

Research Article

An assessment of oxidative stress and antioxidant system activity in alfalfa plant treated with different forms of mineral arsenic

Farnoosh Zargari¹, Latifeh Pourakbar^{1*}, Seyed Yahya Salehi-Lisar², Jafar Razeghi² and Rouhollah Motafakker Azad²

¹Department of Biology, Faculty of Sciences, Urmia University, Urmia, Iran

²Department of Biology, Faculty of Natural Sciences, University of Tabriz, Tabriz, Iran

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Abstract

Arsenic (As), a metalloid and an unnecessary element, is highly toxic to living organisms. It can be accumulated in the environment and enter the food chains through uptake by plants. The present study was designed to assess the response of antioxidant defense system of alfalfa to As (As⁰, As³⁺, and As⁵⁺) induced oxidative stress at four rates (0, 100, 200, and 300 μM) as a completely randomized design with three replications in the laboratory conditions. Tukey test was used for data analysis. The 90-day seedlings after harvesting, were used to evaluate MDA and H₂O₂ contents, antioxidant system, including SOD, POD, APX, CAT activities, contents of phenol, flavonoids and anthocyanin. According to the results, root and shoot H₂O₂ contents of As³⁺-treated plants, were the highest at the rate of 300 μM and the MDA contents were the highest at the rates of 200 and 300 μM in roots and shoots, respectively. However, in 200 μM of As⁰-treated plants, the MDA content showed a significant decline (P ≤ 0.05). The antioxidant responses varied with different treatments. Increased free radical scavenging activity was only observed at lower rates of As⁵⁺ in the shoots. Consequently it can be concluded that all of three forms of As, induced oxidative stress, which As³⁺ (300 μM) was more toxic than As⁰ and As⁵⁺. Nonetheless, the alfalfa plant mitigated As-induced oxidative stress by manipulating the activity of their antioxidant system through increasing contents of total phenol, flavonoids, anthocyanin and changing antioxidant enzymes activities, in different types and rates of As.

Keywords: Arsenic, Enzymatic antioxidant, *Medicago sativa* L., Non-enzymatic antioxidant, Oxidative stress

Introduction

A serious challenge facing humans is the environmental problems arising from heavy metals, e.g. Cu, Ag, Pb, Ni, Cr, and Cd, and metalloids, e.g. arsenic (Choppala *et al.*, 2014). Arsenic (As), a metalloid from the group 5 of the periodic table, is among the most abundant elements on the earth's crust so that it is the 20th abundant element on land with the distribution of 1.5-3 mg kg⁻¹ (Zhao *et al.*, 2010; Drewniak and Sklodowska, 2013), the 14th most abundant element in seawater, and the 12th most abundant in human body (Mandal and Suzuki, 2002). This element can find its way into the environment both through the natural resources (volcanic activity, weathering of rocks and stones, hot springs, and so on) and human activities (mining, the use of wood protectors, fossil fuels, As-containing fertilizers and insecticides, and so on) (Zhou *et al.*, 2016; Khalid *et al.*, 2017) and get into human body through food chains after being absorbed by plants and accumulated in their edible parts. In addition, humans can also be exposed to As through As-contaminated drinking water and the breathing of particles in the atmosphere (Bhattacharya *et al.*, 2010). According to the World Health Organization (WHO) and the US Environmental Protection Agency (EPA), the threshold

level for As in drinking water is 10 μg L⁻¹ (Zhou *et al.*, 2016). Many people around the world are exposed to the risk of As due to the use of As-contaminated groundwater for irrigation or drinking (Chatterjee *et al.*, 2017). This element is unnecessary for living organisms including plants (Khalid *et al.*, 2017; Zhao *et al.*, 2009; Farooq *et al.*, 2015). Furthermore, exposure to As is a serious threat to the health of living organisms, and according to the Agency for Toxic Substances and Disease Registry (ATSDR), As has the leading rank among the top 20 most dangerous substances (Banerjee *et al.*, 2013; ATSDR, 2013). The International Agency for Research on Cancer (IARC) and EPA have classified As and its compounds as group 1 carcinogens that can cause skin, blood and kidney cancers in humans (Mehmood *et al.*, 2017; Abid *et al.*, 2016). Plants are unable to prevent As from entering their cells but can modulate its toxicity by regulating its concentration at a certain level (Srivastava *et al.*, 2015). Arsenic occurs in the Earth crust in organic forms, e.g. monomethyl arsenic acid (MMA), dimethyl arsenic acid (DMA), arsenobetaine, and As-containing sugar compounds, or mineral forms, e.g. arsenates (60%), arsenites, arsenides, silicates, oxides, and elemental form (20%), sulfide and sulfur salts (20%) (Mandal and Suzuki,

*Corresponding Author, Email: l.pourakbar@urmia.ac.ir

2002). In some researches, have reported that mineral types of arsenic (arsenite and arsenate), are more toxic than its organic types and are carcinogenic (Martinez *et al.*, 2011; Finnegan and Chen, 2012). However, some recent researches have been reported contradictory results (Duncan *et al.*, 2017). Arsenate and arsenite are very similar in structure to phosphate and borate ions respectively. Arsenate can replace phosphate in important metabolic reactions and thus can interfere with cellular processes such as photophosphorylation, oxidative phosphorylation, glycolysis, RNA/DNA metabolism, and ATP synthesis, ultimately disrupting energy flow (Finnegan and Chen, 2012; Shahid *et al.*, 2018). Arsenic creates oxidative stress in plants through generation of surplus ROS (Carlin *et al.*, 2016; Rafiq *et al.*, 2017). MDA, a byproduct of lipid peroxidation, and H_2O_2 are biomarkers of As-induced oxidative stress in plant cells. A considerable increase in the level of MDA and H_2O_2 has been reported in different plants (Singh *et al.*, 2018; Talukdar, 2013).

