Heavy metals transformation in arbuscular mycorrhizal assistant constructed wetlands

Ph.D. Thesis

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Thesis

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Abstract

Heavy metals like chromium (Cr) in plants are tolerable to a certain extent, but their excess result in biological toxicity by affecting several physiological and biochemical processes in plants. Moreover, the activity of the antioxidant defense system is reduced due to reactive oxygen species generated as a result of heavy metal stress. Arbuscular mycorrhizal fungi (AMF), as an important group of soil microorganisms, can improve plant tolerance to heavy metal stress in the terrestrial system and also provide a positive effect on antioxidant mechanisms in terrestrial plants under heavy metal stress. However, the factors that affect AMF colonization in wetland plants and the physiological functions of AMF inoculated wetland plants are poorly studied. Furthermore, the antioxidant mechanisms and detoxification capacity of AMF to heavy metals in wetland systems remain unclear. Therefore, the main objective of this thesis was to (1) evaluate the effects of water depths (below the surface of sands: water levels of 5 cm, 9 cm, 11 cm, and fluctuating water depth (9-11 cm)) on AMF colonization in different wetland plants (*Phalaris arundinacea* and *Scirpus sylvaticus*); (2) investigate the influences of AMF on wetland plant (*Iris pseudacorus*) growth and antioxidant response under Cr stress at different water depths; (3) determine the impacts of AMF on Cr distribution and bioavailability in semi-aquatic habitats under different water depths. Results showed that the fluctuating water depth was suitable for the formation of root colonization by AMF. Wetland plant species did not show any significant difference in AMF colonization. In comparison to the non-inoculated plants, the physiological functions of AMF inoculated wetland plants were increased largely. Meanwhile, AMF plays an important role in the antioxidant response of wetland plants under heavy metal stress, and it can improve wetland plants' tolerance to heavy metal stress at fluctuating water depth. Moreover, AMF increased heavy metal accumulation in the roots of wetland plants. Conversely, heavy metal content in the shoots of AMF inoculated wetland plants were lower than those in the non-inoculated controls. Heavy metal concentration and mass content in water were decreased in semi-aquatic habitats with AMF inoculation under the low heavy metal stress. Besides, AMF reduced the heavy metal bioavailability in substrates under the fluctuating water depth. Results indicated that the physiological functions in wetland plants with high AMF colonization might be improved under a specific water depth condition (e.g. depth of fluctuating water regime). AMF can decrease the heavy metal toxicity in semi-aquatic habitats by enhancing the antioxidant activities in wetland plants and modulating the heavy metal distribution and bioavailability. In conclusion, this thesis provides a possibility that AMF can be used to remove heavy metal from polluted wastewater by constructed wetlands (CWs) under fluctuating water depth (e.g. tidal flow CWs).

Abstrakt

Těžké kovy, jako je chrom (Cr), jsou do určité míry tolerovatelné rostlinami, ale jejich přebytek vede k biologické toxicitě ovlivněním několika fyziologických a biochemických procesů v rostlinách. Kromě toho je aktivita antioxidačního obranného systému snížena v důsledku reaktivních forem kyslíku generovaných v důsledku stresu, který způsobují těžké kovy. Arbuskulární mykorhizní houby (AMF), jako důležitá skupina půdních mikroorganismů, mohou zlepšit toleranci rostlin vůči stresu těžkých kovů v suchozemském systému, a také mohou pozitivně ovlivnit antioxidační mechanismy suchozemských rostlin pod stresem těžkými kovy. Faktory, které ovlivňují kolonizaci AMF v mokřadních rostlinách a fyziologické funkce AMF naočkovaných mokřadních rostlin, jsou však studovány jen velmi málo. Antioxidační mechanismy a detoxikační kapacita AMF na těžké kovy v mokřadních systémech navíc zůstávají nejasné. Hlavním cílem této práce proto bylo: (1) vyhodnotit účinky hloubky vody pod povrchem písku: hladina vody 5 cm, 9 cm, 11 cm a kolísající hloubka vody 9-11 cm na kolonizaci AMF v různých mokřadních rostlinách (*Phalaris arundinacea* a *Scirpus sylvaticus*); (2) zkoumat vlivy AMF na růst mokřadních rostlin (*Iris pseudacorus*) a antioxidační odpověď při stresu Cr v různých hloubkách vody; (3) určit dopady AMF na distribuci Cr a biologickou dostupnost v semi-akvatických stanovištích v různých hloubkách vody. Výsledky ukázaly, že kolísající hloubka vody byla vhodná pro vytvoření kolonizace kořenů pomocí AMF. Mokřadní druhy rostlin nevykazovaly žádné významné rozdíly v kolonizaci AMF. Ve srovnání s neočkovanými rostlinami byly fyziologické funkce AMF naočkovaných mokřadních rostlin značně zvýšeny. Bylo zjištěno, že AMF má důležitou roli v antioxidační reakci mokřadních rostlin pod stresem těžkými kovy a může zlepšit toleranci mokřadních rostlin vůči stresu těžkými kovy při kolísající hloubce vody. Kromě toho AMF zvýšila akumulaci těžkých kovů v kořenech mokřadních rostlin. Naopak obsah těžkých kovů ve výhoncích mokřadních rostlin naočkovaných AMF byl nižší než v neočkovaných kontrolách. Koncentrace těžkých kovů a hmotnostní obsah ve vodě byly sníženy na semi-akvatických stanovištích s inokulací AMF při nízkém zatážení těžkými kovy. Kromě toho AMF snížily biologickou dostupnost těžkých kovů v podkladech pod kolísavou hloubkou vody. Výsledky ukázaly, že fyziologické funkce v mokřadních rostlinách s vysokou kolonizací AMF se mohou zlepšit za určitých podmínek hloubky vody (např. při režimu kolísavé hloubky vody). AMF mohou snížit toxicitu těžkých kovů v semi-akvatických stanovištích zvýšením antioxidačních aktivit v mokřadních rostlinách a modulací distribuce těžkých kovů a biologické dostupnosti. Závěrem lze konstatovat, že tato práce poskytuje možnosti, jak lze AMF využít k odstranění těžkých kovů ze znečištěných odpadních vod pomocí vybudovaných mokřadů pod kolísavou hloubkou vody.

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Chapter I

Introduction

1. Introduction

Heavy metals such as chromium (Cr), cadmium (Cd), nickel (Ni), lead (Pb), and copper (Cu), are mainly from human activities and extensive industrialization (e.g. leather tanning industries, pigment production, and electroplating), can inhibit plant photosynthesis, damage skin, liver and kidney of humans, as well as destroy the freshwater ecological balance (Chen et al., 2015a; Mohanty and Patra, 2011). In recent years, heavy metals released into the environment (e.g. rivers, lakes, and soil) due to the mining wastewater, landfill leaches, municipal wastewater, urban runoff, and industrial wastewater discharge, which have become one of the most serious environmental problems (Renu et al., 2017). As a result, they are easily enriched into animals and humans by the food chains and accumulate in the aquatic organisms, plants, and soil (Wei et al., 2020). Due to the high enrichment and difficult biodegradation, they pose a toxicity threat to the environment even at low concentrations. Therefore, it is particularly necessary to decrease heavy metal contamination in wastewater for the protection of the environment.

Constructed wetlands (CWs), as an effective, environmentally friendly, and economical alternative technology, have been used successfully to enhance wastewater purification for at least five decades and are well known for removing suspended solids, nutrients, bacteria, emerging pollutants, and heavy metals (Vymazal, 2005; Vymazal et al., 2010). Previous studies reported that organic matter and nutrients (e.g. nitrogen and phosphorus) removal efficiencies in CWs were over 40%, and the outflow concentrations of these pollutants in CWs systems can meet the wastewater discharge standards (Stottmeister et al., 2003; Vymazal, 2014). However, some researchers reported that the outflow concentrations of emerging pollutants and heavy metals in CWs were still high, although CW technology had a positive effect on their removal (Verlicchi and Zambello, 2014; Vymazal, 2014). Thus, in order to alleviate the impact of heavy metals on human health and the environment, it is of great significance to improve the wastewater purification capacity in CWs.

Arbuscular mycorrhizal fungi (AMF), as an important component of the population of the soil microorganisms, can form symbiotic associations with the roots of most plant species (nearly 85%) in all terrestrial ecosystems (Dodd and Perez-Alfocea, 2012; Liu et al., 2014). They have large amounts of positive effects on terrestrial plants, such as improving the nutrient status of plants (Ansari et al., 2013), and enhancing plant resistance to heavy metals salinity, drought, disease, water-logging, and cold (Camprubi et al., 2012; Wang et al., 2018a). Therefore, AMF play an important role in terrestrial plant resistance to pollutants stress. Sufficient oxygen content in the soil-plant system provides an important advantage for AMF colonization in plant roots (Ramírez-Viga et al., 2018; Xu et al., 2016). However, the soil moisture in CWs is usually under the saturated situation, which leads to the low oxygen content in the substrate, thus affecting AMF colonization in the roots of wetland plants (Stevens et al., 2011). Despite that, more and more studies confirm that AMF also widely exist in many wetland plant species at different wetland systems around the world over the last three decades (Wang et al., 2018b; Xu et al., 2016). Nevertheless, it is not clear how AMF colonization in the roots of wetland plants changes under different water contents in CWs, and whether AMF play important roles in wastewater purification in CWs.

Therefore, this thesis aimed to investigate (1) the effects of water depths on AMF colonization in different wetland plants to select the best candidates for AMF colonization; (2) the influences of AMF on wetland plant growth and antioxidant response under heavy metal stress to explore the relationships between AMF and physiological functions toward wetland plants; (3) the impacts of AMF on heavy metal distribution and bioavailability in CWs to evaluate the capacity of AMF for promoting wastewater purification. The knowledge gained here can provide a new strategy for the treatment of wastewater by CWs technology.

Chapter II

State of the art

Contents

2.1 Heavy metals contamination in aquatic habitats

2.1.1 Sources and toxicity of heavy metals

Sources of heavy metals in the environment are divided into two categories: natural sources and anthropogenic sources.

Natural sources of heavy metals include volcanic eruptions, mineral degradation, forest fires, evaporation from soil and water surfaces (He et al., 2013). Heavy metal dust formed in the natural process of volcanic eruptions and forest fires floats in the atmosphere, and eventually enters the surface water with rainwater, causing pollution of water bodies (river, lake, ocean, etc.) (Zhang and Wang, 2020). In general, the total amount of mercury (Hg) released into the atmosphere in the world is 6500-8200 mg per year, of which 56.7-81.5% comes from natural processes (primary geological emissions plus secondary emissions) (Driscoll et al., 2013). Matschullat (2000) showed that the global arsenic (As) (metalloid) emissions to the environment from volcanic eruptions and the eruption of submarine volcanoes are 1.72×10^{7} kg/yr and 4.87×10^{6} kg/yr, respectively. Heavy metals such as cadmium (Cd), Cu, Pb, As, and Hg in the soil can be enriched in the process of rock (limestone) weathering and soil formation, which may also cause the accumulated heavy metals to enter the groundwater through irrigation and rainfall leaching (Wang and Lei, 2018; Zhang and Wang, 2020).

Global trends towards industrialization and urbanization have led to an increasing proportion of heavy metals in the environment (Nagajyoti et al., 2010). Compared to natural sources, anthropogenic sources are the main cause of heavy metal pollution in the environment (He et al., 2013). The anthropogenic sources consist of three categories: (1) emissions from heavy metal impurities, such as coal-fired power generation, heating, mining, and other metallurgical activities; (2) emissions from man-made extraction and use of heavy metals, such as leather manufacture, electroplating production, and manufacturing of heavy metal products; (3) emissions from waste incineration, landfill, agricultural fertilizer, etc. The detailed anthropogenic sources of main heavy metals are summarized in **Table 2.1**.

Table 2.1: Main anthropogenic sources of heavy metals

Heavy metals	Main anthropogenic sources
Cu	Fungicidal treatments, liquid manure (mainly from pigs), sewage sludge, atmospheric deposition, particles from car brakes, and mining, smelting, fuel combustion and refining industries (Panagos et al., 2018)
Cd	Smelting industry, agricultural fertilization, dye and plastic pigments, batteries, fossil fuels combusting, wood preservatives, chemical production (Kubier et al., 2019)
Cr	Energy plants, metals production and processing, chemical production, mineral industry, wastewater and waste management, paper and wood production processing, tanning, and dyeing (Tumolo et al., 2020)
Pb	Lead smelters, power plants, mining and beneficiation, burning of lead-containing fossil fuels, accumulators, pigments, leaded petrol, shooting and military activities (Charvát et al., 2020)
Zn	Smelting industry, Vehicle emission, sewage sludge and pig slurry spreading, road runoff, battery manufacture, catalysts, printing, galvanization materials, agricultural fertilizer, and ore processing (Yin et al., 2015)
As (metalloid)	Gold and iron mining, metallurgical plants, phosphate fertilizer plants, glass and ceramics industries (Błaszkowski and Czerniawska, 2013)
Hg	Mining and burning of coal, power generation, ore processing, agriculture (herbicides, fungicides), electrochemistry, batteries, medicine (thermometers, dental amalgams) (Charvát et al., 2020)

In the light of the above, industrial application and urban sources are closely related to the heavy metals accumulation in water bodies and sediments. Moreover, the source of each heavy metal in the water bodies is different (Tumolo et al., 2020). For example, according to the latest update of the European Pollutant Release register, the heavy metals released to all river basin districts in the Czech Republic in the year 2017 were mainly from waste and wastewater management, thermal power stations, and other combustion installations (European Environmental Agency, 2017) (**Figure 2.1**).

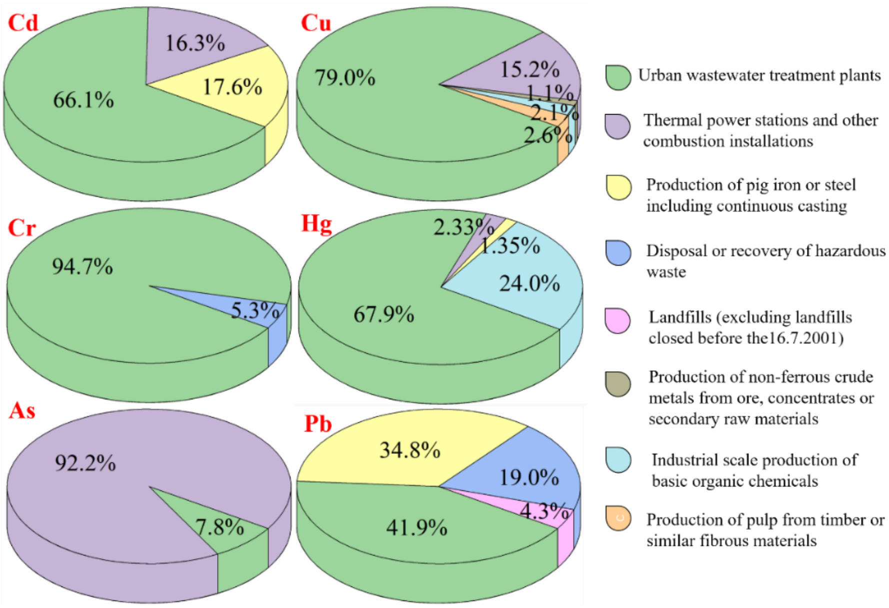

Figure 2.1: Percentage of heavy metals emission in all river basin districts per industrial sector in the Czech Republic. Data source: (European Environmental Agency, 2017)

The release of heavy metals into the aquatic habitats may lead to a variety of physical, chemical, and biological processes, which have a great impact on plants, aquatic organisms (e.g. fish), and even human health (Guo et al., 2018). The influences of heavy metals on plants can be isolated into two classifications which include beneficial effects and harmful effects. Manganese (Mn), iron (Fe), Cu, Zn, and Ni, as the micronutrients needed for plant growth, have advantages for plant structure and its metabolism. However, the excess of these micronutrients (particularly Zn, Ni, and Cu) in water or soil may also result in plant metabolism disorders and even death (Vardhan et al., 2019). Besides, as the non-essential nutrients, Cr, Cd, Pb, Hg, and As are dangerous to plants even at low concentrations (Shahid et al., 2017a). The exposure of heavy metals into human beings mainly observed by the three routes which include oral ingestion, inhalation, and dermal exposure. Among them, oral ingestion is considered as one of the essential routes of these heavy metal exposures compared with inhalation and dermal exposures (Vardhan et al., 2019). Heavy metals can be transferred from aquatic organisms (especially fish, shrimp, and crab) to humans as a result of bioaccumulation and biomagnification in the food chain, thereby causing damage to human health (Ali et al., 2019). The main damages caused by heavy metals to plants and human health are summarized in **Table 2.2**. In addition, heavy metal toxicity is also highly dependent on their valence in the environment. For example, hexavalent Cr is more toxic than trivalent Cr for plants, animals, and microorganisms (Shahid et al., 2017b). Overall, the removal of heavy metals from water or wastewater is important because they pose a serious threat to the health of humans and other organisms.

Table 2.2: The main damages caused by heavy metals to plants and human health

Object	**Damages from heavy metals**
Plants	Interfere with ionic homeostasis and the activity of enzymes (e.g. superoxide dismutase, peroxidase, glutathione reductase, and catalase); increase the generation of reactive oxygen species (e.g. hydroxyl radical, superoxide radical, and hydrogen peroxide); decrease germination, development, and photosynthesis, disorder plant water balance (Adrees et al., 2015).
Humans	Harm or decrease the mental and central nervous activities; damage the lungs, liver, kidneys, blood compositions, and other fundamental organs; muscular dystrophy, Alzheimer's disease, different types of cancers, and multiple sclerosis (Song and Li, 2015).

2.1.2 Heavy metal removal technologies

Heavy metals can easily enter the aquatic system due to industrial wastewater, agricultural runoff, household, and commercial applications. In general, the technologies for removing heavy metals from water or wastewater include chemical precipitation, chemical coagulation/flocculation, electrochemical methods, membrane filtration, ion exchange, adsorption, and bioremediation (**Figure 2.2**).

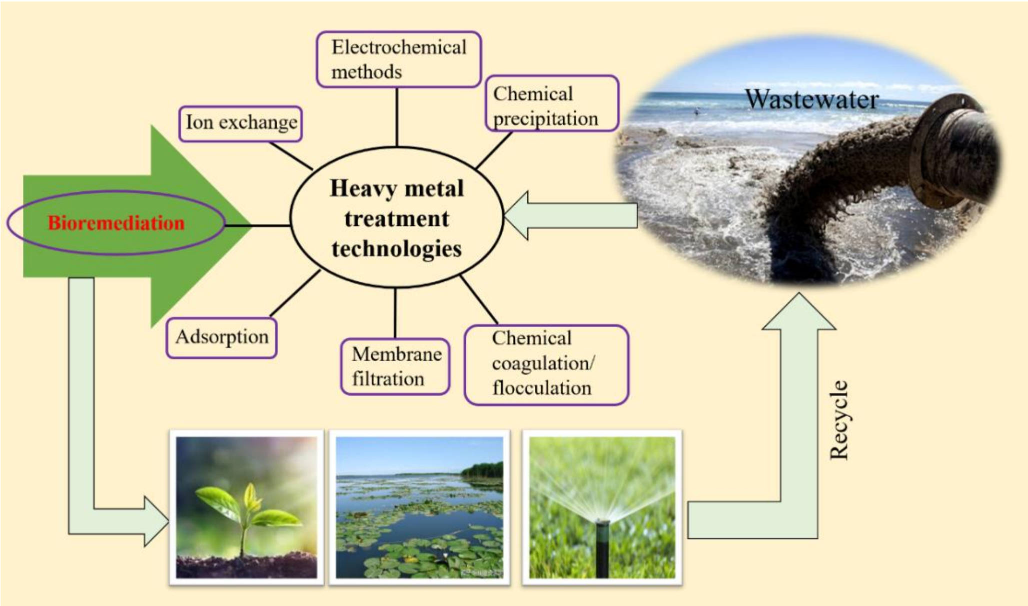

Figure 2.2: Heavy metal treatment technologies in wastewater. Source: (Vardhan et al., 2019)

Chemical precipitation

The chemical precipitation method is widely used for the removal of heavy metals in wastewater due to its low price and relatively simple operation (Xanthopoulos et al., 2017). The main process of chemical precipitation to remove heavy metals includes adjusting the pH of the wastewater to a suitable condition, and then adding a precipitating agent (e.g. CaO, $Ca(OH)_2$, and $NaOH$) to react with the heavy metal ions in the wastewater to form an insoluble precipitate (Balladares et al., 2018; Vardhan et al., 2019). Generally, the main chemical precipitation processes include hydroxide precipitation and sulfide precipitation. Due to the low cost, simplicity, and easy pH control, hydroxide precipitation has become the most widely used chemical precipitation method for the treatment of heavy metals in wastewater (Baltpurvins, 1997). Despite this, the hydroxide precipitation also has limitations in the removal of heavy metals due to the production of large amounts of low-density sludges, easily causing dehydration and disposal problems (Kongsricharoern and Polprasert, 1995). Compared with the hydroxide precipitation, the metal sulfide precipitates obtained by the sulfide precipitation method in wastewater have the characteristics of lower solubility, higher thickening, and dewatering, which is beneficial to improve the heavy metal removal in wastewater (Özverdi and Erdem, 2006). However, this method is prone to produce dangerous hydrogen sulfide vapor, which is easy to cause secondary pollution to the environment (Alvarez et al., 2007). Therefore, the chemical precipitation not only requires a lot of chemicals to increase heavy metal removal in wastewater but also produces a large number of by-products (such as sludge, hydrogen sulfide vapor), which leads to high sludge treatment costs and secondary environmental pollution (Vardhan et al., 2019; Yang et al., 2001).

Chemical coagulation/flocculation

Chemical coagulation/flocculation is the traditional water treatment technology, which also has certain applications in heavy metal polluted wastewater (Johnson et al., 2008). Coagulation treatment is mainly through the use of chemical coagulants (e.g. s alum, ferric chloride, and ferrous sulfate) to destabilize colloidal particles and lead to sedimentation. Among them, the unstable small particles that cannot settle will form massive flocs under the action of flocculants (e.g. poly-aluminum chloride, polyacrylamide, and poly ferric sulfate), thus accelerating the precipitation of compounds containing heavy metals (Teh et al., 2016). Although some heavy metals can be removed quickly by precipitation, heavy metals cannot be removed completely (Bojic et al., 2009; Tokuyama et al., 2010). Moreover, the extensive use of coagulants and flocculants also increases the processing cost.

Electrochemical methods

The electrochemical methods are considered to be extremely effective wastewater treatment technologies, especially for removing heavy metal ions in industrial wastewater. The mechanism of these methods to remove heavy metals in wastewater is mainly to recover heavy metals in the elemental metal state through the anode and cathode reactions in the electrochemical cell (Vardhan et al., 2019). In general, electrocoagulation, electrodeposition, and electroflotation techniques have been widely used in the removal of heavy metal ions from wastewater.

Electrocoagulation combines different mechanisms to remove heavy metal ions in wastewater, which include electrochemical (e.g. metal dissolution and water reduction, pollutant electro-oxidation or electro-reduction), chemical (e.g. acid/base equilibria with pH change, hydroxide precipitation, a redox reaction in the bulk), and physical (e.g. physical adsorption, coagulation, and flotation) processes (Hakizimana et al., 2017). Among them, the main process of electrocoagulation to remove heavy metals involves the production of coagulants in situ by dissolving electrically either iron or aluminum ions from iron or aluminum electrodes (Chen, 2004). Afterward, the flocculated particles float from the water through the hydrogen generated by the anode, thereby achieving the purpose of removing heavy metals (Oden and Sari-Erkan, 2018). Above all, this technology has the advantages of less sludge generation and no need to add chemical agents (Hakizimana et al., 2017). However, the electrocoagulation process still has limitations, such as higher operating and capital investment costs (Song et al., 2017). Electrodeposition is an effective method to recover and remove metal ions from metal plating, metal processing, and electronics industry waste (Mezine et al., 2018). The main advantages of this method are the ability to recover the purest metals, and no sludge disposal problems (Oliveira et al., 2018). Electroflotation is a solid-liquid separation process, which floats the heavy metals to the water surface by small bubbles of oxygen and hydrogen gases generated from the water electrolysis (Adjeroud et al., 2018). Similar to electrocoagulation and electrodeposition technologies, electroflotation is also a fast and controllable method to remove heavy metals in industrial wastewater. Nevertheless, the three electrochemical methods require high capital investment and expensive power supply, which greatly limits their improvement.

Membrane filtration

The application of membrane technology has an excellent effect on heavy metal removal in wastewater (Ali et al., 2017). The main advantages of this technology are high heavy metal removal efficiency, small space requirements, and simple operation (Lam et al., 2018). Different types of membranes, including reverse osmosis, ultrafiltration, nanofiltration, and electrodialysis have been successfully used to remove toxic metal ions in wastewater (Vardhan et al., 2019). Reverse osmosis is composed of a semi-permeable membrane, which allows water to pass through and intercept heavy metals in wastewater, thereby achieving the purpose of separation and concentration of heavy metals (You et al., 2017). Microfiltration and ultrafiltration are currently the most widely used and mature membrane separation technologies, which have the characteristics of simple operation, low energy consumption, and large flux (Song et al., 2017). Both methods are operated at low transmembrane pressures to recover dissolved and colloidal solids (Yousefi et al., 2021). Due to the pore sizes of the metal ions are smaller than the ultrafiltration or microfiltration membrane, inorganic metal ions cannot be intercepted directly. Therefore, the effective removal of heavy metal ions requires other physical and chemical processes such as polymer enhanced ultrafiltration and micellar enhanced ultrafiltration methods (Jana et al., 2018). Nanofiltration is a membrane separation technology between reverse osmosis and ultrafiltration (Al-Rashdi et al., 2013). This method has the advantages of high treatment efficiency, low energy consumption, no secondary pollution, and convenient operation (Salbarde and Bhuyar, 2015). Electrodialysis is a process in which charged ions in a solution selectively permeate ion exchange membranes under an electric field (Nataraj et al., 2007). The electrodialysis membrane device includes both a cation exchange membrane and an anion exchange membrane. Electrodialysis is a relatively mature membrane separation technology, currently mainly used for the recovery of heavy metals in the rinsing water of the electroplating industry (Sadrzadeh et al., 2009). In conclusion, membrane treatment technologies can successfully remove heavy metals in wastewater, but these methods have some ploblems such as complex process, high cost, membrane fouling, and low permeate flux.

Ion exchange

Ion exchange processes have been widely used to remove heavy metals from wastewater due to their many advantages, such as excellent removal rate, high treatment capacity, and rapid kinetics (Kang et al., 2004). Ion exchange resins (synthetic or natural solid resin) have a specific ability to exchange their cations with the heavy metals in the wastewater (Fu and Wang, 2011). The most common cation exchangers are strongly acidic resins with sulfonic acid groups and weakly acid resins with carboxylic acid groups. The hydrogen ion in the resin sulfonic acid group or carboxyl group can be exchanged with heavy metal cations, thereby achieving the purpose of removing heavy metals from wastewater (Vardhan et al., 2019). In addition to synthetic resins, natural zeolite and natural silicate minerals are widely used to remove heavy metals in wastewater due to their high environmental utilization and low cost (Inglezakis et al., 2018; Taffarel and Rubio, 2009). Although there are many reports on the use of zeolites as an ion-exchange resin to remove heavy metals, its application is limited as compared with synthetic resins because it is only used in a laboratory-scale system, not at an industrial-scale level (Fu and Wang, 2011). In addition, the adsorption of heavy metal ions by ion exchange resins is greatly affected by factors such as pH, temperature, initial metal concentration, and contact time (Gode and Pehlivan, 2006). Moreover, this technology requires a high cost to meet the demand for a large number of resins, and chemicals used for ion exchange resin regeneration can produce serious secondary pollutants.

Adsorption

Adsorption is currently considered to be an economical, efficient, and selective treatment method to remove heavy metals in wastewater (Sharma et al., 2019). The method is a solid-liquid mass transfer operation in which heavy metals migrate from wastewater to solid surfaces (adsorbents) and then are combined by chemical or physical adsorption on the adsorbent surface. In general, the method has flexibility in design and operation, and in many cases will produce a high-quality treated effluent (Fu and Wang, 2011). Activated carbon adsorbents (e.g. granular activated carbon, powdered activated carbon, and woven carbon) are widely used in the removal of heavy metals from wastewater because of their high specific surface area and affinity for heavy metals (Ajmal et al., 1998; Kaveeshwar et al., 2018). Despite this, activated carbon also has limited its application due to its high cost and limited regeneration capacity. In order to overcome these problems, agricultural wastes, industrial by-products and wastes, and natural substances have been studied as adsorbents for heavy metal wastewater treatment (Betancur et al., 2009; Sud et al., 2008). Compared with traditional materials, these materials have the advantages of low cost, high efficiency, large adsorption capacity, simple operation, no sludge, and no need for additional nutrients (Fu and Wang, 2011). Based on these advantages, adsorbents can also be used as substrates for bioremediation, for example, activated carbon and wood chips act as substrates applied in the wastewater treatment in constructed wetlands (CWs) (Li et al., 2017; Scholz and Xu, 2002). Unfavorably, adsorption technology also has the risk of desorption of heavy metals from the adsorbent into the environment. Moreover, the adsorption efficiency of heavy metals depends on the type of adsorbents.

Bioremediation

In recent years, bioremediation technology as an environmental-friendly technology (low chemical requirements and operating costs) has focused on more and more attention (Srivastava and Majumder, 2008). The bioremediation process is mainly through the synergistic effects of microorganisms and plants to remove the toxic pollutants from the aquatic environment (Mukherjee et al., 2013). Aquatic plants such as wetland plants (e.g. *Glyceria maxima*, *Phragmites australis, and Typha angustifolia*) have special characteristics to remove heavy metals in wastewater (Vymazal, 2014). Wetlands plants often influence the heavy metal migration and transformation through some processes (such as holding in the rhizosphere, reducing, and complexing) to decrease heavy metal toxicity in the environment (Zeng et al., 2011). Besides, wetland plants can also reduce the concentration of toxic heavy metals through the action of enzymes, thus enhancing plant resistance to heavy metal toxicity. For example, wetland plants can transfer Cr (VI) to Cr (III) by the action of reductase in the body or other reducing organic small molecules (such as glutathione and amino acids) (Shanker et al., 2004). Meanwhile, the rhizospheres in wetland ecosystems provide upgraded supplements supply to the microbial environments of wetland plants, which effectively convert and remove the heavy metals in their biological functions (Agnello et al., 2018). Therefore, CWs, as a bioremediation technology, have been effectively utilized for the treatment of heavy metals in wastewater (e.g. rural overflow, urban sewage, and mine seepage) (Xu and Mills, 2018).

In general, heavy metal removal in water or wastewater is mainly achieved by physicochemical or biological treatment processes. Compared with the bioremediation technology, the conventional technologies (chemical precipitation, chemical coagulation/flocculation, electrochemical methods, membrane filtration, and ion exchange) are normally fast and effective in removing heavy metals. However, high costs and energy consumption are required especially at low heavy metal concentrations (Song et al., 2017). Furthermore, these technologies also easily produce large numbers of toxic chemical by-products, of which disposal is a major problem (Sultana et al., 2014). Therefore, bioremediation technology (especially CWs) might become an attractive alternative for heavy metal removal in the aquatic environment.

2.2 Heavy metal removal in constructed wetlands

2.2.1 Classification of CWs

CWs, as an ecological wastewater treatment technology, have been widely applied to treat various types of wastewater (e.g. domestic wastewater, mine wastewater, landfill leachate, industrial effluents, stormwater, and agricultural runoff) in recent years (Vymazal, 2010). The concentrations of pollutants (e.g. suspended solids, toxic organic matter, heavy metals, pathogenic microorganisms, nitrogen, and phosphorus) in wastewater can be decreased largely by this technology, detailed purification mechanisms for the pollutants removal are shown in **Table 2.3**. The wastewater treatment by CWs is the result of comprehensive action of physics, chemistry, and biology. Among them, substrates, plants, and microorganisms are the three main factors that play an important role in wastewater purification in CWs systems. The wastewater is purified in the CWs systems through the adsorption and filtration of the substrate, the absorption, fixation, transformation, and metabolism of wetland plants, and the decomposition, utilization, and dissimilation of microorganisms (Vymazal, 2014). Meanwhile, the removal efficiencies of pollutants in wastewater are affected by different types of CWs. Therefore, it is necessary to discuss the purification of pollutants in wastewater by various CWs.

Generally, CWs are mainly divided into three types: free water surface CWs (FWS CWs), vertical subsurface flow CWs (VSSF CWs), and horizontal subsurface flow CWs (HSSF CWs) according to the diverse hydrology conditions (open water-surface flow and sub-surface flow), and various water flow direction (horizontal and vertical) (**Figure 2.3**). In addition, hybrid CWs are constructed, based on the different combinations of three types of CWs (such as VSSF- HSSF hybrid CWs), to improve the pollutants (e.g. heavy metals, nitrogen, phosphorus, and chemical oxygen demand) removal efficiencies (Vymazal, 2014).

