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Research Article

Cadmium Tolerance in Alfalfa is Related to the Up-regulation of Iron and Sulfur Transporter Genes along with Phytochelatin Accumulation

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ABSTRACT

Cadmium (Cd) toxicity is a serious limitation for agricultural production. In this study, we explored tolerance mechanism associated with Cd toxicity tolerance in alfalfa plants. We used three distinct alfalfa cultivars *M. sativa* cv. Vernal, *M. sativa* cv. Zhung Mu, and *M. sativa* cv. Xing Jiang Daye in this study. Cd showed declined chlorophyll score in Xing Jiang Daye compared with Zhung Mu and Vernal. No significant change observed among the cultivars for root and shoot length. Atomic absorption spectroscopy analysis demonstrated a significant accumulation of Cd, Fe, S and PC in distinct alfalfa cultivars. However, Zhung Mu and Xing Jiang Daye declined Cd accumulation in root, where Fe, S and PC incremented only in Zhung Mu. It suggests that excess Cd in Zhung Mu possibly inhibited in root by the increased accumulation of Fe, S and PC. This was further confirmed by the response of Fe (*MsIRT1*) and S transporters (*MsSULTR1;2* and *MsSULTR1;3*), and *MsPCS1* genes associated with Fe, S and PC availability and translocation in roots and shoots. It suggests that specially the transcript signal inducing the responses to adjust Cd especially in Zhung Mu. This finding provides the essential background for further molecular breeding program for forage crops.

(**Key words**: Cadmium, Alfalfa, Transporter gene, Phytochelatin)

Ⅰ. INTRODUCTION

Cadmium (Cd) is toxic metal that induces plant injury and human health risk (Genchi et al., 2020). Cd releases in soil environment by improper management of industrial waste and agro-chemicals (Roberts, 2014). Cd absorbed by plant roots and found to be located in distinct organs and tissue (Parrotta et al., 2015). Cd induced toxicity causes plant retardation, and photosynthetic disturbance in plants (Dias at al., 2013). Plans have evolved some tolerance mechanisms, which support it to alleviate heavy metal toxicity.

Phytochelatins (PCs) are small metal-binding peptides found in plants, function as detoxifying agents against Cd toxicity through its ability to bind Cd (Cobbett et al., 2000). Several mineral like Fe and S concentration and their transporter genes are associated with alleviation of Cd toxicity in plants (Lu et al., 2019). In addition, sulfate transporter genes such as *SULTR 1;2,* and *SULTR 1;3* were reported to induced in plants, which are associated S regulation in plants (Cui et al., 2020). Although heavy metals toxicity is known to involved in the inhibition plant growth along with metabolic and molecular disturbance in plants (Kim et al., 2017), the mechanism associated with Cd tolerance in forage crops is not yet to be explored. Therefore, this study was design to explore Cd tolerance mechanism in forage crops.

Alfalfa is considered as queen of forages due to its qualitative and quantitative attributes. Alfalfa is worldwide cultivated forage legumes, improving soil quality by fixing atmosphering N_2 (Zhao et al., 2020). However, these benefits of alfalfa are noted to be disturbing by multiple toxic metals/metalloids. Therefore, it is imperative to explore susceptibility and tolerance mechanism in plants in response to toxic metals/metalloids. In this study, we assessed susceptibility of distinct alfalfa cultivar to Cd toxicity. Further, we investigated the expression of Fe and S transported genes, which are associated with metals/metalloids toxicity tolerance in plants.

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Ⅱ. MATERIALS AND METHODS

1. Plant growth and Cd treatment

Seed of three distance alfalfa (*Medicago sativa* L. cv. Vernal; *Medicago sativa* L. cv. Zhung Mu; *Medicago sativa* L cv. Xing Jiang Daye) seeds were surface sterilized using 70% ethanol for 2 min, and then washed properly by deionized water. Seeds were then placed in a germination tray for 2 days followed by alfalfa seedling were moved to Hoagland nutrient basal salt (Phyto. Tech. Lab, USA). In addition to this nutrient solution, 25 μM $CdCl_2$ was supplemented for three cultivars for 1 week, respectively. Seedlings were treated individually for each treatment with three replications in a plastic container (1 L) and cultivated in a controlled environment with 14 h light and 10 h dark photoperiod (550–560 μmol s^{-1} per μA) at 25°C. After one week alfalfa plants were harvested for physiological and molecular analyses. In several experiments, root and shoot were excised then washed properly to remove excess Cd, and the samples were kept at -80°C until further use.