Plants have several mechanisms to protect themselves against As-induced oxidative stress. Examples are the antioxidant system that includes enzymatic systems such as SOD (superoxide dismutase), POD (peroxidase), APX (ascorbate peroxidase) and CAT (catalase) and non-enzymatic systems such as carotenoids, flavonoids, and phenolic compounds, as well as the modification of damaged compounds (Yoshiyama *et al.*, 2013). Increased activity of superoxide dismutase, catalase and ascorbate peroxidase have been observed by various authors in different plants (Singh *et al.*, 2018; Jin *et al.*, 2010, Talukdar, 2013). They also use the dead cell layers to protect their living tissues against the effects of stress through ROS-induced programmed mechanisms (Ca^{2+} influx and K^+ efflux) (Demidchick *et al.*, 2014). Understanding the biochemical toxicity of As will be helpful to identify arsenic-tolerant plants that are economically important. Alfalfa (*Medicago sativa* L.), is a perennial herb plant from pea family (*Fabaceae*) with a deep root system, so is resistant to drought and its height may be up to one meter. It is one of the medicinal plants which has anticancer, antimicrobial and antioxidant activity. The presence of active constituents (phytochemicals) such as phenolic compounds, flavonoids, alkaloids and saponins in leaves and roots of this plant, could be a cause of its therapeutic properties. As well as it has nutritional importance because of some vitamins (A, K, E, C, B_1 and B_6) and nutrients such as zinc, Iron, Potassium and Calcium in its leaves and roots (Karimi *et al.*, 2013; Okwu, 2005; Duggal *et al.*, 2011). Alfalfa is a globally important crop that is used to feed animals and can transfer arsenic to the human body both directly by consumption of its edible parts and indirectly by consuming As-contaminated milk and meat of livestock. So, the present study was aimed to evaluate and compare the toxicity of different types of inorganic arsenic (As^0 , As^{3+} and As^{5+}) to alfalfa in order to shed light on the antioxidant response of this plant

species to different types of arsenic.

Material and methods

Experimental design: This experiment was conducted in laboratory growth chamber of plant sciences department of Tabriz University in (autumn) 2017. All experiments were carried out as pot culture in a completely randomized design (CRD) with three replications for each treatment at the rates of 0, 100, 200 and 300 μM .

Seed preparation for pot culture: The seeds of alfalfa (*Medicago sativa* L.) were supplied by the Agricultural Research Center of Eastern Azerbaijan province, Tabriz, Iran. Then, healthy and uniform seeds with the same weights were selected to be disinfected with sodium hypochlorite (v/v) 10% for 10 mins. Then, they were washed with distilled water and were dried to get ready for the experiment.

Preparation of treatment solutions: To treat the seedlings, certain amounts of sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$, Merck, Germany), arsenic oxide (As_2O_3 , Merck, Germany) and arsenic (As^0 , Merck, Germany) were used to prepare the aquatic solutions of arsenate, arsenite, and arsenic at the rates of 100, 200 and 300 μM .

Planting conditions: The uniform and healthy seeds of alfalfa that were disinfected with sodium hypochlorite 10% were sown at the rate of 50 seeds in plastic pots containing perlite, with a diameter of 13 cm and a height of 13.5 cm under laboratory conditions at a light/dark period of 16/8 hours, a temperature of 25-30°C, a relative humidity of 60-70%, and a light intensity of 12500 lux. Then they were irrigated with distilled water and after one week, Hoagland nutrient solution was also used. The water content of the pots was adjusted at the field capacity after daily weighing. The 45-day seedlings were treated with arsenate (As^{5+}), arsenite (As^{3+}) and arsenic (As^0) at the rates of 0, 100, 200 and 300 μM . After 45 days, when the symptoms of toxicity emerged at the morphological level, the 90-day seedlings with the same sizes were harvested for the measurements.

The measurement of oxidative stress indexes: Hydrogen peroxide (H_2O_2) content was measured by Harinasut *et al.* (2003)'s procedure. In this way, root and shoot samples were homogenized in 0.1% trichloroacetic acid and were centrifuged at 10000 g for 15 mins. Immediately after that, 0.5 mL of the supernatant was mixed with 0.5 mL of phosphate 10 mM buffer (pH=7) and 1 mL of 1 M potassium iodide and was placed at 25°C for 15 mins. Then plant extract absorption was read at 390 nm with a spectrophotometer (Analytic Jena, Specol 1500, Germany). Finally, H_2O_2 concentration in the samples was calculated based on the standard curve prepared from its different concentrations (0-120 μM) and was recorded in $\mu M g^{-1}$ fresh weight (FW) of the organ.

To explore the peroxidation of lipids, malondialdehyde (MDA) concentration was measured

by Boominathan and Doran (2002)'s method. The plant extract was derived in 0.1% (w/v) solution of trichloroacetic acid (TCA, Merck, Germany) and was centrifuged at 10000 g for 5 mins. Then, the supernatant was mixed with 20% TCA solution containing 0.5% thiobarbituric acid at the rate of 1:4 in a test tube. Then, it was placed in a hot water bath at 95°C for 30 mins. Afterward, the test tubes were quickly cooled down in ice and were centrifuged at 10000 g for 15 mins. Concurrent with the plant extract, standard solutions were prepared from 3,1,1,3 tetraethoxypropane in the range of 0-100 nM, and the absorption of the samples was read at 532 nm with a spectrophotometer. Finally, the MDA content of the samples was calculated in $\mu\text{M g}^{-1}$ FW of the organ.

