Rhizophagus irregularis regulates antioxidant activity and gene expression under cadmium toxicity in *Medicago sativa*

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Abstract

Cadmium (Cd) is a phytotoxic heavy metal (HM) that can induce generation of reactive oxygen species (ROS). Arbuscular mycorrhizal fungi (AMF) are considered as bio-ameliorators that help to mitigate HM-derived oxidative stress. The objective of this study was to assess AM fungus *Rhizophagus irregularis* on changes in enzymatic activity and transcription of antioxidants of *Medicago sativa* under Cd stress. Increased superoxide dismutase (SOD) and ascorbate peroxidase (APX) transcripts accumulation under Cd stress did not lead to improvement in their activities which could be related to inactivation of enzyme resulting from excess ROS or Cd binding to the active site. However, more pronouncedly enhanced the activity of CAT instead of SOD, APX, and GR in the roots of *M. sativa* under Cd stress conditions suggested that antioxidant enzymes differed in sensitivities to Cd exposure**.** AMF symbiosis significantly reduced transcripts abundance of *MsCu/Zn SOD* and *MsCAT* genes compared with NM plants indicating that metal sequestration within hyphal fungi probably made Cd concentration insufficient in root cells for induction of these genes. However, GR activity was not affected by its gene expression under Cd stress. This phenomenon might be attributed to AMF-mediated post-transcription regulation. The results presented here could provide an enlightenment to the capability of Cd-induced ROS-scavenging system in *M*. *sativa* colonized with *R*. *irregularis*.

Keywords: *Rhizophagus irregularis;* alfalfa; antioxidant activities; Cd stress; gene expression

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Introduction

Cadmium (Cd) is a phytotoxic element even at low doses (0.5 µg Cd g^{-1} soil) (Aibibu et al., 2010) which is easily absorbed by plant roots and translocated to shoots and its subsequent entrance to food chain is a serious threat to animal and human health (Gill et al., 2011). A reduction in the Cd concentration in plants is therefore an essential issue for producing safe food. Increasing cadmium (Cd) accumulation more than permissible levels in plants causes stunted growth due to its inhibitory effect on photosynthesis and nitrogen (N) metabolism (Márquez-García et al., 2011). Furthermore, Cd can interact with sulfhydryl (thiol) group of enzymes and hence disrupting their activities (Mendoza-Cozatl et al., 2005). Cd also induces the production of reactive

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oxygen species (ROS) including O_2^-, H_2O_2, and OH^- by perturbing enzyme activities involved in redox status homeostasis resulting in cell membrane permeability disruption, reduced plant nutrient absorption and consequently, yield losses (Singh and Shah, 2014). Under HMs stress, the ratio of saturated/unsaturated fatty acids of membrane lipids is enhanced through conversion of unsaturated fatty acids into small hydrocarbons like malondialdehyde (MDA) which leads to altered membrane permeability (Morsy et al., 2012). In plants, counteracting Cd-induced oxidative burst is achieved by enhancement of antioxidant capacity. Key enzymatic scavengers include Superoxide dismutase (SOD) (Bowler et al., 1992), catalase (CAT), ascorbate peroxidase (APX) (Gill and Tuteja, 2010), and glutathione reductase (GR) (Garg and Kaur, 2013). SOD as an enzyme at the front line of defense against oxidative stress catalyzes the dismutation of reactive O_2 (O_2^-) to H_2O_2 (Irfan et al., 2014). CAT then directly neutralizes H_2O_2 to water and molecular oxygen (Schutzendubel and Polle, 2002). APX is the other H_2O_2-quenching enzyme that uses ascorbate as a reductant (Iturbe-Ormaetxe et al., 2001). GR enzyme uses NADPH as an electron donor to mediate the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) (Garg and Kaur, 2013). It has been found that GSH as an indispensable regulator of redox signaling has an important role in maintenance of cellular redox balance (Zechmann et al., 2008). In addition to described strategies, effect of Cd in the up-regulation of genes involved in sulfur (S) assimilation pathway can increase the synthesis of S-containing antioxidants like cystein (Cys) and GSH which play crucial role in mitigating Cd-induced oxidative stress (Gill and Tuteja, 2011).

