Bangladesh J. Bot. **46**(1): 249-253, 2017 (March) **- Short communication**

RESPONSE OF ANTIOXIDANT DEFENCES TO SELENIUM STRESS IN ALFALFA LEAVES

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Key words: Selenium stress, Alfalfa, Antioxidant defences

Abstract

After three-month cultivation, the plants were treated with three Na_2SeO_3 concentrations of control, 100 and 900 μM for 20, 40 and 60 days. Selenium (Se) induced a increase in SOD, CAT, APX activities and the contents of MDA, soluble protein but an decrease in POD activities and free proline contents in leaves. The activities of SOD, CAT, APX, MDA, soluble protein increased significantly with increased concentration of Se, while the activities of SOD, CAT, and APX increased at 100 μM Se stress and then CAT activities decreased at 900 μM stress. POD activities decreased upon exposure to any levels of Se. Se pretreatment had little effect on the enzymatic components of seedlings grown under normal conditions. The results indicate that the exogenous application of Se at low concentrations increases the tolerance of plants to Se-induced oxidative damage by enhancing their antioxidant defense and Se detoxification systems.

For adult human, the recommended dietary allowance of selenium (Se) - an essential micronutrient is estimated at between 40 and 70 μg per day (World Health Organization) with meat, seafood and cereals being the main sources of dietary Se in China. Selenium is a metalloid that resembles sulphur (S). In trace amounts, Se is considered an essential element for human and animal health (Mehdawi *et al*. 2014). It is a constituent of selenoenzymes such as glutathione peroxidase (GSH-Px), thioredoxin reductases (TR), proteins implicated in Se transport (selenoprotein P) and proteins with unknown functions that are involved in maintaining the cell redox potential (Zeng and Comb 2008, Pazoki *et al*. 2010, Cambrollé *et al*. 2012) because of its critical role in organic antioxidant defense systems (Inostroza-Blancheteau *et al*. 2003) and cancer prevention (Dai *et al*. 2012).

Se is a metalloid that can occur naturally in soils from the Enshi and Ziyang in the western China. Agricultural irrigation and runoff dissolves Se from these soils, causing accumulation of toxic levels of selenate (SeO_4^{2-}) in water and soil. Selenate is the most common species of Se found in the root zone (Bañuelos *et al*. 2010) and can contaminate both water and soil (Mehdawi *et al*. 20111). Therefore, it has negative effects on plants at high concentrations by reducing growth and causing leaf chlorosis, and nutritional disturbances. The deleterious effects of selenium on photosynthesis is associated with Se-induced decreases in electron transport yields in photosystem II (PSII) and pigment biosynthesis (Germ *et al*. 2005, Zahedi *et al*. 2009), as well as Se-induced oxidative damage (Mechora *et al*. 2013) and (Funes-Collado *et al*. 2013). In order to detoxify the excess of reactive oxygen species (ROS), plants have antioxidant enzymatic systems involving superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), guaiacol peroxidase (POD, EC 1.11.1.7), and glutathione reductase (GR,EC 16.4.2). These functionally interrelated enzymes can provide protection by eliminating ROS to minimize oxidative damage in plant when response to environmental stress. A coordinated increase in enzymatic antioxidants (SOD, APX and GR) was noticed in response to different Se concentrations in ryegrass (Hartikainen *et al*. 2000, Cartes *et al*. 2011). Similarly, the antioxidant enzyme activities

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(SOD, CAT and APX) increased in Se-treated sorghum and wheat (Djanaguiraman *et al*. 2010, Inostroza-Blancheteau *et al*. 2013). Redox-active metals (i.e. Zinc) are also known to cause both direct oxidative damage and antioxidative protection (Cambrollé *et al*. 2012), although little is known about the role of non-redox active metals (as such Se) in oxidative pathways.

Alfalfa (*Medicago sativa* L.) is an important leguminous forage and has a wide distribution in the world. In this study, we first constructed Se tolerance and related tolerance mechanisms in alfalfa by evaluating their antioxidant system activities (SOD, CAT, APX and POD) when exposed to increasing Se doses. This study will help us to better understand the physiological regulation mechanisms of forage in response to Se exposure and provide new insights into engineering forage for phytoremediation.

The experiments were performed in the orchard of Shaanxi University of Technology, Hanzhong (33°34′N, 107°28′E), P. R. China. Alfalfa (*Medicago sativa* ssp.) seeds were purchased from Northwest A&F University. Seeds were surface-sterilized with 0.1% (w/v) $HgCl_2$ for 10 min. Alfalfa (*Medicago sativa* ssp.) seeds were sown in plastic pots filled with a peat/vermiculite matter mix (w/w 1 : 1) and grown in a greenhouse under a day/night temperature of 25/15ºC, 500 μmol/m^2/s photosynthetic active radiation and a 12 hrs photoperiod. The nutrient solution includes 5 mM $Ca(NO_3)_2{\cdot}4H_2O$, 5 mM KNO_3, 2 mM $MgSO_4{\cdot}4H_2O$, 1 mM KH_2PO_4, 0.1 mM EDTA-Fe, 46l mM H_3BO_3, 9.1l mM $MnCl_2{\cdot}4H_2O$, 0.32l mM $CuSO_4{\cdot}5H_2O$, 0.76l mM $ZnSO_4.7H_2O$ and 0.5l mM $H_2MoO_4{\cdot}H_2O$ (Dai *et al*. 2013). After three-month cultivation, the plants were treated with 0, 100 and 900 μM Se by adding Na_2SeO_3 into the nutrient solution, respectively. Six repeats were obtained for every treatment.

