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## Modulation of growth and oxidative stress by seed priming with salicylic acid in *Zea mays* L. under lead stress

Roya Zanganeh, Rashid Jamei and Fatemeh Rahmani

Department of Biology, Faculty of Science, Urmia University, Urmia, Iran

### ABSTRACT

Lead is an important environmental pollutant, extremely toxic to plants and other living organisms. In the present study, possible ameliorating effects of salicylic acid (SA) were investigated at biochemical levels in maize exposed to lead stress. The elevation in hydrogen peroxide and malondialdehyde contents and the decline in protein thiol (PT) level were revealed under lead stress. The SA pretreatment prevented a lead-induced decrease in chlorophyll and PT contents, as well as an increase in lipid peroxidation and hydrogen peroxide levels. Lead stressed plants which are pretreated with salicylic acid accumulated more ascorbic acid, glutathione and stimulated more antioxidant enzymes activity than plants treated with lead alone. In addition, SA pretreatment of seed enhanced L-cysteine desulphydrase activity and endogenous hydrogen sulfide content. The results indicate that SA regulates the response of plants to lead stress and suggest hydrogen sulfide as a downstream signal molecule which might be involved in SA-induced lead tolerance.

### ARTICLE HISTORY

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### KEYWORDS

Hydrogen sulfide; lead tolerance; salicylic acid; *Zea mays* L

### Introduction

Contamination by heavy metals is widespread as a result of human agricultural and industrial activities. Among heavy metals, lead (Pb) is an element easily accumulated in soil and sediments. Although lead is not an essential element for plants, it is absorbed and accumulated (Gobran and Huang 2011). After absorption of Pb by the plant's root system, it becomes highly concentrated in the root system as compared to other plant's parts (Sharma and Dubey 2005). Pb phytotoxicity involves a decrease in growth, photosynthetic pigment content, disruption of protein structure, impairment of cell division and DNA synthesis, inhibition in activities of many enzymes (by binding to the sulphydryl groups of various proteins) and oxidative stress (Wang et al. 2011; Srivastava et al. 2014). Plants possess defensive mechanisms to detoxify metals for their survival. Alleviation of oxidative damage and increasing resistance to environmental stresses are often correlated with an efficient antioxidative system. The antioxidative system includes enzymatic and non-enzymatic antioxidants (Mostofa et al. 2015). Metal ion chelation is one of the defense strategies evidenced in plants under metal stress (Hassan and Aarts 2011). Proline, polyamines and non-protein thiols (NPT), e.g. cysteine, glutathione (GSH) and phytochelatins (PCs), are components of the chelating compounds involved in metal chelation in the cytoplasm and contribute to the amelioration of negative impacts (Hall 2002).

Salicylic acid (SA), a phenolic compound, is an endogenous growth regulator and signal molecule whose role is well established in the defense mechanism under stress conditions. Previous studies show that exogenously applied SA effectively alleviated heavy metal stresses like copper (Cu), mercury (Hg), arsenic (As), cadmium (Cd), zinc (Zn) and Pb toxicity (Zhou et al. 2009; Wang et al. 2011; Mostofa and Fujita 2013; Singh et al. 2015; Roychoudhury et al.

2016; Namdjoyan et al. 2017). The exogenous SA could protect *Vallisneria natans* plants against Pb toxicity by preventing Pb-induced oxidative stress (Wang et al. 2011).

The hydrogen sulfide (H<sub>2</sub>S) is a gaseous signal molecule involved in plant response to biotic and abiotic stresses (Li et al. 2015). In parallel, H<sub>2</sub>S was also confirmed to contribute to Pb toxicity in oilseed rape via ROS regulation and improvement of antioxidative defense (Ali et al. 2014). Qiao et al. (2015) showed that SA-induced cadmium tolerance was weakened by H<sub>2</sub>S biosynthesis inhibitor supplementation and hypotaurine as its scavenger. Hence, it might be expected that SA-induced H<sub>2</sub>S generation (as a down-stream) regulates stress tolerance. Nowadays, various exogenous compounds are used to relieve plant stress. Previous studies indicated many roles for SA and H<sub>2</sub>S in alleviating environmental stresses (Wang et al. 2011; Ali et al. 2014). However, there are few reports about the cross talk between these two signaling molecules (Li et al. 2015; Qiao et al. 2015). In this study, the role of SA priming (pre-sowing seed treatment) was assessed in alteration of growth, total chlorophyll content, Pb accumulation and some protective compounds (proline, GSH, and ascorbic acid (AsA), NPT) content and antioxidant enzymes activity in maize plants exposed to Pb. Additionally, the aim of this study was to investigate the effect of SA treatment on L-cysteine desulphydrase (L-DES) activity, a key enzyme in H<sub>2</sub>S biosynthesis. The possibility of the cross talk was also investigated between H<sub>2</sub>S and SA.

