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ANTIOXIDANT DEFENSE RESPONSE OF ARBUSCULAR MYCORRHIZAL FUNGI AND *SETARIA VIRIDIS*

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Abstract

Setaria viridis inoculate with *Funneliformis mosseae* was studied for examining the effects of arbuscular mycorrhizal fungi (AMF) on the growth, antioxidant enzyme activity and metabolites of *Setaria viridis* under Cadmium stress. The growth index, including root length, plant height and biomass of *S. viridis* inoculated with *F. mosseae* under Cd stress were higher than those of *S. viridis* without inoculation. Inoculation with *F. mosseae* can increase activities of CAT, SOD, POD and APX in shoot and decrease the activities of NADPH oxidase in root and shoot. The expression of *SvRbohF* gene encoding NADPH oxidase was down-regulated in roots but up-regulated in shoots. It showed that inoculation with *F. mosseae* induced the shoot of *Setaria viridis* to obtain higher antioxidant enzyme activity and scavenge more reactive oxygen species, thus further weakening the oxidative stress response of shoot and maintaining higher shoot biomass. The GSH and Cd content significantly increased in root inoculated with *F. mosseae* under Cd stress, but the MDA and Cd content in shoot significantly decreased. The changes increasing yield of phytochelatin, immobilizing Cd in root as much as possible, and reducing Cd content in shoot and lipid peroxidation products may be one of the mechanisms of AMF promoting the resistance of *Setaria viridis* to Cd stress. In addition, two-factor interaction analysis showed that Cd stress and AMF had significant interaction effects on aboveground biomass, CAT and other enzyme activities and Cd content of *Setaria viridis*. It confirmed that AMF could reduce the damage of *Setaria viridis* to Cd and promote the growth of *Setaria viridis* by regulating antioxidant defense system.

Key words: Arbuscular mycorrhizal fungi, *Setaria viridis*, Heavy metal, Antioxidant enzymes.

Introduction

Cadmium (Cd) can affect the transport channels of Fe^{2+}, Ca^{2+} and other essential elements, and be absorbed and transported to plants (Alloway, 2013; Kaur & Garg, 2018). Excessive Cd can destroy chlorophyll and make plants wilt. It can also cause the synthesis and accumulation of active oxygen, which induces oxidative damage and inhibit the growth of plants by reducing the absorption of nutrient elements（Menahem & Meni, 2018). The Cd concentration in soil is low under natural conditions, but it will enter the environment with human behavior, including mining, smelting and farming. It will be absorbed into the plant by the plant roots, and accumulated in the edible part, entering the body through the food chain, causing diseases such as cancer, poses a threat to human life and health（Hashem *et al*., 2016).

Arbuscular mycorrhizal fungi (AMF) can obtain Carbon sources from the host plant for self-use, and provide more inorganic mineral nutrition such as water and phosphorus for the host plant by extra-root hyphae. It can promote plant growth, improve plant nutrition and enhance plant resistance and tolerance (Salazar *et al*., 2018). AMF also plays critical role in plants replying to Cd stress and alleviates the harmful effects on plants to Cd stress (Rask *et al*., 2019; Wang *et al*., 2020).

The active oxygen contained in plants is volatile and prone to oxidation, forming O_2. Excessive active oxygen can destroy cellular oxygen balance, cell membrane lipid peroxidation, and finally cause cell injury and even death. The antioxidant defense system is composed of Superoxide dismutase (SOD), Catalase (CAT), Peroxidase (POD), Ascorbic acid peroxidase (APX), and non-enzymatic antioxidants (Glutathione, etc.), which can synergistically remove excess oxygen free radicals and maintain oxygen-free radical dynamic equilibrium in plants (Gallego *et al*., 2012). Plasma membrane NADPH oxidase is the most important enzyme source of H_2O_2. It can transfer electrons from NADPH in cytoplasm to O_2 and finally produces H_2O_2 (Babior, 2004). Plasma membrane NADPH oxidase is also an important signal enzyme in plants under heavy metal stress. When plants are stressed, it can rapidly produce a large amount of H_2O_2, and mobilize CAT, POD to respond, to decrease the harm to plants (Mohammadi & Rannug, 2014).

Plants can produce plenty of active oxygen under Cd stress. The defense system of plants can be activated by AMF to resist the damage of Cd. AMF can increase the activity of SOD (Abdelhameed & Metwally, 2019) and GSH content (Degola *et al*., 2015). It can also up-regulate the corresponding gene expression and eliminate the excessive free radicals in plants caused by Cd stress to avoid oxidative damage to cells (Molina *et al*., 2020). However, further studies have found that the mechanism of AMF involved in plant antioxidant defense response is different, which has not been fully clarified (Neagoe *et al*., 2013). Meier *et al*., (2011) considered that AMF could reduce antioxidant enzyme activity in plants but had the opposite effects reported by Liu *et al*., (2011). The antioxidant defense response induced by AMF is different in different parts (Degola *et al*., 2015). The GSH content in roots of tobacco inoculated by AMF did not change significantly treated with 30μM, but the GSH content in leaves was substantially different from the control. Therefore, the effects of AMF on plant antioxidant defense response are different, and the molecular research to help clarify these response mechanisms is lacking.