Table 2.3: Purification mechanisms of pollutants in CWs

Purification mechanism	**Pollutants**	**Position in CWs**
Settlement	Suspended solids	Between wastewater layer and gravel layer
Filtration	Suspended solids	Substrates or plant interspace
Adsorption, ion exchange, chemical precipitation	Phosphate, heavy metals	Bottom sediment, gravel, plant surface
Microbial mineralization and transformation	Organic matter, ammonia nitrogen, nitrite, nitrate	Plant rhizome, sand medium surface, bottom sediment
Assimilation and uptake	Organic matter, nitrogen, phosphorus, heavy metals	Microbes and plants
Solar radiation	Pathogenic bacteria	The surface of the wastewater
Predation	Pathogenic bacteria	Wastewater layer

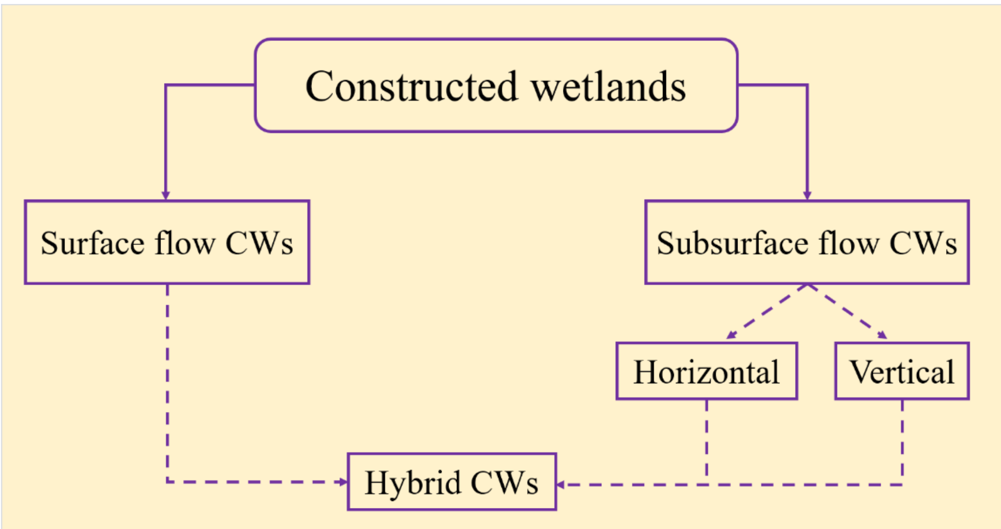

Figure 2.3: Classification of constructed wetlands

Table 2.4: The differences of the wetland plant categories

Categories	Characteristic	Application	Examples
Emergent plants	The roots of plants grow on the base of the substrate, and the aboveground parts of the plant are exposed to the water	FWS CWs, SSF CWs	*Phragmites australis, Juncus effusus, Iris pseudacorus, Glyceria maxima*, *Typha angustifolia*
Submerged plants	The entire plant is submerged in water	Seldom used to remove pollutants	*Euryale ferox salisb, Ceratophyllum demersum, Potamogeton malaianus*
Floating plants	Suspended in water or leaves floating on water	FWS CWs	*Pistia stratiotes, Euryale ferox salisb.*

Note: FWS CWs: free water surface CWs; SSF CWs: subsurface flow CWs.

A typical FWS CWs with wetland plants is a basin, with a water depth of 20-40 cm, containing 20-30 cm of soil or gravel to provide aquatic plants for rooting (Vymazal, 2013). The common wetland plants used in the FWS CWs are emergent plants and floating plants, the differences of the wetland plant categories are shown in **Table 2.4**. Although the FWS CWs are easy to construct and effectively remove organic matter by microbial degradation and colloidal particle sedimentation (Vymazal, 2010), the systems cannot operate properly in winter since the water surface is easy to freeze (**Table 2.5**). Moreover, FWS CWs are often used for tertiary treatment, which showed that the FWS CWs systems have certain limitations in the removal of high-concentration pollutants.

Table 2.5: The advantages and disadvantages of these CWs

Types of CWs	Advantages	Disadvantages
FWS CWs	Simple design, less investment	Easy freeze in winter, easy breed flies, emit an odor
SSF CWs	Good hygienic condition, abundant microorganism, small floor area	High infrastructure requirements, high investment cost

Note: FWS CWs: free water surface CWs; SSF CWs: subsurface flow CWs.

Subsurface flow CWs (SSF CWs) are commonly used wastewater treatment technologies because of their good hygienic conditions, abundant microorganisms, and small floor area (**Table 2.5**). SSF CWs are often filled with 0.4-0.6 m thick water-permeable sand or gravel as substrates to support the plants' growth, the wastewater is fed at the inlet and flows through the substrates and the roots (Vymazal, 2011). Wetland plants in SSF CWs systems are selected as emergent plants due to their large biomass, well-developed root system, strong capacity of oxygen transport, and strong pollution resistance (Shelef et al., 2013). SSF CWs are divided into two types (HSSF CWs and VSSF CWs) according to the water flows path under the surface of the beds. The wastewater flows through the horizontal path in the HSSF CWs (Vymazal, 2011). While the VSSF CWs use a special design such as pipeline and slope to make the wastewater flow vertically distribute in the CWs (Vymazal, 2014). Both SSF CWs are very effective in removing organics and suspended solids. Despite this, VSSF CWs are usually used for primary or secondary treatment, while HSSF CWs are often used for the treatment of wastewater diluted with stormwater runoff (Gorgoglione and Torretta, 2018; Vymazal, 2010). Besides, the aerobics of VSSF CWs are much higher than that of HSSF CWs, which provides suitable conditions for nitrification (Vymazal, 2007). Moreover, compared with the VSSF CWs systems, the influence of substrates on the interception, filtration, and adsorption of pollutants is limited in HSSF CWs due to the wastewater flows horizontally, thereby the lower pollutants (e.g. phosphorus, ammonium, and heavy metals) removal efficiencies may be reported in HSSF CWs.

In summary, various types of CWs differ in their main design characteristics and the types of wastewater that are responsible for purification. For this thesis, the removal mechanisms of heavy metals in wastewater are only considered in the VSSF CWs with emergent macrophytes.

2.2.2 The removal mechanisms of heavy metals in CWs

CWs have been used for heavy metals removal since the early 1980s (Dunbabin and Bowmer, 1992). Since then, these technologies are widely used for heavy metals removal from various types of wastewater, surplus sludge, and even heavy metal-containing aqueous solutions (Michailides et al., 2013; Soda et al., 2012). Generally, one of the most important factors for heavy metals removal is the combined effects of the physicochemical and biological processes including sedimentation, binding to substrates, wetland plant uptake, and precipitation as insoluble forms (mainly sulfides and (oxy-) hydroxides) (Vymazal, 2005; Vymazal et al., 2010). The effective reaction zone in CWs is the root zone (rhizosphere) where physicochemical and biological processes take place through the interaction of substrates, microorganisms, macrophytes, and pollutants (Stottmeister et al., 2003).

Effects of substrates on heavy metals removal

Substrates, which are generally composed of soil, fine sand, gravel, and ash, are the main way to remove heavy metals in CWs. The substrate is also called filler or porous media, which is a bed that can be used for plant growth, hydraulic process, and microbial attachment (Stottmeister et al., 2003). Chen et al. (2009) and Calheiros et al. (2008a) revealed that the substrate plays an important role in heavy metals and nutrient adsorption, growth of emergent plants and microorganisms. The main decontamination processes of the substrate include filtration, ion exchange, obligate and non-specific adsorption, chelation, and sedimentation reaction (Lesage et al., 2007; Lizama Allende et al., 2012). Substrates with poor infiltration ability can decrease the heavy metals removal efficiencies in CWs. Meanwhile, the adsorption capacities of substrates are related to their chemical characteristics. For example, substrates containing Al and Fe ions have the advantage of decreasing the phosphate from wastewater (Batool and Saleh, 2020). The heavy metals are easily adsorbed or settled in the substrate, however, the absorption and accumulation of contaminants by the substrate will reach saturation with the extension of the running time, thus losing the purification function (Stefanakis and Tsihrintzis, 2012). In spite of that, the substrate can still remove more heavy metals in the CWs systems, as compared to emergent plants (Stefanakis and Tsihrintzis, 2012; Sultana et al., 2014; Vymazal, 2014).

Gravel and sand are the most widely used for the substrates because of their low cost and easy to obtain. Rai et al. (2015) showed that the removal efficiencies of Pb, Cu, Zn, As, Cr, Co, Ni, and Mn from urban sewage in CWs filled with gravel in summer were achieved to 86%, 84.01%, 83.48%, 82.23%, 81.63%, 76.86%, 68.14%, and 62.22%, respectively. Aslam et al. (2007) reported that significant heavy metals (Fe, Cu, and Zn) removal efficiencies (35-45%) were obtained in the gravel-based wetland. Besides, limestone, zeolite, and cocopeat can also be used as wetland substrates, which have positive effects on heavy metals removal in VSF CWs (Allende et al., 2011). Moreover, some adsorbents, such as activated carbon, waste materials (e.g. alum sludge, oyster shell, organic wood mulch, quartz, slag, and crushed brick) are also applied in the CWs to improve the adsorption of heavy metals by substrates (Batool and Saleh, 2020). However, these adsorbents may also cause some disadvantages that include increasing operating and maintenance costs, accelerating clogging. Therefore, the selection of suitable substrates in CWs should not only consider the removal efficiency of heavy metals but also evaluate the operating cost and other factors.

Effects of microorganisms on heavy metals removal

Wastewater purification in CWs is also affected by the species, biomass, total group structure, as well as physiological and biochemical properties of microorganisms. The root zone is the most active microbe-heavy metals interaction zone in CWs systems and the interactions are very complicated. The main processes for the microbial enrichment of heavy metals in the environment include: an active process that depends on energy, called bioaccumulation; and a passive process that does not rely on energy, called biosorption (Gadd, 2004). Microorganisms in CWs improve the heavy metals removal from wastewater mainly through the processes of metal speciation, biosorption, precipitation by sulfate reduction, and methylation of heavy metals (Kosolapov et al., 2004; Sultana et al., 2014; Vymazal, 2014). The adsorption of heavy metals by microorganisms in CWs is controlled by a variety of mechanisms and interactions, including ion exchange, chelation, and adsorption (Kosolapov et al., 2004). Meanwhile, heavy metals in CWs can be immobilized by some microorganisms through precipitation, dissimilatory reduction, and microbial metabolism interactions

(Kosolapov et al., 2004). In addition, the wetland plant root systems can also form a symbiotic relationship with bacteria and fungi, thereby reducing the toxicity of heavy metals in wastewater and improving the efficiency of phytoremediation (Marchand et al., 2010). Xu et al. (2016) showed that wetland plants can accumulate higher heavy metal concentrations in the presence of AMF in CWs, resulting in enhancing the heavy metal removal in wastewater. This provides a possibility to improve heavy metal removal efficiency from wastewater in CWs by increasing the activities or types of microorganisms that are beneficial to heavy metal removal (such as AMF).

Effects of wetland plants on heavy metals removal

Wetland plants play an important role in heavy metals removal because they can (a) release root exudates that may affect heavy metals migration and toxicity, (b) provide large surface area for microbial growth, and (c) accumulation of heavy metals in their tissue (shoots, roots, and rhizomes) (Southichak et al., 2006; Zhang et al., 2010). Generally, heavy metal concentrations are commonly higher in the underground parts (roots and rhizomes) of wetland plants as compared to the aboveground parts (flowers, leaves, and stems) (Fawzy et al., 2012; Vymazal, 2016). Roots are the most important sites for heavy metals uptake and further translocation may be limited due to the plant tissue cell walls that can bind or precipitate heavy metal ions (Sultana et al., 2014). Moreover, the accumulation, retention, and fixation of heavy metals in wetland plant roots are mainly carried out by the process of rhizosphere filtration (Sultana et al., 2014). Meanwhile, wetland plants can also promote the deposition of heavy metals in the substrate through rhizodeposition (Kidd et al., 2009).

P. australis and *T. angustifolia* are widely used for heavy metals removal in HSSF CWs systems due to their good growth with properties of accumulation for different heavy metals (Batool and Saleh, 2020). Calheiros et al. (2008b) revealed that the concentrations of Cr in rhizomes, shoots, and leaves of *P. australis* were reached to 4825, 883, and 627 mg/kg after 6 weeks of exposure in the tannery wastewater, respectively. Sultana et al. (2015) reported that Cr (VI) removal efficiency was 87% in the HSSF CWs planted with *P. australis* while the removal efficiency in unplanted CWs only up to 21%. In addition, other emerged plants also have positive effects on heavy metals removal in CWs. Mustapha et al. (2018) indicated that VSSF CWs planted with *Typha latifolia*, *Cyperus Alternifolius*, and *Cynodon dactylon* can be used for the large-scale removal of heavy metals (Cu, Cr, Zn, Pb, Cd, and Fe) from secondary refinery wastewater. *Canna indica* and *Cyperus alternifolius* also have efficient heavy metals (Cu, Cr, Co, Ni, and Zn) accumulation in the underground parts (Yadav et al., 2012). Furthermore, based on the similar properties to *P. australis*, *Phalaris arundinacea*, as a common wetland plant in the Czech Republic and other European countries, is also widely used to remove heavy metals (e.g. Cr, Cu, Mn, Zn, Cd) in CWs (Březinová and Vymazal, 2015). Although wetland plants are vital to the removal of heavy metals from wastewater in CWs, heavy metals also can result in toxicity to the wetland plants (especially under the high concentrations of heavy metals), which may decrease the heavy metals removal efficiencies and even lead to plant death. Therefore, the detoxification mechanism towards heavy metals stresses in CWs needs detailed research, and the improvement of wetland plants' tolerance to heavy metals stress is also needed to explore.

2.2.3 Detoxification mechanisms of wetland plants to heavy metals

The purification effect of CWs on pollutants in wastewater is related to plant growth and plant physiological and ecological changes. However, physiological and biochemical responses in the growth of different wetland plant organs are influenced by heavy metal stress. The high solubility and strong oxidizing capacities of heavy metals can cause phytotoxicity. The excessive heavy metal contents in CWs can damage the cell membranes and easily cause oxidative stress in wetland plants, which results in the decrease of enzyme activities, biomass, chlorophyll and nutrients contents, etc (Sultana et al., 2014). In order to alleviate the toxicity of heavy metals, wetland plants can also resist heavy metal stress through various biochemical reactions. Rai et al. (2015) indicated that the detoxification mechanism to heavy metal stress in CWs is the multigenic adaptability of wetland plants. The main resistance mechanisms of wetland plants to excessive heavy metal stress include (1) precipitation of heavy metals through biomineralization; (2) formation of complexes between glutathione and heavy metals and transport to the vacuoles of roots; (3) production of organic ligands enriched in non-protein thiols (e.g. metallothionein, plant chelating protein, and cysteine), which helps to form chelate complexes with heavy metals, later transporting them to vacuoles (Batool and Saleh, 2020; Verbruggen et al., 2009). Shanker et al. (2005) showed that the trace transport of heavy metals by plant roots may be due to the sequestration in vacuoles, which forms a natural defense to compensate for the potentially toxic effects of heavy metals. Palmer and Guerinot (2009) also observed that metallothionein can protect against oxidative stress, help to bind heavy metals, and maintain homeostasis in plants. Besides, Ryan et al. (2001) indicated that plants can act as a catalyst in biochemical reactions between heavy metals and organic acids (e.g. citrate, oxalate, malate, malonate, fumarate, and acetate), thus resulting in decreasing metal phytotoxicity by the formation of chelate metallic ions. In all, wetland plants have a certain tolerance to heavy metal stress, but the irreversible damage caused by heavy metals still exists, especially under the high heavy metal stress.

2.3. Arbuscular mycorrhizal fungi application in constructed wetlands

2.3.1 AMF status in wetland plants

In recent years, many studies have proven that AMF can exist in the roots of many wetland plants in wetland status (Xu et al., 2016). Wetland plants such as *Scirpus acutus, Scirpus validus, Panicum clandestinum, Typha latifolia, Phalaris arundinacea, Phragmites communis, Juncus effusus, Glyceria maxima, Scirpus triangulatus,* and *Phragmites australis* can be colonized by AMF, and the various percentages of AMF colonization in the roots of wetland plants were observed under different wetland status (e.g. freshwater marsh, fen, floating wetlands, floating mats, coastal wetland, urban wetland, and CWs) (**Table 2.6**). This difference is the result of a combination of many factors, for example, wetland sizes and types, plant species and traits (e.g. salt tolerance, lifestyle, and origin), climate, nutrients, hydraulic conditions, water quality (e.g. pH, salinity, and pollutant concentration), and AMF species (Guo and Gong, 2014). Choudhury et al. (2010) evaluated the AMF distribution in the marshy and shoreline vegetation of Deepar Beel Ramsar Site of Assam, India, observing that most rhizospheric soil samples were found to be dominated by *Glomus morphotypes*, and all total 18 AMF morphotypes were recorded to be composed of four genera: *Glomus* (66.67%), *Acaulospora* (16.66%), *Gigaspora* (11.11%), and *Scutellospora (5.56%)*. Gao et al. (2020) showed that the AMF inoculation percentages of 32.0%, 46.0%, and 40.7% in the roots of *Canna indica* were achieved under the salt concentrations of 0, 2500, and 5000 mg/L in ecological floating beds, respectively. Wu et al. (2014a) observed that low *Funnelliformis mosseae* (16.5%) and *Rhizophagus irregularis*

(18.1%) colonization percentages in the roots of *P. australis* were obtained under the same experimental conditions. Fester (2013) also evaluated the *F. Mosseae* and *R. irregularis* colonization in the roots of *P. australis* under the CWs, the colonization percentage was above 25% and even achieved 85%. On average, AMF colonization in dicots (58%) is generally higher than in monocots (13%) (Xu et al., 2016). Meanwhile, AMF colonization in the roots of wetland plants is regarded as lower in aquatic systems than that in terrestrial ecosystems (Lumini et al., 2011). Despite this, the presence of AMF has a wide range of benefits for their host plants.

The physiological functions toward wetland plants such as biomass, enzyme activities, and nutrient concentrations can be enhanced after AMF colonization successfully in the wetland plants (Hu et al., 2021; Wang et al., 2018b). Gao et al. (2020) showed that AMF increased the total dissolved solids, chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP), and salt ions (Na, K, Mg, and Ca) removal in ecological floating bed with planted *C. indica*, compared to the without AMF inoculation systems in September. Bao et al. (2019) indicated that the concentration and total amount of leaf phosphorus in three flooding regimes (none, intermittent and continuous) were higher in the mycorrhizal rice plants than that in the non-mycorrhizal plants. In addition, AMF can also affect the composition, succession, and diversity of the wetland plant community in aquatic habitats (Weishampel and Bedford, 2006). AMF mainly affects plant diversity by regulating plant competition and affecting community uniformity and helps build plant communities by allowing mycorrhizal plants to compete with non-mycorrhizal plants in wetland habitats (Xu et al., 2016). Therefore, AMF may have important ecological significance even the low colonization in the roots of plants is observed. Moreover, high AMF colonization in the roots of plants is beneficial to improve the physiological functions of wetland plants and enhance the ecological functions of wetland systems (Xu et al., 2016). Hence, it is of great importance to increase the AMF colonization in the roots of wetland plants under aquatic habitats.

Table 2.6: AMF colonization in the roots of wetland plants observed in aquatic habitats

Plant species	Aquatic habitats	Wetland scale	AMF colonization (%)	Mycorrhizae	Sources
Typha angustifolia, Typha x glauca, Typha latifolia	Floating wetlands, floating mats (USA)	Full-scale	4-13	*Glomus albidum, Glomus caledonium, Glomus microcarpum*	(Stenlund and Charvat, 1994)
Typha angustifolia	Aquatic and semi-aquatic environments (USA)	Lab-scale	+	*Funneliformis mosseae*	(Tang et al., 2001)
Lythrum salicaria	Aquatic and semi-aquatic environments (USA)	Lab-scale	+	Unspecified mix	(Stevens et al., 2002)

Scirpus acutus, Scirpus validus, Panicum clandestinum	Freshwater marsh (USA)	Full-scale	3-95	Unspecified mix	(Bauer et al., 2003)
Typha latifolia, Phalaris arundinacea,	Fen and marsh habitats (USA)	Full-scale	0-50	Unspecified mix	(Bohrer et al., 2004)
Carex tribuloides, Phalaris arundinacea, Rumex orbiculatus	Aquatic and semi-aquatic environments (USA)	Lab-scale	4.1-49.0	*Glomus spp., Gigaspora spp.*	(Fraser and Feinstein, 2005)
Typha latifolia, Panicum hemitomon	Aquatic environments (USA)	Lab-scale	1-17	Unspecified mix	(Ipsilantis and Sylvia, 2007a)
Imperata cylindrica, Phragmites karka, Cyperus rotundus	Marshy and shoreline (India)	Full-scale	32.6-46.9	*Glomus, Acaulospora, Gigaspora, Scutel-lospora*	(Choudhury et al., 2010)

Fen plant species (e.g. *Typha latifolia, Carex lasiocarpa*)	Fen (USA)	Full-scale	0-55.5	Unspecified mix	(Twanabasu et al., 2013)
Phragmites australis	Constructed wetland (Germany)	Lab-scale	25-80	*Funneliformis mosseae, Rhizophagus irregularis*	(Fester, 2013)
Phragmites communis, Juncus effusus	Saltwater bay (China)	Full-scale	+	*Glomeromycota*	(Guo and Gong, 2014)
Phragmites australis	Wetlands (China)	Full-scale	5.4-11.7	Unspecified mix	(Wang et al., 2015)
Phragmites australis	Constructed wetlands (China)	Lab-scale	9-18	*Glomus mosseae, Glomus clarum, Glomus claroideum, Glomus etunicatum, Glomus intraradices*	(Zheng et al., 2015)

Phragmites australis	Aquatic environment (Italy)	Pilot-scale	1-11	*Funneliformis mosseae,*	(Lingua et al., 2015)
Phragmites australis	Aquatic and semi-aquatic environments (China)	Lab-scale	+	Unspecified mix	(Liang et al., 2018)
Scleria bracteata, Rhynchospora colorata, Cladium jamaicense	Coastal wetland (Mexico)	Full-scale	20-65	Mix species (e.g. *Funneliformis geosporum, Rhizophagus intraradices, Claroideoglomus claroideum*)	(FabiÁN et al., 2018)
Aquatic plants (*e.g. Typha orientalis, Typha minima, Scirpus validus, Phragmites australis, Juncus effusus*)	Urban wetland (China)	Full-scale	2-72	Unspecified mix	(Wang et al., 2018b)

Canna indica, Canna flaccida, Watsonia borbonica	Constructed Wetlands (Portugal)	Full-scale	+	*Glomus spp, Rhizophagus spp, Acaulospora spp.*	(Calheiros et al., 2019)
Atriplex halimus, Atriplex canescens, Suaeda fruticosa, Marrubium vulgare, Dittrichia viscosa	Wetlands (Algeria)	Full-scale	24-98	*Glomus spp, Funneliformis spp, Rhizoglomus spp, Sclerocystis spp, Septoglomus spp*	(Sidhoum and Fortas, 2019)
Canna indica	Floating wetlands (China)	Lab-scale	32-46	*Glomus etunicatum*	(Gao et al., 2020)
Phragmites australis	Vertical flow constructed wetlands (China)	Lab-scale	21	*Rhizophagus intraradices*	(Xu et al., 2020)

Phragmites australis	Aquatic environment (China)	Lab-scale	18–21	*Rhizophagus irregularis*	(Wu et al., 2020)
Glyceria maxima	Constructed wetlands (Czech Republic)	Lab-scale	61.3	*Rhizophagus irregularis*	(Hu et al., 2021)

Note: “+” means the plant roots are colonization by AMF without an accurate value of colonization percentage.

2.3.2 Factors influencing AMF colonization in CWs

Numerous studies have demonstrated the prevalence and importance of AMF in wetland habitats (Cooke and Lefor, 1998; Huang et al., 2018; Wang et al., 2018b). However, the percentages of AMF colonization in the roots of wetland plants are showed differences under the various wetland systems. According to the previous studies on AMF application in the aquatic environment, the main factors influencing AMF colonization in CWs conclude that phosphorus (Tang et al., 2001), wastewater quality (Wang et al., 2010), hydraulic condition (Vallino et al., 2014), CW types (Wu et al., 2001), and operation modes of CW (Li et al., 2011a). The detailed information is shown in **Table 2.7**.

Table 2.7: Factors influencing AMF colonization in CWs

Factors	AMF colonization in wetland plants
Phosphorus	The high AMF colonization found at low phosphorus concentration (< 30 mg/kg) (Wang et al., 2010)
Wastewater quality	AMF colonization (e.g. arbuscule numbers and spore densities) gradually decreased with the increase of pollutant concentration (Akhtar et al., 2019; Wu et al., 2019)
Hydraulic condition	(a) AMF colonization in wetland plants gradually decreased with the increase of hydraulic loading rate (Miller, 2000; Wang et al., 2011); (b) AMF colonization in the root of wetland plants was not related to the hydrologic loading rates (Ipsilantis and Sylvia, 2007b; Turner et al., 2000)
CW types	The order of AMF colonization in wetland plants in CWs: VSSF CWs > HSSF CWs (Xu et al., 2016)
Operation modes of CW	High AMF colonization in wetland plants could be observed in aeration, summer season, wastewater recirculation, alternation of wetting and drying, and intermittent operation conditions (Li et al., 2011b)

2.3.3 Role of AMF in heavy metal contaminated CWs

AMF have developed various strategies to persist in a heavy metal contaminated environment and to avoid the damage produced by heavy metals. Many studies have proven that the fast-growing hyphae can grow under heavy metal stress, thereby promoting symbiosis with host plants (Cabral et al., 2015; Wu et al., 2019). If AMF successfully symbioses with the host plant, they can induce certain changes in host plants to deal with this heavy metal-polluted environment. AMF have positive effects on the detoxification of plants under heavy metal stress. The possible role of AMF in wetland plants' tolerance to heavy metal stress is shown in **Figure 2.4**. Based on previous studies on the influence of AMF on heavy metals remediation in terrestrial ecosystems (Garg et al., 2018; Riaz et al., 2021), AMF-induced changes in the wetland plants under heavy metal stress may generally be divided into direct effects and indirect effects (Garg et al., 2018). AMF mainly reduce heavy metal toxicity in plants directly through the mycorrhizal pathways, including extraradical mycelium promotes heavy metals uptake and transport to the intraradical fungal structures (Ren et al., 2015), and heavy metals transport to the intraradical fungal structures from root cells that absorb Cr directly from the environment (Wu et al., 2019). Meanwhile, AMF may be used to promote heavy metal remediation in the environment by altering subcellular compartmentalization and chemical forms of heavy metals in plants (Wu et al., 2016a). Besides, AMF indirectly induce tolerance in the plants by increasing shoot and root biomass (Dolinar and Gaberščik, 2010), improving water and nutrient uptake (Chen et al., 2019), promoting antioxidant response (e.g. increasing antioxidant enzyme activities, decreasing the production of reactive oxygen species) (González-Guerrero et al., 2010), and causing changes in root morphology (Riaz et al., 2021).

In conclusion, AMF could effectively improve heavy metal removal in CWs. However, the optimum conditions for AMF survival in CWs are not completely investigated, and the detailed mechanisms of AMF to enhance the removal of heavy metals in CWs are still unclear. Therefore, further studies should be investigated to enhance the wastewater purification in CWs under optimum operating conditions conducive to AMF survival.

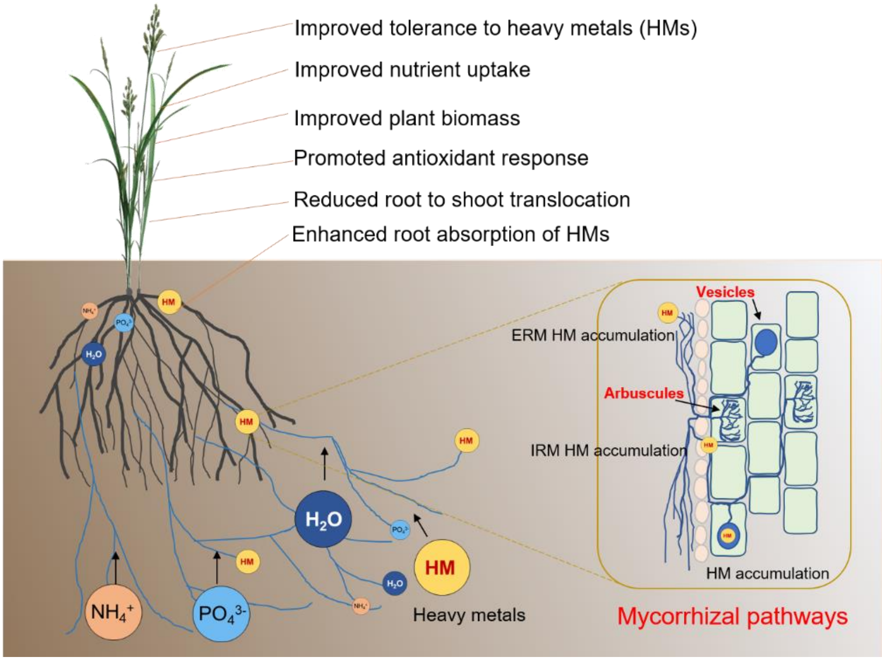

Figure 2.4: The possible role of AMF in wetland plants' tolerance to heavy metal stress (Note: HM: heavy metal; ERM: Extra- radical hyphae; IRM: Intra- radical hyphae)

Chapter III

Arbuscular mycorrhizal fungi colonization and physiological functions toward wetland plants under different water regimes

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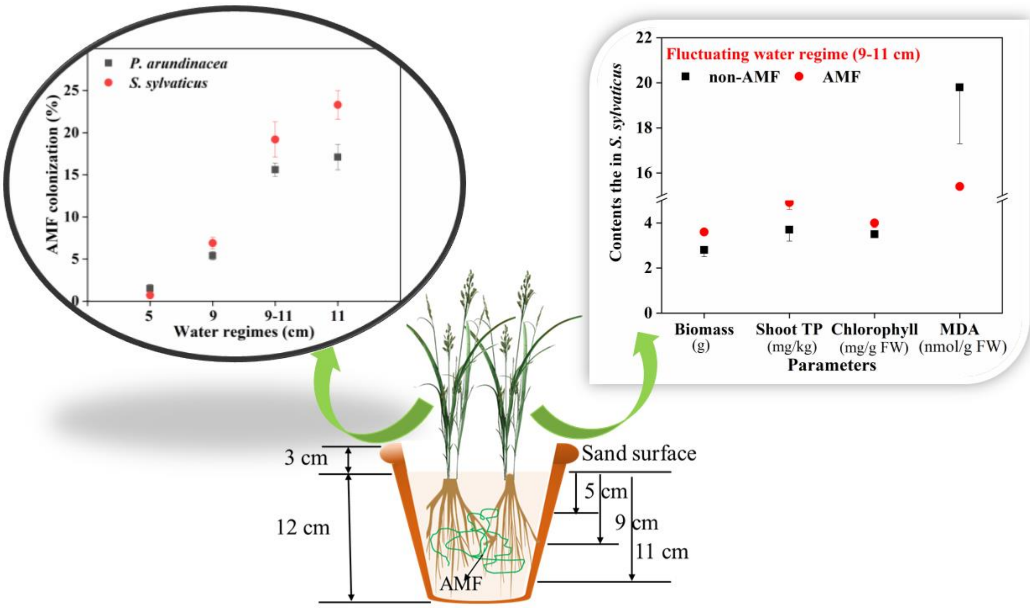
Contents

3.1 Abstract

Arbuscular mycorrhizal fungi (AMF) have been widely reported to occur in the association with wetland plants. However, the factors that affect AMF colonization in wetland plants and physiological functions in AMF inoculated wetland plants are poorly studied. This study investigated the effects of four water regimes (below the surface of sands: water levels of 5 cm, 9 cm, 11 cm, and fluctuating water depth (9-11 cm)) on AMF root colonization in two wetland plants (*Phalaris arundinacea* and *Scirpus sylvaticus*) which are commonly used in a constructed wetland. Results showed that two lower water regimes were the most suitable for the formation of root colonization by AMF. Plant species did not show any significant difference in AMF colonization. The AMF colonization of 15.6-23.3% in the roots of both wetland plants was determined under the water regimes of 11 cm and 9-11 cm. In comparison to the non-inoculated plants, root length, shoot height, biomass, shoot total phosphorus, and chlorophyll contents of both wetland plants under the fluctuating water regimes (9-11 cm) were increased by 35.4-46.2%, 13.1-26.6%, 33.3-114.3%, 25.7-80% and 14.3-24%, respectively. Although malondialdehyde (MDA) contents in both AMF inoculated wetland plants were decreased under the lower water levels, the MDA contents under the water regime of 11 cm were still high. Therefore, these results indicated that the physiological functions in wetland plants with high AMF colonization might be improved under a specific water regime condition (e.g. depth of fluctuating water regime).