### Material and methods

Seeds of *Zea mays* L. cv. 704 were supplied by the Agriculture Research Center of Kerman. Seeds were surface sterilized in 10% (w/v) sodium hypochlorite, rinsed several times in distilled water and pretreated with 0.5 mM SA for 12 h

(concentration was optimized in preliminary experiments). Then, seeds were germinated on moistened filter paper for 3 days. Thereafter, seedlings were grown in pots filled with sand and perlite in the ratio of 2:1, and transferred to a growth chamber with day/night temperature of 29/20°C with a 16 h photoperiod, the relative humidity of 60–80%. The 6-day old seedlings were subjected to lead stress by 2.5 mM Pb(NO<sub>3</sub>)<sub>2</sub>. After 9 days' treatment, root and shoot of plants were harvested and immediately stored at –80°C for further analyses.

### Determination of Pb accumulation

The harvested samples were immediately blotted and oven-dried at 105°C for 24 h. The dried material was ground in a mortar. Powdered samples (shoots and roots) of each plant material were weighed and 500 mg aliquots transferred to tubes and digested for 12 h with 7.5 mL nitric acid (65%) and 2.5 mL hydrochloric acid (36%) at 25°C, then heated at 105°C for 2 h. The content of Pb was determined by atomic absorption spectroscopy (SHIMADZU AA-6300, Japan). Replications were maintained, and their average was used for calculations. The concentrations of elements were expressed as mg/g dry weight (Nóvoa-Muñoz et al. 2008).

### Measurement of growth parameters

Shoot and root lengths, fresh and dry weights of control and treated plants were measured as growth parameters. Shoot and root lengths were determined with a ruler. After the separation of shoots and roots, fresh weights were measured. For the estimation of dry weight, plants were oven-dried at 65–75°C for 48 h.

### Photosynthetic pigment estimation

Chlorophyll (Chl) content was determined using the methods of Lichtenthaler (1987). In this method, Chl was extracted with 80% acetone. Extracts were centrifuged at 3000g and the absorbance of the supernatant was measured at 663.2 and 646.8 nm with a UV-VIS spectrophotometer. Chl a and Chl b contents were calculated by the following formulas:

$$\begin{aligned} \text{Chl a} = & (12.25A_{663.2} - 2.79A_{646.8}) \\ & \times \text{volume of supernatant (mL)} \\ & \times \text{dilutionfactor/samplemass (g)} \end{aligned}$$

$$\begin{aligned} \text{Chl b} = & (21.21A_{646.8} - 5.1A_{663.2}) \\ & \times \text{volume of supernatant (mL)} \\ & \times \text{dilutionfactor/samplemass (g)} \end{aligned}$$

A: Absorbance at specific wavelength.

### Proline content

The proline content was assayed by the method of Bates et al. (1973). Frozen plant samples were homogenized in 10 mL of 3% aqueous sulfosalicylic acid. The homogenate was centrifuged at 5000g for 5 min and supernatant mixed with 2 mL acid-ninhydrin and 2 mL of glacial acetic acid. The mixture was placed in a water bath at 100°C for 1 h. The reaction mixture was extracted with 4 mL toluene. Absorbance was measured at 520 nm. The proline content was assayed using

a standard curve prepared with the known concentration of proline. Results were expressed as  $\mu\text{mol g}^{-1}$  fresh weight.