The measurement of total dissolved protein content and antioxidant enzymes activity: The plant extract was taken from the root and shoot samples (0.1 g) in potassium phosphate buffer at the rate of 50 mM and pH=7. It was, then, centrifuged at 10000 g at 4°C for 15 mins. The supernatant was used to measure total dissolved proteins and the activity of antioxidant enzymes including catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), and superoxide dismutase (SOD).

Total dissolved proteins was measured by Bradford (1976)'s method. The light absorption of the samples was read at 595 nm with a spectrophotometer. At the same time, a standard protein solution was prepared with bovine serum albumin (BSA) at the range of 0-20 μg . Finally, total dissolved proteins was calculated in mg g^{-1} FW of the organ.

SOD enzyme activity was determined by measuring the photo-reduction inhibition of nitro blue tetrazolium (NBT) by the enzymatic extract with a slight modification in the procedure (Winterbourn *et al.*, 1977). Light absorption of the samples was read at 560 nm with a spectrophotometer. The control reaction solution lacked enzymatic extract. One unit of enzymatic activity was used to refer to the amount of enzyme that caused 50% inhibition of photo-reduction of NBT. The specific enzymatic activity was expressed in U mg^{-1} protein per mins.

Peroxidase (POD) activity was measured by Chance and Maehly (1955)'s method. Light absorption of the samples were read at 470 nm with a spectrophotometer for 3 mins. The control reaction solution had no enzymatic extract. Finally, the enzyme activity was measured by the extinction coefficient of tetraguaiacol ($25.5 \text{ mM}^{-1} \text{ cm}^{-1}$). One unit of activity shows the amount of enzyme that is required to oxidize 1 μM of guaiacol into tetraguaiacol. The specific enzyme activity was expressed in U mg^{-1} protein per mins.

Catalase (CAT) activity was measured by Chance and Maehly (1955)'s method according to the decline of light absorption of hydrogen peroxide at 240 nm. Finally, the enzyme activity was measured by the extinction coefficient of hydrogen peroxide ($40 \text{ mM}^{-1} \text{ cm}^{-1}$). One unit of enzyme activity refers to the amount

of enzyme required to decompose 1 μM of hydrogen peroxide in one minute. The specific enzyme activity is expressed in terms of U mg^{-1} protein per min.

To measure ascorbate peroxidase (APX) activity, the method proposed by Boominathan and Doran (2002) was used. It is based on the oxidation of ascorbic acid and the decline of absorption at 290 nm. The enzyme activity was determined by the extinction coefficient of ascorbic acid ($2.8 \text{ mM}^{-1} \text{ cm}^{-1}$). One unit of enzyme activity shows the enzyme required to reduce 1 μL of ascorbic acid in one minute. The specific activity of the enzyme was expressed in U mg^{-1} protein per mins.

The measurement of non-enzymatic antioxidants:

Total flavonoid content of root and shoot samples was measured by the aluminum chloride colorimetric assay with some slight modification (Chang *et al.*, 2002). Light absorption was read at 415 nm versus the control with a spectrophotometer. Total flavonoid content was calculated based on the standard curve prepared from different concentrations of quercetin ($20\text{-}200 \mu\text{g L}^{-1}$) and was recorded in mg of quercetin equivalent per g FW.

Total phenol content was measured by the folin-ciocalteu reagent using Meda *et al.* (2005)'s assay. Absorption was read at 720 nm with a spectrophotometer. Total phenol content was measured based on a standard curve prepared from different concentrations of gallic acid ($0\text{-}100 \mu\text{g L}^{-1}$, sigma aldrich) and was recorded in terms of mg gallic acid (GA) equivalent per g FW.

Mita *et al.* (1997)'s method was employed to estimate total anthocyanin content of the samples. The absorption of the samples solutions was read at 530 and 657 nm versus the control with a spectrophotometer. Finally, the total anthocyanin content of the samples was calculated in mg g^{-1} FW by the equation (1):

$$C (\text{mg}) = A_{530} - (0.25 \times A_{657}) \quad (1)$$

Meanwhile, A represents the absorption by the solution at 530 and 657 nm and C represents the total anthocyanin content of the samples in mg.

Antioxidant activity measurement by DPPH method: The electron or hydrogen donating potential of the methanolic extracts due to the plant parts as measured according to the DPPH (1,1-diphenyl-2-picrylhydrazyl) free radicals scavenging activity (Miliauskas *et al.*, 2004). To do this, 0.1 g of plant sample was ground in a mortar containing 5 mL of 80% methanol and centrifuged at 10000 g for 5 mins. The supernatant was taken to measure antioxidant activity. So, 2 mL methanolic solution of 0.004% DPPH was added to 1900 μL of 80% methanol and 100 μL of the plant extract. The solution was well vortexed and was kept at darkness and room temperature for 30 mins. Then the light absorption of the samples was read at 517 nm by a spectrophotometer. Finally, free radical scavenging percent (I%) of the extracts was determined by the equation (2):

$$I\% = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (2)$$

Meanwhile, A_{control} shows the control solution absorption read at 517 nm and A_{sample} represents the absorption of the samples read at 517 nm.

Statistical analysis: After the parameters were recorded, the data were subjected to the Tukey test at the $P \leq 0.05$ level with the SPSS ver. 16 software package. The graphs of the mean values of the parameters were drawn by the MS-Excel software package.

Results

Effects of arsenic on parameters related to oxidative stress:

The results revealed that H_2O_2 content in the shoot samples was increased by 1.62 and 2 times in the treatments of 200 and 300 μM of As^0 and As^{3+} respectively (figure 1a) and the root samples in plants exposed to the highest rate (300 μM) of the treatments (As^0 , As^{3+} and As^{5+}) showed 2.43, 2.55 and 1.87 times higher H_2O_2 content than control, respectively (figure 1b).