Figure 3.1: Graphical abstract



3.2. Introduction

Arbuscular mycorrhizal fungi (AMF) are an important component of the population of the soil microorganisms, they can form a symbiotic association with the roots of most plant species (nearly 85%) in all terrestrial ecosystems (Dodd and Perez-Alfocea, 2012; Liu et al., 2014). Numerous researchers have indicated that AMF have a large amount of positive effects on terrestrial plants, such as improving the nutrient status of plants (Ansari et al., 2013), enhancing the resistance of the plant to heavy metals, salinity, and cold (Camprubi et al., 2012), as well as increasing the activities of rhizosphere microorganisms (Artursson et al., 2005). Therefore, AMF play a key role in terrestrial ecosystems. In the last two decades, more and more studies have confirmed that AMF are also widely present in many species of wetland plants in different wetland systems around the world (Wang et al., 2011; Xu et al., 2016). However, AMF colonization in wetland plant roots is still low due to a variety of negative factors (e.g. wetland environment, water, plants) (Kohout et al., 2012; Xu et al., 2016), and the knowledge of AMF effect on the aquatic environment is still scarce.

Wetlands are unique habitats at the interface between terrestrial and aquatic ecosystems, which have ecological significance in natural waste purification, nutrient cycling, and flood prevention (Zedler and Kercher, 2005). AMF can easily survive and colonize in plant roots under aerobic conditions, which is why high AMF colonization is detected in terrestrial plants (Ramírez-Viga et al., 2018; Xu et al., 2016). Compared to terrestrial plants, lower AMF colonization is determined in wetland plant roots, which mainly results from low oxygen content in the wetland system (Brundrett, 2009; Stevens et al., 2011). Meanwhile, the roots of wetland plants can uptake nutrients directly from water for their growth, which also results in low AMF colonization in the wetland system (Xu et al., 2016). Therefore, it is very important to control the water content in wetland systems in order to increase AMF colonization in wetland plants. Previous studies reported that AMF colonization in wetland plants gradually decreased with the increase of water regime (Miller, 2000; Wang et al., 2011). Despite that, increasing evidence demonstrates that AMF are observed in the roots of many wetland plants under different hydrologic sites around the world (Sidhoum and Fortas, 2019; Wang et al., 2018b; Xu et al., 2016). Moreover, AMF can even be detected in submerged wetland plants (Sudová et al., 2015), and also can be found in some pollutant stress conditions (e.g. heavy metals) (Ramírez-Viga et al., 2018). In contrast, some researchers revealed that soil moisture had no significant effect on AMF colonization, as well as the percentage of root in AMF colonization was not related to the hydrologic category (Ipsilantis and Sylvia, 2007b; Turner et al., 2000). In addition, previous studies showed that AMF colonization was significantly lower in continuously flooding conditions than in intermittent flood conditions (such as drying-rewetting cycles or fluctuating water depth) (Liang et al., 2018; Stevens et al., 2011). Li et al. (2011a) and Shi et al. (2015) also indicated that alternation of drying-rewetting or intermittent operation can bring oxygen into wetlands, which was beneficial to the growth, abundance, and diversity of AMF in the wetland ecosystem, and wetland plants growth also can be improved. Therefore, it is necessary to explore which water regime condition can improve AMF colonization and also promote wetland plant physiological functions in wetland systems.

AMF may enhance wetland plants tolerance to environmental stresses (e.g. drought, salt, pollutants) and increase vegetation restoration in wetlands by determining the physicochemical indicators of wetland plants (e.g. biomass, malonaldehyde, and chlorophyll) (Bharti and Garg, 2019; Sidhoum and Fortas, 2019; Wang et al., 2018a). However, different wetland plant species colonized by AMF are not consistent, which easily leads to different tolerance of wetland plants to environmental stress (Stevens et al., 2011). Therefore, it is necessary to determine AMF colonization in different wetland plants. In general, AMF colonization was higher in dicots (58%) than in monocots (13%) (Xu et al., 2016), and the frequency of AMF hyphae is significantly higher in angiosperms than in both *Isoetes* species (Kohout et al., 2012). Meanwhile, AMF colonization is also related to well-developed aerenchyma in wetland plant roots (Xu et al., 2016). Wang et al. (2018b) studied AMF status in urban wetland plants and its impact factors, results showed that 87.5% of plants (49 of 56 species including *Costus speciosus*, *Polypogon fugax*, *Adenophora trachelioide*, *Senecio scandens*, *Tephroseris palustris*, *Phragmites australi*, *Typha orientalis*, and *Glyceria maxima*) were colonized by AMF, with colonization ranged from 2% to 72%. Fester (2013) also showed that substantial AMF colonization was observed (colonized root length: 25-80%) in *P. australis* roots although these fungi were exposed to high concentrations of toxic pollutants. In addition, more and more studies have proved that high AMF colonization can provide a wide range of benefits for the physiological functions of wetland plants (Grilli et al., 2014; Ramírez-Viga et al., 2018; Wang et al., 2018a).

Therefore, the aim of this work was to (1) assess AMF colonization in different wetland plants under various water regimes; (2) evaluate the effects of AMF and water regimes on the physiological functions of wetland plants.

3.3. Materials and methods

3.3.1 Host plants

Phalaris arundinacea and *Scirpus sylvaticus* were selected from natural ponds in the Czech University of Life Sciences Prague campus. The roots of each plant were surface sterilized with 75% ethyl alcohol for 10 seconds and 1% sodium hypochlorite (NaClO) for 15 minutes, washed carefully with sterile distilled water five times. Some roots were selected from each plant to detect AMF colonization before transplanted into the sterilized pots. Finally, *P. arundinacea* and *S. sylvaticus* with no AMF colonization were used as experimental plants.

3.3.2 Arbuscular mycorrhizal fungus

The AMF inoculum (*Rhizophagus irregularis* BEG140) was obtained from the Institute of Botany, Czech Academy of Sciences, Průhonice, Czech Republic. The inoculum comprised a mixture of spores, mycelium, sandy soil, and plant root fragments.

3.3.3 Experimental design

The experiment used a factorial design with 2 regimes of AMF (*R. irregularis* BEG140 and non-inoculated), 4 water regimes (below the surface of sands:11 cm depth of water, 9 cm depth of water, 5 cm depth of water, and fluctuating water depth with water depth vary between 9 cm and 11 cm), and 2 wetland plant species (*P. arundinacea* and *S. sylvaticus*), making a total 16 treatments, 5 replicates for each treatment. The cycle of fluctuating water depth was 4 days (2 days with 9 cm depth of water and 2 days with 11 cm depth of water). Considering water loss, we added water every day and keep water in each depth of water. The experiment was carried out in a pot with a dimension of 17 ×15 cm (diameter × height). The experimental setup was shown in **Figure S1**. Sand with a grain size of 0.1-0.5 mm was used as experimental substrate after sterilization at 150 °C for 2 h. For AMF addition systems, 1500 g sterilized sand was first put into the pot, then 1000 g sand containing 50 g fungal inoculum was added. Three ramets of plants (average height of 15 cm) were transplanted into each pot, and make sure roots of each plant were placed in the substrate layer containing fungal inoculum, finally 500 g of sand were added after plants were transplanted. For non-inoculated controls, the fungal inoculum was replaced by 50 g of sterilized inoculum and 10 mL inoculum filtrate (except for AMF) including soil microbial communities. The experiment was conducted at a greenhouse in the Czech University of Life Sciences Prague, with the following climate condition parameters: 25 °C and 14 h light at day time; 20 °C and 10 h at dark time. Distilled water was used to control the water regimes. In addition, each pot was irrigated with 1/4-strength Hoagland solution (100 mL/week/pot) with a P concentration of 7.8 mg/L and N concentration of 42 mg/L to avoid nutrient deficiency, which was diluted with deionized water. The plants were maintained for 3 months before harvest.

3.3.4 Sample analysis

The aboveground heights of plants in the 120 pots were measured every month. Plant shoots and roots were harvested separately, the height and fresh weight were measured afterward, then washed carefully with deionized water for further analysis. Subsamples of about 2 g fresh weight of roots were collected for the determination of AMF colonization. Samples of about 5 g fresh weight of plants were used to determine the root to shoot ratio, relative saturated water content (RWC), chlorophyll, and MDA concentrations. Dry weights of shoots and roots were determined after oven drying at 70 °C for 48 h. The dried samples were used to measure the biomass, total phosphorus (TP), total nitrogen (TN), total carbon (TC), and mycorrhizal dependency.

Samples of about 2 g fresh weight of roots in each pot were cut into approximately 1 cm pieces to determine the root length colonization rate of AMF. Samples of fresh roots in each pot were cleared in 10% KOH (30 min, 90 °C), then washed in water, acidified with 2% HCl solution for 3-5 min, and stained with Trypan blue (30 min, 90 °C), finally destained with 50% glycerol for 2-3 days (Phillips and Hayman, 1970). The roots were cut into approximately 1 cm pieces and mounted onto microscope slides before viewing under a stereomicroscope. Colonization percentage was determined according to the method of MYCOCALC software (National Institute for Agricultural Research) (Trouvelot et al., 1986). Relative saturated water content, root to shoot ratio, and biomass were measured by the gravimetric method. Chlorophyll content was determined by spectrophotometry with acetone extracts (Palta, 1990). MDA content in leaf was determined according to Chen et al. (2013). TP content in the roots and shoots was determined by a colorimetric analysis after digestion in nitric-perchloric acid (Sommers and Nelson, 1972). TC and TN contents in the roots and shoots were directly determined by a Skalar Primacs SNC analyzer (Breda, the Netherlands), NIST 1547 Peach Leaves was used as the standard (National Institute of Standards and Technology, Gaithersburg, MD, USA). Mycorrhizal dependency was calculated by the equation given below:

$$\text{Mycorrhizal dependency (g/g DW)} = \frac{\text{Dry biomass of inoculated fungus plants}}{\text{Dry biomass of controlled plants}}$$

DW: dry biomass of plants.

3.3.5 Statistical analysis

Physiological functions in wetland plants were analyzed using a two-way analysis of variance (ANOVA), with water regime and AMF colonization as main factors and water regime * AMF colonization as an interaction effect. AMF colonization was analyzed using a two-way ANOVA with plant species and water regime as main factors and plant * water regime as the interaction effect. Duncan's multiple range tests were used to compare the effects of plant species and water regime on the AMF colonization, biomass, RWC, mycorrhizal dependency, chlorophyll, MDA, TC, TN, TP, and root to shoot ratio, $p < 0.05$ was set as a significant difference. Principal component analysis (PCA) was completed to display data groupings and correlations for plant parameters. The statistic software Origin 2018 for windows was used to perform the test and to plot the graphs. All statistical analyses were performed using the software package IBM SPSS statistics 21.0 for Windows.

3.4 Results and discussion

3.4.1 Effect of water regime on AMF colonization

The frequency of mycorrhiza in the root system (F%) and the intensity of mycorrhizal colonization (M%) ranged from 34.6% to 88.3% and 0.7% to 23.3%, respectively; while the arbuscule abundance (A%) in all treatments (11 cm, 9-11 cm, 9 cm, and 5 cm) barely existed (**Table 3.1, Figure S2**). In general, AMF colonization was decreased in the following treatments of 11 cm, 9-11 cm, 9 cm, and 5 cm. Meanwhile, AMF colonization in low water regimes (11 cm and 9-11 cm) was significantly higher than that in high water regimes (9 cm and 5 cm) ($p<0.05$), while differences were not significant between the water regime of 11 cm and 9-11 cm ($p>0.05$). The AMF colonization of 15.6-23.3% in both wetland plants was determined under the water regime of 9-11 cm. Furthermore, two-way ANOVA analysis also showed that water regime had a significant effect on AMF colonization (**Table 3.1**).

AMF colonization was affected by water regimes. Although low AMF colonization was determined under water regimes of 9 cm and 5 cm in our study, it was beneficial for AMF colonization under the fluctuating water depth condition (9-11 cm). Oxygen content in wetlands is the main reason that AMF colonization was affected by water regimes (Wang et al., 2015). Wolfe et al. (2007) reported that AMF colonization in the wetland was decreased under high water regimes mainly because the soil lacked available oxygen for aerobic soil microorganisms (such as AMF). Similar results were also reported by (Dolinar and Gaberščik, 2010; Xu et al., 2016). In addition, high AMF colonization can also be observed in fluctuating water depth or drying-rewetting cycle conditions, which was due to oxygen content in the soil might be increased by constantly changing water regime (Fusconi and Mucciarelli, 2018; Wang et al., 2015; Xu et al., 2016). Stevens et al. (2011) studied AMF effects on plant growth under three water regimes, which also showed that hyphal, arbuscular, and vesicular colonization was greater in dry conditions compared with intermediate and flooded treatments. However, Wang et al. (2015) reported that root colonized by hypha ranged from 13% to 20%, and the colonization increased gradually as the hydrologic gradient increased. The reason might be that this research was done in a natural wetland (Sun Island Wetland in Harbin, China) where AMF colonization has already been observed in the roots of wetland plants, hence water regime was no longer the main factors affecting their colonization. Dolinar and Gaberščik (2010) also reported that once AMF symbiosis was established in the roots, the subsequent increase in water regime or even permanent flooding situation did not affect their colonization. Moreover, Bauer et al. (2003) revealed that soil moisture was not solely responsible for AMF colonization in the roots of wetland plants. Physicochemical differences of the substrate (e. g. pH, conductivity, and contents of phosphorous and nitrogen) were important in determining AMF colonization in wetlands (Xu et al., 2016). Therefore, the effect of water regime on AMF colonization in wetland might be reduced by adopting some special methods such as use fluctuating water regime and change physicochemical properties of the substrate.

Table 3.1: Mycorrhizal status under different treatments. These include the frequency of mycorrhiza in the root system (F%), the intensity of mycorrhizal colonization (M%), and arbuscule abundance (A%) in the whole root system. Data are presented as means ± SD (n=5)

Plants	Water regimes (cm)	F%	M%	A%
P. arundinacea	11	87.1 ± 9.9^{a}	17.1 ± 1.5^{a}	0.4 ± 0.1^{a}
	9-11	80.9 ± 10.5^{a}	15.6 ± 0.8^{a}	0
	9	63.5 ± 10.0^{b}	5.4 ± 0.5^{b}	0
	5	40.8 ± 6.5^{c}	1.5 ± 0.5^{c}	0
S. sylvaticus	11	88.3 ± 7.2^{a}	23.3 ± 1.7^{d}	0.5 ± 0.1^{a}
	9-11	83.7 ± 10.1^{a}	19.2 ± 2.1^{a}	0
	9	69.4 ± 9.8^{b}	6.9 ± 0.7^{b}	0
	5	34.6 ± 8.7^{c}	0.7 ± 0.3^{c}	0
Significance of				
Plant		ns	ns	-
Water regime		*	**	-
Plant*Water regime		ns	ns	-

Note: ns: not significant; *$P < 0.05$ and **$P < 0.01$. a, b, c and d show significant differences ($P < 0.05$).

3.4.2 AMF colonization in two plant species

AMF colonization (M%) in the roots of *S. sylvaticus* under the water regimes of 11 cm, 9-11 cm, 9 cm, and 5 cm were 1.5-6.2% higher than those in the roots of *P. arundinacea* (**Table 3.1**). Meanwhile, the highest AMF colonization (23.3%) was observed in the roots of *S. sylvaticus* under the water regime of 11 cm. In addition, two-way ANOVA analysis showed that plant species had no significant effect on AMF colonization (**Table 3.1**), but significant differences in AMF colonization were obtained between *S. sylvaticus* and *P. arundinacea* under the water regime of 11 cm.

Wetland plants can be colonized by AMF in different water regimes, this was mainly due to the aerenchyma in stems and roots, which allowed plants to ventilate their underground tissues through pressurized airflow (Visser and Bogemann, 2006). Wang et al. (2018b) studied AMF colonization status in 56 wetland plants and found that 87.5% of plants can be colonized. Meanwhile, the range (0.7-23.3%) of AMF colonization in our study was broadly similar to the previous study, which reported that most of the wetland plant had low AMF colonization (<25%) (Wang et al., 2018b; Xu et al., 2016). This indicated that most wetland plants might be difficult to be colonized by AMF when roots grow in water-saturated sediment. Nevertheless, high AMF colonization (>40%) in some wetland plants were observed, such as *Costus speciosus*, *Polypogon fugax*, *Oryza sativa*, *Lindernia parviflora*, and *Senecio scandens* (Sathiyadash et al., 2010; Wang et al., 2018b). Bauer et al. (2003) studied the effects of plant species on AMF colonization, results also showed that 21 plant species including *P. arundinacea*, *Typha* spp., and *Scirpus cyperinus* had 3-90% of their root length colonized by AMF. The reason for the discrepancy might be caused by the different environmental factors of each wetland, such as phosphorus availability, redox related processes affecting Fe and Mn chemistry in the sediments, pH, and water regime (Wang et al., 2015; Xu et al., 2016). *S. sylvaticus* and *P. arundinacea* in our study were controlled in the same experimental condition under each water regime, this also might be the reason that AMF colonization did not differ significantly between the two wetland plants ($p>0.05$). Meanwhile, both wetland plants had well-developed aerenchyma, which promoted oxygen diffusion from leaves to the rhizosphere, providing a chance for AMF colonization in the roots of each plant (Ramírez-Viga et al., 2018). Moreover, the reason for the low of AMF colonization in the roots of *S. sylvaticus* and *P. arundinacea* might also be due to their belonging to monocots. Xu et al. (2016) reported that AMF colonization was higher in dicots (58%) than in monocots

(13%). However, some studies showed that whether it is a dicot or a monocot, as long as wetland plants had a good environment for their growth, it might be beneficial to AMF colonization (Bao et al., 2019; Xu et al., 2016). A similar study also reported that more vigorous plants could maintain higher AMF colonization (Wang et al., 2015). In general, plant species did not show any significant difference in AMF colonization under a similar wetland environmental condition.

3.4.3 Physiological functions in wetland plants

Nutrients in wetland plants

TP contents in roots and shoots of two inoculated wetland plants were 0.4-1.1 mg/g and 0.9-1.7 mg/kg higher than that of corresponding non-inoculated control under the low water regimes (9-11 cm and 11 cm), respectively (**Figure 3.2**). TP contents in the shoot of two inoculated wetland plants under the water regimes of 9-11 cm and 11 cm were increased by 25.7-68% and 68-80%, respectively. The differences between inoculated *S. sylvaticus* and non-inoculated control were significant ($p<0.05$). Meanwhile, TP contents in the root of two inoculated wetland plants under the low water regimes were also increased by 9.1-42.3%. The highest TP contents of root and shoot in the inoculated wetland plants (4.8 mg/g and 4.9 mg/g) were determined under the fluctuating water regime (9-11 cm). Meanwhile, the results of two-way ANOVA analysis showed that TP contents in the shoot and root of wetland plants were affected by AMF, water regime, and interaction of AMF* water regime (**Table 3.2**). In addition, TC and TN contents in the shoot of inoculated *S. sylvaticus* under the fluctuating water regime (9-11 cm) were significantly higher than that of non-inoculated *S. sylvaticus* ($p<0.05$), with the TC and TN contents increased by 26.1% and 66.7%, respectively (**Table 3.3**). In conclusion, wetland plant nutrients can be increased by AMF colonization presence under the fluctuating water regime condition.

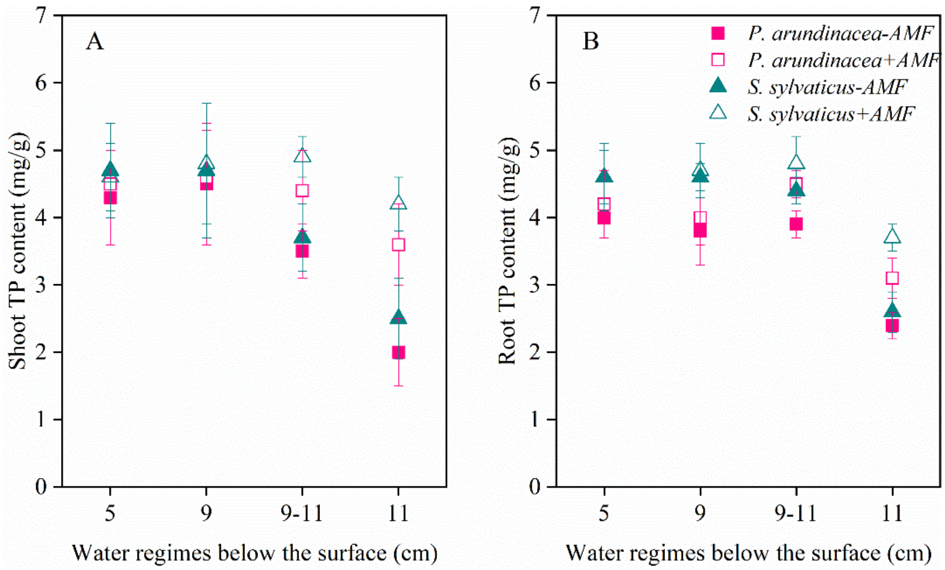


Figure 3.2: TP contents in AMF inoculated and non-inoculated wetland plants under different water regimes

AMF are the non-negligible driver of minerals and nutrients (e. g. TC, TP, and TN) in wetlands, although flooding may decrease their relative importance (Bao et al., 2019; Xu et al., 2016). AMF promotes minerals and nutrients uptake in the substrate through the extra-radical mycelium, in exchange for carbon that plants assimilated (Garcia et al., 2016; Jansa et al., 2019). Meanwhile, high nutrient contents of AMF inoculated plants in our study were related to the well-developed aerenchyma in *S. sylvaticus* and *P. arundinacea*, which provided nourishment for AMF colonization and plant roots growth (Xu et al., 2019). Oxygen radial loss might become the main source of oxygen supply in wetlands, which favored AMF development around wetland roots (Brune, 2000; Lai et al., 2012). Ramírez-Viga et al. (2018) reported that AMF may be able to accumulate in the oxidized rhizosphere of their hosts, which promotes C, N, and P cycles between plants and substrate in wetlands. Mei et al. (2019) also showed that AMF symbiosis can change soil available N and P concentrations, promote plant P uptake, and decrease plant N:P ratios. Similar results were also reported by Püschel et al. (2017), which showed that AMF symbiosis generally improved P uptake by plants and considerably stimulated the efficiency of biological N fixation under a low P availability (below 10 mg/kg water-extractable P). Moreover, the improvement in minerals and nutrients uptake is mainly reflected in the promotion of plant growth (Grilli et al., 2014; Miransari, 2011). Previous studies also reported that AMF significantly improved plant physiological functions, resulting in greater plant height, diameter at ground regime, plant biomass, as well as increased plant absorption of nutrients (e. g. TP, TN, TC, and potassium) (Ingraffia et al., 2019; Miransari, 2011). Similar to the results of our study, Bao et al. (2019) showed that the concentration and total amount of leaf P in three flooding regimes (none, intermittent and continuous) were higher in the mycorrhizal rice plants than that in the corresponding non-mycorrhizal plants. Therefore, AMF had a positive effect on nutrients uptake by wetland plants under fluctuating water regime condition.

Table 3.2: Two-way ANOVA analysis for the effects of AMF and water regime on plants characteristics

Parameters	AMF	Water regime	AMF* Water regime
Height	0.02	0.04	0.02
Biomass	<0.001	0.006	0.001
MDA	0.001	0.001	0.002
Chlorophyll	0.03	0.002	0.01
Root to shoot ratio	0.002	0.02	0.001
RWC	0.04	**0.22**	**0.49**
Shoot TP	0.007	0.03	0.01
Root TP	0.04	0.02	0.006

Note: the values in bold show no significant difference ($p > 0.05$).

Table 3.3: Shoot and root TC and TN contents of *S. sylvaticus* under different water regimes below the surface

Plants	Water regimes below the surface (cm)	TC (%DM)		TN (%DM)	
		AMF-	AMF	AMF-	AMF
Root	11	-	37.1 ± 3.1^{a}	-	2.1 ± 0.4^{a}
	9-11	31.3 ± 6.1^{Aa}	38.7 ± 5.6^{Aa}	2.8 ± 0.8^{Aa}	3.2 ± 0.6^{Abc}
	9	39.2 ± 8.7^{Aab}	38.9 ± 10.1^{Aa}	3.0 ± 0.9^{Aa}	3.2 ± 1.1^{Abc}
	5	38.3 ± 9.8^{Aab}	38.6 ± 6.9^{Aa}	3.1 ± 0.7^{Aa}	2.9 ± 0.4^{Ab}
Shoot	11	-	44.6 ± 10.8^{a}	-	4.1 ± 0.7^{Ac}
	9-11	37.5 ± 7.9^{Aab}	47.3 ± 8.7^{Ba}	3.0 ± 0.5^{Aa}	5.0 ± 0.6^{Bd}
	9	39.9 ± 10.1^{Aab}	42.1 ± 9.8^{Aa}	3.7 ± 1.0^{Aa}	3.9 ± 0.8^{Abc}
	5	44.8 ± 9.7^{Ab}	45.6 ± 9.6^{Aa}	4.4 ± 1.0^{Aa}	4.6 ± 0.9^{Acd}

Note: DW: Dry matters of pants; A, B shows a significant difference between AMF- and AMF; a, b, c, d shows significant difference among the 4 water regimes; -: no data.

Physiological indexes in wetland plants

Compared with non-inoculated wetland plants, root length, shoot height, biomass, and RWC of both AMF inoculated wetland plants (*S. sylvaticus* and *P. arundinacea*) were significantly increased by 35.4-46.2%, 13.1-26.6%, 33.3-114.3%, and 6.7-11.8% under the water regimes of 11 cm and 9-11 cm ($p<0.05$), respectively (**Figure 3.3**, **Figure 3.4**, **Table 3.4**). In addition, mycorrhizal dependency was decreased gradually as water depth increased except water depths of 9 cm and 5 cm in *S. sylvaticus* (**Table 3.4**). Meanwhile, root to shoot ratios in AMF inoculated plants under the water regimes of 9-11 cm were significantly lower than those in non-inoculated controls ($p<0.05$). These indicated that AMF had a positive effect on these physical indicators of both wetland plant growth, and the influence of AMF was significant under the low water regimes. The highest chlorophyll content of 4 mg/g FW was determined in the AMF inoculated *S. sylvaticus* under the water regime of 9-11 cm (**Figure 3.5**). Meanwhile, compared with non-inoculated plants under the condition of 9-11 cm, chlorophyll contents in both AMF inoculated plants were increased by 14.3-24%. Additionally, chlorophyll contents of both non-inoculated plants under the water regime of 11 cm were significantly lower than those under other water regimes ($p<0.05$). It showed that chlorophyll content in wetland plants might be decreased due to the lack of water and AMF. MDA contents in AMF inoculated and non-inoculated wetland plants were stable under the water regimes of 9-11 cm, 9 cm, and 5 cm (**Figure 3.6**). The highest MDA contents were determined in non-inoculated wetland plants under the water regime of 11 cm, accounting for 43.5 nmol/g FW and 51.6 nmol/g FW in *S. sylvaticus* and *P. arundinacea*, respectively. Furthermore, MDA contents of AMF inoculated plants under the water regime of 11 cm were significantly lower than that of non-inoculated plants ($p<0.05$). Meanwhile, the results of two-way ANOVA analysis showed that biomass, MDA, and chlorophyll were affected by AMF, water regime and the interaction of AMF* water regime (**Table 3.2**). Results of PCA indicated that these parameters were largely determined by PCA axis 1, which revealed that there was a strong correlation among them (**Figure 3.7**). Height, biomass, RWC, and chlorophyll had a strong negative correlation with the root/shoot and MDA (r is -0.6 to -0.9), meanwhile, a positive correlation was observed among the height, biomass, RWC, and chlorophyll (r is 0.5 to 0.9). Moreover, the results of scores of 6 treatments on axis 1 and axis 2 showed that AMF had a similar effect on plant physiological functions in the water regimes of 11 cm and 9-11 cm. This indicated that AMF probably can improve wetland plant physiological functions under fluctuating water depth conditions.

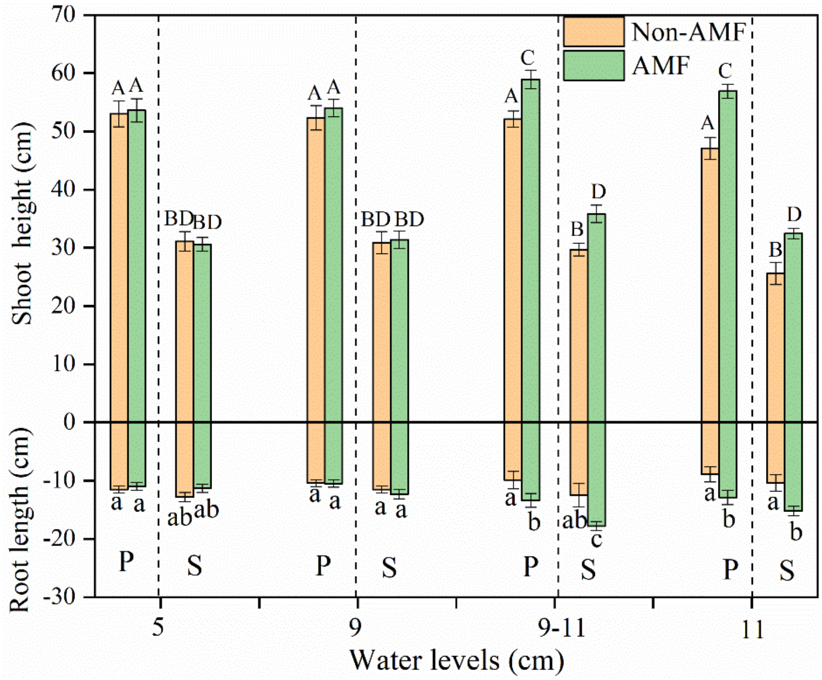


Figure 3.3: AMF inoculated and non-inoculated plant heights under different water regimes (P: *Phalaris arundinacea*, S: *Scirpus sylvaticus*; a, b, c; and A, B, C, D show the significant difference for root length and shoot height, respectively ($p < 0.05$))

Table 3.4: Root to shoot mass ratio, mycorrhizal dependency (MD), and relative saturated water content (RWC) of two wetland plants under different water regime conditions

Plants	water regimes (cm)	MD (g/g DW)	Root to shoot ratio (g/g FW)		RWC (%)	
			AMF-	AMF	AMF-	AMF
P. arundinacea	11	2.1^{a}	2.5^{Aa}	1.5^{Ba}	69.8^{Aa}	74.5^{Ba}
	9-11	1.5^{b}	1.4^{Ab}	1.1^{Bb}	73.2^{Ab}	79.8^{Bb}
	9	1.0^{c}	1.1^{Ac}	1.1^{Ab}	82.3^{Ac}	83.2^{Ac}
	5	1.2^{c}	1.0^{Ac}	0.9^{Ab}	83.1^{Ac}	83.2^{Ac}
S. sylvaticus	11	1.7^{b}	1.5^{Abd}	1.2^{Bb}	70.3^{Aa}	77.6^{Bab}
	9-11	1.3^{c}	1.7^{Ad}	1.4^{Bab}	75.4^{Ab}	84.3^{Bc}
	9	1.1^{c}	1.8^{Ad}	1.6^{Aac}	89.5^{Ad}	89.5^{Ad}
	5	1.0^{c}	1.7^{Ad}	1.6^{Aac}	89.7^{Ad}	89.6^{Ad}

Note: DW: Dry weight of pants; FW: fresh weight of plants; A, B shows a significant difference between AMF- and AMF; a, b, c, d shows significant difference among the 4 water regimes.