### Determination of NPT and protein thiol contents

The NPT content was determined using the method described by Ellman (1959). A known weight (200 mg) of the sample was extracted with 4 mL of EDTA (20 mM) and centrifuged at 5000g for 30 min. The supernatant was divided into two parts. One mL of the supernatant was added to 0.8 mL distilled water and 200  $\mu\text{L}$  TCA (50%). The solution was vortex-mixed for 10 s and incubated at 25°C for 15 min and centrifuged at 3000 g for 15 min. One mL of the supernatant was added to 1.3 mL of potassium phosphate buffer (150 mM) (pH 8.9) and 50  $\mu\text{L}$  of DTNB (10 mM) and incubated for 5 min. Absorbance was measured at 412 nm. To estimate the total thiol content, 0.5 mL of the supernatant was mixed with 1.3 mL of 100 mM potassium phosphate buffer (pH 8.2), 50  $\mu\text{L}$  of DTNB (15 mM) and 3 mL methanol. After incubation at 30°C for 15 min, the yellow color was developed and measured at 412 nm. The protein thiol (PT) content was calculated by subtracting the content of NPT from total thiols and expressed in terms of micromole per gram fresh weight of tissues using an extinction coefficient of  $13.1 \text{ mM}^{-1} \text{ cm}^{-1}$ .

### GSH content

Reduced GSH contents were estimated using the method of Ellman (1959). Fresh tissues (500 mg) were homogenized in metaphosphoric acid (15%) and centrifuged at 5000g at 4°C for 30 min. The supernatant (200  $\mu\text{L}$ ) was mixed with 2.6 mL phosphate buffer and 200  $\mu\text{L}$  of DTNB (6 mM) and incubated for 30 min. The absorbance was read at 412 nm, and glutathione content was calculated from the standard curve.

### Determination of AsA content

The AsA content was assayed as per the method of Omaye et al. (1979). One gram of fresh material was homogenized in 5 mL of 10% TCA. The homogenate was centrifuged at 4000 g for 20 min, reextracted twice, and supernatant made up to 10 mL, and used for the assay. To 1 mL of extract, 2 mL of DTC reagent (2,4-Dinitrophenylhydrazine/thiourea/copper) was added and incubated at 37°C for 3 h. Then, 0.75 mL of ice cold 65% H<sub>2</sub>SO<sub>4</sub> was added, allowed to stand at 30°C for 30 min, and then resulting color was recorded at 520 nm. The AsA content was assayed using a standard curve prepared with AsA, and the results were expressed in  $\text{mg g}^{-1}$  fresh weight.

### Measurement of lipid peroxidation level

The level of lipid peroxidation in plant tissues was measured by the determination of the malondialdehyde (MDA) content (Heath and Packer 1968). Frozen samples were homogenized in 2 mL ice-cold 0.1% (W/V) TCA and centrifuged at 15,000g for 15 min. Assay mixture containing 1 mL of the supernatant and 2 mL of 0.5% (W/V) thiobarbituric acid (TBA) in 20% (W/V) TCA was heated at 95°C for 30 min and then rapidly cooled in an ice bath. After centrifugation (10,000g at 4°C for 10 min), the supernatant absorbance was read at 532 nm, and the values

corresponding to nonspecific absorption (600 nm) were subtracted. For the calculation of MDA, an extinction coefficient ( $\epsilon$ ) of  $1.55 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  was used. Results were expressed as  $\text{mmol g}^{-1}$  fresh weight.

### Determination of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) content

$\text{H}_2\text{O}_2$  levels were determined according to Alexieva et al. (2001). Fresh tissues (0.5 g) were homogenized in an ice bath with 5 mL 0.1% (w/v) trichloro acetic acid (TCA). The homogenate was centrifuged at 12,000g for 15 min, and 0.5 mL of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of KI (1 M). The reaction was carried out for 1 h in darkness and the absorbance was measured at 390 nm. The amount of hydrogen peroxide was calculated using a standard curve prepared with known concentrations of  $\text{H}_2\text{O}_2$ .

### Antioxidant enzyme extraction and activity assay

Shoots and roots (500 mg) were homogenized in 50 mM potassium phosphate buffer (pH 7.0) containing 1% soluble polyvinyl polypyrrolidone (PVP), 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 10,000g for 20 min, and the supernatant used for assay of the activity of enzymes. In the case of extract associated with the activity of ascorbate peroxidase (APX) enzyme, 10 mM AsA was added to the extracts.

### Catalase activity assay

Catalase (CAT) activity was assayed by measuring the initial rate of  $\text{H}_2\text{O}_2$  disappearance at 240 nm using the extinction coefficient of  $40 \text{ mM}^{-1} \text{ cm}^{-1}$  for  $\text{H}_2\text{O}_2$  (Dhindsa et al. 1981).

### APX activity assay

APX was determined spectrophotometrically according to the oxidation of AsA. Hydrogen peroxide-dependent oxidation of AsA was followed by measuring the decrease in absorbance due to AsA oxidation for 1 min at 290 nm using extinction coefficient of  $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$  (Nakano and Asada 1981).