2. Plant growth and photosynthesis parameters

Three cultivars of alfalfa seedlings were gently taken out after treatments, followed by root and shoot length was measured using a metric scale (cm). The chlorophyll score of young alfalfa leaves was measured using SPAD (soil plant analysis development) meter (Minolta, Japan). In the data program, the SPAD device read the differences between a red and an infrared (650-940 nM) through the leaf, producing a three-digit SPAD value.

3. Elemental concentration in root and shoot

Elemental concentration in root and shoot of three distinct cultivars was determined according to the method described previously (Rahman et al., 2020). Root and shoot of three treated cultivars were separated from the root-shoot connection zone. In order to remove excess Cd from root surface, roots were washed with distilled water. The root and shoot of three distinct alfalfa cultivas were separately dried in micro-oven at 80°C for 72 h. Subsequently, digestion of plant samples was performed using a mixture containing $HNO_3/HClO_4$ (3:1 v/v). Elemental (Cd, Fe, and S) concentration in digested solution was measured according to the standard known solution of that specific element by the ICP-MS system (Agilent 7700, ICP-MS).

4. Determinatin of phyochelatins (PCs)

The level of GSH in alfalfa sample was determined according to method described earlier (Hissin and Hilf,1976). Briefly, 100 mg of tissue sample was homogenized with 5% sulfosalicylic acid (1:10 w/v). Subsequently, the solution was mixed well then centrifuged at 4°C with 13,000 rpm for 15 min. The aliquots of supernatant ware used for the assaying for PCs level. A reaction mixture was prepared with 2 mL of 0.2 M Tris buffer solution (TBS, pH 8.2) and 0.15 mL of 10 mM DTNB (5,5′-Dithiobis-2-nitrobenzoic acid). The mixture was incubated at room temperature for 30 min. The absorbance of supernatant was determined at 412 nm. The non-protein thiols (NPTs) and PCs levels were quantified in terms of GSH equivalents, and the level of PCs was estimated from the difference between the NPTs and GSH (PC= NPTs-GSH).

5. Gene expression analysis

Total RNA was isolated from the treated alfalfa samples using RNeasy® plant mini kit (QIAGEN, Germany). Briefly, 100 mg of plant tissue was homogenized with RNA extraction buffer containing 2M Dithiothreitol (DTT) followed by centrifugation (≥12,000 rpm) for 2 min. Total RNA was obtained from the supernatant and RNA yield was recovered by adding 30-50 μL RNase-free water. RNA quantification was carried out using a micro-volume UV/Vis spectrophotometer (UVIS Drop-99, Taiwan). Samples with RNA concentration ≥200 ng/μL were selected for subsequent analysis. cDNA synthesis was performed with 1 μg of total RNA using cDNA synthesis kit (Bio-Rad, USA). Quantitative real time polymerase chain reaction (q-RT-PCR) was conducted by CFX96 Real Time system (BIORAD, USA) for the expression of target genes using gene specific primers: *MsActin2* Forward- ACCGGTGTGATGGTTGGTAT, *MsActin2* Reverse- GCCACACGAAGCTCATTGTA; *MsIRT1* Forward-TTTACCCTTGGCGACACGTT, *MsIRT1* Reverse-CATGAAC CCGGTCCCAAGAA; *MsPCS1* Forward- ACCCTTCCTCCAC CTTCAAT, *MsPCS1* Reverse- CCAGGGTCAATA GCAAGA GC; *MsSULTR1;2* Forward- TATTATCTCCGTGTTGAAGGC, *MsSULTR1;2* Reverse- CAATAAATTTGGCGACCAG, *MsSULTR1;3* Forward- ATTTATGCCGTCATGGGTAG, *MsSULTR1;3* Reverse-TTGGGATCAATCTCATTCTGTA. Total 20 μL reaction mixture consisted of 10 μL of iQ™ SYBR® Green Supermix, 2 μL of template cDNA, 0.8 μL of forward primer (10 μM), 0.8 μL of

reverse primer (10 μM), 6.4 μL of DEPC treated H_2O. The amplification was performed using the following programs: 95 °C for 30 sec, followed by 40 cycles at 95 °C for 5 sec, 60 °C for 30 sec. The relative gene expression were analyzed using the $dd^{-\Delta Ct}$ method (Livak and Schmittgen 2001), where *MsActin* used as internal control.