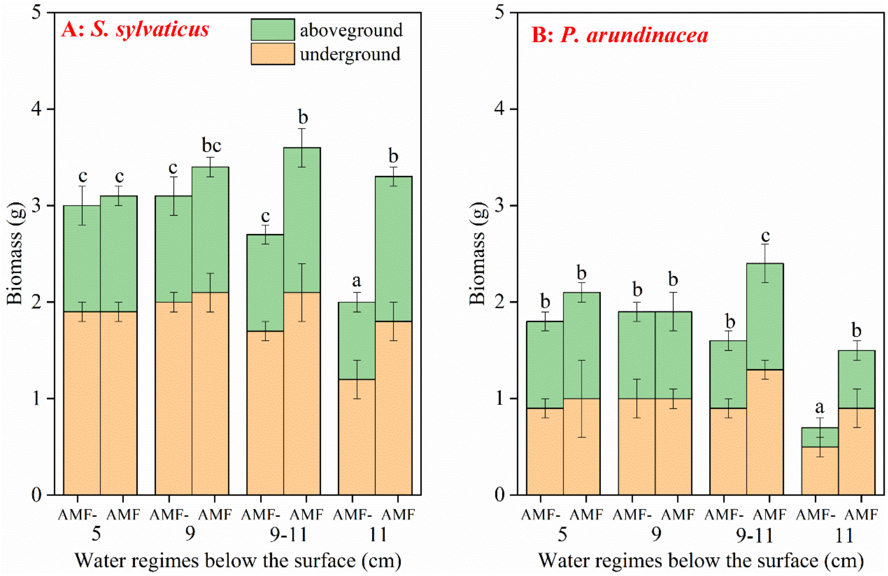


Figure 3.4: AMF inoculated and non-inoculated plant biomass under different water regimes (a, b, c, d show the significant difference for the sum of biomass ($p < 0.05$))

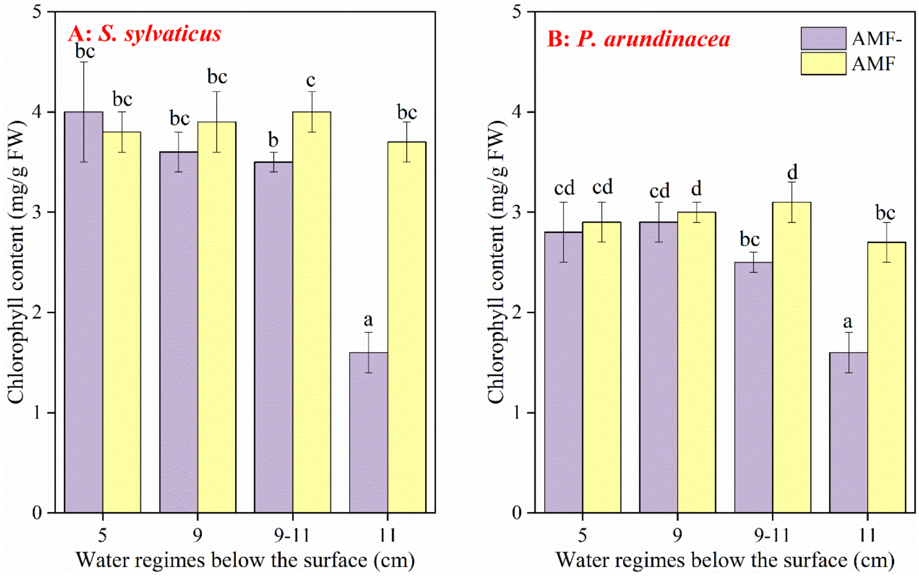


Figure 3.5: Chlorophyll contents in AMF inoculated and non-inoculated wetland plants under different water regimes (a, b, c, d show the significant difference ($p < 0.05$))

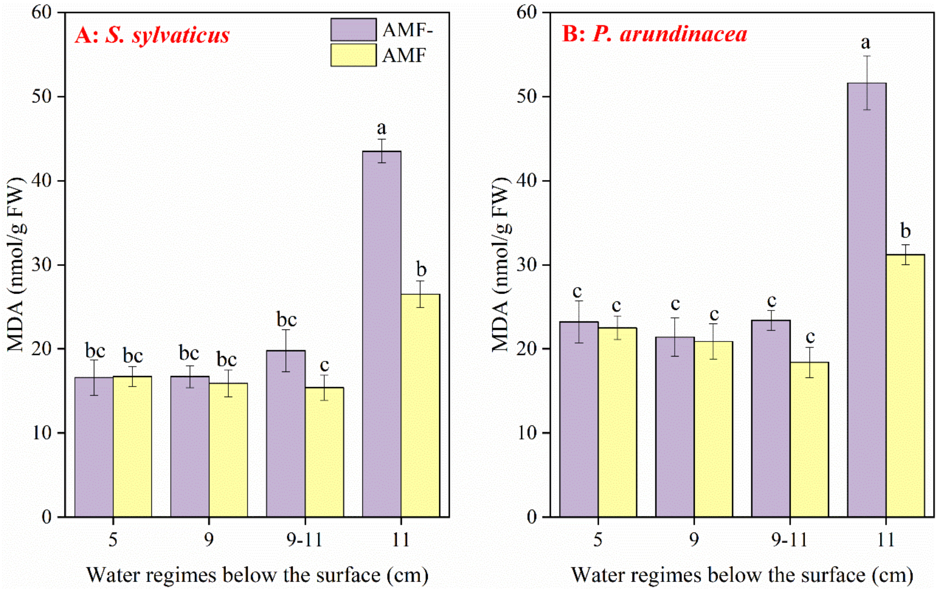


Figure 3.6: MDA contents in AMF inoculated and non-inoculated wetland plants under different water regimes (a, b, c, d show the significant difference (a, b, c show the significant difference ($p < 0.05$))

AMF promote physiological functions of wetland plant in alternate water cycles by increasing plant height, biomass, leaf area, and biomass (Liang et al., 2018; Pii et al., 2015). Fraser and Feinstein (2005) studied AMF and water depth effects on growths of three wetland plants (*Carex tribuloides*, *P. arundincacea*, and *Rumex orbiculatus*) also showing that biomass of three AMF inoculated wetland plants was greater than that of non-inoculated plants, and root/shoot ratios were reduced nearly by 50% in AMF inoculated system. In addition, since the photosynthesis process of plants requires water and nutrients, a series of photosynthesis properties (e. g. chlorophyll concentration, photosynthetic efficiency) are also improved by AMF insistence (Ramírez-Viga et al., 2018). Furthermore, MDA, as a general indicator of lipid peroxidation, is an oxidized product of membrane lipids and accumulates when plants are exposed to oxidative stresses (Soleimani et al., 2010). In our study, the highest MDA contents were determined in non-inoculated plants under the water regime of 11 cm, showing that MDA content was affected by drought stress, as well as MDA content in wetland plants can be decreased by AMF addition. Similar results were also reported by Wu et al. (2006), which indicated that drought stress (55% of relative soil water content) the MDA concentration of plants, and 33% of MDA content were decreased by AMF inoculation. Therefore, AMF might be beneficial for the physiological functions of wetland plants under the fluctuating water depth condition. Furthermore, the regime of lipid peroxidation in wetland plants also can be decreased by AMF inoculation under the low water depth condition.

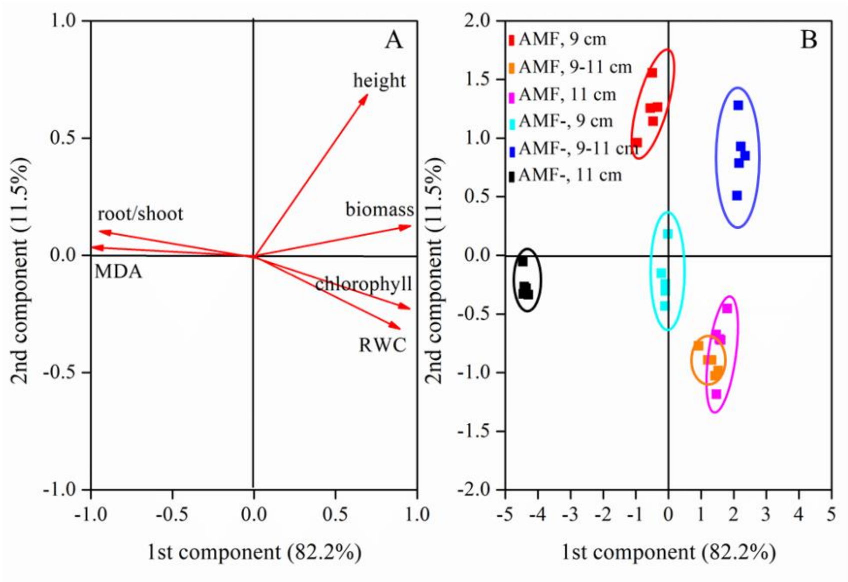


Figure 3.7: Principal component analysis (PCA) of physiological function parameters in plants and scores of 6 treatments on AX1 and AX2

3.5 Conclusion

Water regime had a significant impact on AMF colonization with the highest AMF colonization (23.3%) was observed in the roots of *S. sylvaticus* under the water regime of 11 cm. Meanwhile, physiological functions of AMF inoculated wetland plants (plant height, plant biomass, RWC, and nutrient contents (e.g. TP and TN) can be improved under low water levels or fluctuating water. However, MDA, as a lipid peroxidation product in plants, was accumulated under the water regime of 11 cm. Therefore, the physiological functions of wetland plants might be limited due to lack of water in the wetland system, although high AMF colonization was confirmed. Despite that, specific water regime conditions (e.g. fluctuating water depth) might become a possible method to meet the water requirements of AMF colonization and physiological functions of wetland plants. In addition, the AMF effect on physiological functions of wetland plants was only investigated under different water regimes in our study. In further research, it would be interesting to investigate how AMF affect the physiological functions of wetland plant under pollutant stress (e.g. heavy metal or toxic organic compounds).

3.6 Supplementary Materials

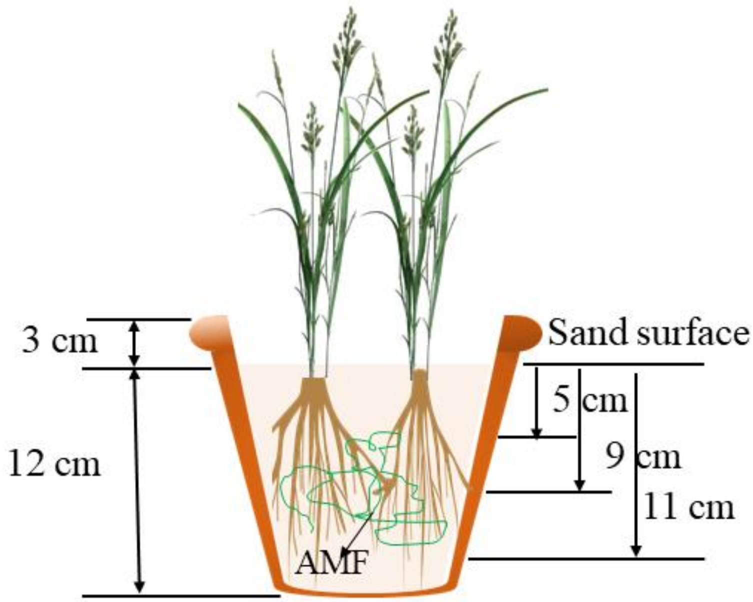


Figure S1: Experimental setup of different water regime conditions

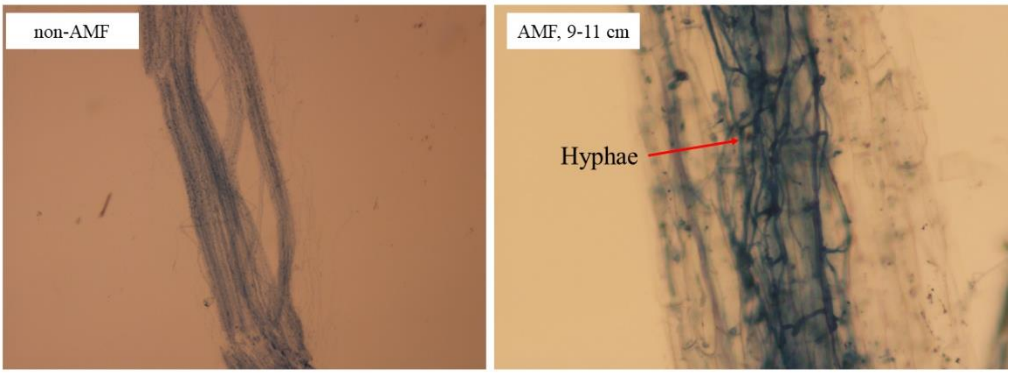


Figure S2: Pictures of non-AMF *S. sylvaticus* roots and mycorrhizal *S. sylvaticus* roots under water regime of 9-11 cm below the surface

Chapter IV

Antioxidant response in arbuscular mycorrhizal fungi inoculated wetland plant under Cr stress

Shanshan Hu, Bo Hu, Zhongbing Chen, Miroslav Vosátka, Jan Vymazal

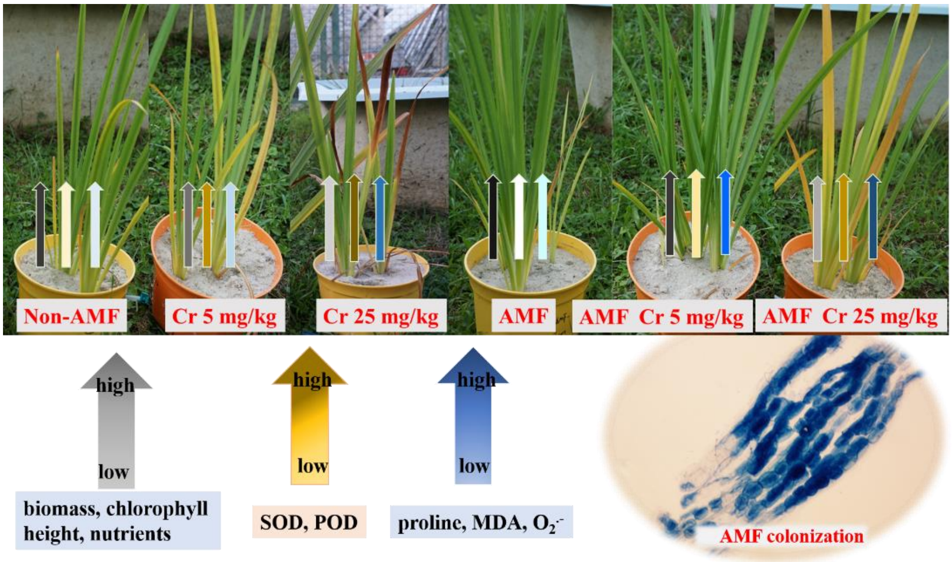
Adapted from Environmental Research 191 (2020): 110203

Contents

4.1 Abstract

Arbuscular mycorrhizal fungi (AMF) provide a positive effect on antioxidant mechanisms in terrestrial plants under heavy metal stress. This study investigated the effects of AMF on wetland plant (*Iris pseudacorus*) growth and antioxidant response under Cr stress at different water depths. Results showed that AMF inoculated *I. pseudacorus* had higher antioxidant response than non-inoculated controls, with shoot superoxide dismutase (SOD), root SOD, shoot peroxidase (POD), and root POD contents increased by 4.7-39.6%, 7.5-29.5%, 11.2-68.6%, 16.8-50.3%, respectively. Meanwhile, shoot (root) proline, malondialdehyde (MDA) and superoxide anion ($O_2^{\cdot -}$) contents in the AMF inoculated *I. pseudacorus* were 10.2-44.3% (2.8-37.2%), 11.5-35.4% (16.9-28.2), and 14.9-30.5% (-0.9-26.3%) lower than those in the non-inoculated controls, respectively. Besides, AMF improved the growth of *I. pseudacorus* with biomass, height, chlorophyll, K, and P contents in the shoots increased by 10.5-32.5%, 17.4-44.9%, 4.7-37.7%, 12.0-30.7%, 13.5-20.6%, respectively. Moreover, the *I. pseudacorus* tolerance to Cr stress was also enhanced under the water depth of 6-3 cm. Therefore, AMF play an important role in wetland plant growth and antioxidant response under Cr stress, and it can improve wetland plants' tolerance to Cr stress at fluctuating water depth.

Figure 4.1: Graphical abstract



4.2 Introduction

Chromium (Cr) is an essential trace element for glucose metabolism in humans and animals, it is widely used in the chemical industries for electroplating, leather tanning, pigment production, paper production, and other industries (Afshan et al. 2015). However, Cr is a non-essential element for plants, which can damage the photosynthesis and respiration processes, cause oxidative destroy, resist important enzymatic activities, and even cause plant death (Singh et al., 2013). Generally, Cr toxicity is highly dependent on its oxidation form, hexavalent Cr (Cr (VI)) is more toxic than trivalent Cr (Cr (III)). Cr (VI) has serious harmful effects on human health including lung cancer, kidney, liver, and gastric damage (Sun et al., 2015). Meanwhile, Cr (VI) can also induce changes in leaf protein profiles and root microRNA expression through interfering with various physiological processes of plants (Bukhari et al., 2015). In order to reduce the Cr (VI) content in the environment, phytoremediation, as a cost-effective and environment-friendly method, can be used to decontaminate heavy metal polluted soils and water bodies through the extraction of heavy metals from soil and water by plants (Robles-González et al. 2008).

Arbuscular mycorrhizal fungi (AMF) represent an important component of the soil microbial community, they can form symbiotic relationships with the roots of most terrestrial plants (Dodd and Perez-Alfocea, 2012). AMF act as a living bridge between soil and plant, which can transport nutrients (e.g. nitrogen and phosphorus) from soil to roots, thus improving host plant growth (Menezes et al., 2016). Meanwhile, AMF can enhance host plant's resistance to various abiotic stresses (e.g. cold, drought, salinity, heavy metal, and emerging pollutant stresses) through decreasing reactive oxygen species (ROS) accumulation, increasing the activities of antioxidant enzymes (e.g. superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD)), increasing the activities of antioxidant enzymes, improving photosynthesis, and promoting the uptake of nutrients (Lin et al., 2017; Wang et al., 2018a). Besides, membrane lipid peroxidation in the host plant also can be reduced by AMF. Malondialdehyde (MDA), as a general indicator of lipid peroxidation, is an oxidized product of membrane lipids when host plants are under abiotic stresses (Soleimani et al., 2010). Generally, MDA content in the AMF inoculated plants were lower than that in the non-inoculation plants under the abiotic stress (Ren et al., 2019). In all, AMF provide a positive effect on antioxidant mechanisms and lipid peroxidation in terrestrial plants. However, it is not clear whether AMF can improve the antioxidative enzyme production and alleviate the lipid peroxidation process in wetland plants.

Constructed wetlands (CWs) have been successfully used to improve the quality of wastewater for at least five decades and are well known for the removal of nutrients and heavy metals (Vymazal, 2014). Wetland plants, especially emergent plants, play an important role in pollutant removal in CWs. AMF colonization in the roots of wetland plants (e.g. *Phragmites australi*, *Typha orientalis*, and *Glyceria maxima*) are observed in recent years (Wang et al., 2018b). Numerous studies indicated that AMF can promote wetland plant growth, increase chlorophyll contents in leaves, and facilitate nutrient uptake under abiotic stresses (Fusconi and Mucciarelli, 2018; Wu et al., 2020). Our previous study also showed that physiological functions (e.g. biomass, nutrients, and MDA content) in AMF inoculated wetland plants (*Phalaris arundinacea* and *Scirpus sylvaticus*) can be improved under fluctuating water depth condition (Hu et al., 2020a). However, few studies focus on the AMF effect on the antioxidant response in wetland plants under Cr stress regarding different water depths.

Therefore, this work aimed to (1) assess AMF inoculated wetland plant growth under Cr stress at different water depths; (2) evaluate the effects of AMF on the antioxidant response in wetland plants to Cr stress and various water depths.

4.3 Materials and Methods

4.3.1 Wetland plant, AMF inoculum, and substrate

Iris pseudacorus was selected as a wetland plant in this study because it’s a commonly used plant in CWs. The seedlings of *I. pseudacorus* were collected from natural ponds on the campus of the Czech University of Life Sciences Prague. The roots of each *I. pseudacorus* were surface sterilized with 75% ethyl alcohol for 10 seconds and 1% sodium hypochlorite (NaClO) for 15 minutes, washed carefully with sterile distilled water five times before transplanted into the sterilized pots. AMF inoculum (*Rhizophagus irregularis* BEG140) was purchased from Symbiom Ltd., Lanškroun, Czech Republic. The isolated AMF was multiplied with host plant *Zea mays L.* in a multi-spore pot culture containing a mixture of zeolite and expanded clay (1:1; v: v) for six months. Thus, the inoculum comprised a mixture of spores, mycelium, zeolite, expanded clay, and plant root fragments. Sand with a grain size of 0.1-0.5 mm was used as substrate after sterilization at 150 °C for 2 h.

4.3.2 Experimental design

The experiment used a factorial design with three Cr levels (0, 5, 25 mg/kg substrate), two AMF treatments (inoculation with *R. irregularis* and non-inoculation), and two water depths (from bottom to top: 3 cm depth of water, and fluctuating water depth with water depth varies between 3 cm and 6 cm (6-3 cm)), making a total of 12 treatments, 3 replicates for each treatment. The experiment was carried out in pot with a dimension of 17 ×15 cm (diameter × height). The cycle of fluctuating water depth was 4 days (2 days with 3 cm depth of water and 2 days with 6 cm depth of water). The sterilized sand of 1500 g was first added into the pot, then 50 g of AMF inoculum mixed with 1000 g of sterilized sand were added in the AMF treatment systems, three seedlings were transplanted into each pot afterward, finally covered by 500 g sterilized sand. The same procedures were also performed in the non-inoculated treatment systems except that 50 g of AMF inoculum was replaced by 50 g of sterilized inoculum and 10 mL inoculum filtrate. Distilled water was used to control the water depths. Meanwhile, each pot was irrigated with 1/4-strength Hoagland solution (100 mL/week/pot) to avoid nutrient deficiency, which was diluted with deionized water. Cr solution was added when the AMF colonization was successfully detected 3 months after planting, with the colonization rate of 58.7% and 48.8% at the water depths of 3 cm and 6-3 cm, respectively (**Figure S1**). Cr solutions in the form of K_2CrO_4 (Cr (VI)) with different concentrations were added in each treatment. The experiment was conducted in the Czech University of Life Sciences Prague with rain protected. The plants were maintained for 5 months before harvest.

4.3.3 Sample analysis

Plant shoots and roots were harvested separately, root length, shoot height, fresh biomass of root and shoot were measured afterward, then washed carefully with deionized water for further analysis. Fresh samples (about 10 g) of roots and shoots were used to determine the contents of chlorophyll, MDA, superoxide anion ($O_2^{\cdot -}$), and proline, as well as the activities of superoxide dismutase (SOD) and peroxidase (POD).

Dry samples of shoots and roots were determined after oven drying at 70 °C for 48 h. The dried samples were used to measure the contents of total phosphorus (TP), and total potassium (TK) in roots and shoots.

Chlorophyll content was determined by spectrophotometry with acetone extraction (Palta, 1990). MDA and $O_2^{\cdot-}$ contents in shoots and roots were determined according to Chen et al. (2013). Proline content was determined by ninhydrin spectrophotometry (Bates et al., 1973). Fresh roots and shoots (1.0 g) were homogenized at 4 °C in 5 mL of 50 mM phosphate buffer (pH 7.8) containing 1mM EDTA and 2% polyvinylpyrrolidone. The homogenate was centrifuged at 10000 g for 20 min at 4 °C, and the supernatant was used for the following enzyme assays. SOD activity was determined by monitoring the inhibition of the photochemical reduction of nitro blue tetrazolium according to the method described by Giannopolitis and Ries (1977). POD activity was determined using the guaiacol oxidation method (Chance and Maehly, 1955). TP and TK contents in roots and shoots were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES).

4.3.4 Statistical analysis

Biomass, height, contents of chlorophyll, MDA, $O_2^{\cdot-}$, proline, TP, and TK, activities of SOD and POD in wetland plant were analyzed using a three-way analysis of variance (ANOVA), with Cr, water depth and AMF colonization as main factors and Cr * water depth, water depth * AMF colonization, Cr * AMF colonization, and water depth *Cr * AMF colonization as interaction effects. Duncan's multiple range test was used to compare the effects of Cr concentration, AMF addition, and water depth on the biomass, height, chlorophyll, MDA, $O_2^{\cdot-}$, proline, SOD, POD, TK, and TP, $p < 0.05$ was set as a significant difference. Principal component analysis (PCA) and cluster analysis were completed to display data groupings and correlations for plant parameters. The ‘Factoextra’ (Kassambara, 2017) and ‘Pheatmap’ (Kolde and Kolde, 2015) packages were used to visualize the experimental data among the plant parameters. The statistic software Origin 2019 for windows was used to perform the test and to plot the graphs. All statistical analyses were performed using the R Software (version 3.6.3) and the software package IBM SPSS statistics 21.0 for Windows.

4.4 Results

4.4.1 Wetland plant growth and chlorophyll

Biomass and height

Fresh root, shoot biomass, shoot height, and root length of AMF inoculated *I. pseudacorus* under the same Cr concentration and water depth were 13.7-34.6%, 10.5-32.5%, 17.4-44.9% and 3.6-55.2% higher than those of non-inoculated controls, respectively (**Figure S2**). Three-way ANOVA analysis showed that AMF inoculation, Cr concentration, and water depth had significant effects on the biomass and height of *I. pseudacorus* ($p<0.05$) (**Table S1**). Meanwhile, compared to the water depth of 3 cm, the fresh root, shoot biomass, root length, and shoot height of AMF inoculated *I. pseudacorus* in the same Cr concentration under the water depth of 6-3 cm were increased by -0.2-34.7%, 4.2-42.9%, 0-50.7%, and 9.3-49.3%, respectively. Furthermore, the biomass and height of AMF inoculated *I. pseudacorus* under the same water depth was gradually decreased with the increase of Cr concentration.

Chlorophyll

Higher chlorophyll content was determined in the AMF inoculated *I. pseudacorus*, with increased by 4.7-37.7% compared to the non-inoculated control under the same Cr concentration and water depth (**Figure S3**). Significant differences were obtained under each water depth and Cr concentration except the water depth of 6-3 cm without Cr addition and water depth of 3 cm with Cr concentration of 25 mg/kg ($p<0.05$). In addition, compared to the water depth of 3 cm, the average chlorophyll contents in the *I. pseudacorus* under the water depth of 6-3 cm with Cr concentrations of 0 and 5 mg/kg were significantly increased by 22.3-34.7% ($p<0.05$).

Table 4.1: K and P contents in roots and shoots of AMF inoculated and non-inoculated wetland plants under Cr stress (6-3: water depth of 6-3 cm; 3: water depth of 3 cm; Cr 0, 5, and 25: Cr concentrations of 0, 5, and 25 mg/kg)

Treatments		Shoot K contents (g/kg)		Root K contents (g/kg)		Shoot P contents (g/kg)		Root P contents (g/kg)	
		Non-AMF	AMF	Non-AMF	AMF	Non-AMF	AMF	Non-AMF	AMF
Cr 0	6-3	23.9±0.3^{aA}	29.8±0.6^{aB}	14.3±0.9^{A}	17.9±1.4^{B}	3.1±0.5	3.9±0.3^{a}	3.3±0.5	4.0±0.2
	3	20.8±0.8^{b}	24.2±1.1^{b}	13.3±1.1	15.1±1.7	2.3±0.3	2.7±0.2^{b}	2.9±1.0	3.9±0.3
Cr 5	6-3	17.9±0.5^{A}	23.9±0.5^{aB}	12.2±1.0	13.9±0.6	2.5±1.2	2.9±0.1	2.8±0.3	3.3±0.1^{a}
	3	17.6±1.4^{A}	20.8±0.7^{bB}	11.2±1.1	13.5±0.4	2.1±0.7	2.4±0.3	2.3±1.2	2.0±0.8^{b}
Cr 25	6-3	15.2±0.3^{aA}	21.0±0.7^{aB}	10.5±0.9	11.7±0.3	2.1±1.5	2.5±0.7	2.4±1.1	2.8±0.7
	3	13.6±0.7^{b}	15.5±0.4^{b}	10.9±1.2^{A}	12.4±1.3^{B}	1.7±1.2	2.1±0.6	1.9±1.3	2.6±0.6

Note: a, b shows significant differences between water depths in the same AMF inoculated condition; and A, B shows significant differences between AMF inoculated and non-inoculated condition under the same water depth ($p < 0.05$)

4.4.2 Nutrients in the wetland plant

The highest K and P contents in the AMF inoculated *I. pseudacorus* were obtained at the water depth of 6-3 cm without Cr addition (**Table 4.1**). Compared to the non-inoculated *I. pseudacorus*, shoot (root) K and P contents in the AMF inoculated *I. pseudacorus* were increased by 12.0-30.7% (10.2-20.3%) and 13.5-20.6% (13.9-27.5%), respectively. In addition, three-way ANOVA analysis showed that AMF, water depth, and Cr had significant effects on the shoot K, root P, and shoot P contents in the wetland plant (**Table S1**). Moreover, no matter in which Cr concentration, higher K and P contents in the *I. pseudacorus* were determined under the water depth of 6-3 cm compared to the water depth of 3 cm.

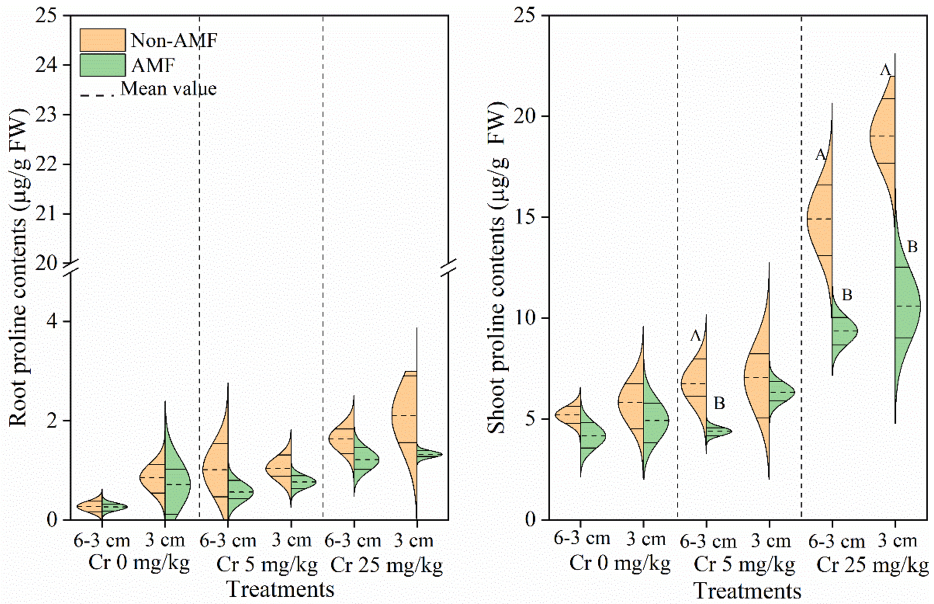


Figure 4.2: Proline contents both in shoots and roots of AMF/non-AMF inoculated wetland plants under Cr stress (a, b shows significant differences between different water depths; A, B shows significant differences between AMF and non-AMF inoculated condition ($p < 0.05$))

4.4.3 Osmotic adjustment substance

Proline, as a main osmotic factor in plants, reflects plant tolerance to stress induced by osmotic adjustment. Proline contents in the shoot of *I. pseudacorus* were 50.0-98.4% higher than those in the root of *I. pseudacorus* (**Figure 4.2**). Meanwhile, AMF decreased the proline contents in the root and shoot of *I. pseudacorus*, with a reduction of 2.8-37.2% and 10.2-44.3%, respectively. In addition, the proline contents in the root and shoot of *I. pseudacorus* under the water depth of 6-3 cm were also 2.0-68.4% and 4.4-30.6% lower than those under the water depth of 3 cm, respectively. These results indicated that AMF and fluctuating water depth provide positive effects on the osmotic regulation of wetland plants. Meanwhile, the interaction of AMF*Cr*water depth also showed a significant effect on the proline content in the root of *I. pseudacorus* (**Table S1**).

4.4.4 Lipid peroxidation and ROS level

MDA and $O_2^{\cdot-}$ contents in the shoot and root of *I. pseudacorus* under the same water depth and AMF inoculation were gradually increased as the Cr concentration raising (**Figure 4.3**, **Figure 4.4**). The highest MDA (32.5 μmol/mg FW) and $O_2^{\cdot-}$ (223.8 μmol/mg FW) contents were determined in the shoot of non-inoculated *I. pseudacorus* under the water depth of 3 cm with the Cr concentration of 25 mg/kg. MDA contents in the shoot and root of AMF inoculated *I. pseudacorus* were 11.5-35.4% and 16.9-28.2% lower than those of non-inoculated controls under the same water depth and Cr concentration, respectively. The $O_2^{\cdot-}$ contents in the shoot and root of AMF inoculated *I. pseudacorus* were also showed similar trends, with significantly decreased by 14.9-30.5% and -0.9-26.3% compared to the non-inoculated controls, respectively ($p<0.05$). Besides, lower MDA and $O_2^{\cdot-}$ contents under the water depth of 6-3 cm were determined compared to the water depth of 3 cm except for the $O_2^{\cdot-}$ content in AMF inoculated *I. pseudacorus* under the Cr concentration of 25 mg/kg. The interaction of AMF* water depth and AMF*Cr*water depth had insignificant differences in the MDA contents ($p>0.05$) (**Table S1**). Meanwhile, the interaction of AMF* water depth and AMF*Cr also had insignificant differences in the root $O_2^{\cdot-}$ contents ($p>0.05$).