Setaria viridis is an annual herb of the genus *Setaria* in the Gramineae (Brutnell *et al*., 2010). *S. viridis* is diploid (2n = 18), with a small genome of less than

520Mb. The plant with fast-growing, short growth cycle, and a large number of seeds, are easy to genetic operation (Brutnell *et al*., 2015). *S. viridis*, closely related to *Setaria italica* and *Panicum virgatum*, is considered the model species of C_4 photosynthesis in xerophytic Gramineae crops and bioenergy plants (Brutnell *et al*., 2015). *S. viridis* is also a pioneer soil ecological restoration in mining areas (Guo & Xia, 2018). It has high plant extraction efficiency, biomass and heavy metal tolerance. AMF can significantly promote the growth of *S. viridis* and enhance the accumulation and extraction of Cd (Zhao *et al*., 2020). Therefore, the study on the symbiosis of AMF and *S. viridis* against heavy metal stress is beneficial to developing phytoremediation and mycorrhizal resistance physiology.

For the above reasons, the effects of AMF on antioxidant enzyme activity and the contents of GSH, MDA and Cd of *S. viridis* under Cd stress were investigated in this study. We also analyzed the expression level of the NADPH oxidase gene to reveal the relationship between AMF and plant antioxidant defense mechanism under Cd stress.

Materials and Methods

Growth matrix, plant material, AMF inoculation: The soil collected from the southern part of the Taihang Mountains was mixed with perlite in a 3:1 ratio to form a substrate. The substrate was sterilized at 121°C for 2 hours and dried in the laboratory for one week. The growing substrate soil was measured, including pH 6.32, total nitrogen 32.13 $mg \cdot kg^{-1}$, available phosphorus 5.34 $mg \cdot kg^{-1}$ and available potassium 40.52 $mg \cdot kg^{-1}$. The seeds of *S. viridis* were gathered from the southern Taihang Mountains in September 2020, and selected after being hung in the shade for one day. The seeds were placed in 0.5% potassium permanganate solution and sterilized for 20 min.

Funneliformis mosseae (BGC BJ01) was provided by the Beijing Academy of Agriculture and Forestry. Maize was used as a host to propagate, and the mixture of fungus hyphae and spores, culture medium and maize root segment were used as inoculants. The pot (diameter 30 cm, height 20 cm) was soaked in 0.5% potassium permanganate solution for 30 min.

First, the substrate was added to the pot about 5 cm from the pot mouth. A layer of inoculants (100 g) was spread on the substrate. The sterilized seeds were seeded on the top. Each pot was planted with 50 seeds covered with about 2 cm of the substrate. A total of 2 Kg of the substrate was added to each pot. The control was spread a layer of sterilized inoculant(100 g), without inoculation treatment, and added 30 mL inoculant filtrate.

Plant culture and experimental treatment: The study was conducted in a natural light glasshouse from September to December 2020, which kept the indoor temperature at 25°C, humidity 60% -70%. The seedlings were watered once a week, about 500 mL each time. Potted plants were randomly consisted of four treatments after growing for two months: Non-mycorrhiza inoculum without Cd stress (NM0), Mycorrhiza inoculum without Cd stress (AM0), Non-mycorrhiza inoculum with Cd stress (NM100), Mycorrhiza inoculum with Cd stress (AM100). $CdCl_2$ solution was added to the pot and finally reached 100 Cd^{2+} $mg \cdot kg^{-1}$ in the soil (Romária *et al*., 2017).

$CdCl_2$ solution was divided into three parts and added to the pot continuously for three days. The pot without Cd stress was irrigated with the same volume of deionized water. Six pots were planted for each treatment.

Sampling and detection of plant growth index: Samples were taken one week after Cd treatment. Three fresh roots of samples respectively were cut into root segments about 1.0 cm in length and soaked in the dark box with FAA fixing solution to detect the infection rate of mycorrhizal fungi. The root length and aboveground height of ten plants were measured, and the average values of ten plants were calculated. The above ten plants samples were dried to constant weight to measure the dry weight of roots, shoots, and the total. The dried samples were also used to test the content of Cd. The matrix of all pots was air-dried under natural conditions and sifted through 100 mesh screens to determine the Cd content in the soil.

Arbuscular mycorrhizal colonization: Thirty root segments soaked with FAA fixative solution were rinsed with clear water and added 10% KOH solution preserving at 90°C for 30 min. Then they were transferred into 5% trypan blue about 1 hour. Finally, the root segments were transferred into glycerol lactate solution after cleaning and observed by microscopical examination (Phillips & Hayman, 1970). The colonization rate of mycorrhizal fungi was determined using Giovannetti and Mosse's method (1980).

$$\text{Colonization rate of mycorrhizal fungi} = \frac{\text{Total number of root infected by mycorrhizal fungi}}{\text{Total number of root detected}} \times 100\%$$

Detection of enzyme activity of plant antioxidant defense system: The leaves (SOD, CAT, POD, APX and NADPH oxidase activity) and roots (NADPH oxidase activity) taken 1g and 300mg respectively were grinded into powder in liquid nitrogen and added into the extraction reagent 1mL and 0.3mL respectively (Tris-HCL 0.05M（pH7.0), 1mM EDTA, 3mM $MgCl_2$) (Chang & Koa, 1988). The NBT method was used to test the activity of Superoxide dismutase in the extract (Han *et al*., 2012). The activity of Catalase, Peroxidase, and Ascorbic acid peroxidase was determined by reffering to the methods of Aebi (1984), Chance & Maehly (1955) and Nakano & Asada (1981), respectively. The plasma membrane NADPH oxidase activity was determined by the Plant NADPH oxidase activity photometric assay Kit (Shanghai Genmed Gene Pharmaceutical Technology Limited Company). The specific operation referred to the product description (Genmed GMS50096.3).

