AMF) to eliminate pollutants. These operations include frequent fluctuations in water levels (tidal flow), passive air pumps (vertical flow), or direct aeration of water in gravel-bed (horizontal flow) (Xu et al, 2016). Therefore, operation modes of CW and appropriate water depth are important for AMF colonization.

3. Materials and methodology

3.1 Experimental design

The experiment was carried out in pots with the size of 17 × 15cm (diameter × height), fine sand (1-2 mm) was filled in as substrate, *Iris wilsonii* was selected as experiment plant, water depth varied between 3cm and 6cm (water depth from bottom to top) (Fig. 5). In total, 18 experimental pots were located in the Czech University of Life Science (CULS) with rain protected. Each pot was cultivated with 100ml 1/4-strength Hoagland solution (diluted with deionized water) once a week to supply nutrients. Hoagland solution contained boron, calcium, copper, iron, potassium (K), magnesium, manganese, molybdenum, zinc, chloride, nitrate (NO_3^-), phosphate (PO_4^{3-}), sulfate, ammonium (NH_4^+), nitrogen. Experimental treatments were blank (0 mg/kg Pb, no AMF), blank with AMF (M), 500 mg/kg Pb (Pb500), 500 mg/kg Pb with AMF (M Pb500), 1000 mg/kg Pb (Pb1000), 1000 mg/kg Pb with AMF (M Pb1000). Each treatment had three replications. *Iris wilsonii* was planted in May 2019. Meanwhile, AMF was added with sand materials, different concentrations of Pb solutions were added in September, and samples were collected in November.

The whole plants including roots and shoots and the sand materials were collected. As for plants, AMF colonization in the roots was determined. The height and biomass of each plant samples were measured as basic growing states. Physiological indexes of plants were determined including superoxide anion ($O_2^{\cdot-}$), activities of peroxidase (POD), superoxide dismutase (SOD), leaf chlorophyll, photochemical efficiency of photosystem II, soluble protein contents, malonaldehyde (MDA). Moreover, Pb, K and phosphorus (P) concentration in both roots and shoots were also measured. As for sand materials, pH and Pb concentration were determined.

Fig. 5 Experimental pot

3.2 AMF colonization

AMF colonization was accessed according to the description of Phillips and Hayman (1970). Firstly, 0.5-1g root samples were washed, cut it to be around 1cm afterward. Then root was heated at 90℃ in 10% KOH for one hour. Rinsed with 2% HCl around 5 minutes. Stained for 5 minutes in 0.05% trypan blue with lactophenol then heated at 90℃ for 30 minutes. Discoloration in a petri dish with lactic acid glycerol. Later taken 30 root sections for slice preparation and observed with a 100×400 microscope. The mycorrhizal colonization (M%), vesicle colonization (V%), and the arbuscular abundance (A%) were calculated with MYCOCALC software.

3.3 Photosynthesis properties

Leaf chlorophyll

The content of leaf chlorophyll was determined with acetone by absorbance at 663nm and 645nm using a spectrophotometer according to Arnon (1949). 0.1g leaves, 5ml of extraction solution (80% acetone) and a small amount of quartz sand was ground in a mortar. The mortar was washed with 5mL of extraction solution. The cleaning solution was transferred to the centrifuge tube, and extracted for 24 hours in the dark. Centrifuged the supernatant for analyzing. Used the extraction solution as a

blank, measured A645 (A1), A663 (A2). Chlorophyll a (Chla) = W*(12.78 *A2-2.69*A1); Chlorophyll b (Chlb) =W*(22.9*A1-4.68*A2); Total chlorophyll CT= W*(8.02*A2 + 20.21*A1), W was the weight of leaf.

Photochemical efficiency of photosystem II

Photochemical efficiency contains potential efficiency (Fm/Fv) and actual efficiency (Yield). It was directly measured by senor machine with plastic clips and steel clips respectively on the same leaf (top of the leaf). The optimal value is around 0.8, and if it is lower than 0.8 means they are under environmental stress.

3.4 Lipid peroxidation and reactive oxygen species

Malondialdehyde (MDA) concentration

MDA is a widely used marker to test environmental stress. 0.5 g leaves, 5 ml 10% TCA (Trichloroacetic acid) and liquid nitrogen were added and grind in a mortar. Then centrifuged at 2000 rm for 10 minutes. The supernatant was the sample. Then 2ml of the centrifuged supernatant (blank is 2 ml of distilled water) were pipetted and added 2 ml of a 0.6% thiobarbituric acid solution in a tube, and then mixed for 15 minutes on a boiling water bath. After cooling quickly, centrifuged again. The absorbances at 532 nm, 600 nm and 450 nm wavelengths of supernatant were measured (Dhindsa et al. 1981; Hodges et al. 1999). MDA= [6.45(A532-A600)-0.56A450]VW-1,V: the whole volume of extraction solution (mL), W: fresh plant weight.

Superoxide anion (O_2^-)

The content of superoxide anion (O_2^-) was measured on the basis of Elstner and Heupel (1976) with some modifications. 1 g sample, 5 mL 50 mmol/L phosphate buffer saline (PBS) (pH 7.8) and a little quartz sand were ground and centrifuged for 10 minutes at 1000 r/min. Kept the supernatant as the sample extraction solution. 2.0 mL of sample extraction solution was added 1.5 mL of PBS and 0.5% hydroxylamine

hydrochloride solution, and reacted for 20 minutes at 25 degrees. Took 2.0 mL of the above reaction solution, then added 4 mL of p-amino benzene sulfonic acid and 0.4 mL of α -naphthylamine in this order, and kept it in a constant temperature water bath for 30min. Then A530 was measured. The concentration of NO_2^- could be calculated with NO_2^- standard curve, which had the same steps as the sample but with a known concentration of NO_2^-. O_2^- content (μ g/g) = 2X*Vt*n/(FW*Vs), Vt was the volume of the sample extraction solution, n was the dilution factor of the sample extract during the measurement; FW was the sample fresh weight (g); Vs was the sample solution volume (mL) for measurement; X was the NO_2^- concentration calculated from the standard curve.

3.5 Antioxidant enzyme activities

3.5.1 Superoxide dismutase (SOD)

SOD is an enzyme that could catalyze superoxide to oxygen and hydrogen peroxide (H_2O_2) by a disproportionation reaction (Giannopolitis et al. 1977). It was measured according to Garcia-limones et al. (2002) and Giannopolitis et al. (1977). The enzyme solution was prepared with 0.2 g samples (fresh leaves and roots separately) and 1.6 mL of 50 mmol / L pre-chilled PBS (pH 7.8), grind and centrifuged and then kept supernatant. Test tube: 3.5 mL buffer solution + 0.5 mL methionine solution + 0.5 mL nitrogen blue tetrazolium solution + 0.5 mL EDTA-Na_2 solution + 0.4 mL distilled water were added in order. Then added 0.1 ml enzyme and 0.5 ml riboflavin solution; two blank tubes were added to the same solutions as the test tube except for the enzyme solution, substituted enzyme solution with distilled water. One test-tube was irradiated and measured as the maximum light reduction tube, and the other one was placed in the dark for zero adjustment. One blank tube was put in a dark place, and the rest of tubes in a light incubator under 400lux light for 20 minutes. After zero adjustment with a blank tube which was put in the dark, measured OD560 without light (when it appeared color then it can be

measured). Total SOD activity = [(A_b-A_e) * v] / (1 / 2A_b*w*v_e); (A_b = OD560 of the blank tube in light condition; A_e= OD560 of test tubes; v is the sample solution volume; v_e is the amount of enzyme solution; w is the fresh weight of the sample).

3.5.2 Activities of peroxidase (POD)

Peroxidase is a group of enzymes which is very essential to biological processes. It was determined according to Fielding and Hall (1978). The enzyme solution was prepared with 0.2 g sample (roots and shoots individually) and 1.6 mL of 50 mmol /L pre-chilled PBS (pH 7.8) grind and centrifuged. Reaction mixture solution was prepared with 1000 mL PBS (100 mM, pH 6.0), 0.56 mL liquid guaiacol and 0.38 mL H_2O_2. 6 ml reaction mixture solution+2 ml H_2O was as a blank sample. Test samples contained 6ml reaction mixture solution+2 ml enzyme solution. OD470 was determined 10 seconds after adding the enzyme solution, and it was read every ten seconds, totally read for 4 minutes, 8 times. Then POD= (△ A*v)/(w*v_e*0.01*t), which △A was the change in absorbance value of 470nm during the reaction time; w was the fresh weight of the sample (g); t was the reaction time (min); v was the volume of the extracted enzyme solution (mL, 1.6 mL); v_e was the volume of the enzyme solution used in the measurement (mL, 30 μL).

3.5.3 Soluble protein content

The soluble protein content of plants was measured with Coomassie Brilliant Blue G-250 according to the Bradford method (1976). 0.5 g fresh sample was ground with 5mL of distilled water or buffer solution. Centrifuged for 10 min at 3000 r/min, the supernatant was the sample extraction solution. Pipette 1.0 mL of sample extraction solution then added 5 mL Coomassie Brilliant Blue reagent, analyses absorbance at 595 nm after shaking 5minutes. The concentration of protein could be calculated with the standard curve. Protein content in the sample = c*v_t/(v_s* w *1000), c was the calculated value of the standard curve (μg); v_t was the volume of the extraction solution (mL); w was the sample fresh weight (g); v_s was the amount of sample added during the measurement (mL).