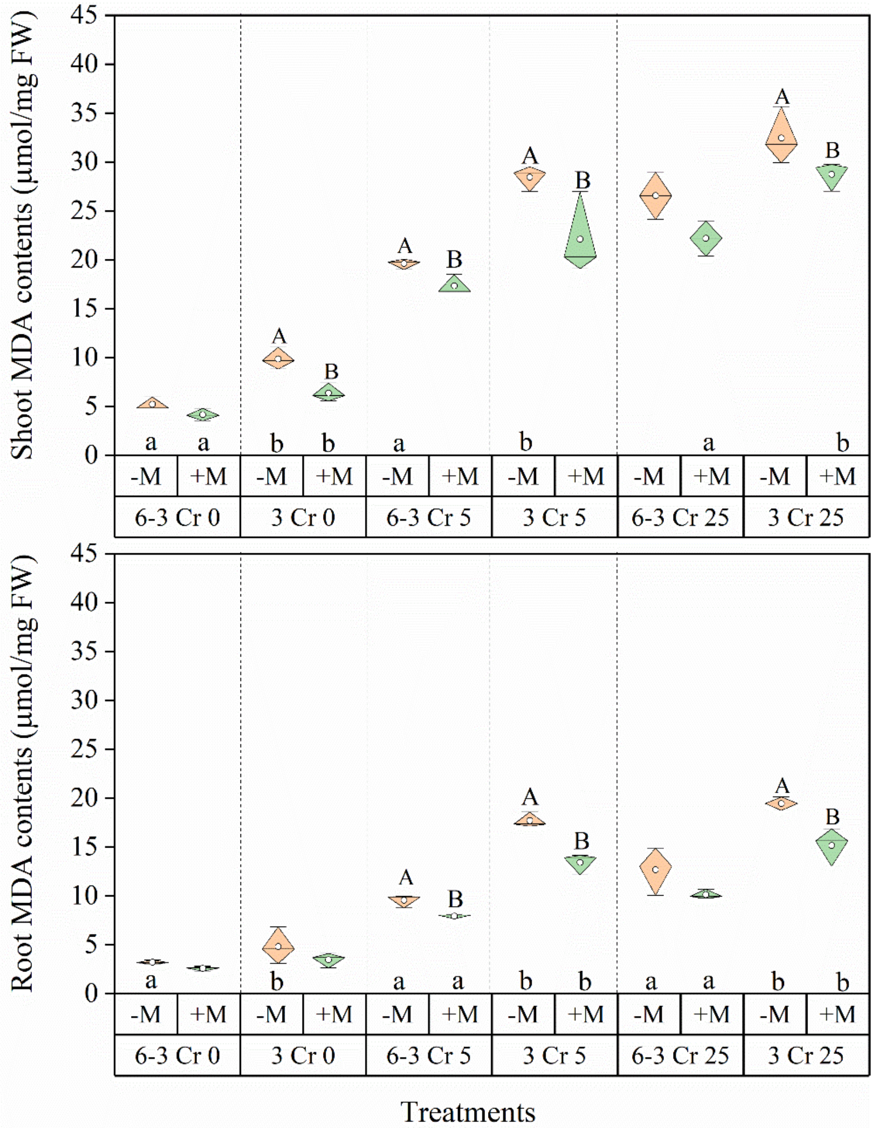


Figure 4.3: MDA contents both in shoots and roots of AMF/non-AMF inoculated wetland plants under Cr stress (a, b shows significant differences between different water depths; A, B shows significant differences between AMF and non-AMF inoculated condition ($p < 0.05$))

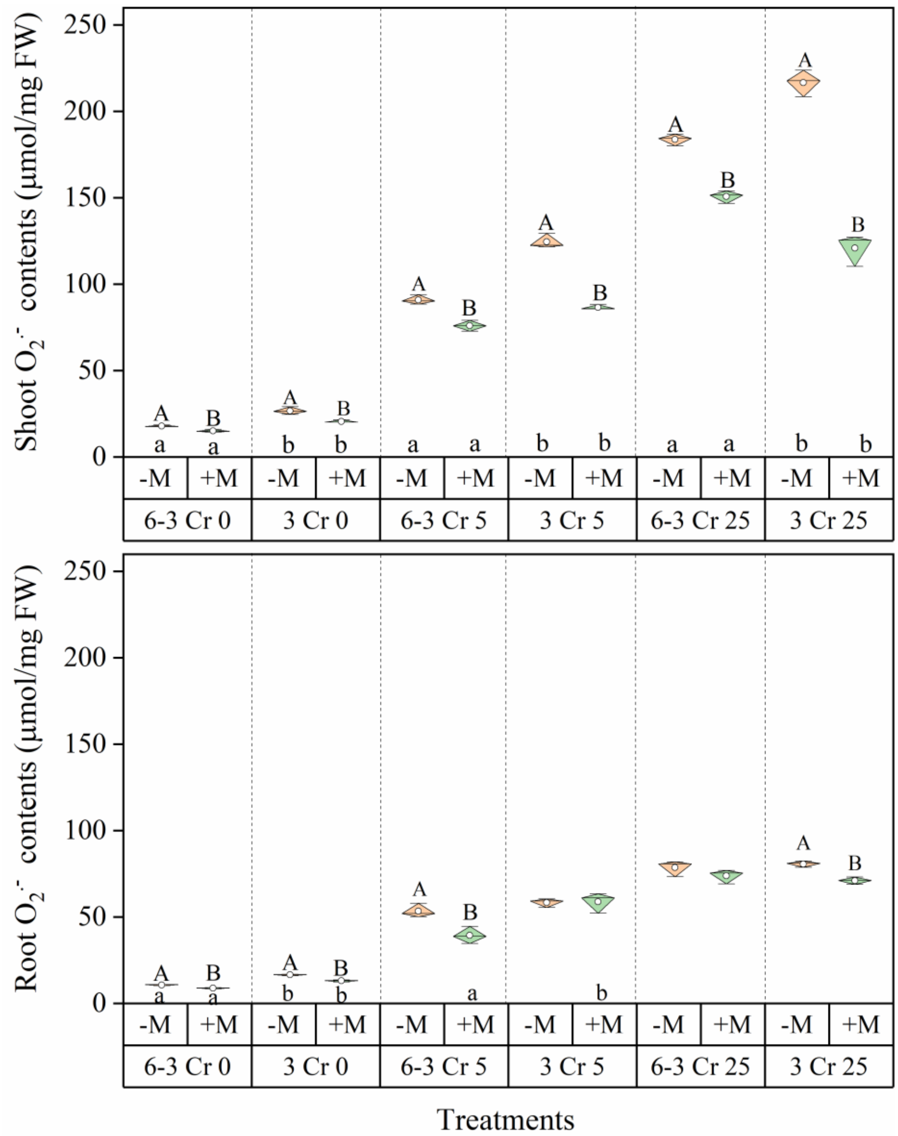


Figure 4.4: $O_2^{\cdot-}$ contents both in shoots and roots of AMF/non-AMF inoculated wetland plants under Cr stress (a, b shows significant differences between different water depths; A, B shows significant differences between AMF and non-AMF inoculated condition ($p < 0.05$))

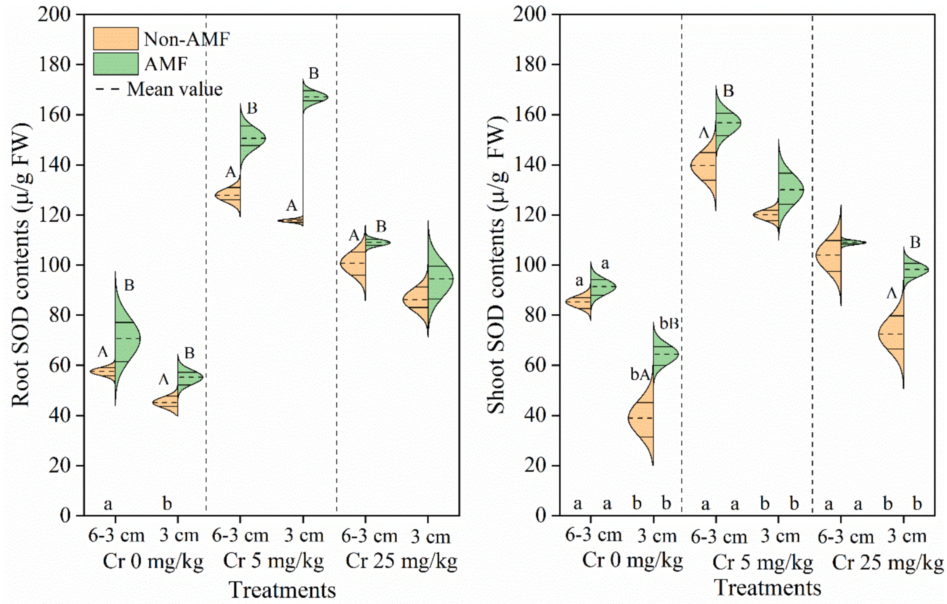


Figure 4.5: SOD contents both in shoots and roots of AMF/non-AMF inoculated wetland plants under Cr stress (a, b shows significant differences between different water depths; A, B shows significant differences between AMF and non-AMF inoculated condition ($p < 0.05$))

4.4.5 Antioxidant enzyme activities

SOD and POD contents in the shoot and root of *I. pseudacorus* under the water depth of 6-3 cm with Cr concentration of 5 mg/kg were significantly higher than those under the condition without Cr addition ($p<0.05$) (**Figure 4.5**, **Figure 4.6**). However, since the Cr concentration increased to 25 mg/kg, the shoot and root SOD and POD contents were significantly decreased compared to the Cr concentration of 5 mg/kg ($p<0.05$). Besides, the SOD contents in the shoot (root) of AMF inoculated *I. pseudacorus* under the same water depth and Cr concentration were 4.7-39.6% (7.5-29.5%) and 11.2-68.8% (16.8-50.3%) higher than those in the non-inoculated controls, respectively. Moreover, the water depth of 6-3 cm also had a positive effect on the SOD and POD accumulation in the wetland plant. Compared to the water depth of 3 cm at the same Cr concentration with AMF inoculation, the shoot (root) SOD and POD contents under the water depth of 6-3 cm were increased by 9.9-54.4% (-10.9-21.9%)

and 24.8-62.8% (28.1-77.4%), respectively. Three-way ANOVA analysis also showed that AMF, water depth, Cr, and the interaction of AMF* Cr* water depth had significant effects on both antioxidant enzyme contents in the *I. pseudacorus* (**Table S1**).

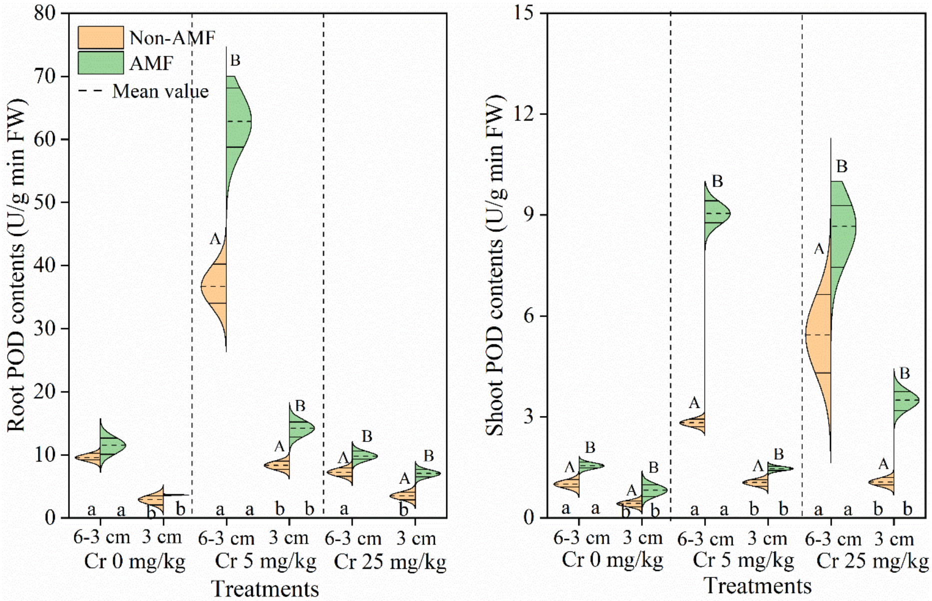


Figure 4.6: POD contents both in shoots and roots of AMF/non-AMF inoculated wetland plants under Cr stress (a, b shows significant differences between different water depths; A, B shows significant differences between AMF and non-AMF inoculated condition ($p < 0.05$))

4.5 Discussion

4.5.1 Cr effect on wetland plant growth and antioxidant response

Excessive heavy metals in plants can cause toxic symptoms due to the destruction of cell structure, imbalance of essential elements, and inhibition of sulphydryl protein

(Maksymiec, 2007). These toxicity symptoms in plants further induce overproduction of ROS and lipid peroxidation, which are harmful to plant growth (Bah et al., 2011). Generally, MDA and $O_2^{\cdot -}$ contents in the *I. pseudacorus* were increased as the rising of Cr concentration in our study. In contrast, the height, biomass, contents of chlorophyll, proline, P, and K in the *I. pseudacorus* were gradually decreased with the increase of Cr concentration. Similar results also reported that the MDA content in the shoot of *Typha angustifolia* under Cr treatment was 37.2% higher than that under the control without Cr addition, and the height, shoot and root dry weights of *T. angustifolia* under the Cr treatment were decreased by 3.3%, 5.7%, and 54.5%, respectively (Bah et al., 2011). Wang et al. (2017) studied the role of AMF on the growth of *Phragmites australis* under Cr stress, indicating that MDA accumulation in plants resulted from the Cd stress-induced oxidative injuries. The increase of $O_2^{\cdot -}$ under Cr stress induced the hydroperoxyl radical ($^{\cdot}OH$, hydrogen peroxide (H_2O_2)) production, which converted fatty acids into toxic lipid peroxides, thus destroying the functionality of biological membranes and changing the protein carbonylation (Bah et al., 2011). Moreover, a possible reason for the plant growth inhibition under heavy metal stress was that heavy metal causes blockage of an apical division, resulting in the reduction of biomass, nutrients, and height in plants (Zhang et al., 2020a). Therefore, the ROS level and lipid peroxides in wetland plants were largely increased in response to Cr stress, thus inhibiting the wetland plant growth.

The increase of ROS in plants under heavy metal stress also induces enzymatic and non-enzymatic ROS scavenging systems to protect against oxidative stress (Zhang et al., 2019). SOD, CAT, and POD are the major antioxidant enzymes for ROS scavenging in plants, they can protect the structure and function of the membrane system and maintain the redox state of cells (Zhang et al., 2019). In general, SOD is a kind of metalloprotein considered to eliminate $O_2^{\cdot -}$ into oxygen and H_2O_2, CAT and POD can break H_2O_2 to water and oxygen (Kapoor and Singh, 2017). The SOD and POD contents in the *I. pseudacorus* under the Cr stress were higher than those under the controls without Cr addition in our study, while a reduction of both contents was determined under the high Cr concentration (25 mg/kg) compared to the low Cr concentration (5 mg/kg). This indicated that the SOD and POD are sensitive to Cr stress, and low Cr stress induced less oxidative damage in plants compared to the high Cr stress. Huang et al. (2017) studied AMF modulate the phytotoxicity of Cd via combined responses of enzymes, thiolic compounds, and essential elements in the roots of *P. australis*, showing that low Cd stress (1 mg/L) caused a significant increase in the activities of SOD, CAT, and POD in the roots of *P. australis*, while the SOD and CAT activities were decreased under the high Cd stress (20 mg/L). In addition, proline can also reduce heavy metal toxicity in plants, a possible reason was that proline (an imino acid) has a chelate ability to bind with metal ions (Choudhary et al., 2007). The proline contents in the *I. pseudacorus* were increased gradually as the increase of Cr concentration in our study. Choudhary et al. (2007) also reported that the increase of proline content was proportional to the concentration of heavy metals (Pb, Cu, and Zn). Therefore, enzymatic and non-enzymatic ROS scavengers play an important role in ROS scavenging in wetland plants under heavy metal stress.

4.5.2 Effect of AMF on wetland plant growth and antioxidant response

AMF promote the acquisition of nutrients and water uptake, thereby increasing the height and biomass of plants (Liang et al., 2019). Hu et al. (2020a) studied the AMF effect on physiological functions in wetland plants (*P. arundinacea* and *S. sylvaticus*), showing that the shoot height, biomass, shoot total carbon contents, and shoot total nitrogen contents of both AMF inoculated wetland plants were 13.1–26.6%, 33.3–114.3%, 26.1%, and 66.7% higher than those of non-inoculated controls, respectively. AMF improved nutrient uptake in plants mainly through the mycelial network around the roots (Liang et al., 2018). Previous studies reported that the extraradical mycelium of AMF can extend >10 cm beyond the root surface, providing the hyphae to absorb polyphosphate, ammonium, and nitrate from the substrate, then quickly transfer phosphate and nitrogen to the host plants (Cavagnaro et al., 2015; Hodge and Storer, 2015). Besides, AMF can directly absorb heavy metals through hypha or reduce abiotic stress in plants (Wu et al., 2020), thereby promoting physiological and biochemical processes in plants. Xu et al. (2019) studied the effect of *Funneliformis mosseae* on *P. australis* under the stresses of water and TiO_2 nanoparticle, showing that chlorophyll content in AMF inoculated plants under the interaction of drought and 500 mg/kg TiO_2NPs were 2.56-2.59 times higher than the non-inoculated controls. Besides, the osmotic adjustment in plants is also influenced by AMF. In addition to being an osmotic regulator, proline is also a component of the cell wall, a free radical scavenger, and an antioxidant stabilizer (Hayat et al., 2012). Lower proline contents were determined in the AMF inoculated *I. pseudacorus* compared to the non-inoculated controls. This indicated that AMF colonization was beneficial to carbohydrate accumulation and reduce the osmotic potential in host plants. Wang et al. (2017) also studied the role of

Rhizophagus irregularis in alleviating Cd toxicity via improving the growth, micro- and macroelements uptake in *P. australis*, indicating that proline content in non-mycorrhizal *P. australis* under the same Cd stress was much higher than that in mycorrhizal *P. australis*. Consequently, AMF may play an important role in the protection or regulation of host plants under heavy metal stress.

AMF promoted the tolerance of the *I. pseudacorus* to Cr stress by enhancing the activities of antioxidant enzymes (POD and SOD), decreasing lipid peroxidation (MDA), and ROS ($O_2^{\cdot-}$) accumulation in our study. The main reason was that AMF symbiosis can coordinate the expression of several proteins (e.g. glutaredoxins and thioredoxins) involved in abiotic responses (e.g. heavy metal, drought), and regulate the induction of oxidative stress-related genes in extraradical mycelium under abiotic stresses (Chen et al., 2015b; Lenoir et al., 2016). Zhang et al. (2019) studied the effect of AMF on the antioxidant response in the roots of *Medicago truncatula* under Pb stress, indicating that SOD activity in AMF inoculated roots was increased by 60% (104%), while $O_2^{\cdot-}$ generation rate was decreased by 38% (20%) compared to the non-inoculated controls under the Pb concentration of 500 (1000) mg/kg. Moreover, Hu et al. (2020) also showed that MDA contents of AMF inoculated *P. arundinacea* and *S. sylvaticus* under the water regime of 11 cm (from the substrate surface to below) were significantly lower than that of non-inoculated controls ($p < 0.05$). AMF cannot counteract the ROS damage under heavy metal stress, although they can enhance the antioxidant response in plants. This may be the reason that the antioxidant enzymes were decreased under the high Cr stress in our study. Therefore, AMF inoculated plants had better ROS scavenging than non-inoculated plants under heavy metal stress. However, the positive effect of AMF on the antioxidant response in plants may be decreased with the increase of heavy metal concentration.

4.5.3 Effect of water depth on plant growth and antioxidant response

Wetland plant growth and antioxidant response are also influenced by water depth. The fluctuating water depth (6-3 cm) promoted the growth of *I. pseudacorus* and decreased the oxidative stress toxicity in the *I. pseudacorus* compared to the low water depth (3 cm) in our study. A previous study also reported that physiological functions (e.g. height, biomass, chlorophyll, and nutrient contents) of wetland plants under low water depth (below the substrate surface: 11 cm) were lower than those under other water depths (9 cm, 11 cm, and 9-11 cm), while the highest MDA content (>50 nmol/g FW) was obtained under the low water depth (Hu et al., 2020a). Low water depth might induce ROS accumulation and lipid peroxidation, resulting in the inhibition of wetland plant growth. Xu et al. (2019) showed that the SOD and POD activities of *P. australis* leaves under drought and flooding conditions (relative water content of substrates: 50% and 100%) at the same concentration of TiO_2NPs were significantly higher than those under normal water condition (70%), the MDA (38.1 μmol/g FW) and ROS (70.0 μg/g FW) contents were the highest under the drought stress. Although low water depth was not conducive to wetland plant growth, AMF enhanced the plant's resistance to drought stress and reduced the ROS level and MDA content (Ren et al., 2019). AMF promoted the tolerance of plants under low water conditions by increasing nutrient uptake and improving antioxidant enzyme activities (Xu et al., 2019). Therefore, fluctuating water depth contributes to the wetland plant growth and promotes the tolerance of plants under heavy metal stress.

4.5.4 The role of AMF on alleviating Cr toxicity in wetland plant

PCA and cluster analyses were carried out to systematically figure out the main factors that AMF responds to Cr toxicity. The relationships among biomass, height, chlorophyll, MDA, $O_2^{\cdot -}$, proline, SOD, POD, K, and P contents in AMF inoculated and non-inoculated wetland plants at the water depth of 6-3 cm under different Cr concentrations were conducted by PCA analysis (**Figure S4**). Results showed that the variations in these parameters under the Cr concentrations of 0, 5, 25 mg/kg were captured by two main axes, together explaining 92.6%, 94.5%, and 86.7% of the variation, respectively. Among them, these parameters were largely determined by PCA axis 1, revealing that there was a strong correlation among them. The biomass, height, chlorophyll, K, and P contents in the AMF/non-AMF inoculated wetland plants under the three Cr concentrations had strong positive correlations with the SOD and POD. Meanwhile, these parameters had negative correlations with MDA, $O_2^{\cdot -}$, and proline. Moreover, the results of scores of 6 treatments on axis 1 and axis 2 showed that AMF had a significant effect on plant growth and ROS scavenging under Cr stress. In addition, hierarchical clustering analysis also indicated that the correlations among plant growth indexes, nutrients, and ROS levels in AMF inoculated plants under the Cr concentration of 5 mg/kg were similar to the treatments without Cr addition (**Figure 4.7**). However, plant growth indexes, nutrients, antioxidant enzymes, and ROS levels had similar correlations under the non-AMF inoculated wetland plants with Cr concentration of 5 mg/kg and AMF/non-AMF inoculated wetland plants with Cr concentration of 25 mg/kg. The results indicated that AMF can significantly improve wetland plant growth and enhance ROS scavenging under low Cr stress.

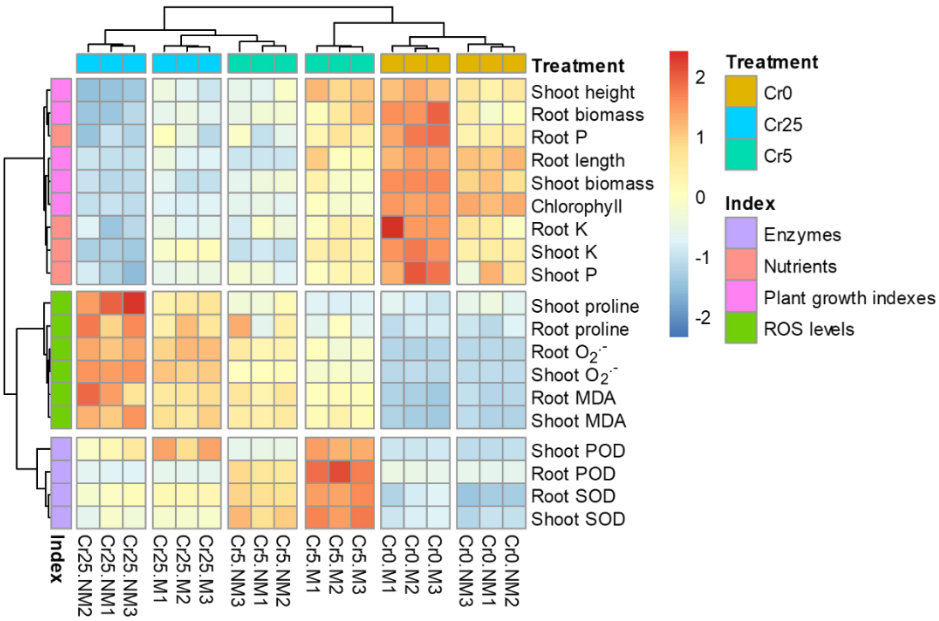


Figure 4.7: Hierarchical clustering of biomass, height, chlorophyll, MDA, $O_2^{\cdot-}$, proline, SOD, POD, K, and P contents in AMF/non-AMF inoculated wetland plants in the 6 treatments under the water depth of 6-3 cm, colors in the heatmap indicate the pairwise Pearson correlation between the different data sets

4.6 Conclusion

Cr had a negative effect on wetland plant growth by increasing ROS and lipid peroxidation accumulation. However, AMF can enhance wetland plant growth and improve ROS scavenging under Cr stress (especially under the condition of Cr concentration of 5 mg/kg). Generally, the biomass, height, the contents of chlorophyll, SOD, POD, K, and P in AMF inoculated wetland plants were higher than those in non-inoculated controls under the same water depth and Cr stress. Conversely, the MDA, $O_2^{\cdot -}$, and proline contents in the AMF inoculated wetland plants were decreased compared with the non-inoculated controls under the same water depth and Cr stress. Meanwhile, AMF inoculated wetland plants had a higher antioxidant response compared to the non-inoculated controls under the low water depth condition (3 cm). Besides, fluctuating water depth (6-3 cm) also contributed to the wetland plant growth and promoted the tolerance of plants under Cr stress. Overall, AMF had good potential in reducing oxidative damage of wetland plants under heavy metal stress. This provided a chance for heavy metal removal in AMF assistant constructed wetlands.

4.7 Supplementary Materials

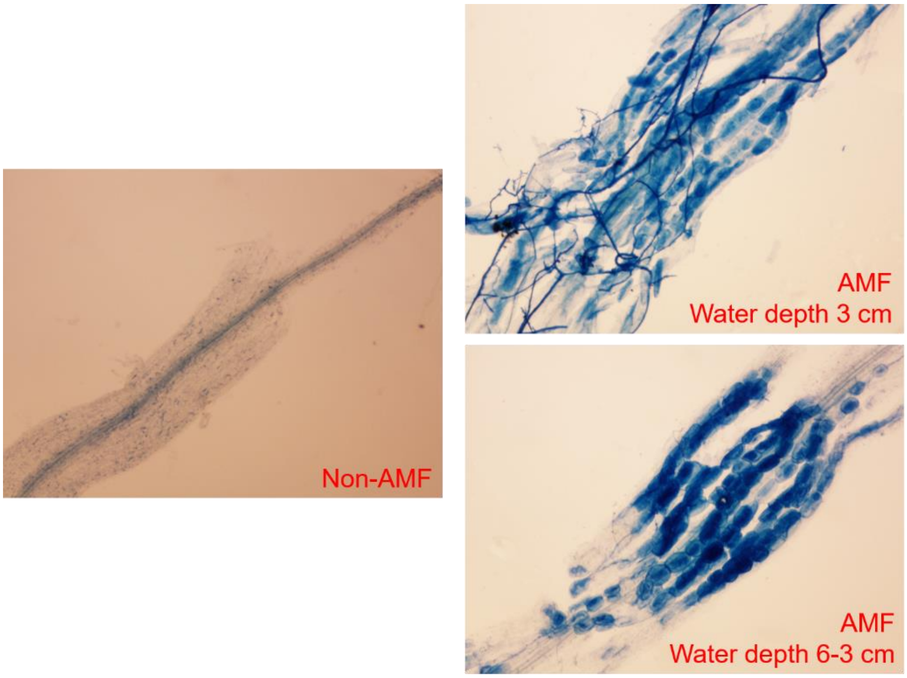


Figure S1: AMF colonization under the water depths of 3 cm and 6-3 cm before Cr solution addition into the systems

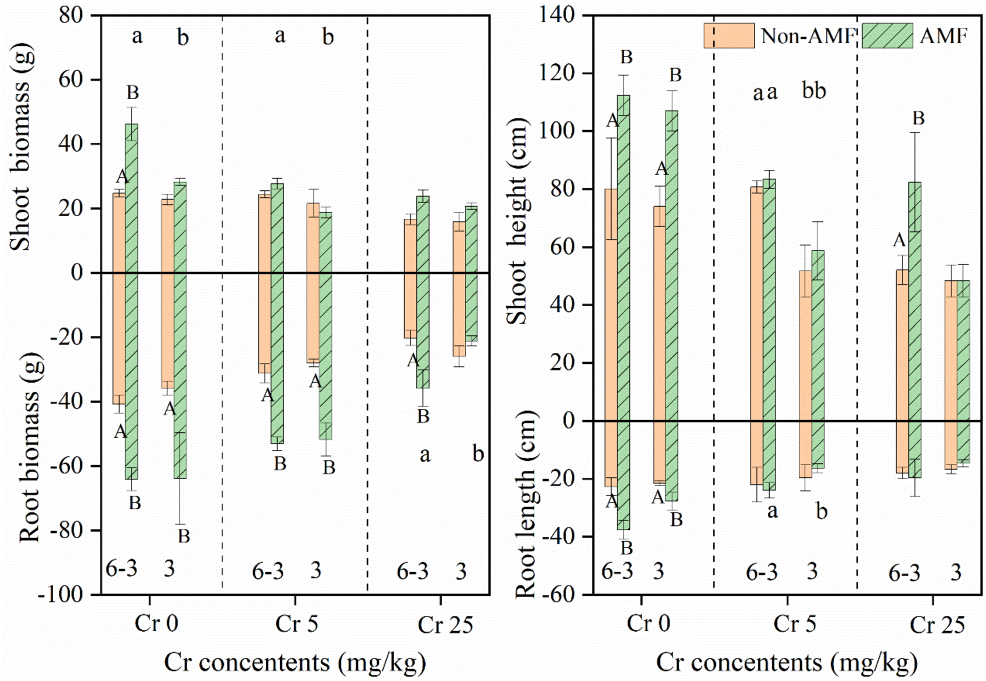


Figure S2: The height and fresh biomass of AMF/non-AMF inoculated wetland plants under Cr stress (a, b shows significant differences between different water depths; A, B shows significant differences between AMF and non-AMF inoculated condition ($p < 0.05$))

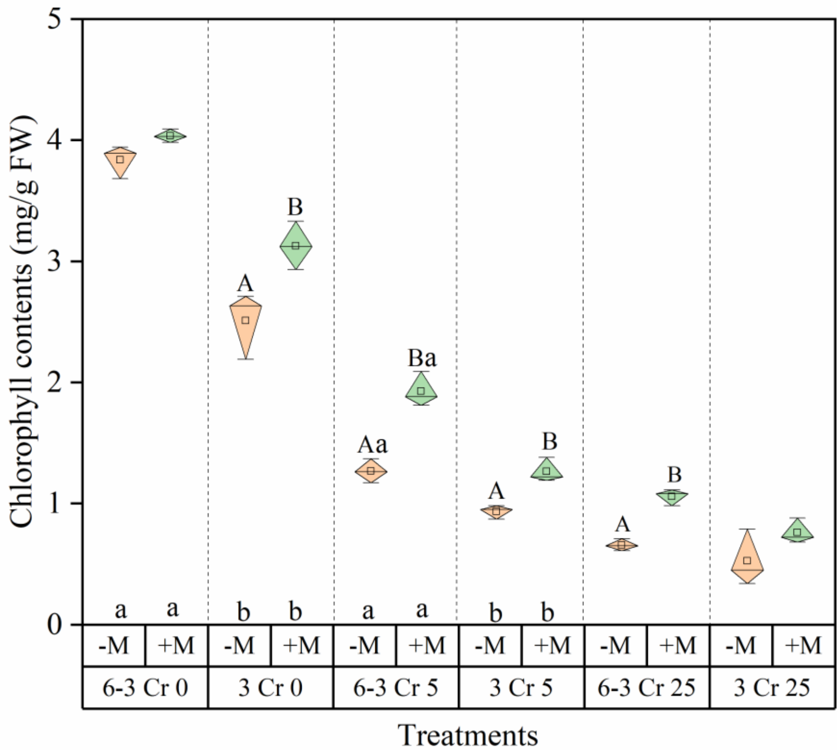


Figure S3: Chlorophyll contents in AMF/non-AMF inoculated wetland plants under Cr stress (a, b shows significant differences between different water depths; A, B shows significant differences between AMF and non-AMF inoculated condition ($p < 0.05$))

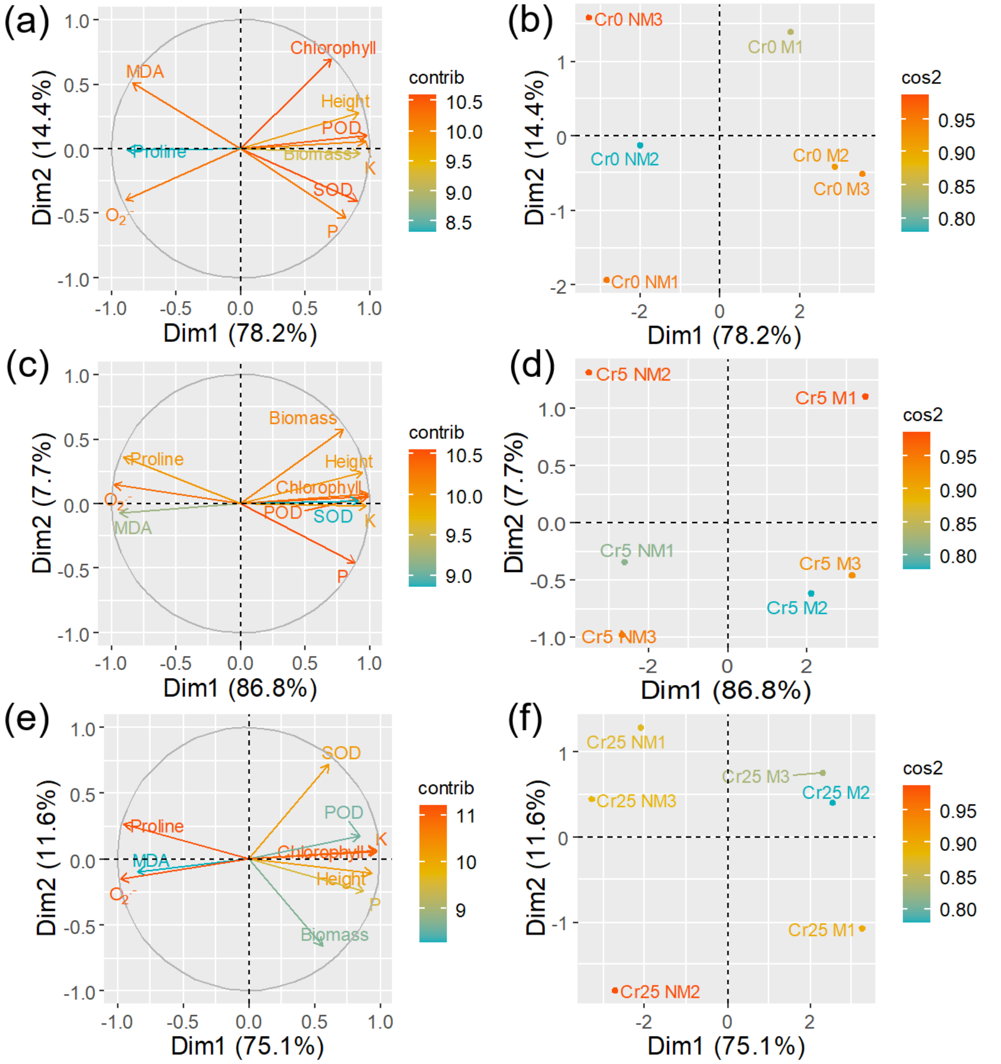


Figure S4: Principal component analysis (PCA) of biomass, height, chlorophyll, MDA, $O_2^{\cdot-}$, proline, SOD, POD, K, and P contents in AMF/non-AMF inoculated wetland plants under the water depth of 6-3 cm, and scores of 6 treatments on AX1 and AX2

Table S1: Three-way ANOVA analysis for the effects of AMF, Cr, and water depth on wetland plants characteristics

Parameters	**AMF**	**Cr**	**Water depth**	**AMF*Cr**	**AMF*Water depth**	**Cr*Water depth**	**AMF*Cr*Water depth**
Root length	<0.001	<0.001	<0.001	0.048	0.001	<0.001	<0.001
Shoot height	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001
Root biomass	<0.001	<0.001	<0.001	0.002	<0.001	0.008	**0.575**
Shoot biomass	0.002	<0.001	<0.001	<0.001	0.001	<0.001	0.042
Root MDA	<0.001	<0.001	<0.001	0.047	0.041	<0.001	**0.608**
Shoot MDA	<0.001	<0.001	<0.001	**0.384**	**0.139**	**0.089**	**0.327**
Root $O_2^{\cdot -}$	<0.001	<0.001	<0.001	**0.200**	**0.233**	<0.001	0.003
Shoot $O_2^{\cdot -}$	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Root POD	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Shoot POD	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Root SOD	<0.001	<0.001	<0.001	<0.001	0.009	<0.001	<0.001

Shoot SOD	<0.001	<0.001	<0.001	**0.826**	0.002	0.001	0.002
Chlorophyll	<0.001	<0.001	<0.001	**0.347**	**0.796**	<0.001	0.012
Root K	<0.001	<0.001	**0.075**	**0.309**	**0.680**	0.036	**0.331**
Shoot K	<0.001	<0.001	<0.001	**0.375**	<0.001	<0.001	**0.14**
Root P	<0.001	<0.001	<0.001	**0.126**	**0.230**	**0.394**	**0.393**
Shoot P	<0.001	<0.001	<0.001	**0.568**	**0.359**	0.028	**0.716**
Root proline	<0.001	<0.001	0.001	<0.001	**0.642**	**0.125**	**0.08**
Shoot proline	0.006	<0.001	0.013	**0.19**	**0.656**	**0.365**	**0.632**

Note: the values in bold show the no significant difference ($p > 0.05$).