Detection of GSH content in plant: The roots (300 mg) and leaves (300 mg) were homogenized in 2 ml 5% (W/V)5-sulfosalicylic acid containing 6.3 mM diethylenetriamine pentaacetic acid (DTPA). The extract was centrifuged for 10 min at 4°C under 10000g centrifugal force (Knecht *et al*., 1994). The supernatant was removed and filtered through the 0.45m membrane (Shanghai Xinya Purification Equipment Limited Company). The GSH content was determined by High-performance liquid chromatography. Based on the principle that 5,5'-disulfide-2-nitrobenzoic acid (DTNB) can be derivatized with sulfhydryl compounds, the absorption chromatographic peaks of TNB and NTB released Quantitatively by RS-TNB at 327 nm were determined with the ultraviolet detector of waters e2695-2489 system. The reversed-phase chromatographic column C18 (Waters, USA) was used to determine by programmed elution with the binary mobile phase. Phase A was an aqueous phase containing 1% formic acid and 0.07% triethylamine, and phase B was an organic phase of acetonitrile containing 1% formic acid and 0.07% triethylamine. Gradient elution procedures: 90% A + 10% B (0 min) , 86% A + 14% B (10 min), 72% A + 28% B (15 min), 10% A + 90% B (40 min), 10% A + 90% B (45 min), 90% A + 10% B (50 min). The sample (10µL) was tested at 25°C under 327 nm wavelength with the flow rate of 0.8 mL/min.

Detection of plant MDA content: The degree of oxidative damage in fresh leaf tissue induced by Cd was evaluated by determining the content of MDA. The leaves (500 mg) were homogenized in a ratio of 1:3 in 0.1% (W/V) trichloroacetic acid solution. The extract was operated referring to Amor's method (Amor *et al*., 2005).

Detection of Cd content in plant and soil: The samples (0.2 g) were accurately weighed in the digestion tube. 8 mL mixed acid (HNO_3: H_2O_2 = 7:1 in plants and HNO_3: HF: H_2SO_4 = 3:2:3 in soil) were added into samples. After soaking overnight, the samples were kept by high-temperature digestion (196°C) in a microwave digestion apparatus (Ethos, Milestone, Italy) until the solution became clear without color. The plant and soil samples were cooled at room temperature and added 0.2% HNO_3 to the scale (25 mL) to prepare the test solution. The graphite furnace-atomic absorption photometer (SUPER AFG, Beijing Purkinje General Instrument Limited Company) was used to test the Cd content.

Transfer coefficients and enrichment coefficients were calculated by the formula (Rafati *et al*., 2011).

$$\text{Transfer coefficient} = \frac{\text{The content of Cd in aerial parts of plants}}{\text{The content of Cd in underground parts of plants}} \times 100\%$$

$$\text{Enrichment coefficient} = \frac{\text{The content of Cd in aerial parts of plants}}{\text{The content of Cd in soil}} \times 100\%$$

Isolation of RNA and synthesis of cDNA: Total RNA of *S. viridis* was extracted by Qiagen RNeasy Plant Mini kit method (Guanidine salt method) developed by Tiangen Biotech Limited Company. Determination of rRNA by agarose gel electrophoresis (Verwoerd *et al*., 1989). The cDNA synthesized by Quanti Tect R reverse transcription kit (Qiagen).

RT-PCR analysis: Real-time PCR experiments were performed using Ariamx Real-Time PCR System of Agilent, which contained SYBR Green iDNA binding dye. Repeat each treatment three times. The total reaction volume was 20 µL PCR system: 20 mg cDNA, 0.5 µL primer, 0.2 mmol dNTPs and 1 U Taq enzyme (Takara).The PCR reaction conditions included an initial denaturation for 5 min at 95°C, followed by 35 cycles of 95°C denaturations for 30 s, 58°C annealing for 30 s, 72°C elongations for 30 s, and a final extension at 72°C for 5 min. According to Tian Baohua *et al*., (2016), the primer sequence of the gene *Rboh F* using encoding NADPH oxidase was forward 5'-TGTATGTTGGGGAGA GGACG-3'; reverse 5'-TTGTAACGGAATGTGGGAGG -3'. Primer was synthesized by Beijing Qingke Limited Company. Gene expression level was analyzed by $2^{-\triangle\triangle CT}$(Livak & Schmittgen, 2001).

Statistical analysis

Six repeated data of each treatment were analyzed by ANOVA using SPSS 18.0. The data were released in mean ± standard error. The results were analyzed by the interaction between mycorrhizal fungi and Cd stress, and multiple comparisons were made by the LSD method. The difference between the different letters was significant (p<0.05).

Results

Arbuscular Mycorrhizal colonization: Arbuscular mycorrhizal symbiosis was formed with *S. viridis* inoculated with AM fungi, but no mycorrhizal colonization was formed in the treatment without inoculation, and the colonization rate was 0 (Fig. 1). The colonization rate of mycorrhizal fungi of *S. viridis* treated by mycorrhizal fungi without Cd stress (AM0) was 61.511%. The colonization rate of mycorrhizal fungi of *S. viridis* treated by mycorrhizal fungi under the Cd stress (AM100) was only 33.527%, which was about half AM0. The Cd stress can significantly reduce the mycorrhizal infection rate of *S. viridis*.

The root length, plant height and plant biomass: The root length, plant height, aboveground biomass and whole plant biomass of *S. viridis* were significantly increased by fungi inoculation. The indexes of *S. viridis* inoculated AMF without Cd stress (AM0) were higher than that non-AM inoculation without Cd stress (NM0). The root length, plant height and root biomass significantly reduced under Cd stress, and NM100 was significantly lower than NM0 (p<0.05). The above-ground and the whole plant biomass had no significant difference under the Cd stress.

Compared with NM100, the root length, plant height, root biomass, shoot biomass and whole plant biomass of AM100 were 1.203, 1.885, 1.553, 1.684 and 1.672 times those of NM100, respectively (p<0.05).