### Guaiacol peroxidase activity assay

Guaiacol peroxidase (GPX) was determined using the method of Plewa et al. (1991). The increase in absorbance at 470 nm was recorded (due to the guaiacol oxidation) for 3 min. One unit of enzyme activity was defined as the amount that causes a change of 0.01 in absorbance per minute.

### Glutathione reductase activity assay

Glutathione reductase (GR) was determined using the method of Foyer and Halliwell (1976) following the decrease in the absorbance of NADPH at 340 nm for GSSG-dependent oxidation of NADPH.

### $\text{H}_2\text{S}$ content

$\text{H}_2\text{S}$  content was determined using the methods of Christou et al. (2013) with modifications. Maize tissues (0.25 g) were homogenized in 1 mL of 100 mM potassium-phosphate

buffer (pH 7.0) containing 10 mM EDTA and centrifuged at 11,200g for 15 min. The supernatant (100  $\mu\text{L}$ ) was mixed with 1880  $\mu\text{L}$  extraction buffer and 20  $\mu\text{L}$  of 20 mM 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) and incubated at 25°C for 5 min. The absorbance was read at 412 nm, and  $\text{H}_2\text{S}$  was quantified using a standard curve developed with known concentrations of NaHS.

### Determination of L-cysteine desulphydrase activity

L-cysteine desulphydrase activity (L-DES, E.C. 4.4.1.1.) was measured by the release of sulfide from L-cysteine as described previously (Riemenschneider et al. 2005). Frozen samples were homogenized in 1 mL 20 mM Tris-HCl, pH 8.0 and centrifuged for 1 min at 13,000g. The assay contained in a total volume of 1 mL: 1 mM dithiothreitol, 1 mM L-cysteine, 100 mM Tris-HCl, pH 8.0 and the enzyme extract. The reaction was initiated by the addition of L-cysteine. After incubation for 15 min at 37°C, the reaction was terminated with the addition of 100  $\mu\text{L}$  of 30 mM  $\text{FeCl}_3$  dissolved in 1.2 N HCl and 100  $\mu\text{L}$  of 20 mM N, N-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 7.2 N HCl. The formation of methylene blue was determined at 670 nm, and the enzyme activity calculated using the extinction coefficient of  $15 \times 10^6 \text{ cm}^2 \text{ mol}^{-1}$ .

### Statistical analysis

The data were statistically analyzed using one-way analysis of variance followed by Duncan's multiple range test (DMRT). All statistical analyses were performed with the SPSS 19 Program. The standard error of means were also calculated for the presentation of the figures.

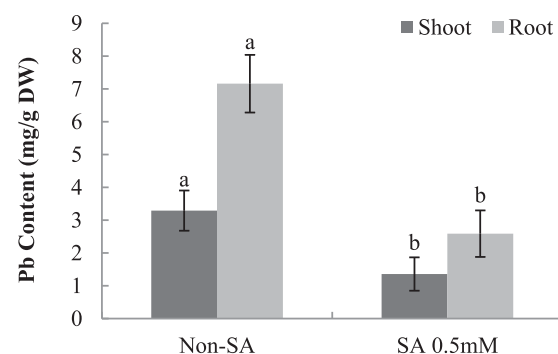
## Results

### Pb accumulation in plant tissues

The concentration of Pb was determined in shoots and roots of maize plants (Figure 1). The content of Pb increased in both shoots and roots of maize plants under Pb treatment. The accumulation of Pb was higher in the roots than shoots. Pre-sowing seed treatment with salicylic acid significantly reduced Pb concentration in both plant tissues.

### Growth parameters

Pb stress decreased shoot and root lengths and root fresh weight, while the shoot fresh weight and both shoot and



**Figure 1.** Effect of seed pretreatment with SA on Pb content (non-stressed plants not detected) of maize plants under lead stress conditions (Mean  $\pm$  SE,  $n = 3$ ),  $P \leq 0.05$ .



**Table 1.** Effect of seed pretreatment with salicylic acid on Pb content, growth parameters and total chlorophyll content of maize plants under lead stress conditions (Mean  $\pm$  SE,  $n = 3$ ).