Chapter V

Arbuscular mycorrhizal fungi modulate the chromium distribution and bioavailability in semi-aquatic habitats

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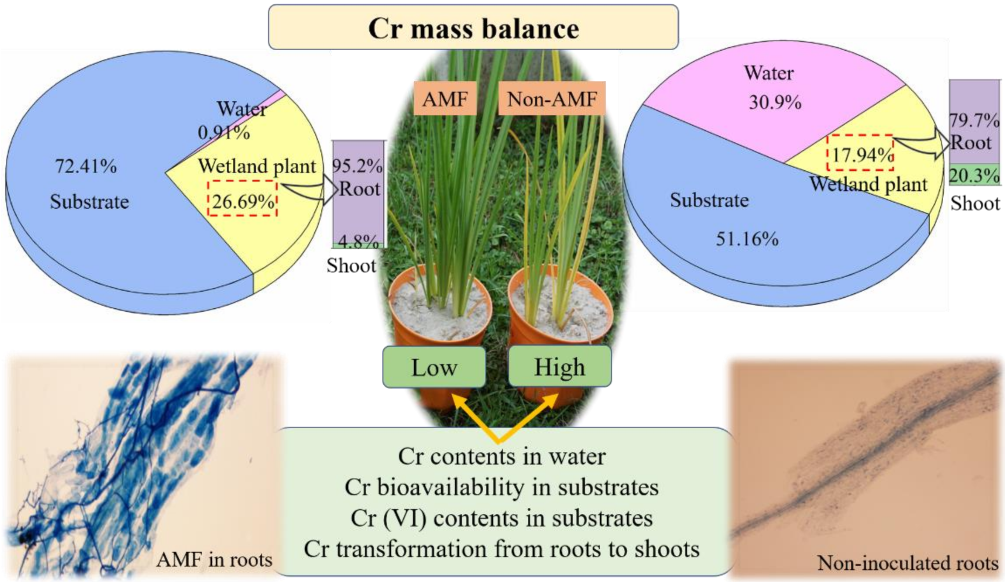
Minor revised manuscript submitted to Chemical Engineering Journal (In process)

Contents

5.1 Abstract

Arbuscular mycorrhizal fungi (AMF) can improve plant tolerance to heavy metal stress in the terrestrial system. However, the detoxification ability of AMF to heavy metals in aquatic systems remains poorly understood. This study investigated the effects of AMF on chromium (Cr) distribution and bioavailability in semi-aquatic habitats under different water depths. Results showed that AMF increased the Cr accumulation in the roots of *Iris pseudacorus*by 10.0-20.0%. Conversely, Cr concentrations in the shoots of AMF inoculated *I. pseudacorus* were 21.7-68.4% lower than those in the non-inoculated controls. Besides, Cr concentrations and mass contents in water were decreased by 34.1-35.3% and 15.4-23.3% under the low Cr stress (5 mg/kg) with AMF inoculation compared to the non-inoculated treatments, respectively. Moreover, Cr (VI) concentrations in substrates under the Cr contents of 5 mg/kg were 27.0-44.0% lower than those under the non-inoculated controls. Meanwhile, AMF reduced the Cr bioavailability in substrates under the fluctuating water depth (water depth of 6-3 cm), with an acid-soluble state of Cr decreased by 4.1-5.6%. Furthermore, AMF also enhanced the biomass, nutrient contents (TC, TN, Ca, Mg, Fe, and Mn) in the *I. pseudacorus* under the fluctuating water depth. Therefore, it provides a possibility that AMF can be used to remove heavy metal from polluted wastewater by semi-aquatic habitats under fluctuating water depth (e.g. tidal flow constructed wetlands).

Figure 5.1: Graphical abstract



5.2 Introduction

Heavy metals (e.g. Cu, Pb, Ni, Cd, and Cr) discharged from wastewater can easily be accumulated in soils, sediments as well as in-ground or surface water, which pose a serious threat to aquatic ecosystems, food safety, and human health (Ali et al., 2020). Therefore, the removal of heavy metals from wastewater is considered to be a major challenge in the field of water pollution. Many techniques (e.g. reverse osmosis, ion exchange, chemical precipitation, adsorption, electrolysis, and solvent extraction) have been established to treat heavy metal polluted wastewater, which provides effective ways for heavy metals removal (Bolisetty and Mezzenga, 2016; Zhang et al., 2020b). However, the application of these technologies also raises additional concerns, such as high construction and operation costs, high energy consumption, complex maintenance, and a large amount of sludge produced (Liu et al., 2020). To reduce the cost of heavy metal processing in wastewater, an environmentally friendly and economical treatment technology is needed.

Phytoremediation is considered as a potentially economical and environmentally friendly method of removing heavy metals, and is particularly attractive for not requiring significant advanced engineering work (Malaviya et al., 2020). Generally, wetland plants (especially macrophytes) represent a suitable tool for heavy metals sequestration and phytoremediation in aquatic habitats (e.g. constructed wetlands (CWs) systems) due to their vigorous growth and high aboveground biomass (Vymazal et al., 2009). Heavy metals can be taken up by roots of wetland plants and could be translocated upwards to the aboveground parts (e.g. shoots, stems, and leaves). Vymazal and Březinová (2016) reported that Zn and Cr accumulated in *Phragmites australis* shoots represented 48.5% and 38.2% of the annual inflow load in the CWs systems, respectively. In addition, heavy metals can be adsorbed on the fine-grained sediment and organic matter in the filtration bed and could be retained by filtration and deposition in the rhizosphere (Vymazal and Březinová, 2016). Although wetland plants have a positive effect on heavy metal removal in aquatic habitats, their resistance to heavy metal toxicity is limited. Batool and Saleh (2020) reported that the high concentration of heavy metals in the environment may have toxic effects on plants, thereby affecting the regulation of important ions by plants, and ultimately destroying the comprehensive activities of plants. Therefore, it is necessary to improve the tolerance of wetland plants to heavy metal stress, which beneficial for heavy metal removal in aquatic habitats.

Arbuscular mycorrhizal fungi (AMF) are one of the important rhizosphere microorganisms which can form symbiotic associations with most terrestrial plants (Akbar Karimi, 2011). They can improve the terrestrial plant's resistance to biotic and abiotic stresses such as salinity, drought, flooding, emerging pollutants, and heavy metals by promoting the nutrients uptake and antioxidant enzyme activities (Agarwal et al., 2017). Nowadays, numerous studies reported that AMF can also establish symbiosis with the roots of some wetland plants in semi-aquatic and aquatic habitats in recent years (Calheiros et al., 2019; Dolinar and Gaberščik, 2010). Some researchers found a decrease in the degree of AMF colonization with the increase of flooding gradients and heavy metal concentrations in aquatic systems (Wang et al., 2010; Xu et al., 2016). Despite that, Ban et al. (2017) indicated that the AMF colonization of *P. australis* living in the heavy metal polluted wetland habitats ranged from 7 to 35%. Zheng et al. (2015) demonstrated that AMF inoculation and the variation of water contents were highly significant effects on the *P. australis* growth, and AMF had protective effects for wetland plants against higher heavy metal concentrations in the aboveground tissues. Our previous studies have also proven that the antioxidant enzyme activities in wetland plants at chromium (Cr) stress were promoted by AMF inoculation under fluctuating water levels (Hu et al., 2020b). Although these studies have indicated AMF had positive effects on wetland plant growth under heavy metal stress in aquatic systems, the response of wetland plants to heavy metal stress under different water levels is still uncertain regarding various heavy metal concentrations. Furthermore, few studies focus on the effects of AMF on heavy metal distribution and bioavailability in semi-aquatic and aquatic habitats.

Therefore, this study aimed to (1) evaluate the effects of AMF on Cr distribution and transformation in semi-aquatic habitats under different water depths; (2) access the response of wetland plants to Cr stress in semi-aquatic habitats under various water depths regarding different Cr concentrations.

5.3 Materials and Methods

5.3.1 Experimental design

The experiment was performed via three Cr levels (0, 5, and 25 mg /kg substrate) applied as K_2CrO_4, two water depths (from bottom to top: 3 cm depth of water, and fluctuating water depth: water depth varies between 3 cm and 6 cm (6-3 cm)), and two AMF treatments (inoculation with AMF and non-inoculation) as three factorial experiments. Each treatment contained three replicates. The period of fluctuating water depth was 4 days (2 days with 3 cm depth of water and 2 days with 6 cm depth of water).

The experiment was conducted in a pot with a dimension of 17 ×15 cm (diameter × height). *Rhizophagus irregularis* (BEG140) was used as an AMF inoculum, which was composed of a mixture of spores, mycelium, zeolite, expanded clay, and plant (*Zea mays L.*) root fragments. *Iris pseudacorus*, as a common wetland plant, was selected in this study. In order to avoid germination failure in the experimental system, the seeds of *I. pseudacorus* collected from a pond on the campus of the Czech University of Life Science Prague were planted in the sterilized sands in April. Similar heights of seedlings (5-7 cm) were selected as experimental plants at the end of May. The roots of seedlings were surface sterilized with 75% ethyl alcohol for 10 seconds and 1% sodium hypochlorite for 15 minutes, washed carefully with sterile distilled water five times before transplanted into the experimental systems. The sterilized sands with grain sizes of 0.1-0.5 mm were used as substrates. 50 g of inoculum mixed with 1000 g of sterilized sand were added after putting 1500 g of sterilized sands to the bottom of the pots. Three seedlings were transplanted into each pot afterward, finally covered by 500 g sterilized sand. The same steps were carried out in the non-inoculated treatment systems except that 50 g of inoculum was replaced by 50 g of sterilized inoculum and 10 mL inoculum filtrate. Detailed information of experimental setup and water depths were shown in **Figure S1**. Each system was irrigated with 1/4-strength Hoagland solution (100 mL/week/pot) to avoid nutrient deficiency, and distilled water was used to control the water depths. Cr (VI) solutions with different concentrations were added when the AMF colonization was successfully detected 3 months after planting. *I. pseudacorus* were maintained for 5 months before harvest.

5.3.2 Sample analysis

Shoots and roots of *I. pseudacorus* were harvested separately, then washed carefully with deionized water for further analyses. Fresh samples of *I. pseudacorus* were collected for the determination of AMF colonization and the root-shoot ratio of biomass. The dry biomass of shoots and roots were determined after oven drying at 70 °C for 48 h. The dried samples in the shoots and roots were used to measure the contents of total carbon (TC), total nitrogen (TN), sulfur (S), sodium (Na), silicon (Si), aluminum (Al), calcium (Ca), iron (Fe), magnesium (Mg), and manganese (Mn). Meanwhile, Cr concentrations were also determined in samples of shoots, roots, water, and substrates. Mycorrhizal dependency, Cr concentrations, and Cr fractions were determined in the wetland plant. Mycorrhizal dependency was calculated by the equation given below:

$$\text{Mycorrhizal dependency (g/g DW)} = \frac{\text{Dry biomass of inoculated fungus plants}}{\text{Dry biomass of controlled plants}}$$

DW: dry biomass of plants.

Translocation factor (TF) was calculated using the following equation to evaluate the effect of AMF on the Cr translocation-absorption capacity in wetland plants.

$$\text{TF} = \frac{\text{Cr content in shoots (mg/kg)}}{\text{Cr content in roots (mg/kg)}}$$

The tolerance of Cr in wetland plants was quantified via the tolerance index (TI) by comparing plant biomass in Cr/non-Cr treatment systems.

$$\text{TI} = \frac{\text{Total dry biomass in Cr treatment systems (g)}}{\text{Total dry biomass in non-Cr treatment systems (g)}}$$

Mycorrhizal Cr response (MCrR) was calculated by using the mean Cr concentration of AMF inoculated wetland plants and mean Cr concentration of non-inoculated wetland plants in each Cr treatment system.

$$\text{MCrR} = \frac{\text{Mean Cr concentration (AMF plants)-Mean Cr concentration (non-AMF plants)}}{\text{Mean Cr concentration (non-AMF plants)}}$$

Fresh roots were cleaned and stained according to the modified procedure of Phillips and Hayman (1970). Briefly, the samples of fresh roots were cleared in 10% KOH (30 min, 90 °C), washed in distilled water afterward, acidified with 2% HCl solution for 3-5 min, then stained with Trypan blue (30 min, 90 °C), finally destained with 50% glycerol for 2-3 days. The roots were cut into approximately 1 cm pieces and mounted onto slides before viewing under a microscope. AMF was assessed using the method described by (Trouvelot et al., 1986). Root-shoot ratio and biomass were measured by the gravimetric method. TC and TN in the roots and shoots were determined by a Skalar Primacs SNC analyzer (Breda, the Netherlands), NIST 1547 Peach Leaves was used as the standard (National Institute of Standards and Technology, Gaithersburg, MD, USA). The dry samples of plants and substrates were digested in HNO_3/HCl (1/3 v/v) at 210°C on an electric heating plate for 12 h, diluted to 50 mL with ultrapure water, and filtered through a 0.22 µm membrane. S, Na, Si, Al, Fe, Ca, Mg, and Mn concentrations in roots and shoots were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES). Cr (VI) concentrations in substrates were determined according to Vitale et al. (1994). Cr concentrations in the roots, shoots, water, and substrates were analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Cr fractions in substrates were extracted by the Community Bureau of Reference (BCR), then analyzed by ICP-MS.

5.3.3 Statistical analysis

AMF colonization was analyzed using a two-way analysis of variance (ANOVA) with Cr concentration and water depth as the main factors and Cr concentration * water depth as the interaction effect. Cr concentration and its fractions were subjected to a two-way ANOVA to examine the effects of AMF colonization and water depths. Biomass, root to shoot ratio, mycorrhizal dependency, and nutrient contents in wetland plants were analyzed using a three-way ANOVA to evaluate the impacts of Cr concentration, water depth, and AMF colonization. The student's t-test was used to compare the effects of Cr concentration, AMF addition, and water depth on the biomass, root to shoot ratio, mycorrhizal dependency, TF, contents of TC, TN, S, Na, Si, Al, Fe, Ca, Mg, and Mn, $p < 0.05$ was set as a significant difference. Principal component analysis (PCA) and cluster analysis were completed to display data groupings and correlations for these parameters. The 'Factoextra' (Kassambara, 2017) and 'Pheatmap' (Kolde and Kolde, 2015) packages were used to visualize the experimental data among these parameters. All statistical analyses were performed using the R software (version 3.6.3) and the software package IBM SPSS statistics 21.0 for Windows.

5.4 Results and discussion

5.4.1 Effects of Cr and water depth on AMF colonization

The intensities of mycorrhizal colonization (M%) and arbuscule abundance (A%) were gradually decreased with the increase of Cr content under both water depths, and significant differences were obtained between the three Cr content treatments (**Table 5.1** and **Figure S2**). The intensities of mycorrhizal colonization under both water depths without Cr addition were 10.0-20.4% and 48.5-53.9% higher than those under the Cr contents of 5 mg/kg and 25 mg/kg, respectively. The arbuscule abundance under the treatments without Cr addition also showed a similar tendency, with an increase by 28.9-54.8% and 68.7-79.8% compared to the Cr contents of 5 mg/kg and 25 mg/kg, respectively. Meanwhile, two-way ANOVA analysis also obtained that Cr contents had significant effects on AMF colonization under both water depths ($p<0.001$) (**Table S1**). These results showed that Cr had a negative effect on AMF colonization in wetland plants, especially under high Cr content. Similar results also reported that AMF colonization (e.g. arbuscule numbers and spore densities) in roots of plants were decreased significantly under high Cr contamination (Akhtar et al., 2019; Wu et al., 2019). Wu et al. (2014b) studied the AMF (*R. irregularis*) effects on Cr (VI) tolerance of terrestrial plants (dandelion and bermudagrass), indicating that AMF colonization in dandelion and bermudagrass were reduced by 33.3% and 46.7% under Cr (VI) content of 20 mg/kg, respectively. However, Kullu et al. (2020) evaluated the roles of *R. irregularis* on Cr bioaccumulation in *Brachiaria mutica* under different Cr (VI) contents, showing that AMF colonization in Cr content of 90 mg/kg was decreased from 67% to 62%, while the colonization in Cr content of 60 mg/kg was increased to 78%. Heavy metals have positive, negative, and neutral influences on AMF colonization in plants, which are related to the host plants, AMF species, heavy metal species, heavy metal concentrations, a season of the year, and environmental conditions (e.g. nutritional status, water depths) (Kullu et al., 2020; Wu et al., 2019).

Table 5.1: Mycorrhizal status under different water depths with various Cr stresses. These include the intensity of mycorrhizal colonization (M%) and arbuscular abundance (A%) in the whole root system. Data are presented as means ± SD

Cr contents	Water depth	M%	A%
0 mg/kg	6-3 cm	45.7±2.7[a]	9.8±0.4[a]
	3 cm	49.7±1.6[b]	14.5±2.1[b]
5 mg/kg	6-3 cm	39.5±3.1[c]	6.6±1.8[c]
	3 cm	40.9±1.5[c]	7.0±2.2[c]
25 mg/kg	6-3 cm	22.9±2.4[d]	2.9±0.6[d]
	3 cm	23.5±1.4[d]	3.1±0.9[d]
Significance of			
Cr		<0.001	<0.001
Water depth		0.060	0.121
Cr*Water depth		0.288	0.078

Note: a, b, c, and d show significant differences among different treatments ($p < 0.05$).

The highest intensity of mycorrhizal colonization and arbuscule abundance of 49.7% and 14.5% were determined under the water depth of 3 cm without Cr addition, respectively (**Table 5.1**). Despite this, an insignificant difference in AMF colonization was obtained between water depths of 3 cm and 6-3 cm under Cr stress ($p>0.05$). This indicated that the fluctuating water depth affects AMF colonization was similar to the low water depth under Cr stress. Although previous studies indicated that AMF colonization can be decreased and the population structures, diversities, and activities of the aerobic microorganisms around the rhizosphere might also be reduced under saturated water depth in the aquatic systems (Garbeva et al., 2008; Ray and Inouye, 2006); some researchers also showed that hyphal and arbuscular colonization in the roots of wetland plants (e.g. *Phalaris arundinacea*, *Typha latifolia*, and *Scirpus sylvaticus*) can be found under the fluctuating water level as a result of oxygen content can increase by constantly changing water level in wetland system (Fusconi and Mucciarelli, 2018; Hu et al., 2020b). Wężowicz et al. (2015) studied the metal toxicity differently affects the *I. pseudacorus*-AMF symbiosis in terrestrial and semi-aquatic habitats, also showing that AMF such as *R. irregularis* had the ability to adapt to flooded habitats. Therefore, the fluctuating water depth provided a possibility for AMF colonization in the roots of wetland plants.

5.4.2 Effects of AMF and water depth on Cr distribution

Cr concentrations in wetland plants

Cr concentrations in the roots and shoots of *I. pseudacorus* were significantly increased as the Cr contents raised from 5 mg/kg to 25 mg/kg ($p<0.05$) (**Figure 5.2**). Meanwhile, a lower MCrR in both water depths were obtained under the Cr contents of 25 mg/kg compared to the Cr contents of 5 mg/kg (**Table 5.2**). This indicated that AMF inoculated wetland plants had a greater capacity to uptake Cr under low Cr (VI) stress. Cr concentrations in the roots of AMF inoculated *I. pseudacorus* under each treatment were significantly higher (10.0-20.0%) than that in the non-inoculated controls ($p<0.05$) (**Figure 5.2**). Conversely, Cr concentrations in the shoots of AMF inoculated *I. pseudacorus* under each treatment were significantly decreased by 21.7-68.4% compared to the non-inoculated controls ($p<0.05$). Meanwhile, TF in the AMF inoculated *I. pseudacorus* under the Cr contents of 5 mg/kg and 25 mg/kg were 70.0-75.0% and 37.6-38.0% lower than those in the non-inoculated controls, respectively. These results showed that AMF can alleviate the transfer of Cr from roots to shoots. Besides, regardless of the water depth of 6-3 cm and 3 cm, Cr contents in the roots and shoots of *I. pseudacorus* had insignificant differences between them except for the AMF inoculated *I. pseudacorus* at the Cr content of 25mg/kg ($p>0.05$).

AMF can promote the plant roots to accumulate heavy metals (e.g. Cr, Cu, Zn, Fe, and Mn) and prevent the transfer of the heavy metals from roots to shoots (Janeeshma and Puthur, 2020). Similar results also showed by Huang et al. (2018), which revealed that Cd concentrations and contents in the roots of AMF inoculated *P. australis* in the Cd concentration of 20 mg/L were higher than that in the non-inoculated controls, while a contrasting tendency was observed in the stems of *P. australis.* Ferrol et al.(2016) reported that AMF improved plants tolerance to heavy metals, mainly because it provided a capacity to chelate and sequestrate heavy metals, leading to the inactivation and removal of toxic heavy metals from sensitive parts, and controlling the acquisition and discharge of heavy metals in metal transport systems. Meanwhile, previous studies also proved that the fungal structures (e.g. arbuscules, intraradical mycelium) together with cell walls in AMF colonized roots could accumulate Cr, and restrain Cr translocation to plant cytoplasm, resulting in relieving Cr toxicity to plants (Wu et al., 2016a; Zhang et al., 2019). Therefore, the reason for the higher MCrR in the treatments

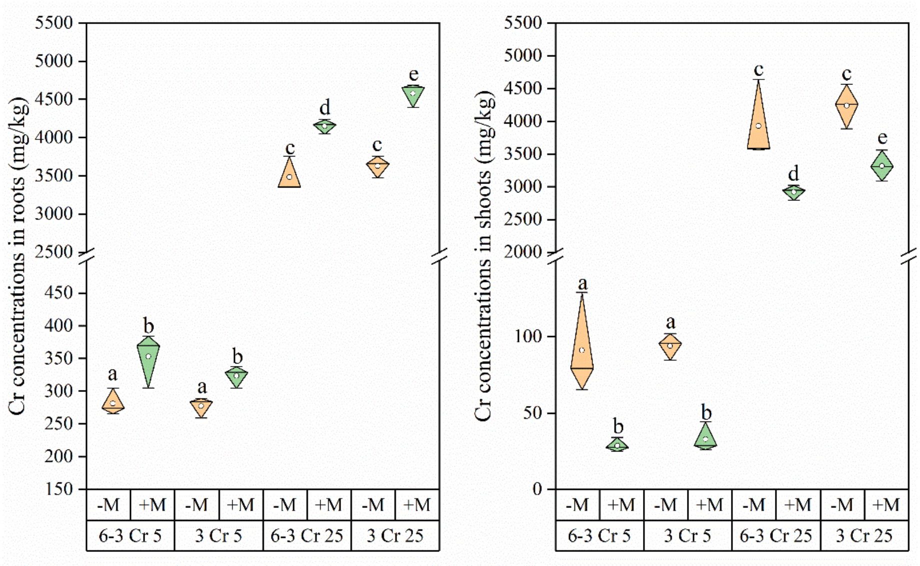


Figure 5.2: Cr concentrations in shoots and roots of AMF inoculated/ non-inoculated wetland plants under different water depths with various Cr stresses (-M: non-AMF; +M: AMF; 6-3 Cr 5: water depth of 6-3 cm with Cr 5 mg/kg; 3 Cr 5: water depth of 3 cm with Cr 5 mg/kg; 6-3 Cr 25: water depth of 6-3 cm with Cr 25 mg/kg; 3 Cr 25: water depth of 3 cm with Cr 25 mg/kg; a, b, c, d, and e show significant differences among different treatments ($p < 0.05$))

with a Cr content of 5 mg/kg might be that significant higher intensities of mycorrhizal colonization and arbuscule abundance were determined under the low Cr stress, which promoted the Cr accumulation in the roots of *I. pseudacorus.* A similar reason might also explain why the Cr concentration was not significantly different under the two water depths in our study. Generally, Cr preferential accumulation in intraradical fungal structures rather than in root cells (Wu et al., 2019). The reasons for the highly accumulated Cr in the intraradical fungal structures are (1) Cr uptake and transport to the intraradical fungal structures by the extraradical mycelium (Ren et al., 2015); (2) Cr transport to the intraradical fungal structures from root cells that absorb Cr directly from the environment (Wu et al., 2019). AMF also enhanced wetland plants' tolerance to heavy metals by improving antioxidant defense. Our study showed that superoxide dismutase and peroxidase activities in the AMF inoculated *I. pseudacorus* were more than 4.7% and 11.2% higher than those in the non-inoculated *I. pseudacorus*, respectively (Hu et al., 2020a). In conclusion, AMF inoculation was beneficial for wetland plants to resist heavy metal stress.

Table 5.2: Mycorrhizal dependency (MD), mycorrhizal Cr response (MCrR), tolerance index (TI), translocation factor (TF), and root to shoot ratio (R/S) of wetland plants under different water depths with various Cr stresses

Cr contents (mg/kg)	**Water depth (cm)**	**MD**	**MCrR**	**TI**		**TF**		**R/S**	
				Non-AMF	**AMF**	**Non-AMF**	**AMF**	**Non-AMF-**	**AMF**
0	6-3	1.27	-	-	-	-	-	1.11	1.23
	3	1.17	-	-	-	-	-	1.14	1.18
5	6-3	1.29	0.49	0.79	0.80	0.32	0.08	1.27	1.61
	3	1.19	0.23	0.81	0.82	0.34	0.10	1.49	1.57
25	6-3	1.05	0.02	0.36	0.30	1.13	0.70	1.15	1.15
	3	1.16	0.19	0.29	0.29	1.17	0.72	1.14	1.15

Cr concentrations in substrates and water

Cr concentrations in substrates under both water depths in the Cr content of 25mg/kg were 52.3-69.4% significantly higher than that in the Cr content of 5 mg/kg ($p<0.05$) (**Figure 5.3**). Meanwhile, Cr concentrations in the Cr content of 5 mg/kg and 25mg/kg with AMF inoculation were 21.2-29.3% and 0-4.1% higher than those non-inoculated controls, and a significant difference was obtained at the Cr content of 5 mg/kg under the water depth of 6-3 cm ($p<0.05$). Cr concentrations in water under the AMF inoculated treatments were 2.3-35.3% lower than those under the non-inoculated controls (**Figure 5.4**), and the Cr concentrations in water under the Cr contents of 5 mg/kg with AMF addition were significantly decreased by 34.1-35.3% compared to the non-inoculated treatments ($p<0.05$). Moreover, Cr concentrations in water under the water depth of 3 cm at the Cr contents of 5 mg/kg and 25 mg/kg were 32.3-33.5% and 24.0-27.8% higher than those under the water depth of 6-3 cm, respectively. Two-way ANOVA analysis indicated that water depth, Cr inoculation, and their interactions had significant effects on the Cr concentrations in water ($p<0.05$) (**Table S1**).

Heavy metal removal in semi-aquatic habitats is mainly due to the combined effects of plants, microorganisms, and substrates. Previous studies reported that the removal and immobilization of heavy metals depend on the pH value, redox state, influent water composition, dominant plant species, and microbial activity (Kosolapov et al., 2004; Marchand et al., 2010). Among them, microorganisms mobilize heavy metals in semi-aquatic systems mainly through autotrophic and heterotrophic leaching, metabolites and siderophore chelation, redox conversion, and methylation (Kosolapov et al., 2004). Meanwhile, some microorganisms (e.g. AMF) can accumulate heavy metals in cells to form amorphous mineral inclusions (Xu et al., 2016). Therefore, it might be the reason that lower Cr concentrations in water and higher Cr concentration in substrates were obtained under the semi-aquatic systems with AMF inoculation compared to the controls without AMF addition. Besides, AMF in the semi-aquatic systems might release some active substances to change the rhizosphere microbial community, thereby promoting the absorption of heavy metals (Xu et al., 2020). He et al. (2020) studied the effects of AMF on cadmium leaching from polluted soils under simulated heavy rainfall, showing that Cd concentrations in the water at the mycorrhizal and hyphal compartments were 38.6%-93.8% lower than those at the soil compartment. Xu et al. (2020) studied the effects of aeration and AMF on copper oxide

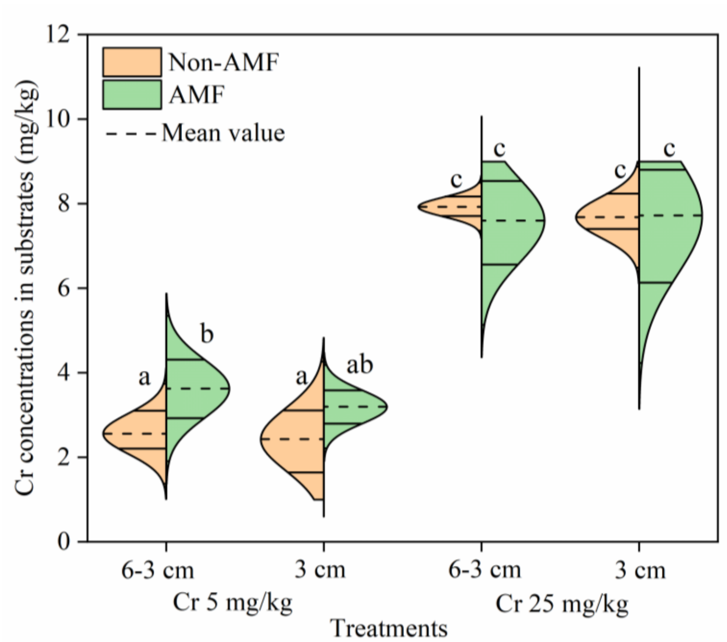


Figure 5.3: Cr concentrations in the substrate under different water depths with various Cr stresses (a, b, and c show significant differences among different treatments ($p < 0.05$))

nanoparticle removal in vertical flow CWs, indicating that the lowest outflow Cu concentration (0.48 mg/L) was determined in CWs under intermittent aeration with AMF inoculation, and the impact of AMF inoculated treatment on Cu purification was better than that of non-inoculated controls. In addition, it might be possible to immobilize Cr in the substrates through the extra radical mycelium, thus reducing the Cr concentration in the water. Kullu et al. (2020) showed that the extra radical mycelium of AMF acted as a plant root extension, which can reach beyond the root depletion zone and may absorb heavy metal from the water or soil. Furthermore, the fluctuating water depth also improved the Cr purification in our study. The reason might be that plant growth and microbial activity were enhanced under the water depth of 6-3 cm, resulting in higher Cr concentrations removed in the water compared to the water depth of 3 cm. Our previous study also obtained that physiological functions of wetland plants (e.g. biomass, TP, and TN) were improved under the water depth of 9-11 cm. In all, AMF can improve Cr purification in semi-aquatic habitats under the fluctuating water depth.