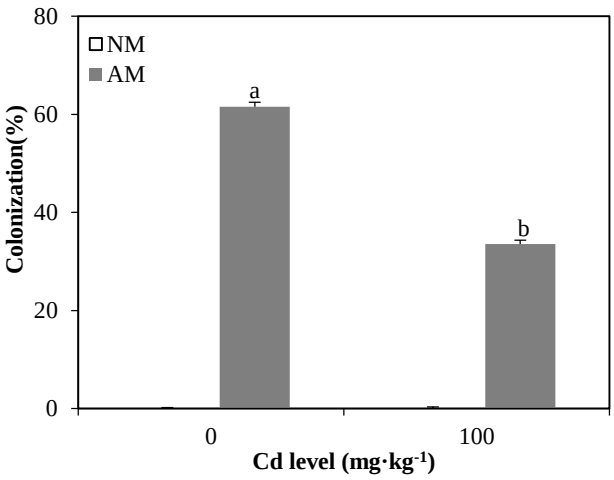

Fig. 1. Arbuscular Mycorrhizal colonization.
Note: Different lower-case letters indicate significant differences among treatments at p<0.05 level.

Two-factor interaction results showed that all the five grow indexes of *S. viridis* had significant effects by AMF (p<0.01). There was a significant interaction between shoot biomass and whole plant biomass (p<0.01), but no interaction between root length, plant height and root biomass (Table 1).

Enzyme activity of plant antioxidant defense system: AMF can improve the activities of CAT and SOD in *S. viridis* and reduce the APX activity. However, POD activity was not affected (Fig. 2).

The activities of CAT, POD, SOD and APX significantly increased under Cd stress. NM100 was 1.566, 1.534, 1.815 and 1.525 times of NM0, respectively. Similar to Cd stress, AMF also improved all indexes under Cd stress. Compared with NM100, the activities of CAT, POD, SOD and APX of AM100 were 1.208, 1.192, 1.168 and 1.139 times of NM100, respectively (p<0.05).

Two-factor interaction analysis showed that the activities of CAT, POD and SOD were significantly affected by AMF except for APX activity. Cd stress had a significant effect on the activities of 4 enzymes (p<0.01).

The activities of NADPH oxidase in root and shoot of AM0 were 15.997 and 37.538 (U $min^{-1}g^{-1}FW$) respectively, which were similar to those of NM0, and the difference was not significant. The activity of NADPH oxidase in the root and shoot of *S.viridis* significantly increased under the Cd stress. The activities of NADPH oxidase in root and shoot of NM100 were 46.135 and 70.581(U $min^{-1}g^{-1}FW$), which 2.884 and 1.880 times of NM0, respectively (p<0.05). Contrariwise, NADPH oxidase activity was significantly decreased by inoculation with AMF under Cd stress. Compared with **NM100**, the activities of NADPH oxidase in root and shoot of **AM**100 were 75.512% and 84.759% of **NM100** respectively, and had significant differences. Inoculation of AMF, Cd stress and their combined effects had significant effects on NADPH oxidase activity (p<0.01) according to two-factor interaction analysis.

AMF significantly improved the expression of *SvRbohF* which was the coding gene of NADPH oxidase in the shoot (Fig. 2g, 2h), but without effect in the roots of *S.viridis*. The relative expression of *SvRbohF* also significantly increased in *S.viridis* under Cd stress. The relative expression of *SvRbohF* was regulated differently in roots and shoots with inoculation of AMF under Cd stress. It was significantly down-regulated in roots but up-regulated in shoots. The results of two-factor interaction analysis indicated that AMF or AMF combined with Cd stress both affected the expression of *SvRbohF* (p<0.01).

The GSH content in plants: The GSH content of *S. viridis* was not influenced by AMF, but it was significantly increased under Cd stress. The GSH content in root and shoot of NM100 was 49.981 and 90.012 (μg·g^{-1} FW), which was 1.907 and 1.940 times of NM0, respectively. Similarly, the GSH content significantly increased in roots and shoots by the inoculation of AMF under Cd stress (Fig. 3). Two-factor interaction analysis indicated that the inoculation of AMF had significant effect on GSH content in root and shoot of *S. viridis*. The effect of Cd stress was extremely significant in root and shoot (p<0.01). The GSH content in roots and shoots significantly was affected by AMF interacted with Cd stress (Fig. 3).

The MDA content in plants: The MDA content of aboveground parts of *S. viridis* was not affected by AMF. The difference was not significant between AM0 and NM0. The MDA content of *S. viridis* significantly increased under the Cd stress. The content of MDA in NM100 was 13.859 (g·mg^{-1} FW), which was 2.071 times of NM0 (p<0.05).

Two-factor interaction analysis showed that inoculation of AMF affected MDA content significantly in aboveground parts of *S. viridis* (p<0.05), while the MDA content was extremely significant effect under the Cd stress (p <0.01).