Parameters	Tissue	Control	SA (0.5 mM)	Pb (2.5 mM)	SA + Pb
Length (cm)	Shoot	33.00 <sup>bc</sup> $\pm$ 1.00	37.00 <sup>a</sup> $\pm$ 0.57	20.33 <sup>d</sup> $\pm$ 0.66	34.00 <sup>b</sup> $\pm$ 0.57
	Root	32.33 <sup>ab</sup> $\pm$ 0.88	34.50 <sup>a</sup> $\pm$ 1.32	19.50 <sup>c</sup> $\pm$ 0.76	31.16 <sup>b</sup> $\pm$ 0.72
Fresh weight (g/plant)	Shoot	0.81 <sup>bc</sup> $\pm$ 0.09	1.27 <sup>a</sup> $\pm$ 0.09	0.55 <sup>c</sup> $\pm$ 0.02	0.95 <sup>b</sup> $\pm$ 0.09
	Root	0.82 <sup>b</sup> $\pm$ 0.09	1.10 <sup>a</sup> $\pm$ 0.11	0.43 <sup>c</sup> $\pm$ 0.02	0.90 <sup>ab</sup> $\pm$ 0.10
Dry weight (g/plant)	Shoot	0.07 <sup>b</sup> $\pm$ 0.004	0.11 <sup>a</sup> $\pm$ 0.02	0.06 <sup>b</sup> $\pm$ 0.003	0.08 <sup>ab</sup> $\pm$ 0.009
	Root	0.06 <sup>ab</sup> $\pm$ 0.007	0.08 <sup>a</sup> $\pm$ 0.007	0.05 <sup>b</sup> $\pm$ 0.008	0.07 <sup>ab</sup> $\pm$ 0.008
Total chlorophyll (mg/g FW)		1.43 <sup>a</sup> $\pm$ 0.04	1.51 <sup>a</sup> $\pm$ 0.10	0.91 <sup>c</sup> $\pm$ 0.02	1.20 <sup>b</sup> $\pm$ 0.07

Note: Values within rows followed by the same letter(s) are not significantly different at ( $P \leq .05$ ) level.

root dry weights displayed a non-significant decrease compared with untreated-Pb plants. Pretreatment of seeds with SA reduced the negative effects of Pb stress on plant height as well as fresh and dry weights. However, the improvement effect of SA on dry weight was non-significant. The application of SA alone increased shoot growth parameters. However, the improvements were non-significant in roots (Table 1).

### Total chlorophyll content

Data presented in Table 1 show that the total chlorophyll content of Pb-treated maize plants was significantly lower compared to the controls. Pre-sowing seed treatment with SA increased total chlorophyll content in plants under Pb stress while SA treatment alone had no significant impact on chlorophyll contents.

### Proline, NPT, PT, GSH and AsA contents

Pb imposition was found to increase significantly proline levels compared to the control. In Pb-stressed plants, SA pretreatment decreased proline content in both shoots and roots (Table 2). Application of Pb resulted in a significant reduction in NPT and glutathione contents of the shoot as well as AsA content of root and PT levels in both shoots and roots compared to the plants grown under the control condition. On the other hand, GSH content remarkably increased in maize roots in response to Pb stress. Pb pretreatment had no significant effect on NPT content of root and AsA level of the shoot. Pretreatment of seed with SA significantly increased the content of NPT, PT, GSH and root AsA under Pb condition; whereas SA pre-treated Pb-stressed plants did not show a significant increase in shoot AsA content compared to the plants subjected to Pb stress without seed pretreatment. Plants, pretreated with SA alone, displayed a dramatic increase in Proline content in shoots as well as GSH level in roots. However, SA pretreatment did not

influence the shoot GSH and root proline contents and both shoot's and root's NPT, PT and AsA levels in non-stressed plants (Table 2).

### MDA and H<sub>2</sub>O<sub>2</sub> content

An increase in MDA and H<sub>2</sub>O<sub>2</sub> contents was detected following Pb treatment in both shoots and roots. Pretreatment with SA, in the absence of Pb stress, did not alter the generation of MDA and H<sub>2</sub>O<sub>2</sub> contents compared with the untreated control plants, while SA application considerably diminished the accumulation of MDA and H<sub>2</sub>O<sub>2</sub> in both shoot and root tissues of Pb-treated plants (Table 3).

### Antioxidant enzymes activity

CAT activity increased under Pb treatment compared with untreated-Pb plants (Table 3). A significant increase in the APX activity of roots was also observed in response to Pb stress. However, the activity of shoot APX and both shoots and roots GPX and GR did not show significant change under Pb stress. SA pretreatment enhanced APX activity in shoots and roots, GPX activity in shoots and CAT and GR activities in roots of plants treated with Pb. APX activity elevated dramatically in roots of plants pretreated with SA alone while SA pretreatment of seeds had no significant impact on the activity of other antioxidant enzymes in control plants.