Similarly, MDA content showed an ascending trend in the most shoot samples. The highest MDA content was observed in the samples exposed to As^0 , As^{5+} and As^{3+} at the rates of 100, 200 and 300 μM , respectively, among which the highest one (46.10 $\mu\text{mol g}^{-1}$ FW) belonged to the treatment of As^{3+} (figure 1c). The MDA content in the root samples was the highest in the treatment of 200 μM As^{3+} and 300 μM As^{5+} , whereas the treatment of 200 μM As^0 showed a significant decline ($P \leq 0.05$) in this trait (figure 1d). Overall, among all of the treatments, As^{3+} at the highest rate brought about a higher rate of H_2O_2 synthesis in the root and shoot samples than the control (155.30% and 104.5%, respectively).

Plants under oxidative stress, have been found to use the antioxidant system (enzymatic and non-enzymatic) to counteract the negative effects of free radicals. The DPPH radical scavenging activity in the shoots and roots of the control plants was $6.85\% \pm 0.09\%$ and $25.86\% \pm 4.15\%$ respectively. In the shoot samples of the plants treated with As^0 and As^{3+} , no significant change was observed in the scavenging activity of this radical. But, the plants exposed to 100 and 200 μM As^{5+} exhibited significant positive changes ($P \leq 0.05$) whose maximum was $11.42\% \pm 0.89\%$ in the treatment with 100 μM (figure 2a). In the root samples of the plants treated with 300 μM As^{3+} and As^{5+} , a reduction was observed in free radical scavenging activity (figure 2b).

Antioxidant enzymes activities: The SOD activity was significantly ($P \leq 0.05$) decreased in all root and shoot treatments, except for a 20.65% increase in the root treatment of 300 μM As^0 versus the control. The greatest decline of activity (89.5%) was related to the application of 300 μM of As^{3+} in root (Table 1). CAT had a descending activity in all root treatments. The lowest activity (0.16 unit mg^{-1} protein) was obtained from the application of 300 μM As^{3+} . Among the shoot samples, the activity of this enzyme was increased in most treatments so that its highest activity was observed

in the As^0 and As^{5+} treatments at the rate of 100 μM and the As^{3+} treatment at the rate of 200 μM (Table 1). The activity of APX differed among the treatments so that it was lower in the root samples treated with 100 and 200 μM of As^0 and those treated with As^{5+} at all rates. The lowest decline was 60.7% observed in the plants exposed to 300 μM of As^{3+} , but at 200 μM of As^{3+} , the activity of this enzyme was maximal (151.5%) versus the control (Table 1). The activity of this enzyme did not significantly change in the shoot samples treated with As^0 , but when they were exposed to 200 and 300 μM of As^{3+} and As^{5+} , they showed the highest APX activity (Table 1). The POD activity was increased in the roots treated with As^0 at all rates and As^{5+} at the rate of 100 μM , but the treatment of As^{3+} had an opposing result. In the shoot samples, the treatment of arsenite at all rates, arsenic at the rates of 100 and 300 μM , and arsenate at the rate of 300 μM had a negative impact on the activity of this enzyme (Table 1). The variation trend of total protein content was ascending in the shoot samples of the plants exposed to As^0 and As^{3+} with the maximum being 1.79 times higher than the control plants observed in the 300 μM treatments of them. The treatment of As^{5+} had an insignificant impact on this trait ($P \leq 0.05$). This trait did not show a certain trend of variations in the roots so that it was insignificant in the treatment of As^0 , but the highest (2.56 ± 0.46 and 4.03 ± 0.30 mg g^{-1} FW) occurred in the plants treated with As^{5+} and As^{3+} at the rates of 200 and 300 μM , respectively (Table 1).

Non-enzymatic antioxidants contents: In the present study, total phenol content in the root samples of the As^0 and As^{3+} treatments was the highest (33.8% and 74%) at the rates of 200 and 300 μM versus the control (2.34 ± 0.07 mg g^{-1} FW), respectively. But, no significant change was observed in the As^{5+} treatment ($P \leq 0.05$). In the shoot samples, total phenol content was the highest (17.82% and 16.76%) in the treatments of As^{3+} and As^{5+} , both at the rate of 300 μM versus the control (3.76 ± 0.13 mg g^{-1} FW) (Table 2). Total flavonoid content in the root samples was the highest in the plants treated with 100 μM of As^0 , 200 μM of As^{5+} , and 300 μM of As^{3+} (28.38%, 29.27% and 29.04%, respectively) versus the control (0.451 ± 12.59 mg g^{-1} FW). There were no significant changes in the shoot samples of the plants treated with As^0 and As^{3+} , but in the treatment of As^{5+} at the rate of 200 μM , the highest total flavonoid content (23.57%) was observed versus the control (1.40 ± 0.06 mg g^{-1} FW) (Table 2). Shoot anthocyanin content was the maximal when the treatments were applied at the rate of 300 μM , among which a 100% increase was observed compared to the control plants (1.04 ± 0.01 mg g^{-1} FW) in the treatment of As^{3+} (Table 2).