Table 1. Effects of AMF and Cd stress on root length, plant height and biomass of *Setaria viridis.*

Treatment	Root length (cm)	Plant height (cm)	Biomass (g×10 DW)		
			Root	Aboveground part	The whole plant
NM0	6.710 ± 0.139^{b}	5.203 ± 0.215^{b}	0.116 ± 0.017^{a}	1.013 ± 0.106^{c}	1.128 ± 0.118^{c}
AM0	7.590 ± 0.261^{a}	8.870 ± 0.324^{a}	0.135 ± 0.017^{a}	2.029 ± 0.267^{a}	2.164 ± 0.279^{a}
NM100	6.030 ± 0.226^{c}	4.503 ± 0.296^{c}	0.082 ± 0.011^{b}	0.806 ± 0.022^{c}	0.888 ± 0.011^{c}
AM100	7.257 ± 0.278^{a}	8.487 ± 0.161^{a}	0.127 ± 0.011^{a}	1.357 ± 0.111^{b}	1.484 ± 0.117^{b}
P_{AMF}	**	**	**	**	**
P_{Cd}	**	**	*	**	**
$P_{AMF×Cd}$	NS	NS	NS	*	*

Note: Different lower-case letters indicate significant differences among treatments at p<0.05 level

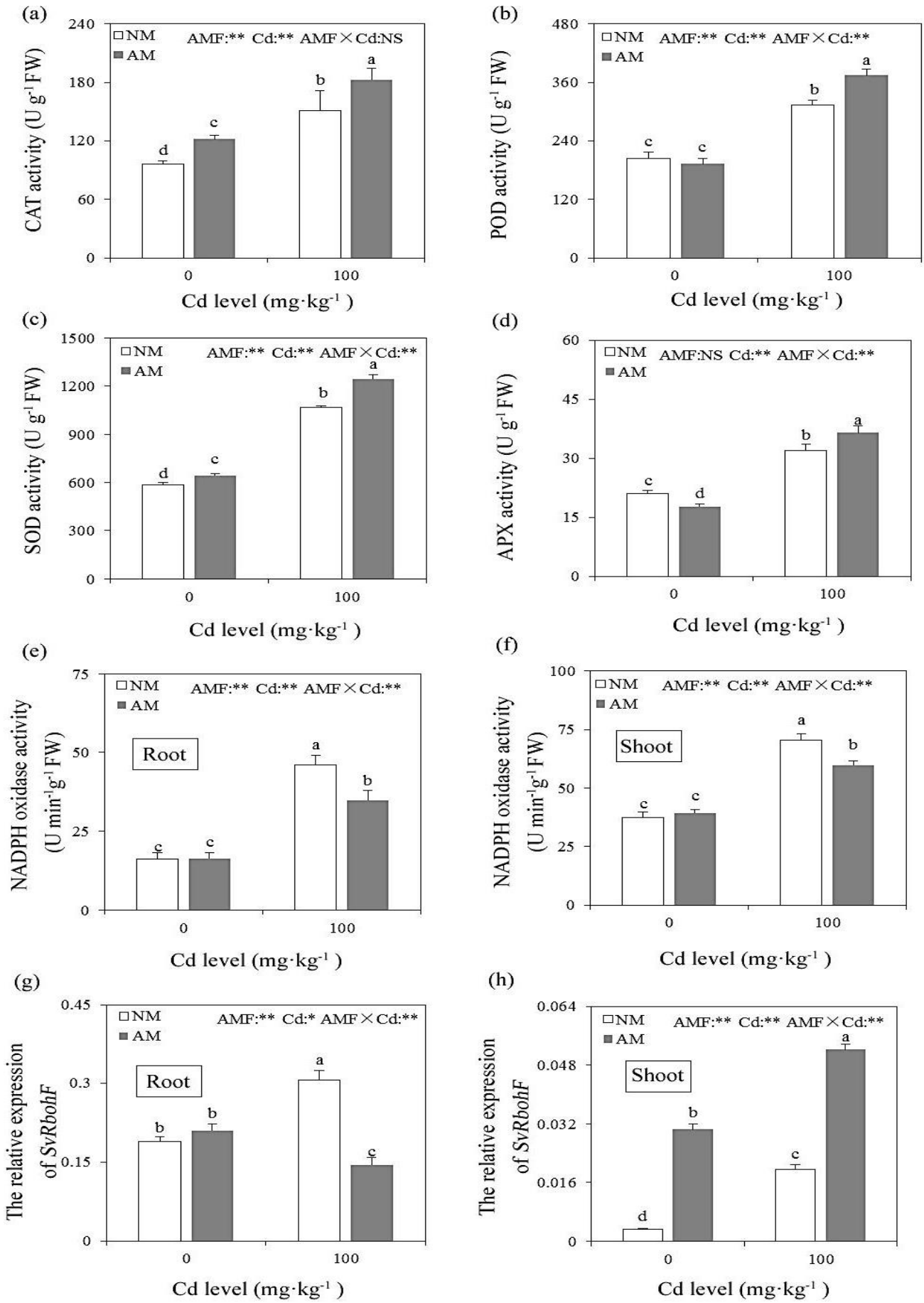

Fig. 2. Effects of AMF and Cd stress on the activities of antioxidant defense enzymes in *S. viridis*.
Note: Different lower-case letters indicate significant differences among treatments at $p<0.05$ level.