3.6 Pb, K and P concentrations in plants

Pb, K and P contents in plants (roots and shoots) were determined with the pseudo-total digestion method according to US EPA Method 3051A with some modifications. 0.2 g grind sample was added 2 ml H_2O_2 and 8 ml HNO_3 in this order then digested with an electric heating plate at 150℃ overnight. Later diluted at 25 ml with distilled water and filtered and then passed the sample for ICP-OES.

3.7 The pH and Pb concentration in the substrate

The pH

The pH values were determined with a pH meter according to Hanlon, E.A. CIR1081. The dried substrate was sieved with 0.710 mm sieve. Added 25 ml deionized water in a beaker. Measured the pH after 30 minutes standing with a pH meter.

Pb concentration

Pb concentration in substrates was determined by the pseudo-total digestion method (US EPA Method 3051A) as analyzed in plants. 500 mg sand materials were added to 2.5 ml hydrochloric acid (HCl) and 7.5 ml nitric acid (HNO_3). Then heated and filtered samples for ICP-OES.

3.8 Statistical analysis

Student t-test was used to analyze if there is a significant difference between two comparable treatments, for example, blank and AMF, M Pb500 and Pb500 and so on. $P<0.05$ was defined as a significant difference. All statistical analysis was performed on R studio Version 1.1.456 for windows.

4. Results

4.1 AMF colonization

The highest arbuscular abundance (A%) in roots was determined in AMF treatment, followed by the treatment of M Pb500 and M Pb1000 (Table 1). The values were 11.34%, 9.47%, 2.96% respectively. Pb decreased A% by 16.49% under Pb500 treatment, by 73.90% under Pb1000 treatment and by 68.74% compared between the treatment of Pb500 and Pb1000. The highest intensity of mycorrhizal colonization (M%) and vesicle colonization (V%) were determined in AMF treatment, and the lowest values were observed in the treatment of M Pb1000. M% and V% decreased with the increase of Pb addition in the treatments. Pb decreased M% by 7.11% under Pb500 treatment, by 50.9% under Pb1000 treatment and by 47.14% compared between the treatment of Pb500 and Pb1000. Pb decreased V% by 24.31% under Pb500 treatment, by 79.08% under Pb1000 treatment and by 72.61% compared between the treatment of Pb500 and Pb1000. In addition, from statistical analysis, the treatment of AMF and M Pb1000, the treatment of M Pb500 and M Pb1000 showed significant difference in M%, V%, A% ($p<0.05$).

Table 1 AMF colonization (mean±SD)

Treatments	M	M Pb500	M Pb1000
M%	64.20±4.13[a]	59.63±5.27[a]	31.52±2.29[b]
V%	19.74±3.89[a]	14.95±0.98[a]	4.13±1.82[b]
A%	11.34±0.90[a]	9.47±1.12[a]	2.96±1.24[b]

a, b shows significant difference among different Pb stress ($p<0.05$)

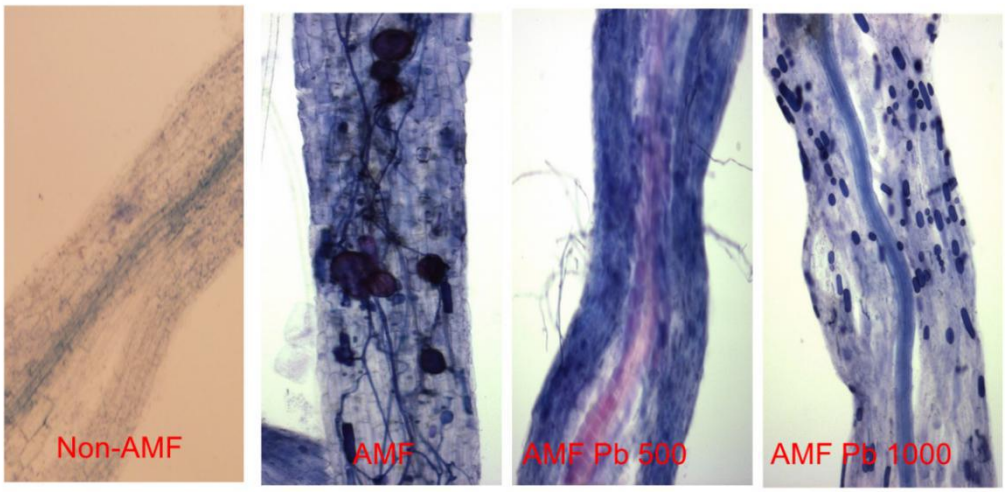

Fig. 6 AMF colonization in different Pb concentrations

4.2 Physiological behaviors in wetland plants under Pb stress

4.2.1 Plant growth

Biomass and height

The results presented in Table 3, the highest values of root length, shoot height, fresh root weight, fresh shoot weight, dry root weight and dry shoot weight were found in the treatment of AMF, the lowest values were observed in the treatment of 1000mg/kg Pb. AMF increased root length by 19% – 39% under Pb stress, increased shoot height by 3% – 16% under Pb stress, increased fresh root weight by 44% – 67%, increased fresh shoot weight by 20% – 86%, increased dry root weight by 54% – 98%, increased dry shoot weight by 26% – 123%. While Pb decreased root length by 16% – 30% without AMF, by 13% – 38% with AMF. It decreased shoot height by 10% – 32% without AMF, by 3% – 24% with AMF. And it decreased fresh root weight by 17% – 58% without AMF, by 3% – 56% with AMF. It decreased fresh shoot weight by 19% – 70% without AMF, by 1% – 55% with AMF. It decreased dry root weight by 6% – 65% without AMF, by 10% – 73% with AMF. It also decreased dry shoot weight 15% – 70% without AMF, by 5% – 47% with AMF. In addition, from statistical analysis plant growth showed a generally significant difference with

AMF and without AMF ($p<0.05$). Pb significantly decreased plant growth with or without AMF at high concentration of Pb addition (1000 mg/kg), but not significant at low concentration of Pb (500 mg/kg).

Table 2 Weight and height of plants (M: AMF) in different treatments

Treatments	Root length ± SD (cm)	Shoot height ± SD (cm)	Fresh root weight ± SD (g)	Fresh shoot weight ± SD (g)	Dry root weight ± SD (g)	Dry shoot weight ± SD (g)
Blank	15.3±1.5^{a}	61.7±3.8^{a}	43.2±3.0^{a}	22.0±2.6^{a}	7.3±0.7^{a}	5.4±0.4^{a}
M	20.3±1.5^{b}	63.7±4.9^{a}	62.3±3.7^{b}	26.4±1.8^{a}	14.4±2.4^{b}	6.9±0.5^{b}
Pb500	12.7±1.5^{a}	55.7±5.1^{a}	35.9±3.8^{a}	17.9±4.7^{a}	6.8±0.4^{a}	4.6±0.1^{a}
M Pb500	17.7±1.5^{b}	62.0±4.6^{a}	60.1±6.6^{b}	26.2±1.4^{a}	13.1±1.1^{b}	6.5±0.6^{b}
Pb1000	10.7±1.2^{c}	41.7±2.9^{b}	18.1±2.6^{c}	6.5±1.8^{b}	2.5±0.9^{c}	1.6±0.3^{c}
M Pb1000	12.7±0.6^{c}	48.3±1.5^{c}	27.7±3.1^{d}	11.9±0.8^{c}	3.9±0.9^{c}	3.6±0.3^{d}

a, b, c, d shows significant difference ($p<0.05$)

Leaf chlorophyll

The contents of leaf chlorophyll showed that the highest content of leaf chlorophyll of 3.57 mg/g was determined in blank with AMF addition, the lowest content was determined in the treatment of Pb1000. The difference of chlorophyll content between the treatment of blank and AMF were 0.19 mg/g, between the treatment of M Pb500 and Pb500 were 0.36 mg/g, between the treatment of M Pb1000 and Pb1000 were 0.33 mg/g. AMF increased leaf chlorophyll by 6% without Pb, by 14% under Pb500 treatment, by 39% under Pb1000 treatment. While Pb decreased leaf chlorophyll by 22% under Pb500 treatment without AMF, by 75% under Pb1000 treatment, by 68% compared between the treatment of Pb500 and Pb1000 without AMF. And Pb decreased leaf chlorophyll by 16% – 67% with AMF. AMF inoculated plants did not show a significant difference in chlorophyll content between non-inoculated plants, but Pb showed a significant difference with non-Pb

addition plants.