### H<sub>2</sub>S content

Upon Pb treatment, the content of H<sub>2</sub>S decreased in shoots and roots, compared with untreated-Pb plants. SA pretreatment of seeds increased H<sub>2</sub>S content of shoots and roots in stressed plants. The H<sub>2</sub>S content was also induced under SA pretreatment in non-stressed plants, while pre-sowing seeds with SA treatment had no significant alteration on root H<sub>2</sub>S content under control condition (Figure 2).

**Table 2.** Effect of seed pretreatment with salicylic acid on proline, NPT, PT, GSH and AsA contents of maize plants under lead stress conditions (Mean  $\pm$  SE,  $n = 3$ ).

Parameters	Tissue	Control	SA (0.5 mM)	Pb (2.5 mM)	SA + Pb
Proline ( $\mu$ mol/g FW)	Shoot	75.26 <sup>c</sup> $\pm$ 4.06	124.30 <sup>b</sup> $\pm$ 6.20	165.16 <sup>a</sup> $\pm$ 1.24	113.54 <sup>b</sup> $\pm$ 5.68
	Root	131.46 <sup>c</sup> $\pm$ 3.79	156.55 <sup>bc</sup> $\pm$ 6.20	215.34 <sup>a</sup> $\pm$ 11.53	171.61 <sup>b</sup> $\pm$ 8.95
NPT ( $\mu$ mol/g FW)	Shoot	0.37 <sup>a</sup> $\pm$ 0.02	0.35 <sup>ab</sup> $\pm$ 0.02	0.27 <sup>b</sup> $\pm$ 0.03	0.40 <sup>a</sup> $\pm$ 0.02
	Root	0.19 <sup>c</sup> $\pm$ 0.01	0.18 <sup>c</sup> $\pm$ 0.02	0.24 <sup>bc</sup> $\pm$ 0.02	0.35 <sup>a</sup> $\pm$ 0.02
PT ( $\mu$ mol/g FW)	Shoot	0.40 <sup>a</sup> $\pm$ 0.05	0.44 <sup>a</sup> $\pm$ 0.01	0.25 <sup>b</sup> $\pm$ 0.02	0.39 <sup>a</sup> $\pm$ 0.05
	Root	0.38 <sup>a</sup> $\pm$ 0.01	0.39 <sup>a</sup> $\pm$ 0.02	0.22 <sup>b</sup> $\pm$ 0.03	0.34 <sup>a</sup> $\pm$ 0.03
GSH ( $\mu$ mol/g FW)	Shoot	0.20 <sup>b</sup> $\pm$ 0.008	0.25 <sup>ab</sup> $\pm$ 0.03	0.11 <sup>c</sup> $\pm$ 0.01	0.33 <sup>a</sup> $\pm$ 0.03
	Root	0.09 <sup>c</sup> $\pm$ 0.02	0.20 <sup>b</sup> $\pm$ 0.02	0.19 <sup>b</sup> $\pm$ 0.03	0.30 <sup>a</sup> $\pm$ 0.03
AsA ( $\mu$ mol/g FW)	Shoot	7.14 <sup>b</sup> $\pm$ 0.13	7.90 <sup>ab</sup> $\pm$ 0.42	7.79 <sup>ab</sup> $\pm$ 0.26	8.81 <sup>a</sup> $\pm$ 0.26
	Root	5.43 <sup>a</sup> $\pm$ 0.06	6.30 <sup>a</sup> $\pm$ 0.53	4.12 <sup>b</sup> $\pm$ 0.26	6.08 <sup>a</sup> $\pm$ 0.10

Note: Values within rows followed by the same letter(s) are not significantly different at ( $P \leq .05$ ) level.

**Table 3.** Effect of seed pretreatment with salicylic acid on MDA and H<sub>2</sub>O<sub>2</sub> content and antioxidants activities of maize plants under lead stress conditions (Mean  $\pm$  SE,  $n = 3$ ).