Discussion

According to the results of this experiment, contents of H_2O_2 and MDA had ascending trend at higher concentrations of treatments. Metalloids such as arsenic

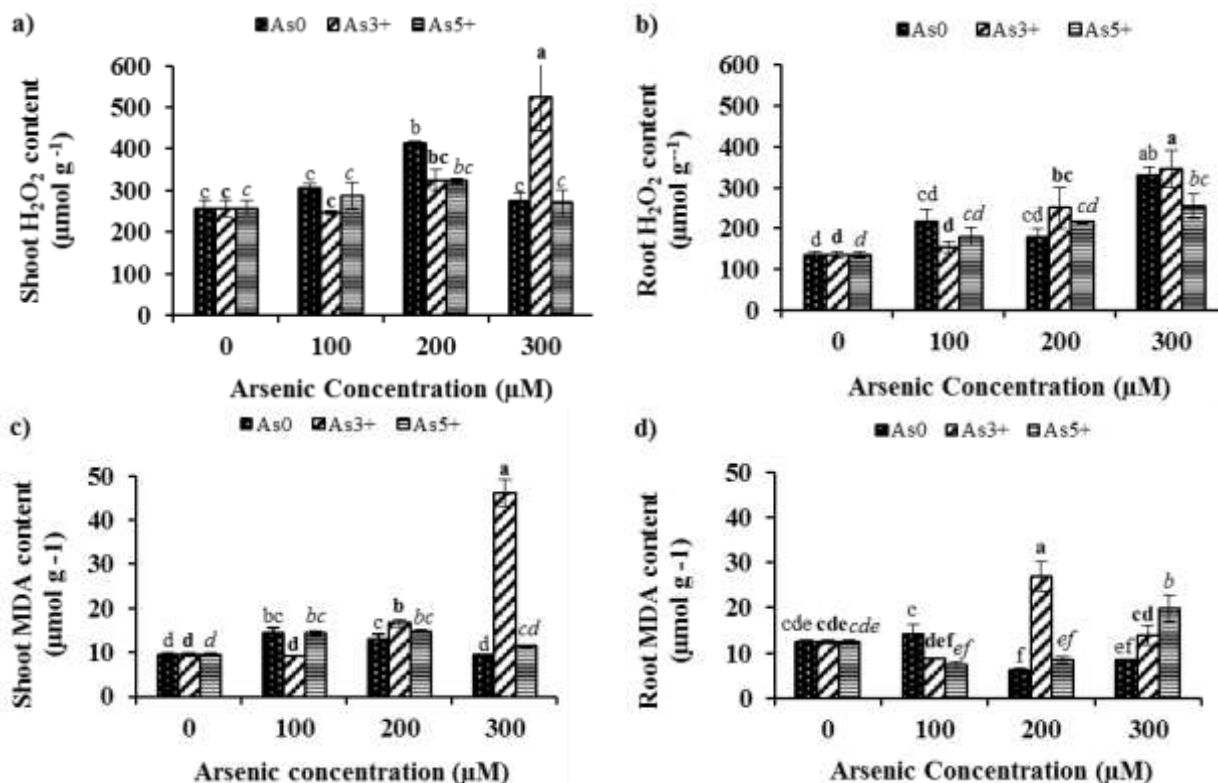


Figure 1. Effect of different concentrations of arsenic solutions (As^0 , As^{3+} and As^{5+}) on the contents of H_2O_2 in the shoots (a) and roots (b), MDA in shoots (c) and roots (d) of alfalfa. The data represent mean \pm SD, (n=3). Different letters above the bars show significant difference at the $P \leq 0.05$ according to the Tukey test

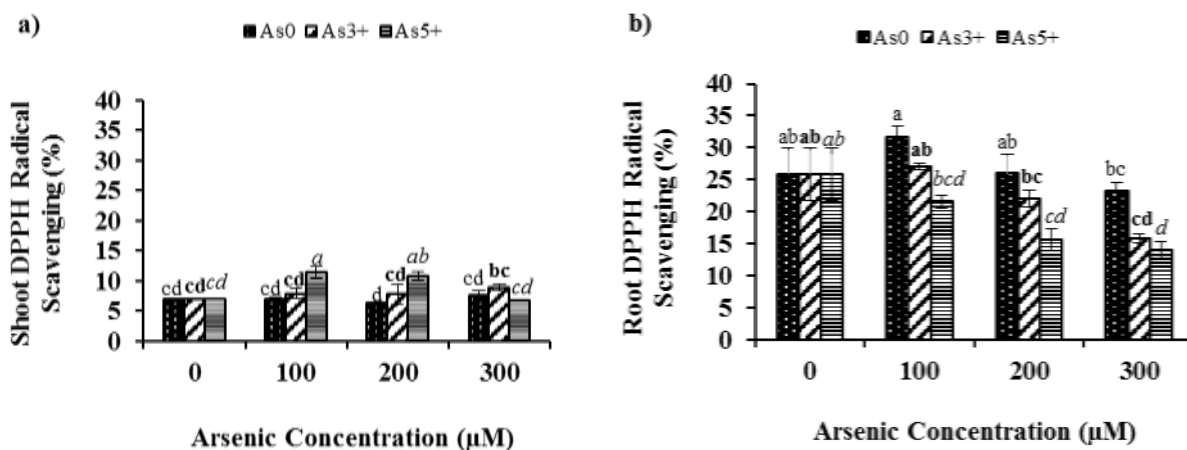


Figure 2. Effect of different concentrations of arsenic solutions (As^0 , As^{3+} and As^{5+}) on the free radical scavenging (%) in shoots (a) and roots (b) of alfalfa. The data represent mean \pm SD, (n=3). Different letters above the bars show significant differences at the $P \leq 0.05$ according to the Tukey test

can oxidize fatty acids as the skeleton component of cell membranes and can disrupt the order and organization of membrane lipids by synthesizing surplus H_2O_2 and MDA, thereby causing numerous physiological impacts such as inactivation of receptors and membrane enzymes, electrolyte leakage, plasmolysis, and serious damages to membranes (Raychaudhuri and Ray, 2017). H_2O_2 is involved in many stresses, such as arsenic stress (Armendariz *et al.*, 2016; Srivastava *et al.*, 2017; Kazemi *et al.*, 2010). The plants exposed to arsenic stress, the oxidation of lipids is a toxic phenomenon