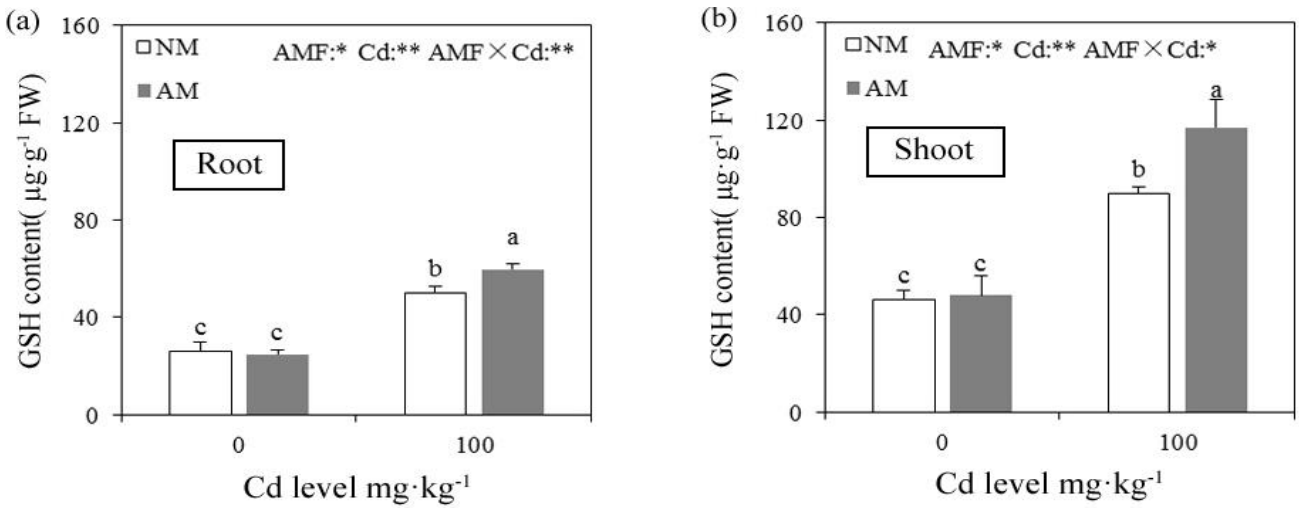

Fig. 3. Effects of AMF and Cd stress on the GSH content in *S. viridis.*
Note: Different lower-case letters indicate significant differences among treatments at $p<0.05$ level.

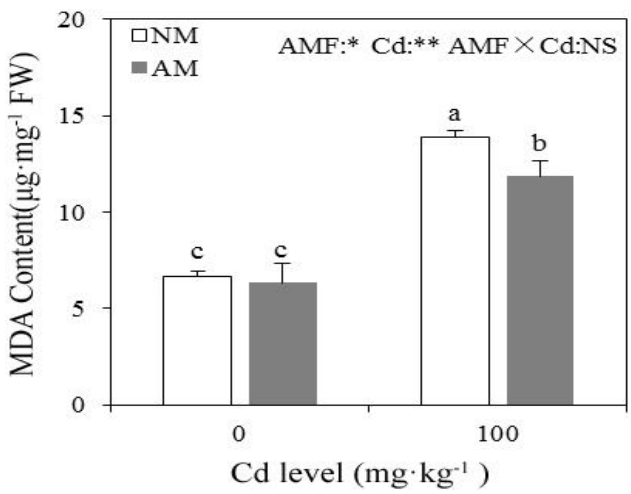

Fig. 4. Effects of AMF and Cd stress on the MDA content of aboveground parts in *S. viridis.*
Note: Different lower-case letters indicate significant differences among treatments at $p<0.05$ level.

The Cd content in plants: The inoculation of AMF had no effect on Cd content in *S. viridis*. The Cd content significantly increased in root and shoot of *S. viridis* under the Cd stress (Fig. 5a and 5b). The Cd content in roots was significantly improved by the inoculation of AMF under Cd stress, but the content of Cd in shoots decreased. The Cd content in the root of AM100 was 1.318 times higher than that of NM100, while that in the shoot of AM100 was 84.502% of NM100. The inoculation of AMF, Cd stress, and their interaction were highly significant to the Cd content in root and shoot of *S. viridis* according to the two-factor interaction analysis ($p<0.01$).

The content of Cd in the soil had no difference between inoculation of AM fungi and inoculation of fungi with Cd stress, but it was affected significantly with Cd stress alone. Two-factor interaction analysis also showed the same result in soil for Cd content (Fig. 5c).

The transfer coefficient and enrichment coefficient of *S. viridis* significantly increased with the inoculation of AMF or Cd stress (Fig. 5d and 5e). However, they were significantly decreased with the interaction of inoculation of AMF and Cd stress. Two-factor interaction analysis showed that AMF, Cd stress, or their interaction had highly significant effects on the transfer coefficient and enrichment coefficient ($p<0.01$) except the effect on the enrichment coefficient treated with AM fungi inoculation.

Discussion

AMF colonization: The heavy metal pollution and other harsh habitats often affect the colonization of AMF to plants and reduce the colonization rate of AMF (Rivera-Becerril *et al*., 2013). The colonization rate of *S. viridis* inoculated with *F. mosseae* was more than 60% without Cd stress, forming an excellent symbiotic relationship in this study. The colonization rate was affected by Cd stress, and decreased significantly to about half with Cd^{2+} (100 $mg \cdot kg^{-1}$) (Fig. 1). Similar to some researchs, heavy metal stress hindered the colonization of AMF, so the colonization rate in roots of plants was generally low (Oliveira *et al*., 2019; Bedini *et al*., 2010).

The growth of plant: The excessive Cd in soil could reduce plant biomass, which was mainly due to the inhibition of plant physiological and metabolic processes induced by Cd, or the damage of plant root activity and nutrient absorption (Sharma *et al*., 2010). In addition, the response of plant growth to Cd stress was also related to the interaction of many factors. Prasad *et al*., (2011) found that the biomass of *Ocimum basilicum* was significantly reduced by inoculation with AMF at a lower concentration of Cd, but improved under the treatment of high concentration.

We found that the root length, plant height, root biomass, aboveground biomass and whole plant biomass of *S. viridis* significantly increased with the inoculation of AMF under Cd stress. Both the AMF and Cd affected the growth of *S. viridis*, and they had significant interaction in aboveground biomass and whole plant biomass (Table 1). AMF could significantly promote the growth of *S. viridis* under the high concentration Cd stress.