The seedlings were harvested after 20, 40 and 60 days of exposure. The roots were immersed in 5 mM $CaCl_2$ for 15 min and then the whole plants were rinsed with deionized water. The leaves were divided and dried in an air oven at 80°C until a constant dry weight was gained. The effects of zinc treatment on the dry weight of leaf were assessed by two-way ANOVA.

The activity of ascorbate peroxidase (APX, EC 1.11.1.11), superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and guaiacol peroxidase (POD, EC 1.11.1.7) after Dai *et al.* (2012) were determined.

Free proline and soluble protein were measured by the method of Dai *et al.* (2015a). The malonaldehyde (MDA) concentrations in plant materials were analyzed spectrophotometrically at 450, 532 and 600 nm as described previously.

A completely randomized design was used for each time point with six replicates. Data were subjected to analysis of variance (ANOVA) to examine the effects of time, treatment and species. Statistical analysis was conducted by using STATISTICA 5.1 software (Statsoft Inc., United States of America). Separation of means was carried out by using Fisher's LSD test at $p < 0.01$ and $p < 0.001$ significance levels.

After 20 days of Se exposure, the contents of MDA, soluble protein and free proline significantly increased when the Se stress increased to 100 and 900 μM, compared to control (non-Se) (Fig.1a,b,c), the MDA concentration in alfalfa leaves increased after 0, 100 and 900 μM Se exposure, respectively (Fig. 1a). After 40 days, under 100 and 900 μM Se treatment led to 40.6 and 62.7% increase in MDA levels, and after 60 days, 23.2 and 32.3% enhancement was observed over the untreated control (Fig. 1a). However, after 60 days, free proline content in alfalfa leaves decreased by 4.1% with 100 μM Se and by 31.6% with 900 μM Se as compared with control leaves, respectively ($p < 0.01$). Exposure to 100 and 900 μM Se also resulted in an increase in the soluble protein content, compared to control (Fig. 1c). After 60 days of Se treatment, the soluble protein level was further increased by 6.3 and 20.3% in comparison with the control, respectively ($p < 0.01$).

As selenium application increased from 100 and 900 µM, the activities of SOD and CAT in leaves (Table 1) showed increasing trend during the days 20. After 60 days, SOD activities in alfalfa leaves increased by 3.7% with 100 µM Se and by 13.4% with 900 µM Se as compared with control leaves, after 100 µM Se exposure, the CAT activity in leaves increased 11.3%, however, exposure to 900 µM Se, the CAT activity was decreased 37.3%, compared to control. After 20 days, APX activity decreased linearly with decreased Se levels, compared to control. In contrast, after 60 days, when compared to the control, the APX activity was increased with the 100 and 900 µM Se treatment of leaves. According to the results, POD activity decreased linearly with increasing Se levels. The highest concentration of Se (900 µM) proved to be extremely toxic resulting in a decline of POD activity (Table 1).