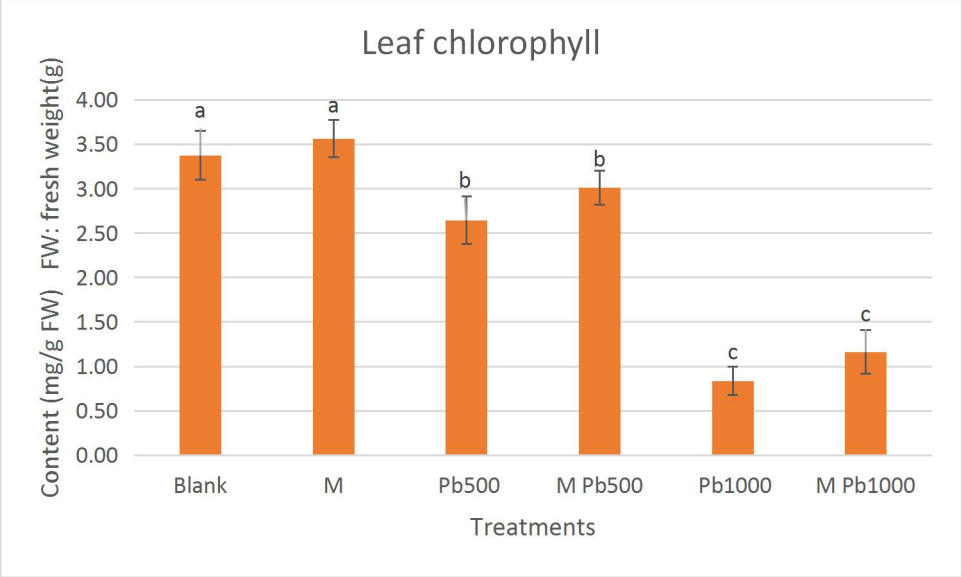

Fig.7 Leaf chlorophyll contents in different treatments

(a, b, c, show the significant difference ($p<0.05$))

4.2.2 **Photosynthetic efficiency of plants**

Photochemical efficiency of photosystem II

For potential efficiency (Fig.8), the highest efficiency was determined in the treatment of AMF, the lowest efficiency was determined in the treatment of Pb1000. The potential efficiency of the treatment in blank, Pb500 and M Pb500 were around 0.8. The values of Pb1000 treatment and M Pb1000 treatment were around 0.6. For actual efficiency (Fig.9), the value of AMF treatment was 0.78, which was the highest value. The actual efficiency of Pb1000 treatment was 0.36, which was the lowest value. AMF increased potential efficiency by 0.5% – 8% under Pb stress, and increased actual efficiency by 4% – 65% under Pb stress. While Pb decreased potential efficiency by 23% without AMF, and by 8% – 24% with AMF. And Pb decreased actual efficiency by 2% – 49% without AMF, by 6% – 23% with AMF. In addition, from statistical analysis, AMF significantly increased potential efficiency without Pb addition ($p=0.03$). While Pb significantly decreased potential efficiency

and actual efficiency ($p<0.05$).

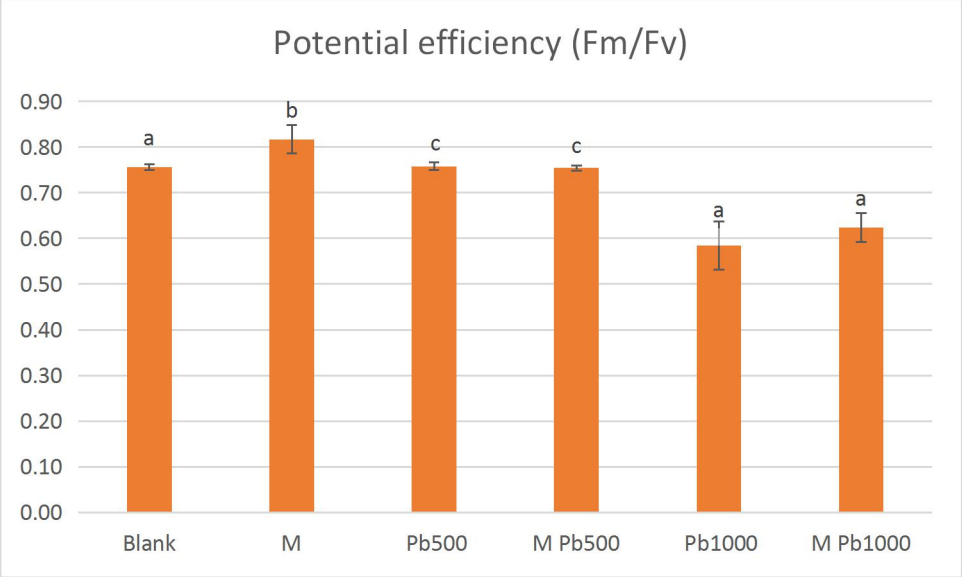

Fig.8 Photochemical efficiency of photosystem II (Potential efficiency (Fm/Fv)

(a, b, c, show the significant difference ($p<0.05$))

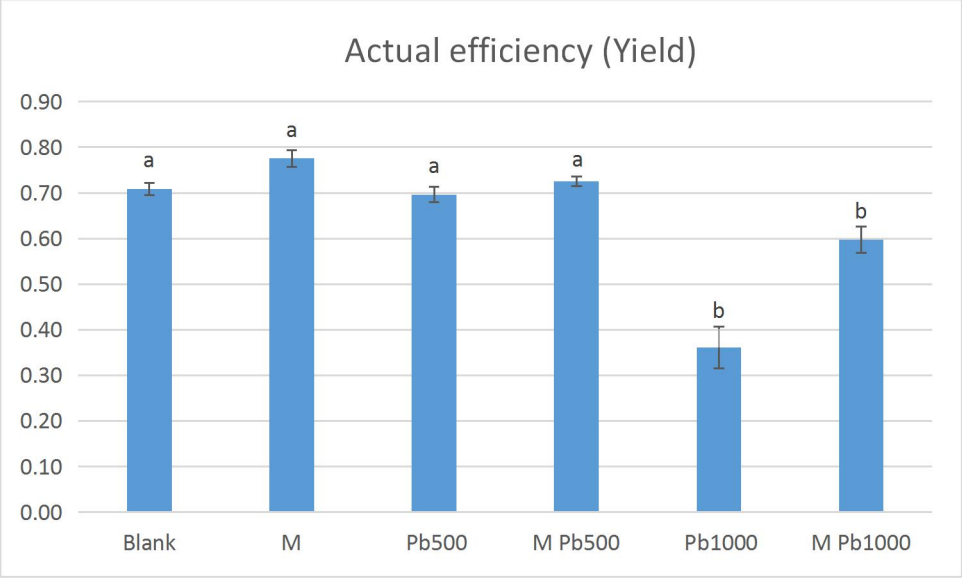

Fig.9 Photochemical efficiency of photosystem II (Actual efficiency (Yield))

(a, b show the significant difference ($p<0.05$))

4.2.3 Nutrients in plant

The highest contents of K and P in roots and shoots were determined in the

treatment of AMF, and the lowest concentrations were found in the treatment of Pb1000. The concentrations of K in shoots were higher (9% – 64%) than in roots. The concentrations of P were higher (28% – 38%) in roots than shoots, but the concentrations in the treatments of AMF and blank were lower in roots than shoots. AMF increased K and P contents in both shoots and roots, while Pb decreased the concentrations. AMF increased K concentration in shoots and roots by 11% – 19% under Pb stress compared with non-inoculated plants, and increased P concentration by 11% – 23% under Pb stress in shoots and roots. While Pb decreased K concentration in shoots by 19% – 37% with AMF compared with non-Pb addition samples, and in roots by 5% – 12% with AMF. Pb decreased K concentration in shoots by 21% – 38% without AMF compared with blank samples, in roots by 3% – 7% without AMF. And Pb decreased P concentration in shoots by 6% – 46% with AMF compared with non-Pb addition samples, in roots by 9% – 22% with AMF. In addition, Pb decreased P concentration in shoots by 5% – 51% without AMF compared with blank samples, in roots by 3% – 20% without AMF. Furthermore, from statistical analysis, AMF significantly increased K and P contents in both shoots and roots ($p<0.05$), Pb significantly decreased the concentrations in shoots ($p<0.05$), but Pb did not significantly decrease the contents in roots ($p>0.05$).

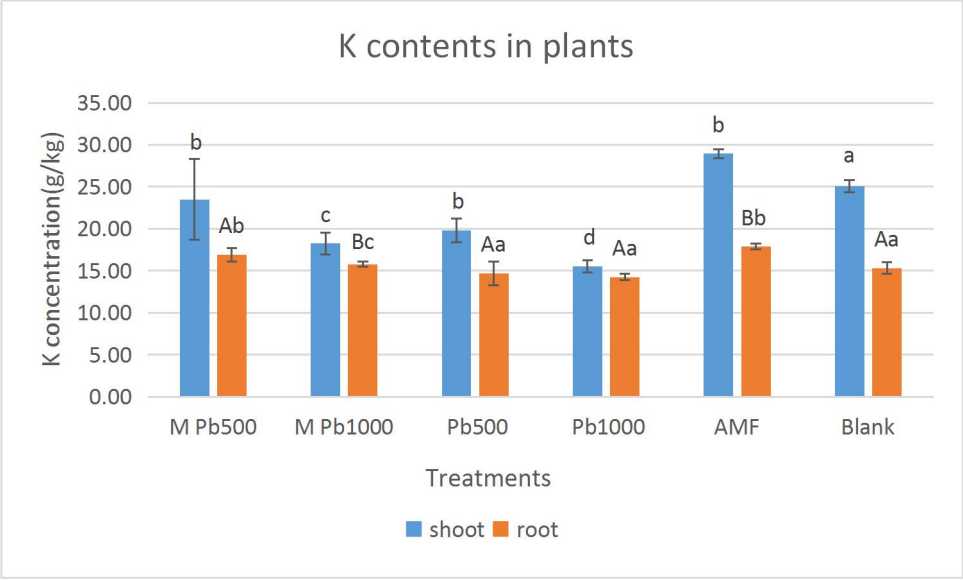

Fig.10 K concentrations in plants in different treatments (in root:A,B shows significant

difference between AMF- and AMF; a,b shows significant difference between Pb addition) (in shoot:a,b,c,d shows significant difference($p<0.05$))