with the most destructive damages (Parkhey *et al.*, 2012; Raychaudhuri and Ray, 2017). Lipid peroxidation products such as MDA can bind to DNA and proteins (Parkhey *et al.*, 2012). The increase in free radicals can lead to the synthesis of more MDA as a marker of cell membrane lipid peroxidation (Gawel *et al.*, 2004; Sharma *et al.*, 2014; Hassan and Mansoor, 2014). Researchers have recently documented an increase in MDA levels in arsenic-induced peroxidation reactions of lipids (Raychaudhuri and Ray, 2017; Singh *et al.*, 2018). A high level of lipid peroxidation (high levels of

Table 1. Effect of different concentrations of arsenic solutions (As⁰, As³⁺ and As⁵⁺) on the content of protein and activity of SOD, POD, CAT and APX in roots and shoots of alfalfa

Compound	Treatment (μM)	Protein (mg g ⁻¹ FW)		Enzymatic antioxidants (Unit mg ⁻¹ Protein)			
				SOD		POD	
		Root	Shoot	Root	Shoot	Root	Shoot
Arsenic (As ⁰)	0	1.55±0.09 ^{cd}	2.11±0.01 ^d	28.77±0.47 ^b	70.30±0.87 ^a	8.13±0.64 ^c	21.49±5.69 ^a
	100	1.75±0.01 ^{cd}	3.52±0.18 ^{ab}	24.95±0.38 ^c	37.53±0.88 ^e	12.46±0.23 ^{ab}	8.78±0.40 ^d
	200	1.72±0.03 ^{cd}	2.85±0.04 ^{bc}	23.38±1.52 ^c	56.13±1.78 ^{bc}	13.12±1.59 ^a	12.89±0.49 ^{abcd}
	300	1.43±0.02 ^{cd}	3.78±0.52 ^a	34.71±1.39 ^a	42.34±1.87 ^{de}	12.72±0.31 ^a	5.67±0.14 ^d
As ₂ O ₃ (As ³⁺)	0	1.55±0.09 ^{cd}	2.11±0.01 ^d	28.77±0.47 ^b	70.30±0.87 ^a	8.13±0.64 ^c	21.49±5.69 ^a
	100	2.02±0.14 ^{bc}	3.36±0.19 ^{ab}	23.14±0.83 ^c	47.81±0.80 ^d	5.45±0.57 ^d	11.14±0.76 ^{bcd}
	200	1.42±0.07 ^{cd}	2.53±0.20 ^{cd}	19.48±0.73 ^d	59.18±1.89 ^b	3.86±1.31 ^{de}	10.27±0.49 ^{cd}
	300	4.03±0.30 ^a	3.78±0.30 ^a	8.01±0.70 ^f	7.41±0.37 ^f	2.08±0.15 ^e	6.82±0.75 ^d
Sodium Arsenate (As ⁵⁺)	0	1.55±0.09 ^{cd}	2.11±0.01 ^d	28.77±0.47 ^b	70.30±0.87 ^a	8.13±0.64 ^c	21.49±5.69 ^a
	100	1.36±0.18 ^d	2.55±0.04 ^{cd}	24.27±0.26 ^c	49.25±0.20 ^{cd}	14.17±0.98 ^a	20.32±0.67 ^{ab}
	200	2.56±0.46 ^b	2.55±0.11 ^{cd}	14.37±0.73 ^e	47.50±6.71 ^d	9.82±0.48 ^c	19.54±1.41 ^{abc}
	300	1.65±0.20 ^{cd}	2.43±0.12 ^{cd}	19.94±1.58 ^d	47.25±2.67 ^d	10.16±0.06 ^{bc}	8.14±0.61 ^d

The data represent mean ± SD, (n=3). Different letters in each column show significant differences at P≤0.05 according to the Tukey test.

Continue of table 1.

Compound	Treatment (μM)	Enzymatic antioxidants (Unit mg ⁻¹ Protein)			
		CAT		APX	
		Root	Shoot	Root	Shoot
Arsenic (As ⁰)	0	1.43±0.09 ^a	0.49±0.01 ^{de}	9.31±0.28 ^c	1.89±0.42 ^{cd}
	100	0.61±0.08 ^c	0.85±0.05 ^{bc}	6.33±0.82 ^{ef}	1.36±0.45 ^d
	200	0.48±0.05 ^{cd}	0.74±0.07 ^{cd}	7.11±0.38 ^{de}	2.22±0.35 ^{abcd}
	300	0.42±0.01 ^{cd}	0.74±0.16 ^{cd}	9.10±0.25 ^{cd}	1.42±0.02 ^d
As ₂ O ₃ (As ³⁺)	0	1.43±0.09 ^a	0.49±0.01 ^{de}	9.31±0.28 ^c	1.89±0.42 ^{cd}
	100	0.34±0.01 ^{de}	0.37±0.11 ^e	20.52±1.50 ^b	2.10±0.11 ^{bcd}
	200	0.51±0.02 ^{cd}	0.87±0.19 ^{bc}	23.42±0.97 ^a	2.95±0.27 ^{ab}
	300	0.16±0.01 ^e	0.57±0.05 ^{cde}	3.66±0.03 ^g	1.65±0.07 ^{cd}
Sodium Arsenate (As ⁵⁺)	0	1.43±0.09 ^a	0.49±0.01 ^{de}	9.31±0.28 ^c	1.89±0.42 ^{cd}
	100	1.20±0.03 ^b	1.33±0.18 ^a	5.91±0.57 ^{ef}	2.60±0.31 ^{abc}
	200	0.31±0.09 ^{de}	0.82±0.02 ^{bcd}	4.25±0.59 ^{fg}	2.31±0.15 ^{abcd}
	300	0.60±0.00 ^c	1.09±0.06 ^{ab}	5.46±0.47 ^{efg}	3.18±0.03 ^a