Parameters	Tissue	Control	SA (0.5 mM)	Pb (2.5 mM)	SA + Pb
MDA ( $\mu\text{mol/g FW}$ )	Shoot	1.09 <sup>cd</sup> $\pm$ 0.04	0.97 <sup>d</sup> $\pm$ 0.04	1.74 <sup>a</sup> $\pm$ 0.05	1.31 <sup>bc</sup> $\pm$ 0.10
	Root	1.00 <sup>d</sup> $\pm$ 0.05	0.91 <sup>d</sup> $\pm$ 0.04	1.83 <sup>a</sup> $\pm$ 0.06	1.39 <sup>c</sup> $\pm$ 0.03
H <sub>2</sub> O <sub>2</sub> ( $\mu\text{mol/g FW}$ )	Shoot	0.74 <sup>c</sup> $\pm$ 0.06	0.75 <sup>c</sup> $\pm$ 0.04	2.71 <sup>a</sup> $\pm$ 0.08	1.88 <sup>b</sup> $\pm$ 0.05
	Root	0.38 <sup>c</sup> $\pm$ 0.07	0.35 <sup>c</sup> $\pm$ 0.08	3.50 <sup>a</sup> $\pm$ 0.27	2.32 <sup>b</sup> $\pm$ 0.16
APX activity (Unit/mg protein in 150 $\mu\text{l}$ extract)	Shoot	32.39 <sup>b</sup> $\pm$ 3.87	41.50 <sup>ab</sup> $\pm$ 6.50	27.86 <sup>b</sup> $\pm$ 5.27	53.01 <sup>a</sup> $\pm$ 2.68
	Root	32.36 <sup>b</sup> $\pm$ 4.67	51.78 <sup>d</sup> $\pm$ 5.10	76.33 <sup>c</sup> $\pm$ 5.61	240.84 <sup>a</sup> $\pm$ 3.48
CAT activity (Unit/mg protein in 100 $\mu\text{l}$ extract)	Shoot	35.25 <sup>b</sup> $\pm$ 1.56	38.75 <sup>b</sup> $\pm$ 2.64	75.25 <sup>a</sup> $\pm$ 2.38	92.50 <sup>a</sup> $\pm$ 8.05
	Root	20.25 <sup>c</sup> $\pm$ 1.56	18.25 <sup>c</sup> $\pm$ 3.25	33.12 <sup>b</sup> $\pm$ 2.60	41.65 <sup>a</sup> $\pm$ 3.00
GPX activity (Unit/mg protein in 20 $\mu\text{l}$ extract)	Shoot	54.41 <sup>ab</sup> $\pm$ 3.05	66.66 <sup>a</sup> $\pm$ 5.65	40.49 <sup>b</sup> $\pm$ 4.51	68.92 <sup>a</sup> $\pm$ 7.42
	Root	71.13 <sup>a</sup> $\pm$ 4.39	78.37 <sup>a</sup> $\pm$ 2.48	75.72 <sup>a</sup> $\pm$ 4.93	82.31 <sup>a</sup> $\pm$ 4.12
GR activity (Unit/mg protein in 300 $\mu\text{l}$ extract)	Shoot	2.39 <sup>b</sup> $\pm$ 0.40	2.98 <sup>b</sup> $\pm$ 0.43	4.38 <sup>ab</sup> $\pm$ 0.90	5.37 <sup>a</sup> $\pm$ 0.74
	Root	8.76 <sup>b</sup> $\pm$ 1.53	10.18 <sup>b</sup> $\pm$ 1.44	13.13 <sup>b</sup> $\pm$ 1.19	17.74 <sup>a</sup> $\pm$ 1.28

Note: Values within rows followed by the same letter(s) are not significantly different at ( $P \leq .05$ ) level.