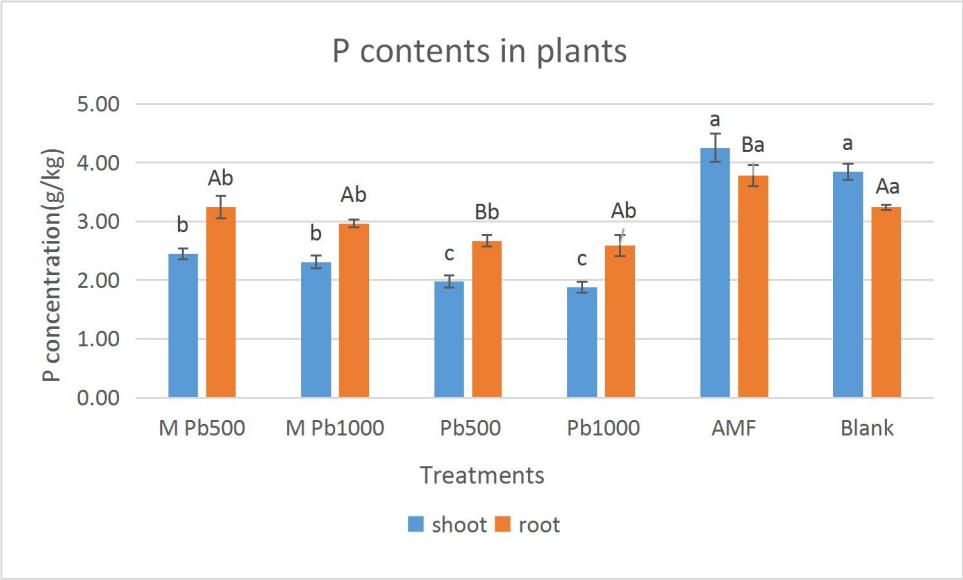

Fig.11 P concentrations in plants in different treatments (in root:A, B shows significant difference between AMF- and AMF; a,b shows significant difference between Pb addition) (in shoot:a,b,c shows significant difference($p<0.05$))

4.3 Antioxidant response in wetland plants under Pb stress

4.3.1 Lipid peroxidation and reactive oxygen species

Malondialdehyde (MDA) concentration

MDA is a widely used parameter to evaluate lipid peroxidation in plants. High MDA concentration in plants indicates a high degree of lipid peroxidation. The highest MDA in roots and shoots were determined in Pb1000 treatment, and the lowest values were determined in the treatment of AMF (Fig. 12). And the contents of MDA were higher (18% – 126%) in shoots than in roots. AMF decreased MDA by 8% – 44% in shoots and roots under Pb stress. While Pb increased MDA by 118% – 1120% in shoots and roots without AMF, and by 150% – 1214% with AMF.

In shoots, a significant difference presented between the treatment of AMF and M Pb500 ($p=0.029$). Pb significantly affected MDA contents no matter with AMF or without AMF ($p<0.05$). In roots, Pb and AMF both significantly affected MDA

concentration ($p<0.05$). And between shoots and roots, there were significant differences observed in the treatment of AMF, Pb1000, M Pb1000, $p=0.027$, $p=5.54e^{-5}$, $p=0.008$, respectively.

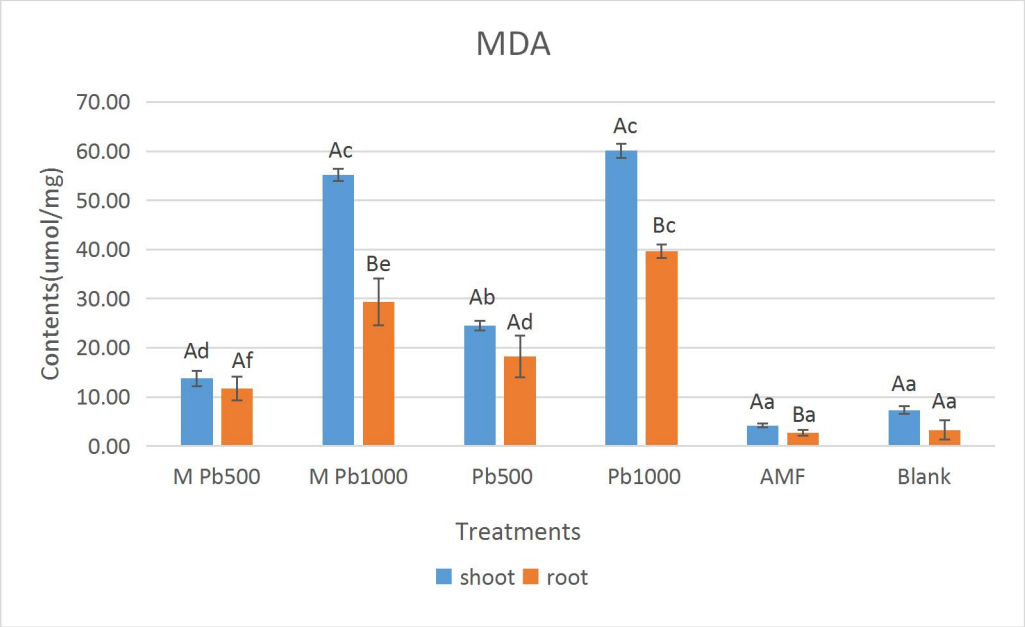

Fig.12 Malondialdehyde (MDA) concentration

(a, b, c, d, e, f shows significant difference between treatments ($p<0.05$);

A, B shows significant difference between roots and shoots ($p<0.05$))

Superoxide anion ($O_2^{\cdot-}$)

The highest contents of $O_2^{\cdot-}$ in shoots and roots were found in the treatment of Pb1000, the lowest contents were determined in the treatment of AMF (Fig.13). The concentrations of $O_2^{\cdot-}$ in shoots were higher (38% – 266%) than in roots. The content of $O_2^{\cdot-}$ in roots and shoots decreased with AMF inoculated by 19% – 42% under Pb stress. Pb increased the content of $O_2^{\cdot-}$ in roots and shoots by 49% – 396% with AMF and by 26% – 554% without AMF compared with non-Pb addition samples. In addition, from statistical analysis, AMF and Pb significantly affected the contents of $O_2^{\cdot-}$ in shoots and roots ($p<0.05$). And it showed significant differences between roots and shoots in $O_2^{\cdot-}$content ($p<0.05$).

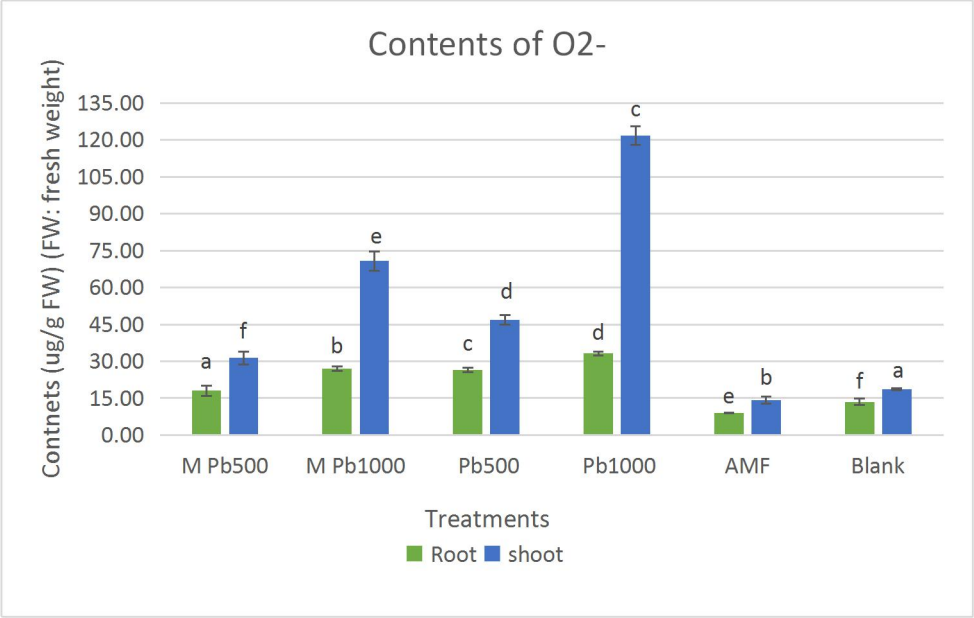

Fig.13 Contents of $O_2^{\cdot-}$ in plants (a, b, c, d, e, f shows significant difference between treatments also between roots and shoots ($p<0.05$))

4.3.2 Antioxidant enzyme activities

SOD

In shoots, the most active SOD was determined in the treatment of M Pb500 and the least active SOD was observed in the blank (Fig.14). In roots, the most active SOD was determined in the treatment of M Pb1000 and the least active SOD was observed in the blank. The activities of SOD in shoots were higher (4% – 29%) than in roots. AMF increased SOD activity by 27% – 89% in shoots and roots under Pb stress. And Pb increased SOD activities in shoots and roots by 139% – 195% without AMF compared with blank samples, but SOD activities decreased (1% – 6%) when comparing between the treatment of Pb500 and Pb1000. And Pb increased SOD activities in shoots and roots by 5% – 112% with AMF, but SOD activities decreased 1% when comparing between the treatment of M Pb500 and M Pb1000 in the shoot.

According to statistical analysis, AMF significantly increased SOD activity in shoots and roots under Pb stress ($p<0.05$). And Pb significantly increased SOD activity compared with blank samples in shoots and roots no matter with AMF or

without AMF ($p<0.05$). However, between the treatment of Pb500 and Pb1000 presented insignificant difference no matter with AMF or without AMF in both shoots and roots ($p>0.05$). And the activity of SOD showed a significant difference between roots and shoots in treatments of blank ($P=0.047$), AMF ($P=0.04$) and M Pb500 ($P=0.049$).