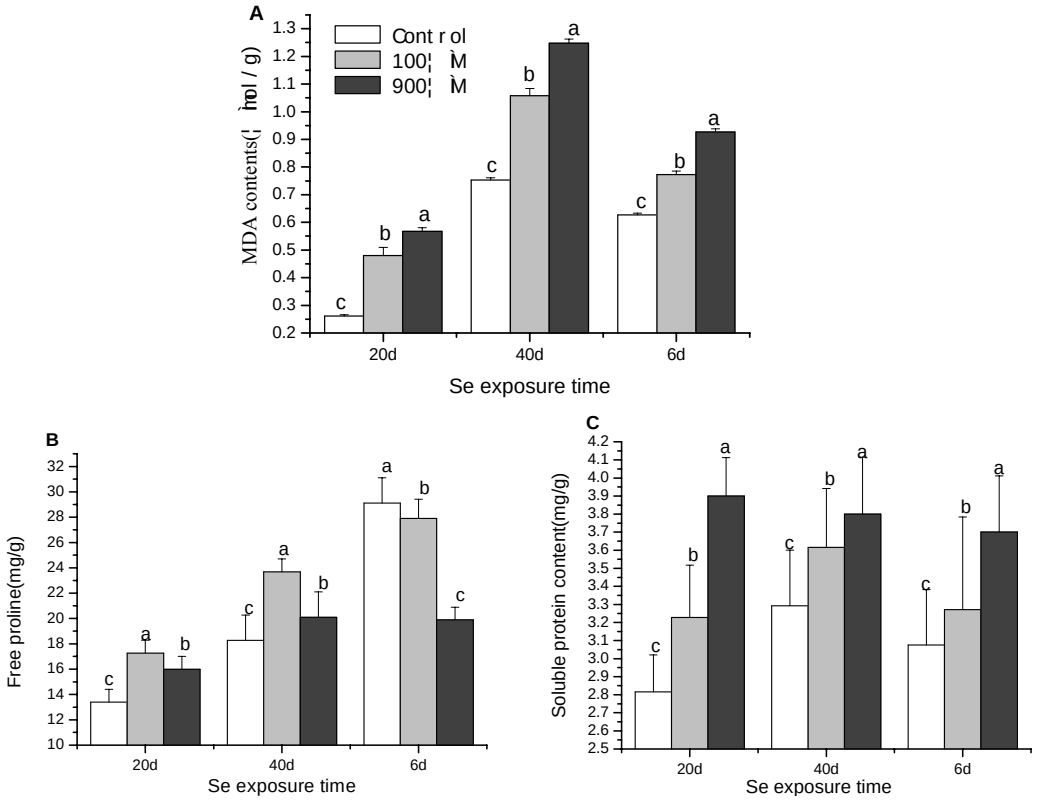

Fig. 1. Changes of MDA, free proline and soluble protein contents in alfalfa leaves under Se stress. Values are means of six replicates ± standard deviation. Different small alphabets are representing the significant difference at $p \leq 0.01\%$.

Plant stress tolerance to heavy metals is often correlated to an increase in the activity of antioxidant enzymes (Dai *et al*. 2012, Dai *et al*. 2015b). Plants which do not have strong antioxidative enzymes system may experience oxidative stress. In addition to nonenzymatic antioxidants, antioxidative enzymes are of great importance for the plants against ROS, such as APX, SOD and CAT (Pazoki *et al.* 2010, Freeman and Bañuelos 2011). The increased activity

Table 1. Changes of SOD, CAT, APX and POD activities (U/mg FW) of alfalfa leaves grown with 0 (control), 100 and 900 μM Se for 20, 40 and 60 days.

Se (μM)	Time (days)	SOD	CAT	POD	APX
	20	307.7 ± 30.1b	21.2 ± 2.1d	35.3 ± 3.9b	257.1 ± 22.2c
Control	40	252.8 ± 23.8d	48.4 ± 4.6bc	47.4 ± 4.1a	385.7 ± 32.6b
	60	257.5 ± 23.3cd	56.5 ± 5.1b	37.6 ± 3.2b	128.6 ± 11.2e
	20	370.2 ± 29.1a	35.3 ± 3.3c	37.1 ± 3.1b	118.6 ± 10.2e
100	40	265.1 ± 26.6c	42.4 ± 4.1bc	51.8 ± 5.1a	259.2 ± 23.5c
	60	267.1 ± 23.6c	50.1 ± 4.8b	34.3 ± 3.5bc	158.6 ± 13.8d
	20	332.3 ± 31.2ab	49.4 ± 5.0ab	28.2 ± 2.9c	214.3 ± 19.6cd
900	40	273.3 ± 28.1c	54.5 ± 5.3b	32.9 ± 3.1c	514.3 ± 49.2a
	60	292.0 ± 28.3b	77.6 ± 7.2a	25.9 ± 2.2d	200.6 ± 18.8d
p values	Se	**	***	***	**
	Time	***	**	**	***
	Se × time	***	***	ns	***

Each point represents the mean of six replicates ± S.E. Data were presented as mean ± St (n = 6). Different letters behind the values in the same column indicate significant difference between the treatments, p values of ANOVA of Na_2SeO_3 (Se), time and their interactions were also shown. Represents the significance level at *p ≤ 0.05%, **p ≤ 0.01%; *** p ≤ 0.001%. ns, not significant.

of antioxidative enzymes in a plant indicates the formation of ROS, and the (Dai *et al*. 2015a, Zhao and Dai *et al*. 2015) indicated the generation of oxidative stress in plants since all enzyme activities increased with high Se levels, which was consistent with our experimental results. Here, we showed that alfalfa responded to Se exposure with increases in concentrations of soluble protein in leaves and with enzymatic activities of APX, SOD and CAT in leaves (Table 1). These results indicated that the antioxidant system is enhanced and well-orchestrated in the leaves of alfalfas under different Se concentrations.

Acknowledgements

This work was financially supported by Key Laboratory of Scientific Research Program Foundation of Shaanxi Provincial Education Department (15JS019), Natural Science Foundation of Shaanxi Province (2015JM3086), supported by Foundation of Gansu Key Laboratory of Biomonitoring and Bioremediation for Environmental Pollution (GBBL 2015006), supported by National Natural Science Foundation of China (41571300), and supported by Qinling-Bashan Mountains Bioresources Comprehensive Development, Collaborative Innovation Center (QBXTZ(P)-15-7).

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(Manuscript received on 30 December, 2015; revised on 28 February, 2016)