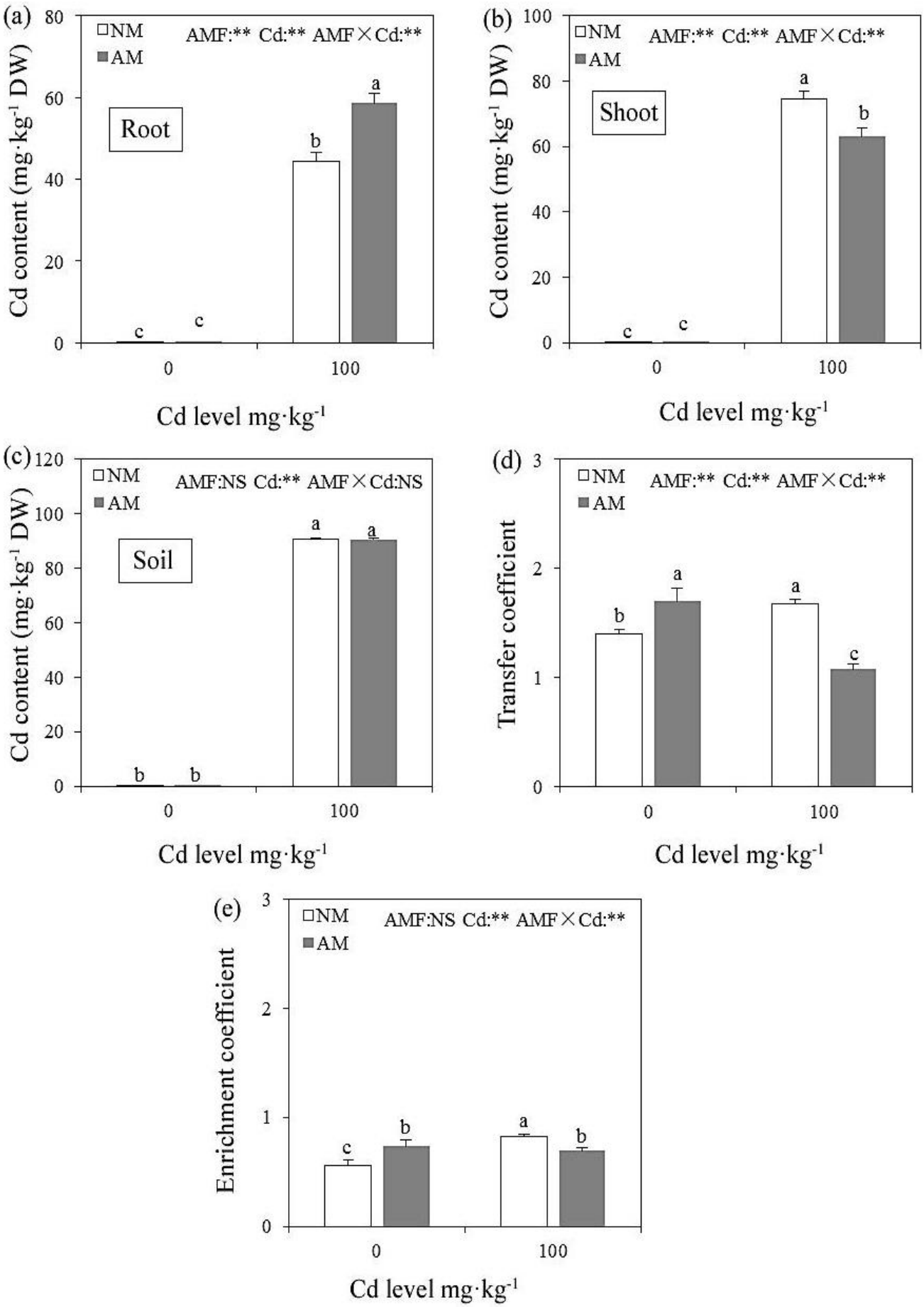

Fig. 5. The Cd content of *S. viridis* effected by AMF and Cd stress.

The effects on plant antioxidant enzyme defense system: Studies have shown that the POD activity of *Helianthus annuus* inoculated with *Glomus intraradices* decreased significantly (Andrade *et al*., 2008). AMF can reduce the production and accumulation of oxygen free radicals in plants, and prevent oxidative damage (Garg & Singla., 2012). On the contrary, some studies indicated that AMF could improve the activities of CAT, APX and other related enzymes, which could help inhibit the oxidative damage caused by heavy metals (Azcón *et al*., 2009). Other studies indicate that different AMF and Cd treatments had different effects on the antioxidant enzyme activities of plants. Liu *et al*. found that the activities of SOD, POD and CAT in rice were reduced by treating Cd with inoculation of *Glomus mosseae*. However, the enzyme activity of SOD and CAT in rice inoculated with *Rhizophagus intraradices* was significantly increased, and POD activity decreased significantly. Therefore, the study thought that the activity of SOD and CAT was related to the species of AMF, the level of Cd treatment, and the types of the enzyme (Liu *et al*., 2011). Our study found a similar situation. Under high concentration of Cd stress, the antioxidant enzyme activities significantly increased with inoculation of AMF, and the activities of NADPH oxidase significantly decreased. The two-factor interaction analysis also supported the above view.