6. Statistical analysis

All physiological and molecular data was statistically analyzed using analysis of variance (ANOVA). Duncan's Multiple Range Test (DMRT) or *t*-test was done to measure the significant differences at $P \leq 0.05$. We used software SPSS 20.0 and GraphPad Prism 8.4.3 for the analyses. Three or more independent replications were considered for the analysis.

Ⅲ. RESULTS AND DISCUSSION

In this study, morphological, physiological and molecular responses were obvious, which were associated with Cd tolerance in distinct alfalfa cultivars. Chlorophyll score was significantly reduced in Xing Jiang Daye compared to Vernal and Zhung Mu. Chlorophyll score was significantly higher in Zhung Mu. The root and shoot length were not showed any notable changes among the three alfalfa cultivars (Fig. 1 a-c). Elemental concentration was significantly changes under Cd stress. Cd analysis showed higher accumulation of Cd in Vernal root compared with Zhung Mu and Xing Jiang Daye where no significant change has been found in shoot among the cultivars (Fig. 2a). Fe analysis showed a significant up-regulation of Fe in Zhung Mu root and shoot compared with Vernal and Xing Jiang Daye (Fig. 2b). Zhung Mu cultivar showed a significant up regulation of S accumulation in root and shoot, where Zhung Mu showed the highest S accumulation compared to the rest of the cultivars (Fig. 2c). Phytochelatin (PC) showed a significant accumulation in Zhung Mu root compared with vernal and Xing Jiang Daye though PC showed no change due to Cd in shoots in three distinct alfalfa cultivars (Fig. 2d).

The expression of Fe and S transporter genes were induced differentially in alfalfa. The expression of *MsIRT1* was significantly up regulated in root and shoot where regulation was higher in shoot of Zhung Mu (Fig. 3a). The expression of *MsPCS1* showed