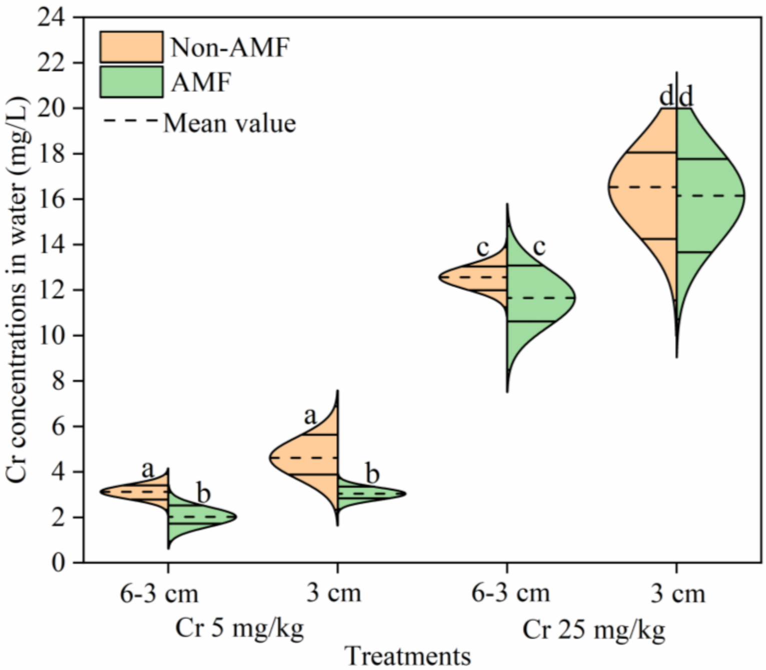


Figure 5.4: Cr concentrations in water under different water depths with various Cr stresses (a, b, c, and d show significant differences among different treatments ($p < 0.05$))

Cr mass balance

Cr mass contents in the wetland plants and substrates under the AMF inoculated treatments were higher than those under the non-AMF controls (**Figure 5.5**). Conversely, lower Cr mass contents in the water under the AMF inoculated treatments were determined compared to the non-AMF inoculated systems, with decreased by 58.1-97.0% and -2.0-10.7% at the Cr contents of 5 mg/kg and 25 mg/kg, respectively. Meanwhile, Cr mass contents in water under the water depth of 6-3 cm at the Cr contents of 5 mg/kg and 25 mg/kg were 7.9-93.5% and 2.4-14.5% lower than that under the water depth of 3 cm, respectively. Besides, Cr mass contents in AMF inoculated plants were largely distributed in roots (more than 60%), which was 11.5-15.5% higher than those in the roots of non-inoculated plants.

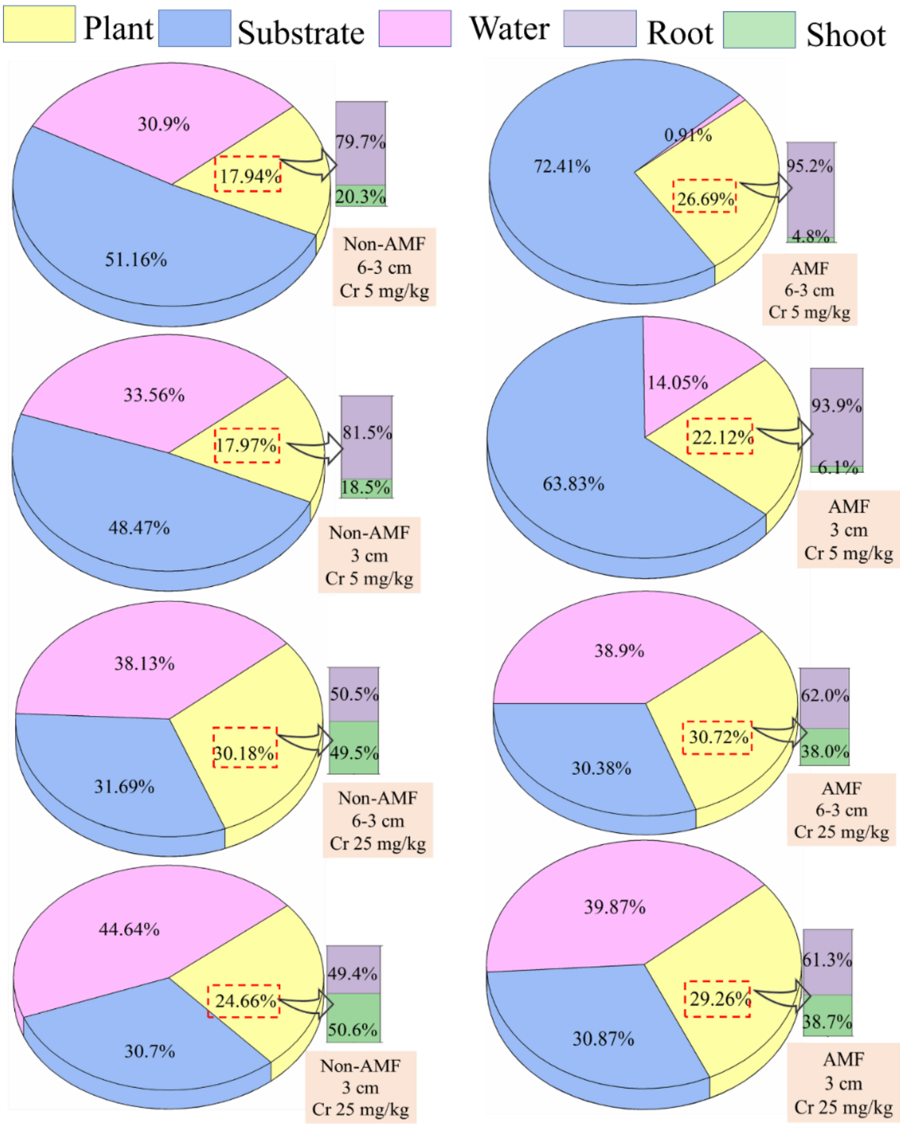


Figure 5.5: Cr mass balance under different water depths with various Cr stresses

AMF might decrease the Cr contents in water by promoting the Cr absorption in substrates and improving Cr uptake in the roots of plants according to the Cr mass balance in our study. Moreover, Cr contents in water were decreased largely by AMF inoculation at the low Cr stress under the fluctuating water depth. A similar result was demonstrated that AMF decreased Cd concentration in the soil interflow and reduced

Cd leaching from polluted soils, owing to the AMF induced an increase in the uptake of Cd by host plants (He et al., 2020). Zheng et al. (2015) evaluated the role of AMF in heavy metal contaminated wetlands with different soil moisture levels, showing that metal species, metal concentrations, and soil moisture level should be very important factors influencing the uptake of the heavy metal by wetland plants in AMF assistant wetland systems. The fluctuating water depth affected the Cr mass balance in AMF inoculated semi-aquatic habits mainly resulted from the enhancing of the activities of microorganisms that influenced heavy metal absorption and improving the uptake of elements by wetland plants. In addition, AMF also inhibited the Cr transformation from roots to shoots in semi-aquatic systems, which reduced the toxicity of Cr to wetland plants and alleviated the Cr contamination in semi-aquatic habitats. Zheng et al. (2015) indicated that AMF inoculation into the aquatic systems provided a potential ecological significance by decreasing the heavy metal concentrations in the shoots of *P. australis*. Due to the aboveground heavy metal pool in wetland plants can be a source for the transport of toxic metals into the water bodies, a lower heavy metal concentration in the aboveground of wetland plants can decrease the heavy metal pollution in aquatic habitats (Weis and Weis, 2004). Therefore, Cr mass balance in semi-aquatic habits might be changed by AMF inoculation and water depths.

5.4.3 Effects of AMF and water depth on Cr bioavailability

Cr bioavailability in substrates

The sum of residual and oxidation states of Cr in substrates under both water depths with AMF inoculation accounted for 52.2-64.3%, with 0.1-5.1% higher than that under the non-AMF inoculated controls (**Figure 5.6**). Meanwhile, the acid-soluble state of Cr in substrates under the water depth of 6-3 cm with AMF inoculation was 4.1-5.6% lower than that without AMF addition. These revealed that AMF mainly decreased the Cr bioavailability in substrates (especially under the water depth of 6-3 cm), thereby alleviating the absorption of Cr in the substrate by wetland plants.

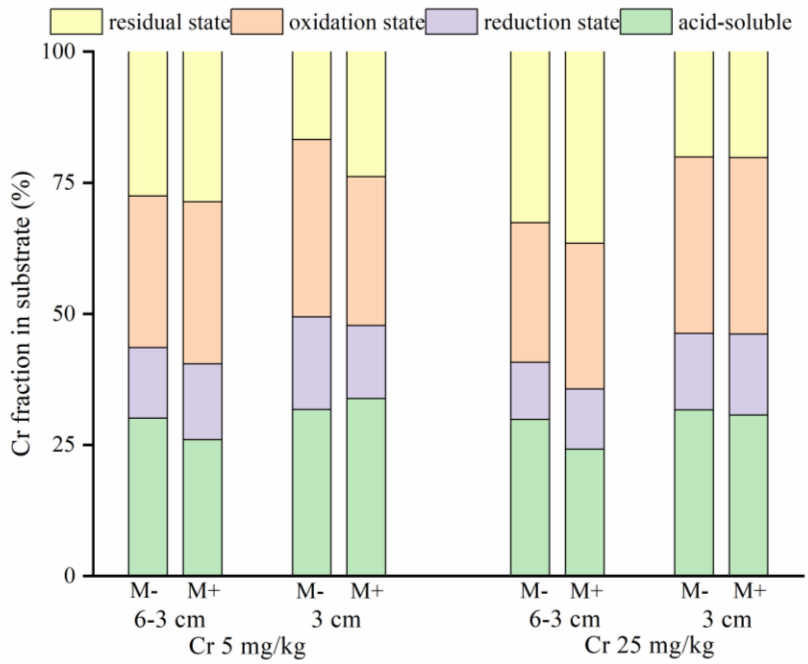


Figure 5.6: Cr fractions in the substrate under different water depths with various Cr stresses (a, b show significant differences between different water depths; A, B show significant differences between AMF and non-AMF inoculated condition ($p < 0.05$))

AMF decreased the acid-soluble state of Cr in the substrates, which might reduce the Cr uptake by wetland plants. Similar results were reported by Wu et al. (2014b), indicating that the acid-extractable Cr concentration in soil was significantly influenced by AMF inoculation ($p<0.01$), while AMF did not influence the reducible and oxidizable Cr. AMF could directly interact with heavy metals to change their bioavailability, thereby reducing the toxic effects of plants (Audet and Charest, 2007). Moreover, AMF can also stimulate microbial activity and acidify the rhizosphere by releasing simple organic acids (e.g. carboxylic, citric, and oxalic acids), which may solubilize carbonate and (hydro) oxide-bound heavy metals, probably increasing their accumulation in the residual fraction (Aghababaei et al., 2014; Giasson et al., 2005). Aghababaei et al. (2014) studied the influence of AMF on Cd speciation in contaminated soil, also showing that AMF declined the inorganic bound Cd fraction by increasing its residual fraction in soil. Therefore, heavy metal bioavailability may be decreased by AMF inoculation, thereby reducing the toxicity of heavy metals to wetland plants.

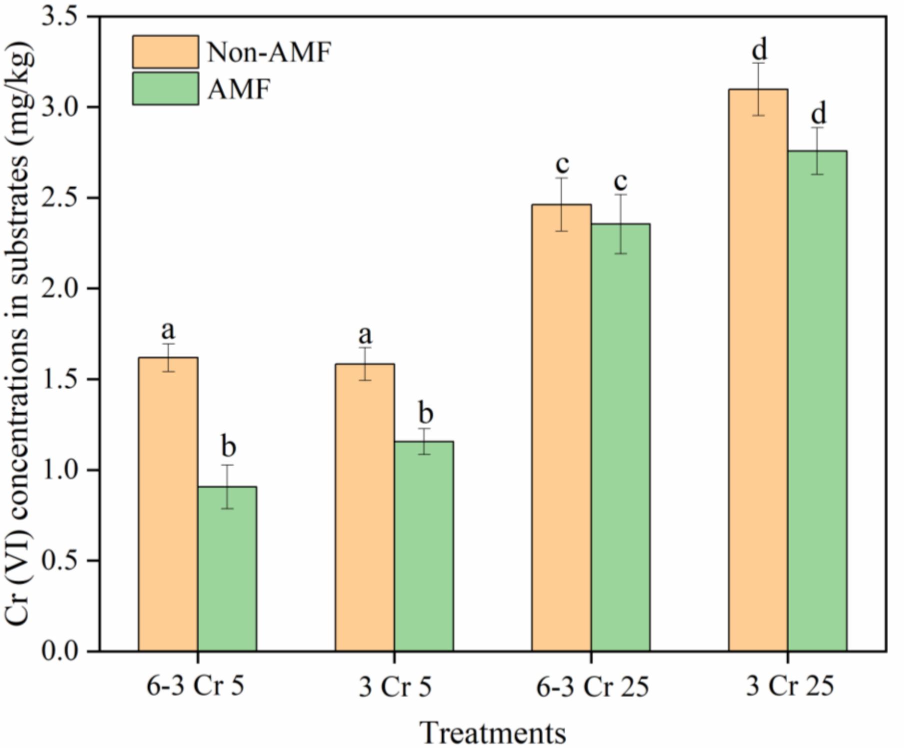


Figure 5.7: Cr (VI) contents in the substrate under different water depths with various Cr stresses (6-3 Cr 5: water depth of 6-3 cm with Cr 5 mg/kg; 3 Cr 5: water depth of 3 cm with Cr 5 mg/kg; 6-3 Cr 25: water depth of 6-3 cm with Cr 25 mg/kg; 3 Cr 25: water depth of 3 cm with Cr 25 mg/kg; a, b, c, and d show significant differences among different treatments ($p < 0.05$)

Cr (VI) in substrates

Cr (VI) concentrations in substrates under the Cr contents of 5 mg/kg and 25 mg/kg with AMF inoculation were 27.0-44.0% and 4.3-11.0% lower than those without AMF addition, respectively (**Figure 5.7**). Meanwhile, Cr (VI) concentrations in substrates under the water depth of 3 cm were -2.2-21.5% higher than that under the water depth of 6-3 cm. AMF symbiosis influence Cr transformation in substrates through redox changes to reduce Cr (VI) to Cr (III). This may be due to the influence of AMF on microorganism activities in substrates, thereby promoting the change of heavy metal speciation in the rhizosphere and further affect metal uptake by plants (Wu et al., 2014b). Vymazal et al. (2010) also reported that the decrease of Cr (VI) in wetland sediments may result from the combined effects of dissolved organic matter, Fe (II), and pH changes. Besides, vegetation also proves to improve the Cr (VI) removal efficiency and can be identified as the main removal mechanism in CWs (Sultana et al., 2015). The higher Cr concentrations were determined in the AMF inoculated plants under the water depth of 6-3 cm in our study, probably resulting in Cr (VI) reducing in substrates with AMF inoculation. Therefore, Cr (VI) toxicity in substrates might be decreased by AMF inoculation under the fluctuating water depth.

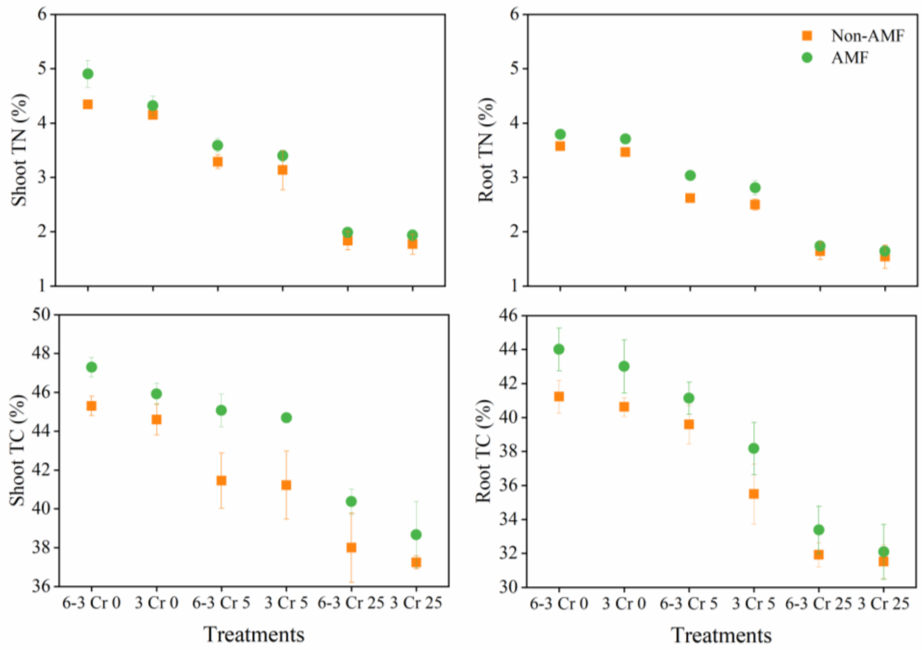


Figure 5.8: TC and TN in shoots and roots of AMF inoculated/ non-inoculated wetland plants under different water depths with various Cr stresses (6-3 Cr 5: water depth of 6-3 cm with Cr 5 mg/kg; 3 Cr 5: water depth of 3 cm with Cr 5 mg/kg; 6-3 Cr 25: water depth of 6-3 cm with Cr 25 mg/kg; 3 Cr 25: water depth of 3 cm with Cr 25 mg/kg)

5.4.4 Biochemical responses in wetland plants

The dry biomass in shoots (roots) of AMF inoculated *I. pseudacorus* under the Cr contents of 0 mg/kg, 5 mg/kg, and 25 mg/kg were 13.5-16.7% (16.0-24.5%), 10.6-13.3% (17.8-29.4%), and 4.9-13.8 (4.6-14.3%) higher than those of non-inoculated controls, respectively (**Figure S3**). Besides, higher root to shoot ratios of biomass in AMF inoculated *I. pseudacorus* under Cr stresses were determined compared to the non-inoculated controls (**Table 5.2**). Tolerance indexes under the low Cr stress with AMF inoculation were higher than those under the non-inoculated controls. Moreover, mycorrhizal dependencies were also decreased under the water depth of 3 cm under the Cr contents of 0 mg/kg and 5 mg/kg. TC and TN in AMF inoculated *I. pseudacorus* were higher than those in the non-inoculated controls, with shoot TC, root TC, shoot TN, and root TN increased by 2.9-8.0%, 1.8-7.0%, 3.9-11.5%, and 5.4-13.7%, respectively (**Figure 5.8**). Meanwhile, the shoot TC, root TC, shoot TN, and root TN under the water depth of 6-3 cm were 0.6-4.2%, 1.2-10.3%, 2.5-12.0%, and 2.1-7.4% higher than those under the water depth of 3 cm, respectively. Generally, Cr had a significant effect on the nutrient contents in wetland plants ($p<0.05$) (**Table S1**), the TC, TN, Ca, Fe, Mg, Mn, Na, and S contents in the shoots and roots of *I. pseudacorus* were decreased gradually with the increase of Cr stress (**Table 5.3**). AMF also had a significant effect on the root Ca, shoot Fe, shoot Mg, shoot S, and roots contents in wetland plants ($p<0.05$), with higher nutrient contents determined in the AMF inoculated wetland plants.

Biochemical parameters in AMF inoculated wetland plants under the fluctuating water depth can be improved at the low Cr stress. Similar results also obtained by Hu et al. (2020a), showing that shoot height, biomass, and shoot P contents of AMF inoculated wetland plants under the fluctuating water depth were 13.1-26.6%, 33.3-114.3%, and 25.7-80% higher than those of non-inoculated controls, respectively. Previous studies proved that AMF improved physical and biochemical responses in wetland plants mainly through the mycelial network around the rhizosphere, resulting in increasing the nutrient contents, height, and biomass of plants (Liang et al., 2018, 2019). The biomass and nutrient contents in AMF inoculated *I. pseudacorus* at high Cr stress (25mg/kg) were insignificantly higher than the non-inoculated controls, the main reason was that AMF colonization was decreased significantly at the high Cr stress. Xu et al. (2020) evaluated the correlation between root colonization and plant (*P. australis*) growth through Pearson correlation analyses and found that *P. australis* with higher

AMF colonization demonstrated better growth in CWs. Moreover, the high water level in semi-aquatic systems also enhanced the water uptake and acquisition of nutrients, thereby increasing the height and biomass of wetland plants (Hu et al., 2020a). In conclusion, AMF contributed to the wetland plant growth under the fluctuating water depth and promoted the tolerance of plants under low Cr stress.

5.4.5 The role of AMF on alleviating Cr toxicity in wetland plant

PCA and cluster analyses were carried out to systematically figure out the main factor that AMF influences on the Cr distribution in semi-aquatic habitats and wetland plants respond to Cr stress. The variations in the parameters (biomass, nutrients, Cr contents in water, substrates, and plants) under the water depth of 6-3 cm at the Cr contents of 0 mg/kg, 5 mg/kg, 25 mg/kg were captured by two main axes, together explaining 71.5%, 81.6%, and 69.2% of the variation, respectively (**Figure S4**). Meanwhile, the biomass and nutrient contents (except Na) at the Cr contents of 0 mg/kg and 5 mg/kg with AMF inoculation were largely determined by PCA axis 1, indicating that there was a strong correlation between them. The results of scores of the treatments with/without AMF inoculation at the Cr contents of 0 mg/kg and 5 mg/kg had the same distribution on the PCA axis, with AMF inoculated treatments occupied on the right of PCA axis 1. In addition, hierarchical clustering analysis also showed that the correlations among plant growth indexes in AMF inoculated plants under the water depth of 6-3 cm at the Cr content of 5 mg/kg were similar to the treatments without Cr addition (**Figure 5.9**). Similar results also indicated that the correlations of wetland plant biomass, height, chlorophyll contents, and antioxidant enzyme activities in AMF inoculated plants under the Cr content of 5 mg/kg were similar to the controls without Cr addition (Hu et al., 2020b). Therefore, the statistical analyses further determined that AMF enhanced the wetland plant growth under low Cr stress, while the effects of AMF on wetland plant growth might be ignored under high Cr stress.

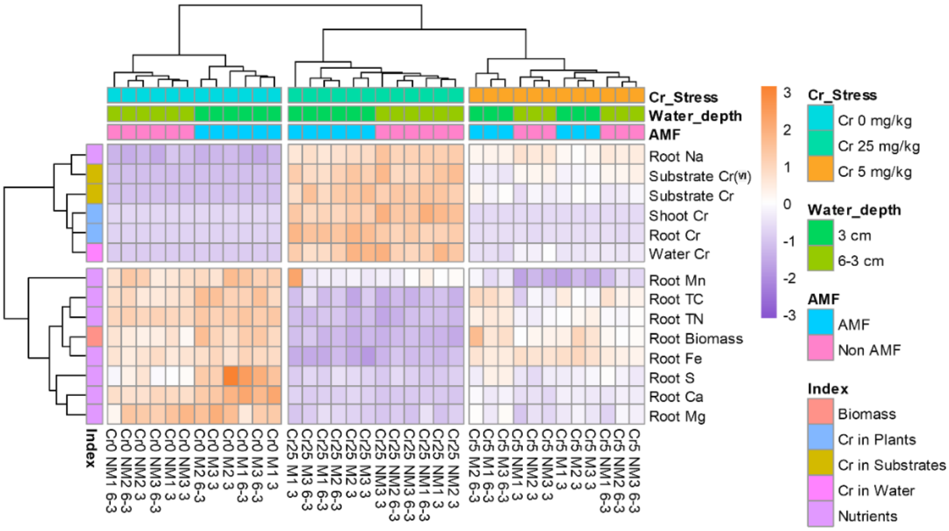


Figure 5.9: Hierarchical clustering of Cr contents in water, biomass, nutrient, Cr contents in AMF/non-AMF inoculated wetland plants, Cr and Cr (VI) contents in the substrate under the treatments with the water depth of 6-3 cm, colors in the heatmap indicate the pairwise Pearson correlation between the different data sets

Table 5.3: Nutrient contents (mg/kg) in shoots and roots of AMF inoculated/ non-inoculated wetland plants under different water depths with various Cr stresses

Nutrients (mg/kg)			**Cr 0 mg/kg**		**Cr 5 mg/kg**		**Cr 25 mg/kg**	
			6-3 cm	**3 cm**	**6-3 cm**	**3 cm**	**6-3 cm**	**3 cm**
Shoot	Ca	M-	14379.8±957.6^{Aa}	13833.7±468.5^{Aa}	10440.2±670.8^{Ba}	9660.0±345.4^{Ba}	9750.4±169.2^{Ba}	9303.2±545.7^{Ca}
		M+	14815.9±1184.1^{Aa}	14786.8±602.1^{Aa}	10747.9±1177.8^{Ba}	10406.6±786.8^{Ba}	9788.9±109.9^{Ca}	10089.4±677.9^{Ca}
	Fe	M-	213.0±18.0^{Aa}	210.1±18.9^{Aa}	125.3±6.0^{Ba}	121.3±7.6^{Ba}	124.2±11.5^{Ba}	113.0±8.3^{Ba}
		M+	235.6±28.0^{Aa}	229.6±18.3^{Aa}	146.3±10.2^{Bb}	169.6±15.3^{Bb}	120.9±16.8^{Ca}	111.2±11.6^{Ca}
	Mg	M-	2773.7±86.8^{Aa}	2934.2±107.5^{Ba}	2089.5±133.2^{CDa}	2222.6±150.3^{Ca}	1957.8±50.6^{Da}	1606.6±165.4^{Da}
		M+	2944.5±64.6^{Ab}	2999.0±102.9^{Aa}	2205.4±211.6^{BCa}	2385.7±93.7^{Ba}	2102.1±33.6^{Cb}	1616.3±68.1^{Da}
	Mn	M-	173.5±5.7^{Aa}	171.5±9.1^{Aa}	165.1±15.7^{Aa}	167.3±11.7^{Aa}	138.0±20.9^{Ba}	135.5±31.5^{Ba}
		M+	171.2±7.2^{Aa}	168.8±10.6^{Aa}	143.6±36.2^{Aa}	139.2±33.1^{Aa}	121.6±21.9^{Aa}	122.9±13.8^{Aa}
	Na	M-	197.5±8.7^{Aa}	243.2±4.0^{Ba}	305.0±13.9^{Ca}	323.7±15.4^{Ca}	621.4±38.4^{Da}	642.4±19.4^{Da}
		M+	190.4±7.2^{Aa}	203.2±10.0^{Ab}	207.0±12.5^{Ab}	271.8±20.8^{Bb}	550.7±12.9^{Cb}	562.8±15.9^{Db}
	S	M-	1614.6±63.1^{Aa}	1508.2±17.1^{Ba}	974.6±61.3^{Ca}	933.2±101.1^{Ca}	836.5±39.6^{Da}	807.9±13.0^{Da}
		M+	2151.6±184.4^{Ab}	2059.3±159.2^{Ab}	1416.5±99.0^{Bb}	1136.6±143.7^{Bb}	937.6±32.1^{Cb}	884.7±26.4^{Db}
Root	Ca	M-	8896.9±402.0^{Aa}	8775.2±229.1^{Aa}	5106.7±255.4^{Ba}	4305.2±311.9^{Ca}	3251.8±155.2^{Da}	3111.4±121.4^{Da}
		M+	11581.1±467.2^{Ab}	11823.3±1280.6^{Ab}	5495.3±458.6^{Ba}	5131.5±173.0^{Bb}	3501.1±123.7^{Ca}	3836.7±148.7^{Cb}
	Fe	M-	579.2±19.3^{Aa}	576.6±6.9^{Aa}	550.7±9.9^{Ba}	582.5±5.3^{Aa}	398.3±11.4^{Ca}	387.4±12.4^{Ca}

	M+	603.4±19.3^{Aa}	595.6±15.0^{Ba}	565.9±11.4^{Ca}	595.8±14.7^{Ba}	370.2±16.7^{Ca}	351.7±19.6^{Da}
Mg	M-	1404.7±217.2^{Aa}	1607.2±84.6^{Aa}	942.5±120.0^{Ba}	813.1±88.3^{BCa}	782.9±93.5^{Ca}	738.4±45.5^{Ca}
	M+	1471.4±217.2^{Aa}	1647.5±131.5^{Aa}	1041.9±95.8^{Ba}	956.5±47.5^{Ba}	815.0±53.6^{Ca}	759.5±38.2^{Da}
Mn	M-	68.3±6.3^{Aa}	68.3±4.1^{Aa}	45.9±4.6^{Ba}	36.5±2.6^{Ca}	56.8±4.5^{Da}	56.0±2.9^{Da}
	M+	72.3±6.3^{Aa}	72.7±7.2^{Aa}	51.6±3.6^{Bb}	37.1±2.9^{Ca}	52.1±1.1^{Ba}	64.1±19.9^{ABa}
Na	M-	280.9±17.4^{Aa}	346.2±27.1^{Ba}	705.9±15.4^{Ca}	740.8±23.1^{Ca}	842.0±19.3^{Da}	883.4±34.5^{Da}
	M+	280.0±17.4^{Aa}	316.6±10.7^{Ba}	656.9±7.7^{Cb}	627.8±25.1^{Cb}	751.7±19.2^{Db}	767.4±36.4^{Db}
S	M-	1083.0±104.3^{Aa}	1158.8±131.7^{Aa}	1004.6±84.4^{Aa}	886.2±62.3^{Ba}	763.7±55.3^{Ca}	728.6±30.8^{Ca}
	M+	1762.7±114.8^{Ab}	1772.8±387.2^{Ab}	1107.3±149.6^{Ba}	980.1±41.6^{Bb}	808.3±53.7^{Ca}	784.8±52.7^{Ca}

Note: a, b shows significant differences between AMF inoculated and non-inoculated conditions under the same Cr stress and water depth ($p < 0.05$); A, B, C, and D show significant differences among different treatments under the AMF inoculated or non-inoculated condition ($p < 0.05$).

5.5 Conclusion

Cr had a negative effect on AMF colonization in wetland plants, especially under high Cr content (25 mg/kg). Meanwhile, the fluctuating water depth provided a possibility for AMF colonization in the roots of wetland plants. AMF symbiosis improved the tolerance of wetland plants to Cr stress by alleviating the Cr transformation from roots to shoots. Besides, Cr concentrations and contents in water were decreased by AMF inoculation under the fluctuating water depth (water depth of 6-3 cm). Conversely, higher Cr concentrations and contents in substrates and wetland plants inoculated with AMF were determined at the low Cr stress compared to the non-inoculated controls. Nonetheless, AMF inoculation decreased the Cr (VI) concentrations and Cr bioavailability in substrates at the low Cr stress. Furthermore, biomass, nutrient contents (TC, TN, Ca, Mg, Fe, and Mn) in the AMF inoculated wetland plants were improved under the fluctuating water depth at the same Cr stress. Therefore, AMF inoculation in semi-aquatic systems can decrease Cr contents in water by promoting the Cr absorption in substrates and wetland plants under the fluctuating water depth. Additionally, this study may be a good starting point for the application of AMF in the removal of heavy metals from wastewater under semi-aquatic and aquatic habitats (e.g. constructed wetlands).