Arbuscular mycorrhizal fungi (AMF) improve the efficiency of nutrient delivery system to the host plants through the entrance of their hyphae in to roots cortex layer (Gutjahr and Parniske, 2013). AMF plants may also affect the expression and activity of ROS- scavenging enzymes. However, antioxidant protective capacity varies depending on host, fungus species and the dosage and period of HM exposure (Shahabivand et al., 2016; Yang et al., 2015).

Medicago sativa (alfalfa) is widely cultured for animal feed and medicinal purposes. In our earlier work, we found that allocating more Cd to the above-ground tissues is a significant threat to safe production of the plant in Cd-polluted soils. However, *Rhizophagus irregularis*-colonized plants could reduce Cd translocation from root to shoot (Motaharpoor et al., 2019). The aim of the present study was to determine whether *R. irregularis* symbiosis could modulate Cd-induced ROS-scavenging system at both enzymatic activity and transcript levels in alfalfa.

Material and Methods

Biological materials

Seeds of *M. sativa* var. Baghdadi were obtained from Agricultural Science and Natural Resources University of Khuzestan (Iran). Seeds were disinfected with 10% (v/v) sodium hypochlorite for 20 min, washed three times with distilled water and then germinated on moist filter papers at 28˚ C for about one week. Inoculum of *R. irregularis* was obtained from pot cultures of Berseem clover (*Trifolium alexandrinem* L.) described by Nadian et al. (2009). Five alfalfa plantlets were transplanted into each pot. Before planting, a loamy-sand soil was autoclaved for 2 h at 121 ˚C to remove all microorganisms. The chemical properties of the soil were characterized with a pH (7.5), ECe 2.6 dS m^{-1}, total N 0.05 %, organic C 0.76 % and available P and K 7.2 mg kg^{-1} and 267 mg kg^{-1}, respectively. In inoculation treatments, 0.1 g fresh inoculum was added at 1-2 cm below seedlings in each planting hole. Each pot of non-mycorrhizal treatments received an equivalent of autoclaved inoculum. Pots were transferred to a greenhouse for an average daily temperature of 25 ˚C and the relative air humidity of 50-75%. All pots were well irrigated every two days and 10 ml Smith's nutrient solution (Smith, 1982) was added to each pot per week throughout the experimental period. Two weeks after transplanting, 45 ml Cd (as $CdCl_2$) at concentrations of 100 mg kg^{-1} was added to each pot. The no Cd treatment only received distilled water in equivalent volume to the Cd treatment. Three weeks later, the plants were harvested for analysis of gene expression and antioxidant

Table 1
Primer sequences designed for qRT-PCR

Target gene	Accession number	sequence (5'-3')	Product size (bp)
Actin	XM-003621971.2	F: TCCCAGGCATTGCTGATAG R:GAACCTCCGATCCAGACAC	115
MsCu/Zn SOD	AF-056621.1	F: ACGAAAGTCCAACAACAGTC R: ATGTGGTCCTGTCGAGATAC	116
MsCAT	XM-013606823.2	F: CTGCTAATGCTCCCAAGTG R: TTTCTGCATGACGAACAGG	123
MsAPX1	XM-003606462.3	F: GGAACCATCAAGCACCAAG R: CAGCCAACTGGTAGAAATCAG	130
MsGR1	AM-407889.2	F: GGACGGTGAACCTGATTTG R: GAATCTAGCAGCACGAACAC	105

enzymatic activities. Root samples were frozen in liquid nitrogen and stored at -80˚ C until use.

Antioxidant enzyme assay

Three hundred (300) mg of root tissue was ground in 1.5 ml phosphate buffer (100 mM, pH 7) and centrifuged at 12000 g for 10 min. the supernatant was collected and then used for SOD, CAT, APX, and GR assays (Goud and Kachole, 2012). The SOD activity was determined by the method of Sun and Zigman (1978). One unit of SOD was defined as the amount of enzyme causing 50% inhibition of autoxidation of epinephrine to adrenochrome. The Nakano and Asada (1987) method was used for the APX activity. The activity of the enzyme was calculated by a reduction of absorbance at 290 nm for 1 min due to H_2O_2-dependent oxidation of ascorbate (extinction coefficient 2.8 mM $^{-1}$ cm $^{-1}$). CAT activity was assayed by the consumption rate of H_2O_2 at 240 nm (extinction coefficient 0.036 mM $^{-1}$ cm $^{-1}$) according to the method described by Aebi (1984). The GR activity was tested by the reduction of absorbance at 340 nm due to NADPH consumption (extinction coefficient 6.2 mM $^{-1}$ cm $^{-1}$) according to the method described by Schaedle and Bassham (1977).