Plants can rapidly increase or decrease reactive oxygen through the activation or inactivation of NADPH oxidase, which acts as a signal molecule to activate the body in response to stress under heavy metal stress (Lherminier *et al*., 2009). The expression of the NADPH oxidase gene *Rboh* was different in different plants. Cheng *et al*., (2013) found that *AtRbohE was* expressed in the roots, while *AtRbohF* was expressed in all tissues. *SvRbohF* was found to be expressed in root and aboveground part of *S. viridis*, but it had differnces in different tissues. *SvRbohF*expression level in roots was significantly down-regulated while in the aboveground parts was up-regulated significantly under Cd stress, and the interaction was obvious. The expression level of *SvRbohF* was not completely correlated with its activity, which was similar to Tian Baohua's research (Tian Baohua *et al*., 2016). It may be that *SvRbohF* expression only reflected the change of mRNA transcription level, while the enzyme activity showed the protein level. They were two different mechanisms (Kumar *et al*., 2015; Aloui *et al*., 2011). Therefore, the high expression of *SvRbohF* in the aboveground parts does not necessarily show the enhancement of NADPH oxidase activity. In addition, the antioxidant enzyme activity increased significantly under a high concentration of Cd, which indicated that plants could enhance their tolerance to Cd by activating the antioxidant defense system (Aghababaei & Raiesi, 2015). The CAT, POD, SOD and APX enzyme activities of *S. viridis* significantly increased under high concentration of Cd stress in the present study, which indicated that *S. viridis* could well tolerate Cd stress. NADPH oxidase and SOD can produce H_2O_2 in plants. At the same time the activities of POD, APX and CAT increased, which can enhance the ability to scavenge H_2O_2, indicating that plants can alleviate the oxidative damage of H_2O_2 to some extent (Zhang *et al*., 2009). Therefore, we considered that *S. viridis* could regulate reactive oxygen metabolism through NADPH oxidase under Cd stress and respond to the damage of Cd stress.

The effects on GSH and MDA: Plants may adopt different strategies to resist the toxic effects of heavy metals at the cellular, molecular and biochemical levels. The chelation of heavy metals by synthetic chelators was one of the common mechanisms. Glutathione (GSH) was one of the chelators of phytochelatins (Yadav *et al*., 2010). We found that the GSH content in *S. viridis* inoculated with AMF was significantly increased (Fig. 3) under Cd stress, which was beneficial to chelate Cd and neutralized harmful effects to a certain extent. Degola *et al*., (2015) also found that mycorrhizal fungi increased the GSH content in tobacco. They believed that inoculation with *Funneliformis mosseae* could affect the expression of plant sulfur transporter, which eventually led to the increase of GSH content. GSH was a good ion chelator (Verbruggen *et al*., 2009), which directly combined with Cd and blocked the aboveground transport of Cd.

Malondialdehyde (MDA) was the product of lipid peroxidation of the membrane, which usually reflected the degree of lipid peroxidation of the cell membrane (Sun *et al*., 2019). We found that the MDA content of *S. viridis* inoculated with AMF under Cd stress was significantly reduced, and the degree of membrane peroxidation was reduced to a certain extent. He (2020) took celery as the research object to analyze the effects of AMF inoculation on the growth, Cd absorption, distribution and accumulation of celery on Cd contaminated in soil. The MDA content of celery was significantly reduced by 30.37% under Cd stress. The inoculation of AMF was helpful to reduce the degree of membrane peroxidation and alleviate the damage caused by Cd.

The effects on Cd content: The Cd content in roots of *S. viridis* with inoculation of *Funneliformis mosseae* significantly increased and significantly decreased in aboveground parts under Cd content. The interaction between them was highly significant under Cd stress. Degola *et al*., (2015) indicated that AMF significantly reduced the accumulation of Cd in leaves, and the Cd content in root was higher than that of leaves in tobacco with inoculation of *Funneliformis mosseae* for 28 days. It was indicated that the isolation effect of AMF on the fixation of Cd in the mycelium of the root limited the transport of Cd to the aboveground parts. In this study, the increase of GSH synthesised in *S. viridis* with inoculation of *Funneliformis mosseae* may be another important reason for the accumulation of Cd in roots and the interception of Cd transport to aboveground parts. The further research was needed to confirm.

The transfer coefficient and enrichment coefficient reflected the ability of plants to absorb and transport Cd. In this study, the transfer coefficient and enrichment coefficient of Cd in *S. viridis* were reduced by inoculation with *Funneliformis mosseae* under Cd stress, and the interaction between them was extremely significant. In the study of He (2020), AMF reduced the transfer

coefficient of Cd in celery, but increased the enrichment coefficient. They believed that AMF could improve the growth of plants and enlarge the absorption area of roots, thus increasing the absorption of Cd by celery roots, reducing the Cd content in plant rhizosphere soil, and increasing the enrichment coefficient. AMF mycelium itself had a fixation effect on Cd, which could retain Cd in roots and inhibit upward transport, thus reducing the transfer coefficient. However, the ability of plants to absorb and transport Cd was also related to the treatment time of Cd. After treatment for more than two weeks, the Cd content accumulated in plant leaves increased, higher than that in roots (Degola *et al*., 2015), or even twice as high as that in roots (Janoušková *et al*., 2007). The enrichment factor also increased with Cd content in aboveground parts. The treatment time of Cd in this study was one week, shorter than that of He (2020) for two months, which may be the reason for the decrease of both transfer coefficient and enrichment coefficient.

Conclusion

The symbiotic relationship between *Funneliformis mosseae* and *S. viridis* resulted in higher Cd tolerance of *S. viridis*. AMF can effectively reduce the aboveground Cd content of *S. viridis*, and protect plants from the impact of Cd stress through promoting the growth of *S. viridis*, improving the antioxidant enzymes activities, reducing the MDA content, reducing the degree of membrane peroxidation, and alleviating the oxidative damage induced by Cd stress. However, more in-depth experimental studies are needed to understand the mechanism of AMF involved in plant resistance to Cd stress, especially the production and elimination of reactive oxygen in aboveground parts.

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