5.6 Supplementary Materials

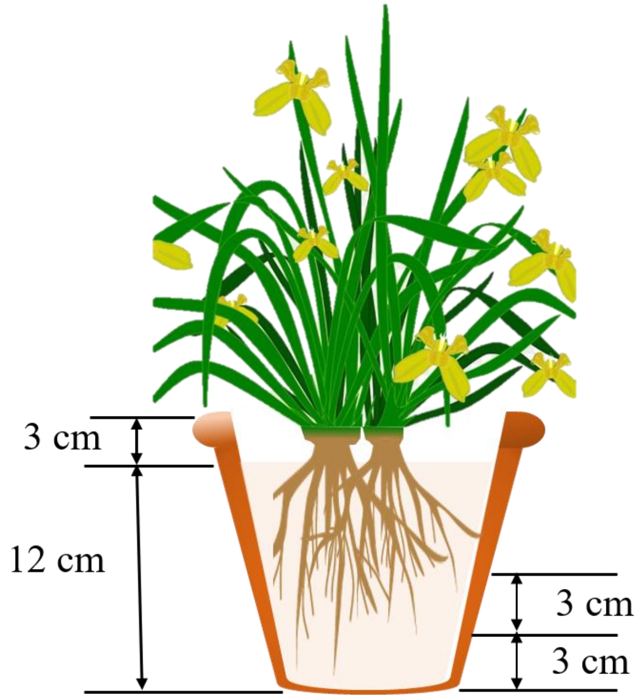


Figure S1: Experiment setup

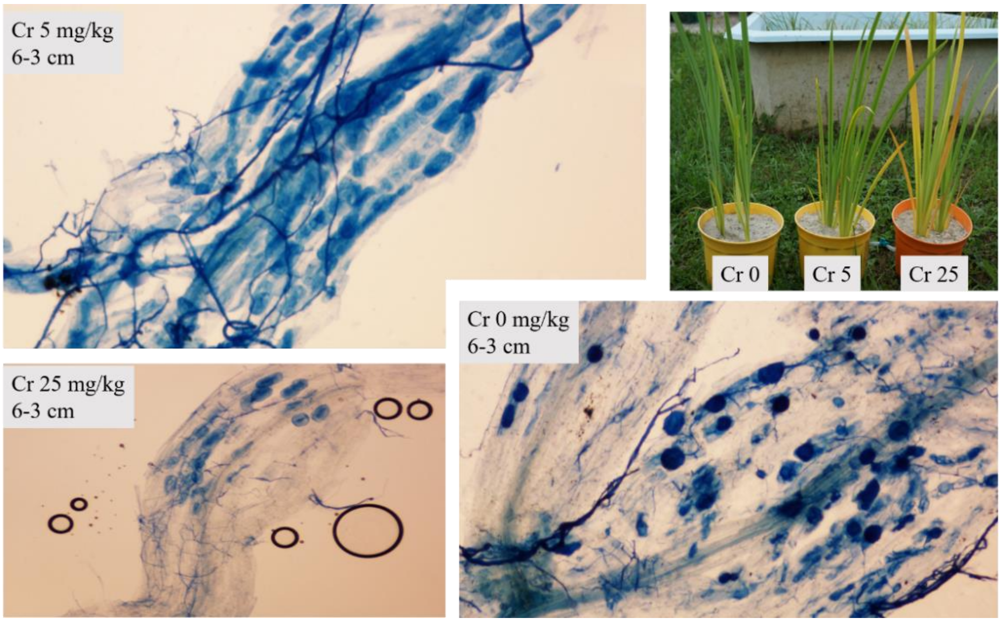


Figure S2: AMF colonization and experimental setup under the water depths of 6-3 cm

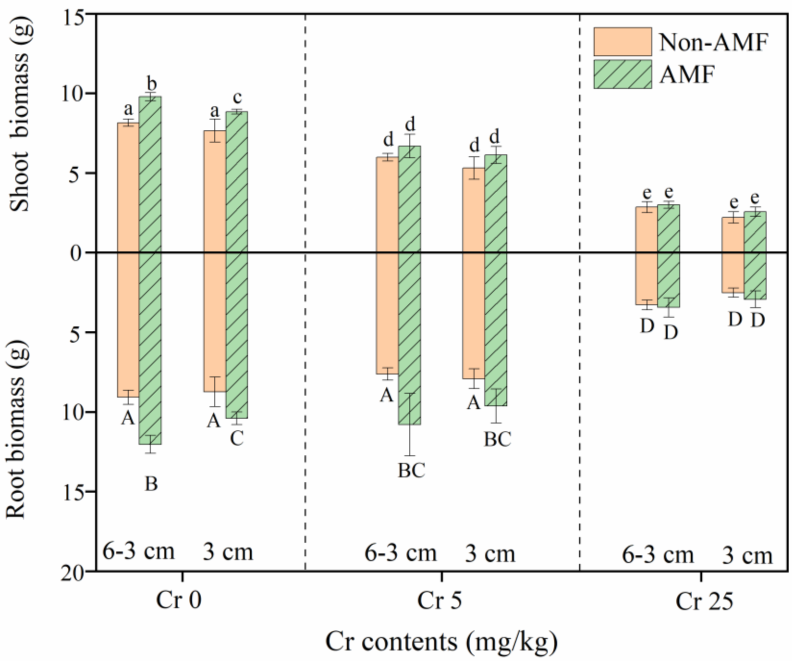


Figure S3: Shoot and root biomass in wetland plants under different water depths with various Cr stresses (a, b show significant differences between different water depths; A, B show significant differences between AMF and non-AMF inoculated condition ($p < 0.05$))

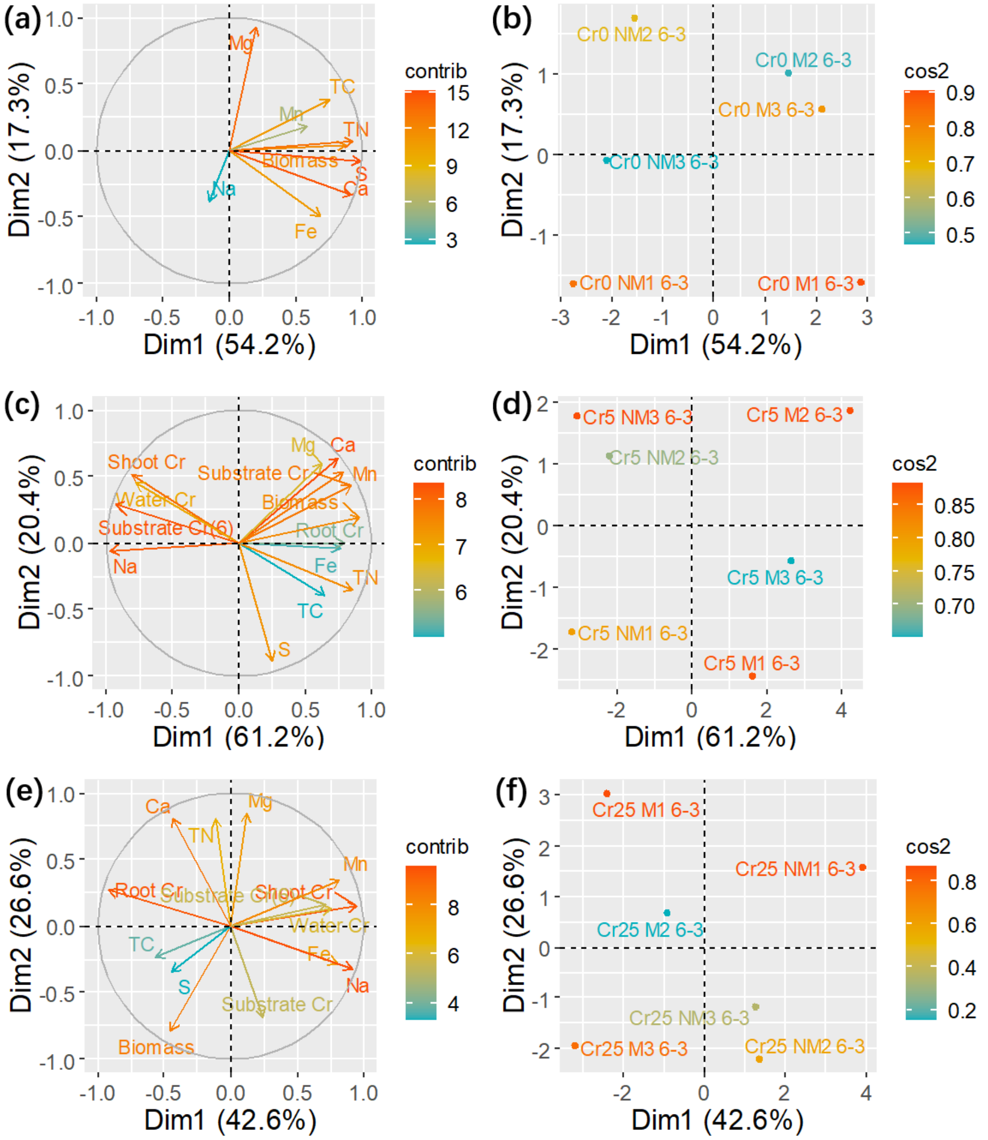


Figure S4: Principal component analysis (PCA) of Cr contents in water, biomass, nutrients, Cr contents in wetland plants, Cr and Cr (VI) contents in substrates under the water depth of 6-3 cm with/without AMF inoculation at the Cr concentrations of 0, 5, 25 mg/kg, and scores of 6 treatments on AX1 and AX2

Table S1: Three-way ANOVA analysis for the effects of AMF, Cr, and water depth (WD) on wetland plant characteristics

Parameters	**AMF**	**Cr**	**WD**	**AMF*Cr**	**AMF*WD**	**Cr*WD**	**AMF*Cr*WD**
Shoot biomass	**<0.001**	**<0.001**	**<0.001**	**0.023**	0.882	0.646	0.679
Root biomass	**<0.001**	**<0.001**	**0.018**	**0.005**	0.137	0.700	0.373
Shoot TC	**<0.001**	**<0.001**	**0.026**	0.088	0.421	0.555	0.897
Root TC	**<0.001**	**<0.001**	**<0.001**	0.311	0.955	**0.020**	0.596
Shoot TN	**<0.001**	**<0.001**	**0.001**	0.347	0.236	0.069	0.303
Root TN	**<0.001**	**<0.001**	**0.004**	**0.029**	0.748	0.660	0.743
Shoot Ca	0.167	0.381	0.174	0.573	0.193	0.584	0.578
Root Ca	**<0.001**	**<0.001**	0.477	**<0.001**	0.288	0.300	0.993
Shoot Fe	**<0.001**	**<0.001**	0.331	**0.004**	0.150	0.724	0.106
Root Fe	0.774	**<0.001**	0.426	**<0.001**	0.588	**0.001**	0.967
Shoot Mg	**0.009**	**<0.001**	0.199	0.803	0.415	**<0.001**	0.597
Root Mg	0.085	**<0.001**	0.779	0.574	0.977	**0.008**	0.922
Shoot Mn	0.057	**<0.001**	0.844	0.426	0.948	0.993	0.952
Root Mn	0.214	**<0.001**	0.389	0.913	0.570	**0.017**	0.296
Shoot Na	**<0.001**	**<0.001**	**<0.001**	**0.001**	0.900	0.219	**0.027**
Root Na	**<0.001**	**<0.001**	**0.001**	**<0.001**	**0.014**	**0.048**	0.517
Shoot S	**<0.001**	**<0.001**	**0.005**	**<0.001**	0.210	0.331	0.244
Root S	**<0.001**	**<0.001**	0.473	**<0.001**	0.835	0.410	0.948

Cr in root	**<0.001**	**<0.001**	**0.014**	**<0.001**	0.201	**0.007**	0.131
Cr in shoot	**<0.001**	**<0.001**	0.120	**0.001**	0.839	0.127	0.844
Cr in water	0.065	**<0.001**	**<0.001**	0.495	0.978	**0.009**	0.619
Cr in substrate	0.232	**<0.001**	0.589	0.109	0.954	0.728	0.600
Cr (VI) in substrate	**<0.001**	**<0.001**	**<0.001**	**0.003**	0.805	**0.001**	**0.020**

Note: The values in bold show a significant difference ($p<0.05$).

Chapter VI

Summary

Bioremediation technology as an environment-friendly technology has focused more and more attention on heavy metal removal from wastewater (Srivastava and Majumder 2008; Mora-Ravelo et al. 2017). Among them, wetland plants play an important role in heavy metal removal from wastewater. Generally, wetland plants remove heavy metals from wastewater mainly through the accumulation, retention, and fixation process (Barya et al., 2020). However, the accumulation of heavy metals by wetland plants is limited, and excessive heavy metals in wastewater can easily result in stunted growth and sometimes death of the wetland plants (Deng et al., 2006; Yang and Ye, 2015). In order to enhance the wetland plants' resistance to heavy metals toxicity and improve heavy metal remediation, previous studies reported that AMF can establish symbiosis with the roots of some wetland plants in semi-aquatic and aquatic habitats in recent years (Calheiros et al., 2019; Dolinar and Gaberščik, 2010), which may provide an opportunity for AMF application in heavy metals remediation in CWs systems. Although AMF can be found in the roots of wetland plants, AMF colonization in CWs is affected by some factors such as phosphorus (Tang et al., 2001), wastewater quality (Wang et al., 2010), hydraulic condition (Vallino et al., 2014), CW types (Wu et al., 2001), and operation modes of CW (Li et al., 2011a).

Phosphorus level in the rhizosphere is one of the main abiotic factors that affect AMF colonization in plants (Wang et al., 2010). The negative correlation between phosphorus availability and AMF colonization well-documented in terrestrial ecosystems, and may also prevail in aquatic habitats (Stevens et al., 2002). Tang et al. (2001) studied the effect of phosphorus availability on AMF colonization of *T.angustifolia*, indicating that the proportional colonization in the roots was significantly greater for plants grown at 1 and 10 uM phosphorus than for the 100 uM phosphorus treatment. Stevens et al. (2002) studied the phosphorus, AMF, and performance of the wetland plant (*Lythrum salicaria*) under inundated conditions, results showed that the levels of AMF colonization generally decreased with increasing phosphorus supply. Tang et al. (2001) also evaluated the effect of phosphorus availability on AMF colonization of *T. angustifolia*, indicating that AMF colonization was present in the three lowest phosphorus (1, 10, 100 uM) treatments, but was absent from the 500 uM phosphorus control. The reduced colonization of AMF at high phosphorus concentrations may be due to the increased availability of phosphorus in the environment, which directly inhibited the growth and spread of external hyphae (Graham et al., 1982). Some researchers also found that the extent of AMF colonization of plants had no significant difference in wetlands under different soil phosphorus concentrations (Miller and Bever, 1999; Van Hoewyk et al., 2001). Cornwell et al.

(2001) studied the occurrence of AMF in a phosphorus-poor wetland and mycorrhizal response to phosphorus fertilization, showing that *T. latifolia* and *Carex lasiocarpa* were nonmycorrhizal in phosphorus-poor condition. Hence it can be predicted that the AMF colonization in wetland ecosystems may be inhibited under high or low phosphorus concentrations. Overall, more attention should be paid to phosphorus concentration when AMF is used in wastewater treatment CWs.

AMF colonization could be gradually decreased as the increase of pollutant (e.g. phosphorus, nitrogen, and heavy metals) concentrations in wastewater. Similar to the factor of phosphorus, nitrogen is also one of the main factors influencing AMF colonization in CWs. The nitrogen concentration can affect the allocation of carbon in plant roots to AMF. Generally, plants could reduce the carbon allocation toAMF under a high nitrogen concentration, thereby inhibiting the growth of AMF (Xu et al., 2016). Blanke et al. (2005) observed that plant roots can absorb nitrogen without the help of AMF under the high nitrogen concentration in the environment, which results in that the negative impact on plants caused by AMF absorption carbon from plant roots was higher than the contribution of AMF transporting nutrients to the plant roots. Some researchers also suggested that a higher incidence of AMF colonization in CWs was due to the less organic matter and lower nutrient availability as compared with those in the natural wetlands (Confer and Niering, 1992). Besides, some studies reported that AMF colonization (e.g. arbuscule numbers and spore densities) in roots of terrestrial plants were decreased significantly under high heavy metals contamination (Akhtar et al., 2019; Wu et al., 2019). However, Wu et al. (2020) evaluated the AMF effect on the growth and photosynthesis of *P. australis* under copper stress, indicating that AMF colonization levels (18-21%) had no significant difference between the Cr stress and non-heavy metal addition treatments. Ban et al. (2017) also found that AMF diversity in heavy metal polluted wetlands was higher than most of the records of upland habitats. In conclusion, wastewater quality has influences on the AMF colonization of wetland plant roots in CWs, but further study needs to be evaluated especially for the heavy metal removal in CWs.

AMF can easily survive and colonize the roots of plants under aerobic conditions, this is why higher AMF colonization is detected in terrestrial plants (Ramírez-Viga et al., 2018; Xu et al., 2016). However, the soil moisture in CWs is usually under the saturated situation, which leads to the low oxygen content in the substrate, thus affecting AMF colonization in the roots of wetland plants (Brundrett, 2009; Stevens et al., 2011). Previous studies reported that AMF colonization in wetland plants gradually decreased with the increase of hydraulic loading rate (Miller, 2000; Wang et al., 2011).

Despite that, increasing evidence demonstrates that AMF was observed in the roots of many wetland plants under different hydrologic sites around the world (Wang et al., 2018b; Wang et al., 2011; Xu et al., 2016). In addition, AMF can even be detected in the submerged wetland plants in some polluted (e.g. heavy metals) wetlands (Sudová et al., 2015; Wang et al., 2018b). Furthermore, some researchers revealed that hydraulic loading rate had no significant effect on AMF colonization, as well as the percentage of the root in AMF colonization was not related to the hydrologic category (Ipsilantis and Sylvia, 2007b; Turner et al., 2000). Moreover, Dolinar and Gaberščik (2010) found that once AMF symbiosis was established in the roots, the hydraulic loading rate did not affect their colonization. Therefore, it is necessary to evaluate whether AMF colonization can be affected by the hydraulic loading rate in CWs.

AMF spore germination and hyphal growth can be influenced by oxygen content directly. Thus, the different CWs types (FWS CWs, HSSF CWs, and VSSF CWs) that affect AMF colonization in the roots of wetland plants are mainly related to the oxygen contents in each CWs system. The main pathways of oxygen transfer in FWS CWs are atmospheric diffusion and plant-mediated (well-developed aerenchyma in wetland plant roots) oxygen transfer (Xu et al., 2016). The limited oxygen-transfer capability in SSF CWs is determined. To solve this problem, some enhanced treatment CWs systems (e.g. aeration, tidal flow CWs, hybrid CWs) are constructed to provide sufficient oxygen for the respiration of microorganisms (e.g. bacteria and AMF), resulting in improving the pollutants removal (Austin et al., 2003; Li et al., 2019; Vymazal and Kröpfelová, 2015). Nevertheless, compared to the HSSF CWs, the VSSF CWs are also exposed to the atmosphere, easing to transfer of oxygen from the atmosphere, thereby leading to higher oxygen concentrations in the systems. Therefore, AMF should easy to colonize the roots of wetland plants in the VSSF CWs.

Aeration, season, wastewater recirculation, alternation of wetting and drying, and intermittent operation can bring oxygen into CWs, which is beneficial to the growth, abundance, and diversity of AMF, thus enhancing pollutants purification in wastewater (Li et al., 2011b). Liang et al. (2018) studied the effects of AMF and soil nutrient addition on the growth of *P. australis* under different drying- rewetting cycles (1, 2, or 4 cycles), indicating that AMF increased leaf area and decreased belowground to aboveground biomass ratio in the medium frequency of drying-rewetting cycles. Dolinar and Gaberščik (2010) showed that the highest level of AMF colonization in the roots of *P. australis* was obtained at the end of the growing season under an intermittent wetland. Xu et al. (2020) also evaluated the effects of aeration on the formation of AMF under a flooded state and copper oxide nanoparticle removal in VFSS CWs, founding that the highest AMF colonization with values of 67% and 21% for frequency and intensity in *P. australis* roots was observed in the treatment with intermittent aeration for 4 h day^{-1}. Therefore, a suitable operation mode of CWs would improve the AMF colonization and enhance wastewater purification.

Many studies have shown that AMF can significantly enhance the tolerance of terrestrial plants to heavy metals such as Zn, Cd, Cu, As, U, and radioactive elements, as well as can change the distribution of heavy metals in terrestrial plants (Bai et al., 2008; Wu et al., 2019; Wu et al., 2014b). In most cases, AMF can enhance the retention of heavy metals in terrestrial plant roots and reduce the transport of heavy metals to aboveground parts (Janeeshma and Puthur, 2020). Compared to the AMF application in the heavy metal contaminated soil system, few studies focus on the role of AMF in alleviating heavy metal toxicity in CWs. Recent studies have found that AMF can colonize the roots of wetland plants in heavy metals-contaminated CWs, this provides a chance that they may play an important role in wastewater purification (He et al., 2020; Weishampel and Bedford, 2006; Xu et al., 2016). Xu et al. (2020) studied the effects of aeration and AMF on copper oxide nanoparticle removal in VSSF CWs, the results observed that the impact of AMF inoculated treatment on Cu purification was better than that of non-inoculated controls. He et al. (2020) evaluated the Cd leaching from polluted soils under simulated heavy rainfall, showing that Cd concentrations of the water in the mycorrhizal and hyphal compartments were 38.6%-93.8% lower than those in the soil compartment. Meanwhile, Huang et al. (2018) studied the effects of AMF on the uptake, translocation, and distribution of cadmium in *P. australis*, revealing that Cd concentrations and contents in the roots of AMF inoculated *P. australis* at the Cd concentration of 20 mg/L were higher than that in the non-inoculated controls, while a contrasting tendency was observed in the stems of *P. australis.* Therefore, AMF may alleviate heavy metal toxicity in CWs by decreasing heavy metal concentration in wastewater and improve heavy metal accumulation in wetland plants.

Although previous studies have proven that AMF could enhance heavy metal polluted wastewater purification in CWs, the mechanisms of AMF alleviating heavy metal toxicity in CWs are still unclear. Based on the AMF application in terrestrial ecosystems, we suggest that the AMF improve the tolerance of wetland plants to heavy metals in CWs mainly divided into direct and indirect influences. Direct influence mainly refers to the direct adsorption, absorption, and transformation of heavy metals by AMF. AMF mycelial cell wall components such as chitin, cellulose, and its derivatives can be combined with metal ions through carboxyl group, amino group, hydroxyl group, thiol group, and other groups, thus have a strong adsorption capacity for metals (Dhalaria et al., 2020; Wang et al., 2020). Moreover, AMF also have effects on the morphology and bioavailability of heavy metals in the rhizosphere, thereby affecting the uptake and transport of heavy metals by host plants (Wu et al., 2019). Xu et al. (2020) indicated that AMF in the CWs may release some active substances to change the rhizosphere microbial community, thereby promoting the absorption of heavy metals. Besides, Xu et al. (2016) also reported that some microorganisms (e.g. AMF) can accumulate heavy metals in cells to form amorphous mineral inclusions. On the other hand, indirect influence means that AMF enhance plant tolerance to heavy metals by promoting plant absorption of mineral nutrients (such as nitrogen, phosphorus) and enhancing plant production reactive oxygen species scavenging (e.g. decrease malondialdehyde and superoxide anion contents, increase antioxidant activities) (Ferrol et al., 2016). Chen et al. (2007) showed that AMF increased the uptake of heavy metal contents in plants, probably due to improving plant nutrition and increased biomass. Meanwhile, heavy metals could be immobilized in the substrates through the extra radical mycelium, thus reducing the heavy metal concentrations in the water or soil. Kullu et al. (2020) showed that the extra radical mycelium of AMF acted as a plant root extension, which can reach beyond the root depletion zone and may absorb heavy metal from the soil. The extra radical mycelium of AMF can also secrete organic acids to activate mineral nutrients in the soil, which enhance plant absorption of mineral nutrients, thereby improving plant tolerance to heavy metals (Subramanian et al., 2009). Besides, Wu et al. (2019) showed that heavy metal preferential accumulation in intraradical fungal structures rather than in root cells, which decreases the heavy metals toxicity to host plants. In all, numerous studies focused on exploring the effects of AMF on detoxification of terrestrial plants to heavy metal stress, but the mechanisms of AMF alleviating the toxicity of wetland plants to heavy metals still need further study in wetland systems.

To determine the optimum candidates for AMF colonization in roots of wetland plants under the wetland systems, we evaluated the effects of water depths (four water levels) on AMF colonization in different wetland plants (two wetland plant species). Although some studies indicated that AMF can promote wetland plant growth, increase chlorophyll contents in leaves, and facilitate nutrient uptake under abiotic stresses (e.g. heavy metal) (Fusconi and Mucciarelli, 2018; Wu et al., 2020), few studies focus on the AMF effect on the antioxidant response in wetland plants under heavy metal stress. Besides, heavy metals distribution and transformation in AMF assistant wetland systems are also unclear. To solve these problems, we investigated the influences of AMF on wetland plant growth and antioxidant response under heavy metal stress (three

Cr concentrations) regarding two water levels to explore the relationships between AMF and physiological functions toward wetland plants. Furthermore, the impacts of AMF on heavy metal distribution and bioavailability in semi-aquatic habitats were determined to evaluate the capacity of AMF for promoting wastewater purification. The detailed research steps are shown in **Figure 6.1**. The results are as follows:

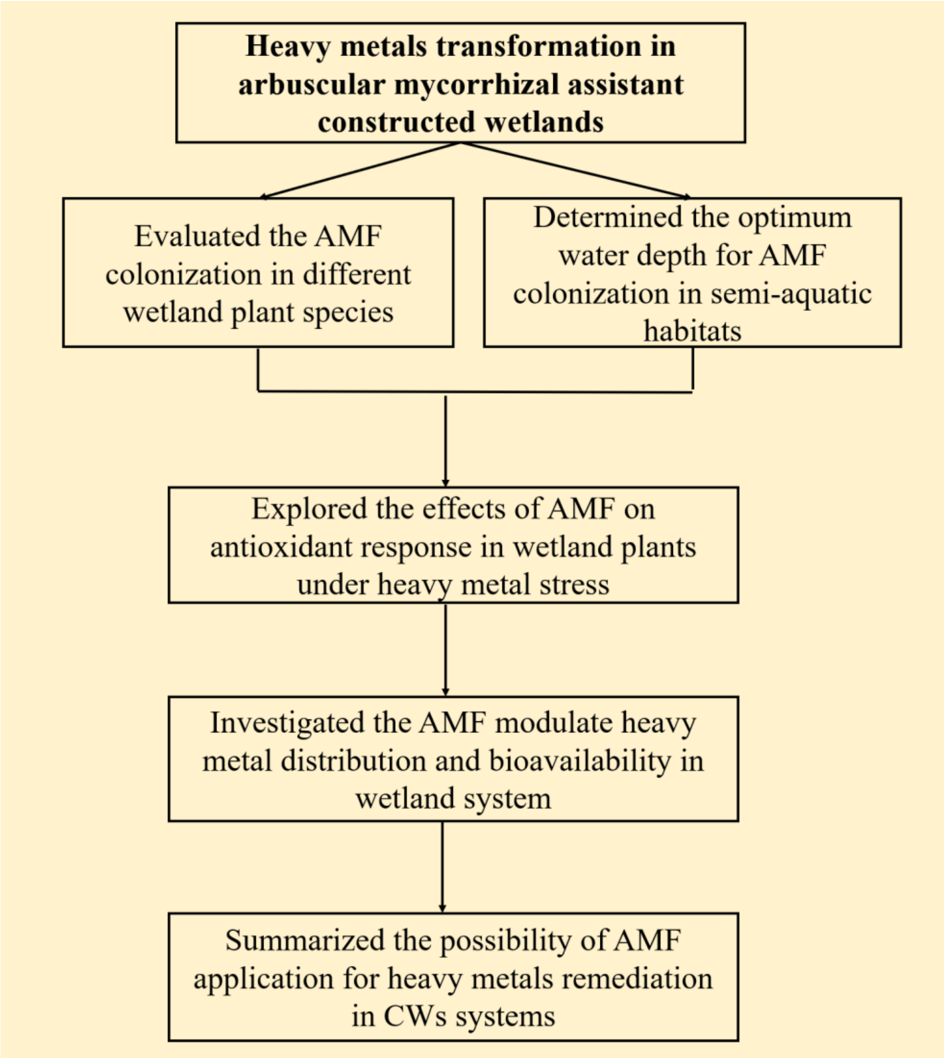


Figure 6.1: Experimental steps in this Ph.D. thesis

AMF colonization and physiological functions toward two wetland plants (*P. arundinacea* and *S. sylvaticus*) under different water depths (below the surface of sands: water levels of 5 cm, 9 cm, 11 cm, and fluctuating water depth (9-11 cm)) were studied. In this context, the best condition for AMF colonization and wetland plant growth were determined under the fluctuating water level. As expected, wetland plant species had no significant difference in AMF colonization under semi-aquatic systems. AMF formation in the roots of wetland plants was inhibited under high water depths, which indicated that AMF application in semi-aquatic or aquatic habitats (such as CWs systems) might be limited under high hydraulic loading rates. Meanwhile, the production of lipid peroxidation in wetland plants was determined under the low water depth (11 cm), resulting in the growth of wetland plants was stressed by water shortage. Moreover, physiological functions toward two wetland plants were improved by AMF inoculation under the fluctuating water level. Therefore, AMF application can be recommended for wetland systems under an optimum water level such as the fluctuating water depth.

While high AMF colonization in the roots of wetland plants can be found in the semi-aquatic systems under the fluctuating water depth, the effect of AMF on wetland plants' growth under heavy metal stress needed to be investigated as well. For this reason, the influences of AMF on wetland plant (*I. pseudacorus*) growth and antioxidant response under various Cr stresses (Cr concentration of 0, 5, 25 mg/kg) at different water depths (low water depth and fluctuating water depth) were evaluated. Generally, Cr had a negative influence on wetland plant growth by increasing ROS and lipid peroxidation accumulation, but AMF inoculation can improve wetland plant growth and enhance ROS scavenging under Cr stress (especially under the low Cr stress). Meanwhile, AMF promoted the antioxidant response in wetland plants, with higher biomass, height, the contents of chlorophyll, SOD, POD, K, and P in AMF inoculated wetland plants were determined compared to the non-inoculated controls. Besides, the wetland plants' tolerance to Cr stress was also enhanced under the fluctuating water depth. In conclusion, AMF provided an opportunity to improve the wetland plants' tolerance to heavy metal stress in wetland systems.

AMF had good potential in reducing oxidative damage of wetland plants under heavy metal stress. However, it is unclear whether AMF can modulate the heavy metal transformation in wetland systems. For this purpose, the impacts of AMF on heavy metal (Cr) distribution and bioavailability in semi-aquatic habitats were performed. It showed that AMF can regulate the Cr distribution in wetland plants by increasing the Cr accumulation in the roots of wetland plants and decreasing the transfer of Cr from roots to shoots. In addition, Cr concentrations and mass contents in water can also be decreased by AMF inoculation. Meanwhile, AMF can reduce the Cr bioavailability in substrates under the fluctuating water depth. Overall, the results indicated a chance for heavy metal removal in AMF assistant wetland systems.

The application of microorganisms generally represents a viable solution for improving heavy metal remediation in semi-aquatic or aquatic habitats such as CWs systems. Although it is a challenge for AMF to colonize the roots of wetland plants due to the high water content in wetland habitats, the obtained results in this Ph.D. thesis have proven that high AMF conization (the intensity of mycorrhizal colonization of 15.6% - 49.7%) in the roots of wetland plants can be observed under the fluctuating water level, which may provide the basis for the application of AMF in tidal flow CWs. Moreover, AMF promoted the antioxidant response in wetland plants and enhanced the heavy metal remediation in semi-aquatic systems, which may establish a good starting point for the application of AMF in the removal of heavy metals from wastewater under semi-aquatic and aquatic habitats (e.g. CWs). The knowledge gained here thus provides an efficient and environmentally friendly new strategy for the treatment of wastewater in CWs technology.

Although this thesis concluded that AMF can be applied in the CWs systems to purify heavy metal contaminated wastewater, this method is still in the preliminary research stage. Wu et al. (2016b) evaluated the cellular distribution and speciation of Cr in both extraradical mycelium and mycorrhizal roots exposed to Cr(VI) in terrestrial ecosystems by using field emission scanning electron microscopy equipped with energy dispersive X-ray spectrometer, scanning transmission soft X-ray microscopy, and X-ray absorption fine structure spectroscopy techniques, which revealed the mechanisms underlying Cr immobilization in cell walls and intraradical fungal structures in mycorrhizal roots of terrestrial plants. Compared to the effect of AMF on heavy metal remediation in terrestrial ecosystems, the mechanism of AMF promote the heavy metal distribution and transformation in wetland systems remains unclear. Therefore, it is necessary to reveal the microscopic migration and transformation processes of heavy metals in the AMF assistant wetland systems by using synchrotron radiation X-ray spectroscopy analysis technology. In addition, pot experiments were carried out in this thesis to evaluate the effect of AMF on wetland plant growth and heavy metal distribution. Thus, long-term pilot-scale and full-scale experiments could be required to directly investigate the influence of AMF on wastewater removal in wetland systems. Moreover, pollutants removal efficiencies in AMF assistant wetland systems are easily affected by some factors such as climate, nutrient concentrations (especially phosphorus contents), pH, hydraulic loading rates, and the size of the wetland system, which indicated that the application of AMF in wetland systems should consider these factors. In all, AMF inoculation is beneficial for heavy metal remediation in wetland habitats, but further studies are still needed to explore the effect of AMF on wastewater purification (e.g. nitrogen, phosphorus, organic carbon, and emerging pollutants removal) in CWs systems.

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Curriculum vitae

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Publications

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Grants and projects

IGA 20184235 (Internal Grant Agency of the Faculty of Environmental Sciences, CULS Prague).

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