Statistical Analysis

The experiment was arranged in a 2×2 factorial completely randomized design which consisted of two Cd concentrations (0 and 100 mg k^{-1}) and two inoculation treatments (non-mycorrhizal control and inoculation with *R. irregularis*) with three replicates per treatment for a total of 12 experimental units. Antioxidant enzymatic activities were analyzed by two-way ANOVAs to evaluate the significance of the effects of mycorrhizal colonization and Cd addition and their interactions. Mean comparisons were performed by Duncan's test, $P<0.05$ (SAS, Inc., 1999).

RNA extraction and qRT-PCR

Total RNAs were extracted from frozen root tissues using Monarch® Total RNA Miniprep Kit (NEB #T2010) and quantified by NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific Inc., USA). The first strand complementary DNA (cDNA) was synthesized by using Revert Aid ™ First Strand cDNA Synthesis Kit (Fermentas, USA), according to the manufacturer protocol. Gene-specific primers of *Actin, MsCu/Zn SOD*, *MsCAT, MsAPX*, and *MsGR1* were designed from the available coding DNA sequence (CDS) of *M. sativa* using online IDT Primer Quest™ Software Integrated DNA technology (Table 1). Quantitative real time-PCR (qRT-PCR) was performed using SYBR® Premix Ex Taq ™ II (TaKaRa Bio Inc, Tokyo, Japan) Master mix kit and Step One Plus® (ABI, USA) machine under the following conditions: an initial denaturation of 10 min at 95° C followed by 40 cycles, with one cycle consisting denaturation at 95° C for 20 s, annealing 60° C for 20 s and extension at 72° C for 15 s. The threshold cycle (Ct)

for each gene was normalized with *M. sativa Actin* gene as a reference gene.

Fold changes in transcript expression were calculated by the $2^{-\Delta\Delta CT}$ comparative method (Livak and Schmittgen, 2001). Relative expression software tool (REST) ® software (Pfaffl, 2001) was used to analyses the obtained data. For qRT-PCR analysis, three individual plants were used as biological replicates. For each biological replicate, two technical replicates were run**.**

Results

Gene expression in roots

Transcript expression analysis of antioxidant enzymes showed no significant change regarding the expression of *MsCAT* in roots of non-inoculated plants under Cd stress compared to 0 Cd treatment while the expression of *MsGR1* was significantly down-regulated in roots of NM plants with rising Cd concentration in soil. *MsCu/Zn SOD* and *MsAPX* transcripts were up-regulated under Cd stress compared to Cd 0 in NM plants (Fig. I. a). On polluted soil, AMF colonization distinctly reduced the level of *MsCu/Zn SOD* and *MsCAT* genes compared to NM counterparts however, transcript levels of *MsAPX* and *MsGR1* were not affected by mycorrhizal symbiosis under Cd stress (Fig.I. b).

Antioxidant enzymes activity

Enzymatic activity of CAT markedly increased ($p<0.05$) under Cd stress in non-mycorrhizal (NM) roots. However, SOD and GR activities significantly down-regulated with increasing Cd concentration. Further, Cd stress had no impact on APX activity. On polluted soils, AMF colonization reduced SOD and CAT activities compared to NM whereas GR activity was significantly enhanced by AMF symbiosis under Cd stress. No difference was observed in the activity of APX between NM and M plants due to Cd stress (Fig. II).

Discussion

The stimulated generation of ROS resulted from Cd causes an imbalance in cellular redox