The data represent mean ± SD, (n=3). Different letters in each column show significant differences at P≤0.05 according to the Tukey test

H₂O₂ and MDA), induced by arsenic stress, has already been reported for various plant species (Raychaudhuri and Ray, 2017; Srivastava *et al.*, 2015; Singh *et al.*, 2007 and 2018; Stoeva and Bineva, 2003), which is consistent with some results of the present study, especially those related to the treatment of 300 μM As³⁺. Maksymiec and Krupa (2006) reported the increased H₂O₂ level under Cd and Cu stress in *Arabidopsis thaliana* and mainly related to the activity of NADPH oxidase and induction of jasmonate signaling pathways. This implies a higher toxicity of arsenite than arsenate and arsenic. Decreasing of MDA content in 200 μM As⁰ of root samples, can be attributed to the better performance of the root antioxidant system. High level of H₂O₂ may be due to the direct function of these arsenic compounds or to the disturbance of the function of antioxidant enzymes which is responsible for the H₂O₂ removing. Increased MDA content at high rates of As treatments, could be related to the expressing As-induced genes that results in production of peroxidase enzymes such as alpha-

dioxygenase and lipoxygenase. Reportedly, high activity of lipoxygenase led to peroxidation of cell membrane fatty acids and caused membrane damage (Molassiotis *et al.*, 2006; Singh *et al.*, 2007).

Metalloids like arsenic activate sensitive signaling pathways of oxidative stress and cause cellular damages by inducing oxidative stress and producing excessive free radicals (Raychaudhuri and Ray, 2017). DPPH is a free radical that is used to determine the ability of plants to scavenge free radicals (Sakanaka *et al.*, 2005). Plants exposed to oxidative stress use an antioxidant system (enzymatic and non-enzymatic) to counteract the negative effects of free radicals (Michalak, 2006). Free radical scavenging activity was only observed at lower rates of As⁵⁺ in the shoots. While at the most rates of treatments in roots, this activity was descended. It seems, since root is the first site that interacts with this toxic element, thus it was more affected and lower free radical scavenging activity in this organ could be attributed to the poor performance of its antioxidant system. Overall, it can be concluded

Table 2. Effect of different concentrations of arsenic solutions (As⁰, As³⁺ and As⁵⁺) on the level of total phenol, total flavonoid and anthocyanin content in roots and shoots of alfalfa

Compound	Treatment (μM)	Non-Enzymatic antioxidants				
		Total phenol (mg g ⁻¹ FW)		Total flavonoid (mg g ⁻¹ FW)		Anthocyanin (mg g ⁻¹ FW)
		Root	Shoot	Root	Shoot	Shoot
Arsenic (As ⁰)	0	2.34±0.07 ^c	3.76±0.13 ^c	0.451±12.59 ^{de}	1.40±0.06 ^{bcde}	1.04±0.01 ^b
	100	2.77±0.24 ^{bc}	3.98±0.12 ^{abc}	0.579±26.52 ^{bc}	1.31±0.02 ^{cde}	0.93±0.07 ^b
	200	3.13±0.14 ^b	3.82±0.04 ^{bc}	0.543±11.13 ^{abc}	1.49±0.09 ^{abcd}	0.75±0.04 ^b
	300	2.63±0.29 ^{bc}	4.20±0.40 ^{abc}	0.456±15.07 ^{de}	1.26±0.03 ^{de}	1.99±0.10 ^a
As ₂ O ₃ (As ³⁺)	0	2.34±0.07 ^c	3.76±0.13 ^c	0.451±12.59 ^{de}	1.40±0.06 ^{bcde}	1.04±0.01 ^b
	100	2.62±0.13 ^{bc}	3.73±0.03 ^c	0.492±18.02 ^{cd}	1.56±0.06 ^{abc}	0.96±0.04 ^b
	200	2.84±0.07 ^{bc}	4.21±0.23 ^{abc}	0.412±9.01 ^e	1.29±0.07 ^{cde}	1.02±0.06 ^b
	300	4.07±0.20 ^a	4.43±0.20 ^a	0.582±8.50 ^a	1.20±0.08 ^e	2.13±0.24 ^a
Arsenate Sodium (As ⁵⁺)	0	2.34±0.07 ^c	3.76±0.13 ^c	0.451±12.59 ^{de}	1.40±0.06 ^{bcde}	1.04±0.01 ^b
	100	2.58±0.25 ^c	3.79±0.17 ^c	0.542±3.18 ^{abc}	1.58±0.09 ^{ab}	0.94±0.10 ^b
	200	2.59±0.04 ^{bc}	3.65±0.10 ^c	0.583±25.12 ^a	1.73±0.14 ^a	0.73±0.02 ^b
	300	2.84±0.17 ^{bc}	4.39±0.07 ^{ab}	0.573±30.13 ^{ab}	1.26±0.13 ^{de}	1.97±0.15 ^a

The data represent mean± SD, (n=3). Different letter in each column show significant differences at P≤0.05 according to the Tukey test

that all of three forms, induce oxidative stress, that which As³⁺ (300 μM) was more toxic than the other two forms. This may be associated with the activity of antioxidant mechanisms, excessive synthesis of phenol compounds, and/or less toxicity of As⁵⁺ at lower dosages. Some researchers such as Tiwari and Sarangi (2017), Ryachaudhuri and Ray (2017) have reported a positive antioxidant response in *Petris vittata* L. and *Momordica charantia* L., respectively.