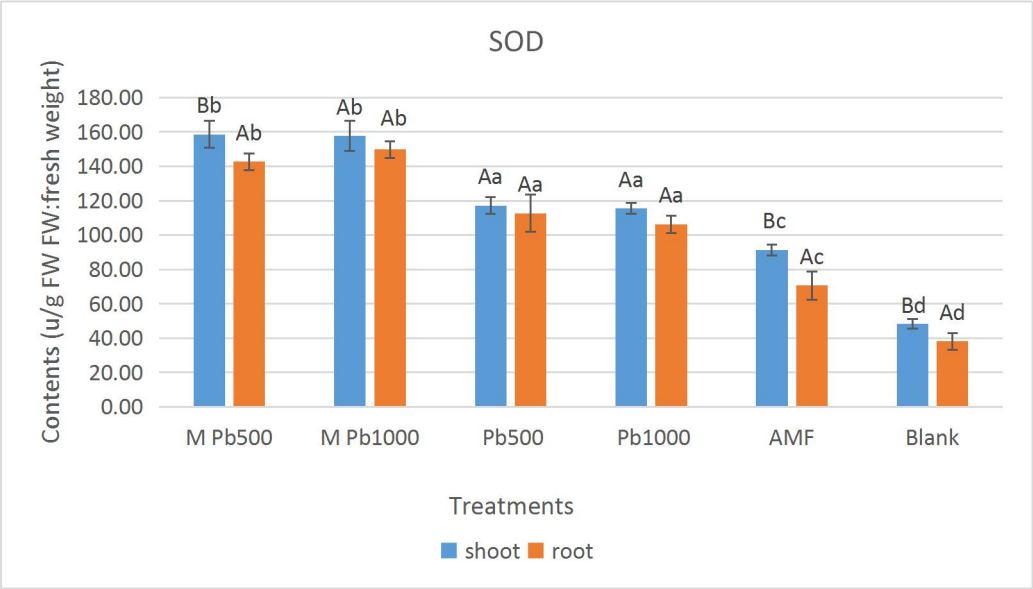

Fig.14 Activity of SOD in plants (A, B shows significant difference between roots and shoots; a, b, c, d shows significant difference between treatments ($p<0.05$))

Activities of peroxidase (POD)

The most active POD in shoots and roots were determined in the treatment of M Pb1000, the least active POD was observed in the blank (Fig.15 and Fig.16). The activities of POD were higher (386% – 1023%) in roots than in shoots. The activities of POD were higher with AMF than without AMF by 44% – 457% in shoots and by 0.5% – 53% in roots, but the activity of POD was lower 1% with AMF when compared between the treatment of M Pb1000 and Pb1000 in roots. And Pb increased POD activity by 35% – 586% with AMF in shoots and roots and by 87% – 2449% without AMF. In addition, AMF and Pb significantly affected the activity of POD in shoots and roots ($p<0.05$), especially more significant in roots. The activity

of POD showed a significant difference between shoots and roots ($p<0.05$).

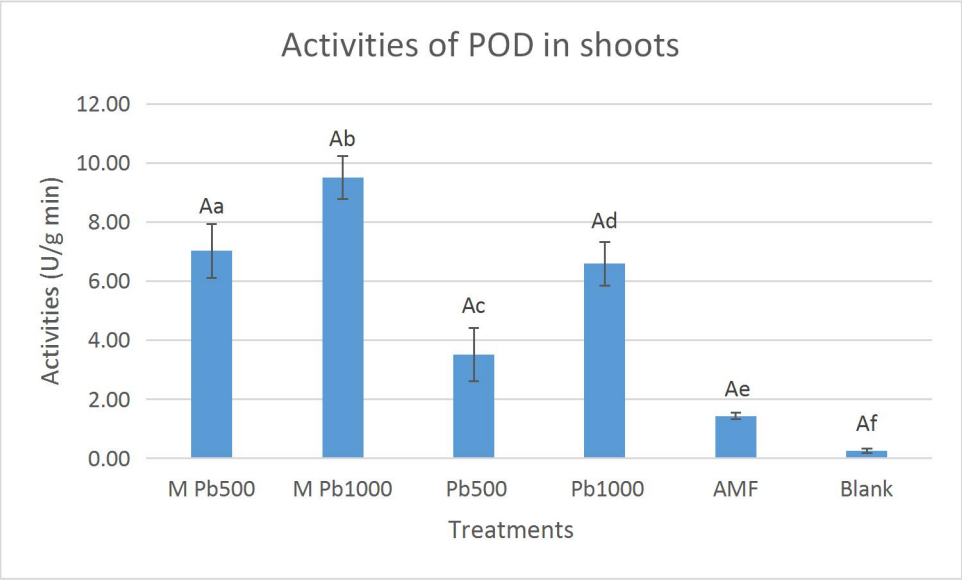

Fig.15 Activities of POD in shoots under different treatments

(a, b, c, d, e, f shows significant difference between treatments; A, B shows significant difference between roots and shoots ($p<0.05$))

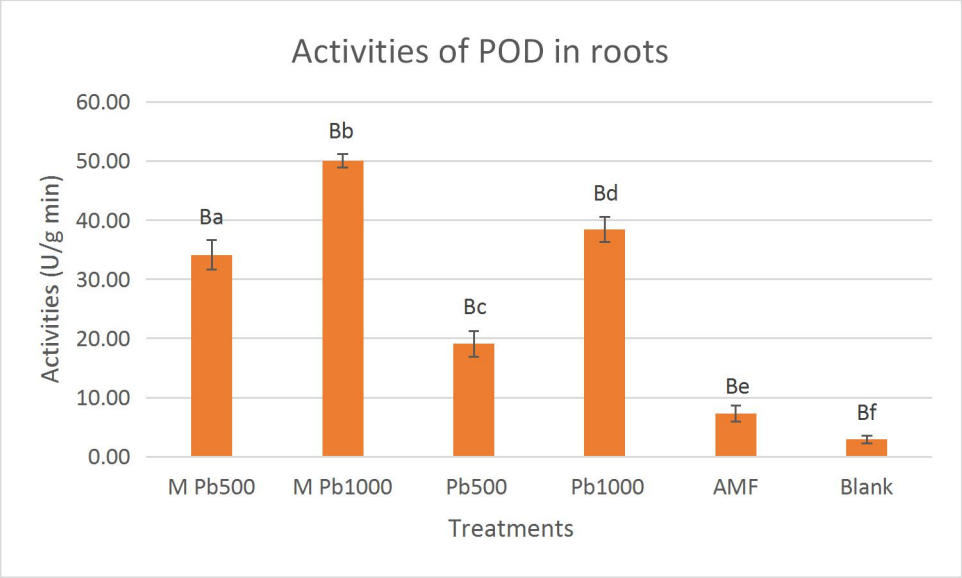

Fig.16 Activities of POD in roots under different treatments

(a, b, c, d, e, f shows significant difference between treatments; A, B shows significant difference between roots and shoots ($p<0.05$))

Soluble protein content

The highest soluble protein content was determined in the treatment of M Pb500 in roots and shoots, and the lowest content was determined in the treatment of Pb1000 in roots and shoots. The soluble protein contents in shoots were higher (13% – 73%) than in roots. AMF increased soluble protein contents by 36% – 103% under Pb stress in both roots and shoots. Pb addition generally decreased soluble protein contents by 13% – 49% in roots and shoots with AMF, but increased contents by 43% in roots and by 13% in shoots when compared between the treatment of M Pb500 and AMF. And Pb generally decreased soluble protein contents by 4% – 31% in roots and shoots without AMF, but increased 3% when compared between the treatment of Pb500 and blank in shoots. From static analysis, significant differences appeared in the treatments of AMF ($p=0.002$), Pb1000 ($p=0.04$) and M Pb1000 ($p=0.0006$) between roots and shoots. AMF significantly increased soluble protein content in both roots and shoots ($p<0.05$). And Pb significantly decreased soluble protein contents in both shoots and roots with AMF ($p<0.05$), but showed an only significant difference between the treatment of Pb500 and Pb1000 in root when without AMF ($p<0.05$).

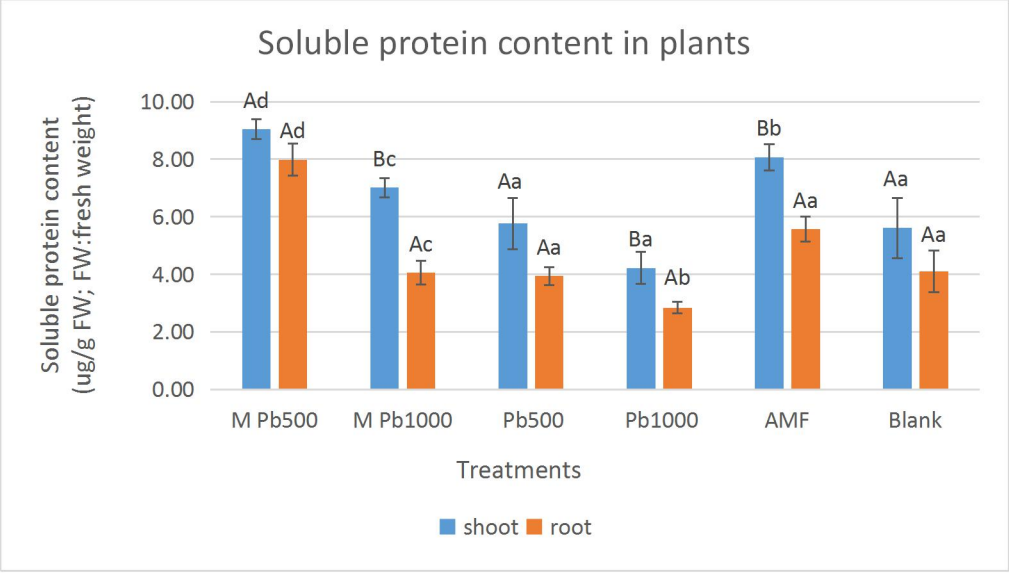

Fig.17 Soluble protein content in plants under different treatments

(a, b, c, d shows significant difference between treatments; A, B shows significant difference between roots and shoots ($p<0.05$))

4.4 Pb transformation in wetland systems

4.4.1 Pb in wetland plants

The highest Pb concentration was determined in the treatment of Pb1000 in shoot and in the treatment of M Pb1000 in root. The lowest Pb contents was observed in the treatment of M Pb500 in shoot and in the treatment of Pb500 in root. In addition, roots contained more Pb (27% – 6633%) than shoots. AMF decreased shoot/root concentration ratio from 0.04 to 0.01 under Pb500 treatment compared with non-inoculated, and from 0.79 to 0.43 under Pb1000 treatment. AMF decreased Pb concentration by 53% under Pb500 treatment in shoots, and 45% under Pb1000 treatment. But AMF increased 19% Pb content under Pb500 treatment and 1% under Pb1000 treatment in roots. However, Pb concentration increased 6658% in shoot and increased 219% in root when compared between the treatment of Pb500 and Pb1000.