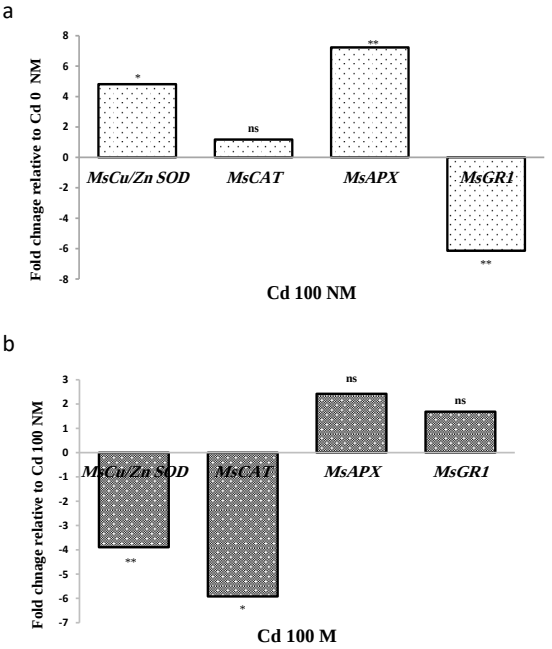

Fig. I. (a) Expression pattern of *MsCu/Zn SOD, MsCAT, MsAPX*, and *MsGR1* genes in roots of non mycorrhizal plants (NM) of *M. sativa* grown on 100 mg k^{-1} Cd (Cd 100) relative to those of control NM plants grown on 0 mg kg^{-1} Cd (Cd 0); (b) Expression pattern of *MsCu/Zn SOD, MsCAT, MsAPX*, and *MsGR1* genes in roots of mycorrhizal plants of *M. sativa* grown on 100 mg k^{-1} Cd (Cd 100M) relative to those of NM plants grown on 100 mg kg^{-1} Cd (Cd 100 NM). * and ** indicate that the mean values are significantly different between M and NM plants grown in 100 mg kg^{-1} Cd, respectively at 5% and 1% levels.

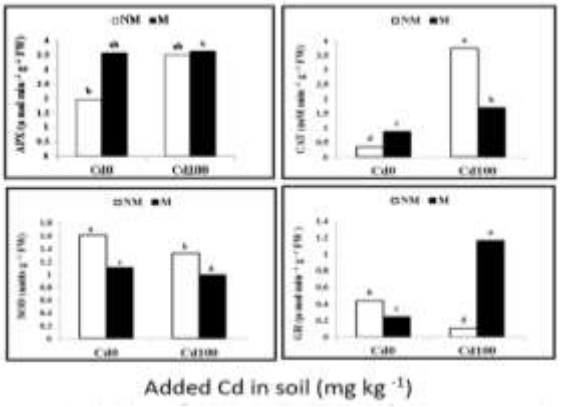

Fig. II. Activities of APX, CAT, SOD, and GR enzymes in roots of non-mycorrhizal (NM) and mycorrhizal (M) plants of *M. sativa* at Cd addition levels of 0 and 100 mg Kg^{-1}; different letters indicate significant difference between means according to the Duncan test at the 5% level.

status, lipid peroxidation, thereby leading to the disruption of cell membrane integrity (Singh and Shah, 2014). To minimize oxidative stress, plants stimulate ROS-scavenging systems by enhancing antioxidant enzymes activity (Morsy et al., 2012).

HMs have been shown to have different effects on gene expression and activity of different antioxidant enzymes in different plant species. Shahabivand et al. (2016) found that a reduction in CAT transcription is in agreement with change in wheat CAT enzyme activity as Cd concentrations increased. However, no correlation was observed between APX activity and its gene expression. Wu and Lee (2008) reported that high copper concentration significantly enhanced the enzyme activities and mRNA levels of *mnSOD*, *FeSOD*, GR, and CAT in *Ulva fasciata*. In contrast, in higher Cu concentrations, there was no coincidence between APX activity and its transcript abundance. Smeets et al. (2008) found that CAT activity was not significantly affected by excess Cd in leaves of *Arabidopsis thaliana* while its expression was transcriptionally up-regulated. On the other hand, GR enzymatic activity was reduced despite the accumulation of a high level of its transcripts. The data from our study indicated that among the antioxidant enzymes studied, only the activity of CAT was significantly increased under Cd stress while its transcriptional level was not influenced by the presence of Cd in soil suggesting that the induction of CAT activity by Cd was not under transcriptional control. In contrast, APX enzymatic activity did not change significantly whereas its transcript increased in response to Cd. As shown by Pourrut et al. (2011), an increase in the level of superoxide radicals resulting from Pb stress led to the induction in transcription of the APX antioxidant gene. In the case of SOD, Cd caused an induction in its transcriptional level, on the other hand, its enzymatic activity decreased in roots. It is likely that inactivation of the enzyme resulted from Cd binding to the active site (Fatima and Ahmad, 2005). In addition, it has been found that excess of ROS induced by Cd was likely to lead to protein denaturing and proteolytic degradation (Cuypers et al., 2010; Semane et al., 2010). Cd-led induction of enzymes involved in sulfur (S) assimilation pathway in higher plants was found to be associated with increased synthesis of cysteine (Cys) which acts as a rate-limiting factor for tripeptide GSH synthesis (Zechmann et al., 2008). Cys and GSH play vital role in Cd sequestration and its compartmentalization in plant vacuoles by phytochelatin (PC) biosynthesis (Gill and Tuteja, 2010). However, it has been reported that the first step of GSH biosynthesis which is catalyzed by γ-glutamylcysteine synthetase (γ-ECS) is inhibited by a highly ratio of reduced glutathione through the activity of GR. Therefore, reduced GR activity results in decreased GSH content and increased γ-ECS activity and subsequently an increased Cd detoxification capacity due to increased PCs synthesis (Noctor and Foyer, 1998). Similarly, a significant decrease in GR activity was observed in our study by increasing soil Cd concentration compared to control plants. In addition, the positive correlation between GR activity and its transcript abundance indicated that the reduced enzyme activity under Cd exposure is related to decreased expression of GR suggesting that regulation may occur at the level of transcription. However, Cd stress only enhanced the activity of CAT instead of SOD, APX, and GR in the roots of *M. sativa* under Cd stress suggesting that antioxidant enzymes differed in sensitivities to Cd contamination.