In the present study, we observed different responses in antioxidant enzymes activities at different concentrations of the used compounds. The SOD activity, except for in the root treatment of 300 μM As⁰ versus the control, was decreased in all treatments. SOD is a key enzyme in the defensive system of living organisms, which counteracts reactive oxygen species (ROS) by converting superoxide radicals into hydrogen peroxide. So, it is perceived as a major marker of the antioxidant efficiency. Its high activity may be associated with the excessive expression of the SOD gene and/or the excessive synthesis of O₂⁻ radicals induced by arsenic (Shahid *et al.*, 2018; Sayantan, 2017). In addition, Souri *et al.* (2017), reported the high activity of this enzyme in *Isatis cappadosica*. It seems that the over-toxicity of As³⁺, which leads to the death of the treated cells, is related to enzyme damage and/or the suppression of enzymes involved in the biosynthesis path of this enzyme. Despite of high level of H₂O₂, especially at high rates of root treatments, CAT activity had a descending trend in all root treatments, that might be ascribed to inactivation and/or inhibition of synthesis of this enzyme by excesses generated ROS. The activity of this enzyme was increased in the most treatments of shoot. Likewise, Souri *et al.* (2017) reported the

increased activity of CAT along with the less SOD activity. It has been established that CAT scavenges H₂O₂ that is produced by the oxidation of fatty acids and the photorespiration in peroxisomes (Karuppanapandian *et al.*, 2011). The effect of arsenic on CAT activity varies with the type and dosage of arsenic (Mallick and Mohn, 2000). The APX activity differed among the treatments. CAT and APX both decompose H₂O₂ into water molecules. Both enzymes scavenge the surplus ROS synthesized in cells. The vital role of APX in mitigating the harmful effects of H₂O₂ that is produced under arsenic exposure, has been well proven (Singh *et al.*, 2017; Saidi *et al.*, 2017; Dave *et al.*, 2013). The H₂O₂ content of apoplast, cytoplasm, and organelles are detoxified with cytosolic APX. APX as a signaling molecule, along with CAT, plays a significant role in controlling the ROS levels (Mittler, 2002; Singh *et al.*, 2017; Kumar *et al.*, 2015). By occurring in cytosol and chloroplast, this enzyme acts as a part of the glutathione-ascorbate cycle and uses two molecules of ascorbate to reduce H₂O₂ into one molecule of water and two molecules of monodehydroascorbate (Rai *et al.*, 2013). In this study, the POD activity was varied in different treatments, so that most shoot treatments showed declined activity of this enzyme. At the same time, in several shoot treatments, CAT had increased activity that it can be concluded that with CAT activity and reduction levels of free radicals, there was no need for high POD activity. Generally, negative changes in the activity of antioxidant enzymes in arsenic-affected alfalfa plant, may have various reasons such as the structural damage to the these enzymes, the reduced expression of the genes related to these enzymes and/or the disruption in the uptake of nutrients that are key to

the structure and performance of these enzymes, such as Cu, Mn and Fe, as constituents of the SOD, as well as Fe as constituent of POD and CAT. Some studies have indicated that these elements deficiency causes a remarkable reduction in these enzymes activity (Leao *et al.*, 2014). Many researchers have reported the escalated activity of these enzymes with the increase in arsenic dosage in some plant species (Singh *et al.*, 2017; Saidi *et al.*, 2017; Hasanuzzaman *et al.*, 2017). Their findings were partially consistent with our results.

We found, out that total phenol, flavonoid and anthocyanin content in the root and shoot samples with high rates of treatments, had positive changes. Non-enzymatic antioxidants such as phenolic compounds were considered effective antioxidants because of their redox properties. They act as reducing factors, i.e., free radical scavenger, hydrogen donors, singlet oxygen extinction agent, and metal chelators (Raychaudhuri and Ray, 2017). Some of these compounds, such as flavonoids, are bound to the hydrophilic end of membrane phospholipids by hydrogen bonds and are stored on the outer and inner surface of the cell membrane (Toyama *et al.*, 2008). According to Lavid *et al.* (2001), when plants are exposed to heavy metal stresses, the increase in phenolic compounds results in the enhanced activity of enzymes involved in the metabolism of these compounds, which can be attributed to their de novo synthesis. Flavonoids are organic molecules that act as compounds with antioxidant activity and protect plants from various stresses alone or through attaching to peroxidase (Jaakola *et al.*, 2004; Mika *et al.*, 2004).

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Conclusion

The results showed that under different types of inorganic arsenic stress, alfalfa (*Medicago sativa* L.) became the tolerant of oxidative stress induced by excess ROS synthesis, especially at high rates, which altered its biochemical properties such as the ability of free radical scavenging, lipid peroxidation, the activity of antioxidant enzymes such as SOD, POD, CAT and APX and non-enzymatic antioxidant contents such as total phenol, total flavonoids, and anthocyanin. In general, according to the results, it can be concluded that, out of the three types of mineral arsenic used in this research, arsenite (As^{3+}) had a more negative effect on the alfalfa plant, especially at higher rates, that indicates the chemical state of As compounds affects on their toxicity in plants. It seems that this plant species has a moderate antioxidant potential that could partially counteracted the stress by altering the activity of the antioxidant system through various mechanisms. It is important that more studies on metabolism of antioxidant system are needed to obtain a better understanding of As-induced stress in this plant. Further, our results are based on laboratory conditions. Field studies also is recommended. This could be our future research.

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