The Pb contents in roots and shoots presented a significant difference ($p<0.05$). AMF significantly affected Pb contents in shoots and roots under Pb500 treatment addition ($p=0.002$ and $p=0.001$ respectively), but it only presented significant difference in shoots under Pb1000 addition ($p=0.0006$). Between treatments of Pb500 and Pb1000 presented a significant difference in roots and shoots no matter with AMF or without AMF ($p<0.05$).

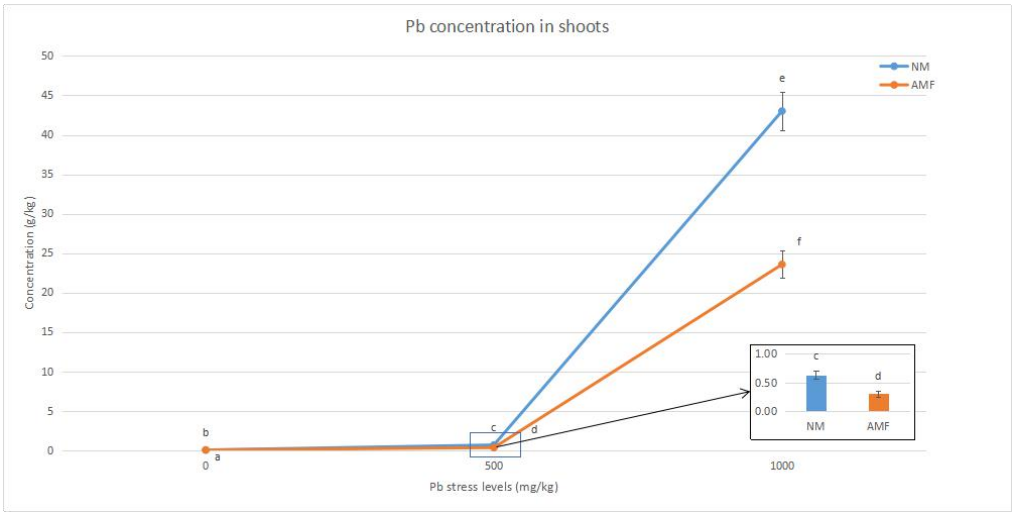

Fig.18 Pb concentration in shoots under different Pb stress level (NM:no AMF)

(a, b, c, d, e, f shows significant difference between treatments and between roots and shoots ($p<0.05$))

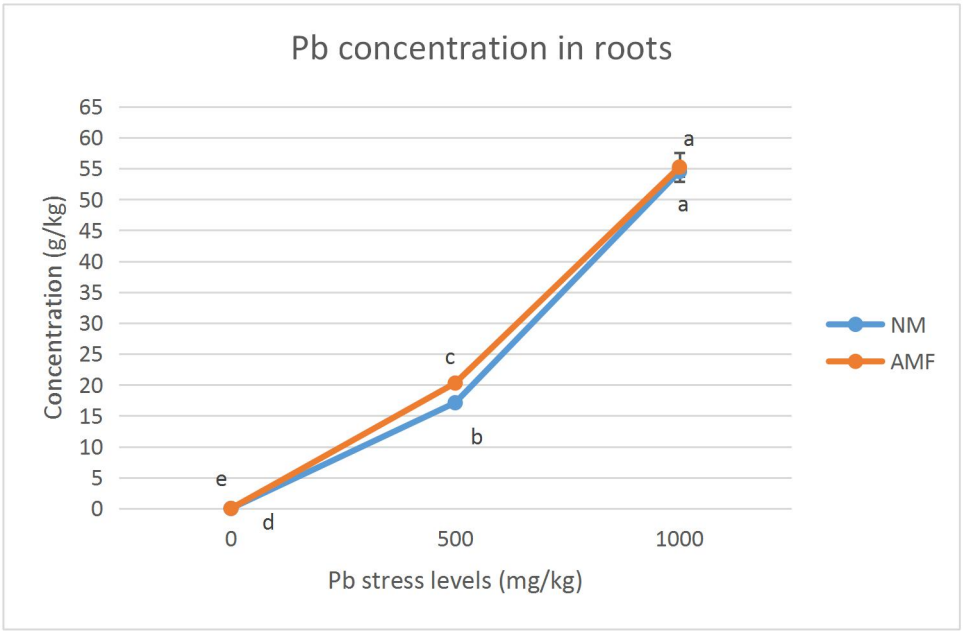

Fig.19 Pb concentration in roots under different Pb stress level (NM:no AMF)

(a, b, c, d, e shows significant difference between treatments and between roots and shoots ($p<0.05$))

4.4.2 Pb in substrate

The pH values

The pH of the substrates was around 6, rather acidic. The pH of M Pb500 treatment was a little bit higher than M Pb1000 treatment. AMF increased the pH of the substrate slightly. Pb decreased pH value slightly.

Table 3 The pH of sand materials

Treatments	M Pb500	M Pb1000	Pb500	Pb1000	AMF	Blank
pH	6.92±0.02	6.44±0.03	6.35±0.03	6.22±0.02	7.01±0.05	6.95±0.08

Pb concentration

Pb concentration of substrates in the treatment of Pb1000 was 369.01 mg/kg, which was the highest contents. The concentration in M Pb500 treatment of 122.92 mg/kg was the lowest. AMF decreased Pb concentration by 26% under Pb500 treatment, and by 11% under Pb1000 treatment compared with non-inoculated samples. Pb addition increased 122% Pb concentration in substrates without AMF when compared between the treatment of Pb500 and Pb1000, and increased 168% with AMF. However, Pb concentrations in substrates with AMF inoculated didn't show a significant difference with non-inoculated samples ($p>0.05$). But treatments between Pb500 and Pb1000 presented significant difference no matter with AMF or without AMF ($P<0.05$). Pb addition significantly increased Pb concentration in substrates ($P<0.05$).

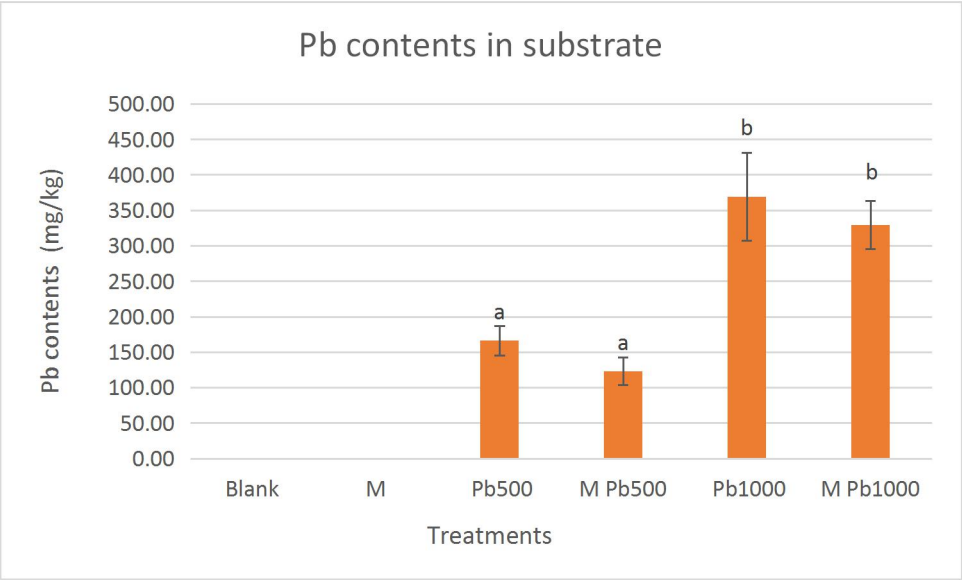

Fig.20 Pb concentration in substrate

(a, b shows significant difference between treatments ($p<0.05$))

5. Discussion

5.1 Pb decreased AMF colonization

The information of AMF colonization status under Pb stress is an important foundation to understand AMF mechanisms of reacting Pb stress (Yang et al. 2015). In the present study, AMF colonization significantly decreased by 7.11% – 50.9% with Pb concentration increasing in the treatment ($p<0.05$), but at 500 mg/kg addition of Pb treatment, not obvious decreasing observed. Zhang et al. (2019b) and Yang et al. (2015) also observed AMF colonization decreased with Pb stress, and Zhang et al. (2019b) found it decreased by 23% – 28% under Pb stress. Ning et al. (2019) obtained Cd addition decreased AMF colonization. This indicated heavy metal stress may inhibit AMF colonization. Wężowicz et al. (2015) claimed in waterlogged conditions, arbuscular abundance (A%) induced with the presence of toxic metals might be a result of the modification of toxic metals. Induced colonization revealed plant limited hyphal growth to optimize its needs when meet environmental stress. Chen et al. (2005) (Yang et al. 2015 cited) found decreased germination of spore and extra-root hyphae growth in AMF with environmental stress. Therefore, Pb stress

might inhibit the germination of spore and extra-root hyphae growth of AMF to decrease AMF colonization. In addition, Bago et al. (2000) (Yang et al. 2015 cited) suggested around 4% and 20% of photosynthetic products were transformed into AMF to provide nutrition. As our study showed Pb decreased photosynthetic activity, so this might be a reason for the decrease in AMF colonization. However, AMF colonization did not disappear even in high Pb concentration indicated high Pb tolerance of AMF (Yang et al. 2015).