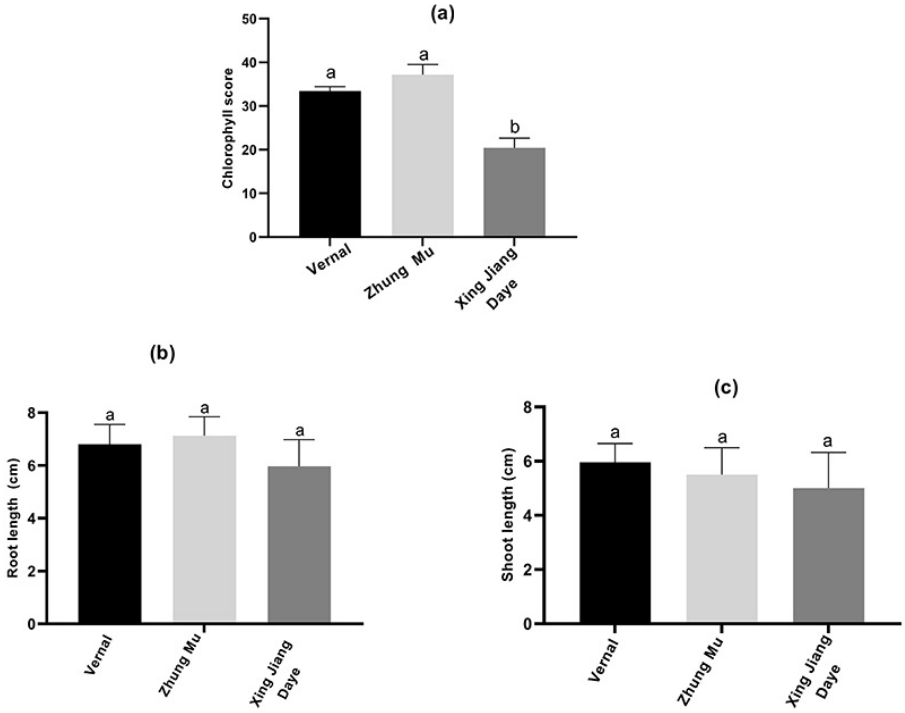

Fig. 1. Morpho-physiological changes of alfalfa under cadmium stress. Chlorophyll score (a), root length (b), shoot length of vernal, Zhung Mu and Xing Jiang Daye alfalfa cultivars under cadmium stress. Data represent means±SD of three independent biological samples. Different letters indicate statistically difference at P(0.05 level.

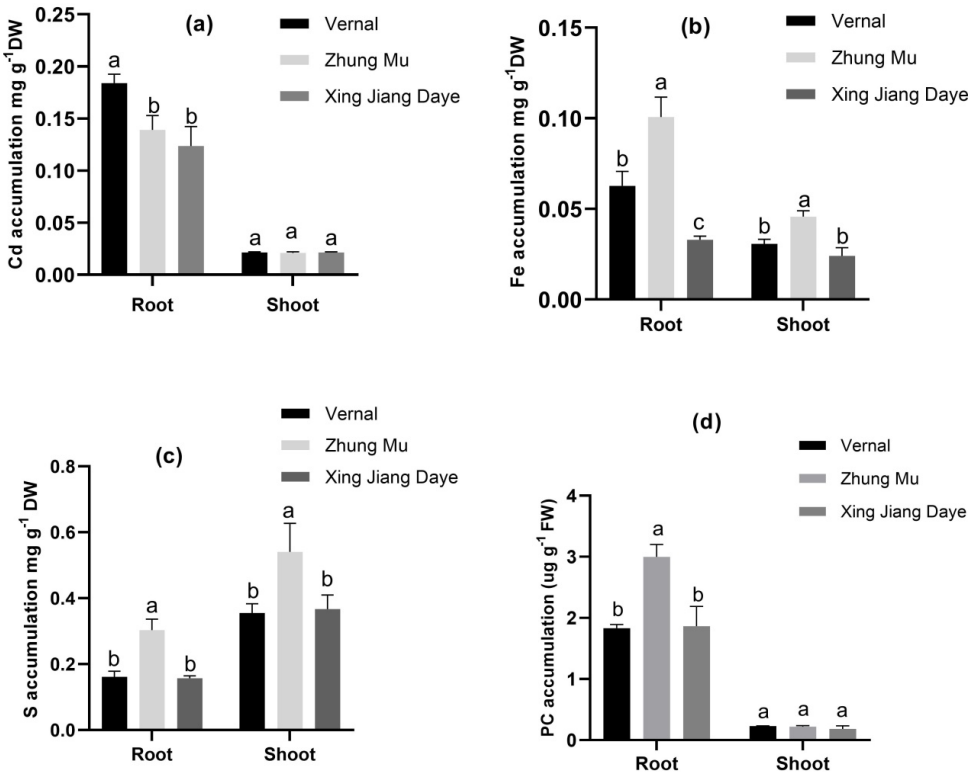

Fig. 2. Elemental concentration in alfalfa roots and shoots. Cd accumulation (a), Fe accumulation (b), S accumulation (c), PC accumulation (d) in roots and shoots of Vernal, Zhung Mu and Xing Jiang Daye cultivars under cadmium stress. Data represent means±SD of three independent biological samples. Different letters indicate statistically difference at P<0.05 level.