It is found that colonization with AMF can effectively alter expression of plant genes involved in alleviating metal toxicity (Rivera-Becerril et al., 2005). Our finding showed that inoculation of *R. irregularis* decreased enzymatic activities and transcription of SOD and CAT in comparison with un-inoculated plants. It is likely that metal sequestration in the vacuolar compartment of AMF structures probably made metal availability insufficient for the induction of ROS in plant roots, thereby decreasing SOD and CAT expression and enzymatic activities. In addition, AMF symbiosis could not induce the enzyme activity and transcription of APX. Similarly, AMF symbiosis had no significant impact on POD, CAT, and GR activities (Yang et al., 2015) probably due to buffering effect of mycorrhizal colonization on immobilization of the heavy metal within fungus vacuolar structures thereby, reducing HMs availability in plant cells (Azcón et al., 2009; Kaldorf et al., 1999). Shahabivand et al. (2016) also showed that GST activity was reduced under Cd stress after fungal inoculation. The coincidence

between enzymatic activities and transcript abundance of SOD, CAT, and APX implies that these antioxidant enzymes are regulated transcriptionally after fungal inoculation. In contrast, AMF colonization did not affect the expression of GR while it significantly enhanced its enzymatic activity under Cd stress. This phenomenon might be attributed to AMF-mediated post-transcription regulation. Similar results from induction of GR enzymatic activity in Cd contaminated soils due to AMF colonization have been reported by Jiang et al. (2016) in *Lonicera japonica* and Garg and Kaur (2013) and Garg and Aggarwal (2012) in *Cajanus cajan*.

In conclusion, in parallel with the down-regulation of *MsCu/Zn SOD* and *MsCAT* genes in root tissue of *R. irregularis* colonized plants grown in Cd contaminated soils, we showed a reduction in their enzymatic activities in the presence of AMF. It seems that buffering effect of mycorrhizal colonization on immobilization of Cd within fungal vacuoles reduced Cd availability in root cells. Therefore, the concentration of Cd may not suffice to induction of these genes in root tissues that contain fungal mycelium and arbuscules structures. Comparative analysis of mRNA levels and enzymatic activities revealed that among antioxidant genes, in non-colonized plants, expression of only GR was regulated at transcriptional level under Cd stress. In contrast, in comparison with other enzymes that showed a coincidence between enzymatic activities and their transcript abundance in inoculated plants, GR activity was not affected by its gene expression under Cd. It might be possible that AMF inoculation mediated post-transcription regulation of this enzyme. However, detailed molecular mechanism of these findings need more studies. Furthermore, improved activity of GR by AM symbiosis has probably had positive impact on regulation of the ascorbate-glutathione cycle and redox balance maintenance. The results presented here may stimulate further studies for better understanding of the molecular mechanism of alleviation of plant Cd toxicity by AMF.

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