5.2 AMF promoted wetland plant growth under Pb stress

5.2.1 Biomass

AMF significantly improved the growth of plants. The results showed AMF significantly increased root length, shoot height, fresh root weight, fresh shoot weight, dry root weight, dry shoot weight under Pb stress when compared with non-inoculated samples by 19% – 39%, 3% – 16%, 44% – 67%, 20% – 86%, 54% – 98%, 26% – 123% respectively ($p<0.05$). Pb significantly affected plant growth at Pb1000 addition no matter with AMF or without AMF ($p<0.05$), but no significant difference appeared under Pb500 addition ($P>0.05$).

Our study showed Pb had a toxic effect on plant growth. Bai et al. (2015) also found Pb treatment decreased plant biomass. Yang et al. (2015) observed Pb declined the shoot height and the leaf, stem and root dry weights. Huang et al. (2018) also found biomass decreased with Cr stress increasing. These may because heavy metal stresses restricted apical division (Xin et al. 2017). However, results revealed plants grew better with AMF under the low concentration of Pb addition. AMF might help plants to grow better by improving the heavy metal tolerances of plants (Wężowicz et al. 2015) and the uptake of nutrition as well as water (Zhang et al. 2020). And AMF has been demonstrated as bio-protectors, bio-fertilizers and biocontrol agents, and is significantly good for plant growth (Jeffries and Barea 2012). Gunathilaka et al. (2017) and Zhang et al. (2019a) also gained a high growth rate and high biomass

of roots and shoots in AMF inoculated plants. Zhang et al. (2019b) and Yang et al. (2015) both observed higher dry weight of shoots and roots with AMF under all Pb treatments as well.

5.2.2 Photosynthesis properties

Leaf chlorosis is a common outcome under Pb stress (Sharma and Dubey, 2005). In the present study, Pb stress significantly declined leaf chlorophyll contents ($p<0.05$). Yang et al. (2015) also observed lower leaf chlorophyll contents with Pb stress. Zhang et al. (2020) also gained Pb inhabited photosynthesis capacity. Huang et al. (2018) observed heavy metal (Cr) decreased leaf chlorophyll contents and it might disturb chloroplast homeostasis. This might because heavy metals disturbed the absorption of Fe, therefore decreased the Fe accumulation which is the need for leaf chlorophyll biosynthesis (Huang et al. 2018 ex. Gopal et al. 2009). And heavy metals could replace Mg^{2+} of chlorophyll in plants with Ni^{2+}, Cd^{2+}, Co^{2+} and so on thus decreased leaf chlorophyll contents (Xu et al. 2014 ex. Pandey and Sharma, 2002). However, AMF could help plants to uptake Mg^{2+} which leads to higher leaf chlorophyll contents (Xu et al. 2014 ex. Stobart et al. 1985). Our study results also proved that, leaf chlorophyll contents were also higher by 6% – 39% in AMF inoculated plants than non-inoculated samples though it did not show a significant difference ($P>0.05$). Zhang et al. (2019b) and Yang et al. (2015) both also proved AMF increased leaf chlorophyll contents under Pb stress. Higher chlorophyll contents may lead to a larger leaf area (Yang et al. 2015 ex. Sheng et al. 2008), thus enhanced photosynthesis and photosynthetic activity (Zhang et al. 2020), which revealed by increased photochemical efficiency in our study. The photochemical efficiency of the photosystem included potential efficiency and actual efficiency. The value of photochemical efficiency is also a useful tool for indicating damage caused by environmental stress (Yang et al. 2015 ex. Krause and Weis, 1991). In our study, both of efficiencies were higher in AMF inoculated plants than non-inoculated control (by 0.5% – 8% for potential efficiency, by 4% – 65% for actual efficiency). And the value of treatment only with AMF was the highest and was around 0.8

means their photochemical efficiency was good and they were not under environmental stress. These indicated AMF increased photochemical efficiency under Pb stress. Ramírez-Viga et al. (2018) also gained similar results. Zhang et al. (2020) also reported AMF could decrease Pb damage to photosynthesis by increasing nutrients uptake.

5.2.3 Nutrients in plant

Our study showed Pb decreased K (3% – 38%) and P (3% – 51%) contents in both shoots and roots no matter with or without AMF. Zhang et al. (2020) also observed lower P contents under Pb stress in shoots with non-inoculated plants. This might because Pb stress disturbed nutrient absorption (Zhang et al. 2020) by decreasing the availability of nutrients in the substrate and created more barriers for root and AMF to absorb nutrients (Zhang et al. 2020 ex. Seshadri et al. 2017). And Pb stress decreased the exchange of nutrients and organic carbon in plants (Zhang et al. 2020). Pb also had an opponent competition with microelements and then disturbed assimilation metabolism (Sharma and Dubey, 2005).

However, AMF significantly increased K (11% – 19%) and P concentration (11% – 23%) in both shoots and roots ($p<0.05$) in our results. Wang et al. (2010) (Xu et al. 2016 cited) also reported that AMF enhanced plant uptake K and P. As known, K and P are the main nutrients for plants. AMF increased the nutrition uptake and absorption for plants by increasing the root surface (Zhang et al. 2020). In addition, AMF maintains mutualistic symbiosis between AMF and host plants by exchanging nutrients (Zhang et al. 2020). Various studies also obtained higher P contents with AMF under Pb stress in shoots and roots compared with non-inoculated plants (Chen et al. 2015; Zhang et al. 2020). Solaiman and Hirata (1997) also observed increased P transfer through hyphae from soils to roots or shoots. Therefore, AMF could increase nutrition like K and P uptake for plants to increase Pb resistance, and this might be a reason for AMF increasing plant growth. Furthermore, the increasing nutrients in plants under Pb stress may also connect further with enzyme activities and reactive oxygen species (Chen et al. 2015).

5.3 AMF improved wetland plant antioxidant capacity

5.3.1 Lipid peroxidation and reactive oxygen species (ROS)

MDA is a bio-marker to reveal environmental stress. In our study, Pb significantly increased MDA contents by 118% – 1214% no matter with or without AMF ($p<0.05$). Huang et al. (2018) and Yang et al. (2015) also obtained a similar result that MDA increased with heavy metals addition. This might because Pb decreased membrane permeability and formed short-chain alkanes and lipid aldehydes repetitively (Wang et al. 2012). However, AMF decreased MDA concentration by 8% – 44% under Pb stress compared with non-inoculated samples in our study. The contents of MDA were higher (18% – 126%) in shoots than in roots. These indicated AMF could decrease environmental stress in plants, especially in roots. And AMF could decrease oxidative damage caused by Pb stress (Yang et al. 2015). Zhang et al. (2019b) also observed decreased MDA contents with AMF under Pb stress. Hu et al. (2020) obtained similar results under low water depth when investigated the effect of AMF on the physiological functions of plants under different water depths. Lu et al. (2014) also pointed out AMF could restrain the increase of MDA under abiotic stress like low-salinity.

$O_2^{\cdot-}$

$O_2^{\cdot-}$ is one of the main productions of reactive oxygen species (ROS) in plants under Pb stress (Corpas and Barroso, 2017). ROS is a product when plants meet environmental stress (Allen, 1995), which is continually produced in chloroplasts during photosynthesis (Asada, 1994), and overabundance ROS would cause oxidative damage to plants (Chen et al. 2015). In the present study, Pb increased the content of $O_2^{\cdot-}$ by 26% – 554% in shoots and roots compared with blanks, and the concentrations of $O_2^{\cdot-}$ in shoots were higher (38% – 266%) than in roots. Zhang et al. (2019b) and Chen et al. (2015) also observed Pb enhanced the $O_2^{\cdot-}$ production rate compared to controls. Huang et al. (2018) observed Cr increased $O_2^{\cdot-}$ contents.

However, AMF decreased $O_2^{\cdot-}$ contents by 19% – 96% in roots and shoots under Pb stress compared with non-inoculated samples in our study, which indicated AMF can decrease the ROS damage in plants and improved $O_2^{\cdot-}$ removing ability under Pb stress (Zhang et al. 2019b). Zhang et al. (2019b) and Chen et al. (2015) also observed AMF declined the $O_2^{\cdot-}$ production rate.

5.3.2 Antioxidant enzyme activities

The production and activity of antioxidative enzymes would increase when plants meet oxidative stress (Zhang et al. 2019a ex. Hossain et al. 2015). Antioxidative enzymes are important for removing ROS (Noctor and Foyer, 1998). SOD is a major cleaner of $O_2^{\cdot-}$, and its enzymatic action product H_2O_2 and O_2. Catalase and POD then scavenged H_2O_2. Catalase changed H_2O_2 into water and oxygen, while POD oxidated H_2O_2 (Noctor and Foyer, 1998). AMF increased the activity of antioxidative enzymes as the symbol of improving plant growth and tolerance of oxidative stress (Yang et al. 2015 ex. Latef and Chaoxing, 2011).