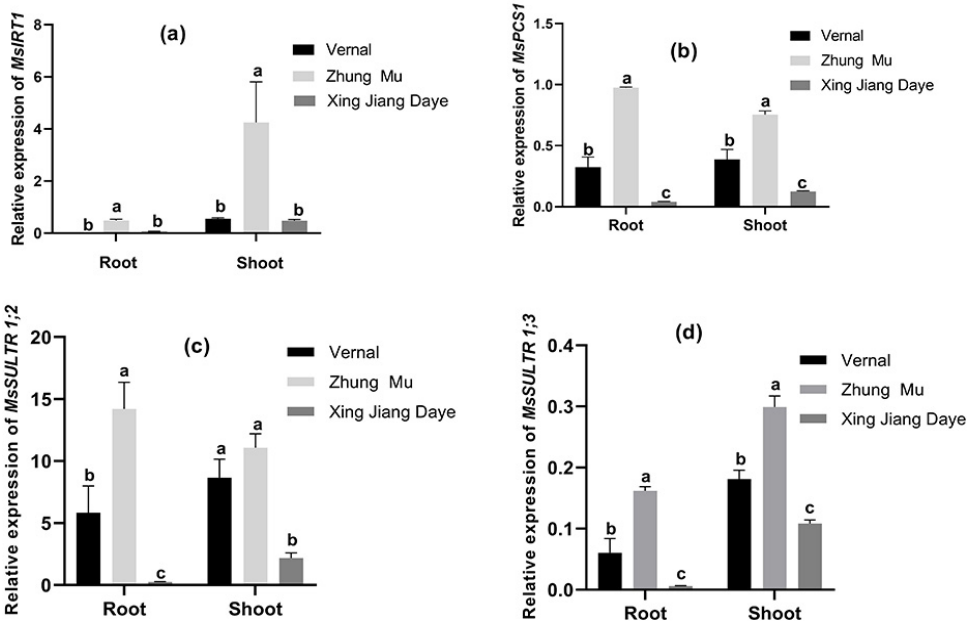

Fig. 3. Relative expression of candidate genes in roots and shoots of distinct alfalfa cultivars. *MsIRT1* (a), *MsPCS1* (b), *MsSULTR1;2* (c), *MsSULTR1;3* (d) in roots and shoots of Vernal, Zhung Mu and Xing Jiang Daye cultivars under cadmium stress. Data represent means±SD of three independent biological samples. Different letters indicate statistically significant difference at P<0.05 level.

a significant upregulation in root and shoot in Zhung Mu. However, the upregulation significantly higher in root (Fig. 3b). S transporter *MsSULTR 1;2* and *MsSULTR 1;2* were significantly induced in distinct cultivars, where Zhung Mu showed notable changes of expression compared with Vernal and Xing Jiang Daye (Fig. 3c-d).

Although heavy metals are known to cause in plant growth inhibition with metabolic and molecular disturbance in plants (Kim et al., 2017), the mechanism related to Cd tolerance in forage crops is little in known. Therefore, to explore Cd tolerance mechanism in fodder is highly desirable. In this study, we noticed that root growth was significantly effected but in case of a comparative observation, no change was observed among the cultivars. Photosynthesis was found to disturbing by the phytotoxicity of heavy metals (Patra et al., 2004). In this study, the alteration of photosynthesis was observed by Chl florescence under Cd stress in alfalfa cultivars. The decline of chlorophyll in Xing Jiang Daye indicate that this cultivar was more sensitive compared to Zhumg Mu and Vernal. It has been found that Cd stress declines chlorophyll reduction in plants (Muradoglu et al., 2015).

The ICP-MS analysis showed that Cd contents reduce in Zhung Mu. In contrast, Fe and S concentration were increased. This suggests that accumulation of Fe and S actively function on the inhibition of Cd uptake and toxicity homeostasis in Zhung Mu cultivar. To explore more insight into mechanisms related to gene expression, we found up regulation of Fe and S transporter (*MsIRT1, MsSULTR1;2* and *MsSULTR1;3*) and PC synthesis related *MsPCS1* genes suggesting that Fe and S triggering Cd toxicity regulation. Lu et al (2019), reported that *MsSULTR1;2* and *MsSULTR1;3* involved in Cd homeostasis in plants.

Ⅳ. CONCLUSION

The Zhung Mu cultivar showed substantial tolerance of Cd compared with Vernal and Xing Jiang Daye. As a consequence, photosynthesis efficiency was declined considerably in Vernal and Xing Jiang Daye. The metabolic and molecular analysis consistently indicated elevation of Fe and S transporter genes in Zhung Mu. This study also demonstrated that *MsPCS1* was correlated with phytochelatin accumulation that supports Zhung Mu to induce tolerance against Cd toxicity.

Ⅴ. ACKNOWLEDGEMENTS

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