SOD

SOD is regarding as an important enzyme for dealing with oxidative stress by transforming $O_2^{\cdot-}$ to O_2 and H_2O_2 (Kanwar et al. 2015). $O_2^{\cdot-}$ was produced in chloroplasts (Asada, 1994). These may be the reason for the activities of SOD in shoots were higher (4% – 29%) than in roots in our study. And Pb significantly increased SOD activity by 5% – 195% compared with blank in shoots and roots no matter with or without AMF ($p<0.05$). Huang et al. (2018) and Zhang et al. (2020) also observed heavy metals increased SOD activity. And Meloni et al. (2003) found environmental stress like sanity increased SOD activity. And AMF significantly increased SOD activity by 27% – 89% in shoots and roots compared with non-inoculated samples in our study ($p<0.05$). Zhang et al. (2019a) also found AMF could increase SOD activity showing low ROS accumulation.

POD

POD is an important enzyme for removing H_2O_2 (Kanwar et al. 2015). In the

current study, Pb significantly increased POD contents by 35% – 586% with AMF and by 87% – 2449% without AMF in shoots and roots. Zhang et al. (2020) also observed Pb^{2+} stress increased POD activity. Scalet et al. (1995) found POD activity was higher in tolerant plants to protect themself from oxidative stress. Meloni et al. (2003) also found oxidative stress like salt stress produced a higher capacity for removing ROS. And AMF significantly increased POD activity by 0.5% – 457% in shoots and roots, especially more significant in roots ($p<0.05$). The values of POD were significantly higher in roots than in shoots. This means root was less affected by Pb stress than shoot, this might because AMF protected root more directly.

Soluble protein content

Soluble protein content, an oxidative stress indicator (Yang et al. 2015 ex. Moran et al. 1994), was observed to decrease in both shoots and roots under Pb stress in the present study. Pb interrupted metabolism equilibrium (Aziz et al. 2015), directly interact with protein (Sharma and Dietz, 2009), and it breakdown proteins by oxidative reactions or proteolytic activity (Sharma et al. 2005). However, AMF could increase the soluble protein content to enhance Pb stress resistance. The present study also confirmed that AMF significantly increased soluble protein content by 36% – 103% in both roots and shoots compared with non-inoculated plants ($p<0.05$). Liu et al. (2011) also observed AMF improved soluble protein contents in marigold plants under Cd stress. It indicated the protective role of AMF expressed at protein in plants (Lingua et al. 2012). In addition, Bona et al. (2010) described the significant effect of AMF on protein expression. AMF increased amino acid metabolism in plants to respond to heavy metal stress (Cicatelli et al. 2014). Amino acids are organic compounds to form the protein (Zhang et al. 2020 ex. Tegeder and Masclaux-Daubresse, 2018). Zhang et al. (2020) also observed AMF increased amino acids in shoots and roots and suggested higher amino acids are good for Pb resistance. Talaat and Shawky (2014) (Zhang et al. 2020 cited) also reported higher amino acids with AMF under salinity stress.

In general, heavy metal stress increased ROS accumulation and ROS

scavenging (Chen et al. 2015 ex. Gupta et al. 2013). The balance between accumulation and scavenging of ROS is an important mechanism for enhancing plant tolerance for abiotic stress (Mittler, 2002). Under normal conditions, the balance is in equilibrium (Ferrer et al. 2018). However, when plants meet environmental stress, it can overwhelm the antioxidant system and cause oxidative stress (Berni et al. 2019). In the current study, Pb significantly increased all the parameters related to ROS ($P<0.05$). Various studies also reported Pb stress increased ROS production (Chen et al. 2015). In addition, ROS is very active, it can damage proteins which leads to damage normal metabolism of plants (Rout and Shaw, 2001). Lingua et al. (2012) also observed protein decreased under heavy metal stress related to the oxidative stress response.

However, the enzymatic antioxidant system is one of the efficient mechanisms for plants against toxic stress induce ROS injuries (Nieves-Cordones et al. 2019). AMF even increased more activity of antioxidant enzymes than non-inoculated plants, which kept ROS at a controlled level (Lingua et al. 2012). AMF can decrease Pb toxicity such as ROS damage and oxidative stress to plants by enhancing the activity of antioxidant enzymes (Zhang et al. 2010; Lingua et al. 2012).

AMF inoculated plants with increasing of Pb stress, cell structure did not increase more injuries, which revealed by the parameters of ROS, this mainly because AMF could provide a biological barrier by fungal structures and increase tolerance to heavy metals by changing the activity of enzymes (Chen et al. 2015).

5.4 AMF influenced Pb transformation in wetlands

5.4.1 Pb in wetland plants

For the aboveground part, AMF significantly decreased Pb contents by 45% – 53% compared with non-inoculated plants ($p<0.05$). “Dilution effect” may explain increased plant biomass and decreased heavy metal concentration in shoots (Nielsen and Jensen, 1983). Wu et al. (2016b) also observed lower Pb contents (20.6% –

67.5%) in shoots with AMF and lower Cd concentration (14.3% – 54.1%) with AMF in shoots compared with non-inoculated plants. Various studies also reported AMF decreased heavy metal contents in shoots (Zheng et al. 2014; Yang et al. 2015; Chen et al. 2015).

For the underground part, AMF can be a sink of heavy metal for plants. It can provide a larger surface to absorb nutrients and maintain metals in their mycelium (Carvalho et al. 2006; Schneider et al. 2013). The present study also confirmed that Pb concentration was higher (1 – 19%) in AMF inoculated plants than non-inoculated plants, especially significantly higher at Pb500 addition ($p=0.001$). Carvalho et al. (2006) also observed heavy metal concentration was higher in roots with AMF than non-inoculated plants, indicated plant especially root can be a hyperaccumulator of heavy metals and has a higher binding capacity of heavy metal with AMF. Chen et al. (2015) and Yang et al. (2015) also reported AMF enhanced Pb uptake and accumulation in roots compared with non-inoculated plants. Furthermore, the pH of substrates was rather acidic in the study, and the Pb accumulation in roots was rather high, which as Sawalha et al. (2005) suggested in an acidic environment, heavy metal mobility was higher, and heavy metal contents in roots were higher. And a negative relationship between pH in substrate and accumulation of Pb in roots was observed in the study when compared between the treatment of M Pb500 and M Pb1000. Sidhoum et al. (2019) also reported the relationship.

5.4.2 Pb in the substrate

AMF played an important role in decreasing the toxicity of heavy metal in substrates by developing extramatrical hyphae and enhance the degradative limit of plants (Chen et al. 2015 ex. Joner et al. 2000). The present study also confirmed that AMF decreased Pb accumulation by 11% – 26% in substrate compared with non-inoculated plants. AMF increased Pb accumulation in roots, thus decreased Pb accumulation in the substrate which declined the Pb contamination in substrates. Joner and Leyval (1997) also observed that Cd transformed from the substrate to the fungal structures of *G. mosseae* within roots, therefore declined the heavy metal

concentration in the substrate. Previous studies found that AMF could moderate metals (Zn, Ni, Cu, Cr and Cd) concentration in substrates (Gunathilaka et al. 2018; Sidhoum et al. 2019).

Generally, AMF increased Pb concentration in roots, while decreased Pb concentration in shoots and substrate compared with non-inoculated plants. So Pb contents were higher in roots than shoots and substrate. Wu et al. (2016b) and Gunathilaka et al. (2018) also observed similar results that heavy metal contents in roots were higher than in shoots. And AMF decreased shoot/root concentration ratio from 0.04 to 0.01 under treatment of Pb500 compared with non-inoculated, and from 0.79 to 0.43 under Pb1000. These indicated AMF accumulated most Pb in roots and inhabited the transformation from roots to shoots (Schneider et al. 2013). In addition, Weis et al. (2004) suggested the shoots can be a source for metals to transport into the water, low heavy metal contents in shoots can decrease heavy metal contamination in the wetland. So AMF may decrease heavy metal contamination in wetlands through controlling heavy metal transformation from roots to shoots. Studies also reported AMF decreased heavy metal stress by immobilizing heavy metal in the roots and mycorrhizosphere of plants and limiting the transformation to the shoots (Joner and Leyval, 1997). Kaldorf et al. (1999) suggested the immobility of heavy metals might because of the structure of fungal and the chelation of metal by polyphosphate particles in fungal vacuoles. And AMF could bind and absorb Pb in roots (Meier et al. 2012).

6. **Conclusion**

AMF colonization in CWs gradually decreased with Pb addition, but an insignificant difference was obtained between the blank and the low Pb addition (500 mg/kg). It indicated AMF was sufficient enough to be functional for plants even the colonization decreased, suggested the protective role of AMF for plants. AMF improved photosynthesis properties such as photochemical efficiency and leaf chlorophyll contents by increasing nutrition and improving growth in plants. Furthermore, ROS like MDA and $O_2^{\cdot-}$ produced when plants were under the Pb stress, which caused damage to protein in plants. While AMF increased antioxidative enzymes like POD, SOD to decrease oxidative stress. In addition, AMF promoted plants to accumulate most of Pb in the roots in a rather acidic environment by binding and absorbing it. It decreased Pb transformation from roots to the shoots and decreased Pb concentration in substrates, thereby reduced Pb contamination in wetlands. Therefore, AMF played an important role in Pb transformation and improving plant growth in wetlands.

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