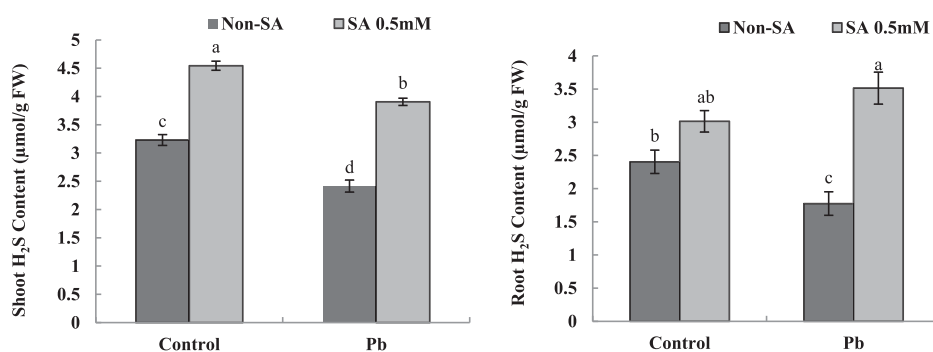
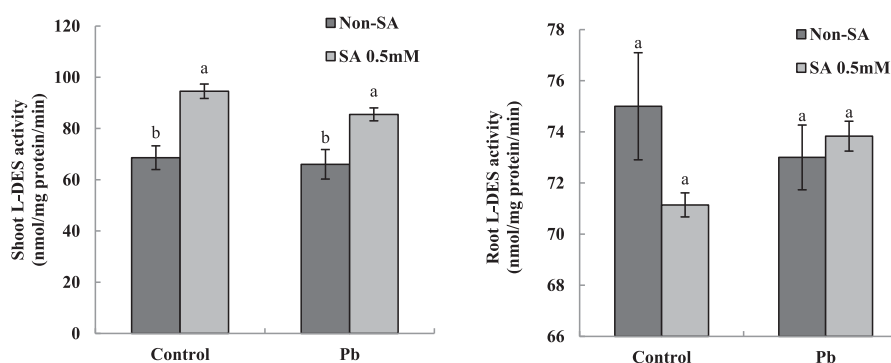
### L-DES activity

The non-significant decrease in L-DES activity was observed under Pb stress in the absence of SA. Upon pre-incubation of seeds with SA, the shoot L-DES activity elevated under stress and control conditions, while SA pretreatment had no significant change on L-DES activity of roots in non-stressed and stressed plants (Figure 3).

### Discussion

Pb is easily taken up by plants and exerts its toxicity effects; thus, numerous studies attempted to improve this problem. As shown in Figure 1, our results demonstrated the accumulation of Pb in roots and shoots of maize plants exposed to Pb stress, especially in roots. However, SA pretreatment significantly lowered the accumulation of Pb in shoots and roots of maize plants with more reduction in the roots, indicating that SA might ameliorate Pb toxicity via preventing Pb uptake

in plants. Kohli et al. (2018) also reported a decline in Pb uptake in *Brassica juncea* plants following exogenous application of SA. In this work, Pb stress led to enhancement in MDA and H<sub>2</sub>O<sub>2</sub> levels (Table 3), which ultimately inhibited growth and caused damage to chlorophyll and oxidize PT groups (Tables 1 and 2). Similarly, higher rates of oxidative damage and lipid peroxidation were found in pea under cadmium stress (Jan et al. 2018). In contrast, pretreatment of seeds with SA reduced the levels of Pb-induced MDA and H<sub>2</sub>O<sub>2</sub> contents, indicating that SA helps to overcome Pb mediated oxidative stress. The ability of ROS to oxidize PT groups and cause damage to organic molecules could probably explain the reduction of PT and chlorophyll contents in this research. Depletion of protein bound thiol groups has been previously reported in Ni-treated rice seedlings (Maheshwari and Dubey 2009). In contrast, seed soaking with SA displayed more growth and higher chlorophyll and PT contents in Pb-stressed plants, suggesting improvement of these parameters in SA pretreated plants.

**Figure 2.** Effect of seed pretreatment with SA on H<sub>2</sub>S content of maize plants under lead stress (Mean  $\pm$  SE,  $n = 3$ ),  $P \leq 0.05$ .**Figure 3.** Effect of seed pretreatment with SA on L-DES activity of maize plants under lead stress conditions (Mean  $\pm$  SE,  $n = 3$ ),  $P \leq 0.05$ .

In the present research, an elevated level of proline in plants exposed to Pb could be a biochemical adaptation for scavenging ROS and maintaining water balance. A significant increase in proline accumulation has also been reported in Pb stressed *B. juncea* L. plants (John et al. 2012). SA pretreatment reduced proline accumulation of stressed plants. Similarly, the application of SA in the salt-stressed rice plants reduced the accumulation of proline (Mostofa et al. 2015). In this study, SA likely alleviated Pb stress and, hence, high level of proline accumulation was not needed.

The previous study indicates that SA plays an important role in stress alleviation through participation in sulfur assimilation (Nazar et al. 2015). Our data showed enhancement of the NPT content in plants pretreated with SA, thus enabling the plant to detoxify heavy metal (Zhou et al. 2009). Induction in NPT by SA pretreatment may indicate stimulation of glutathione synthesis and metal-bound peptides, such as phytochelatin which play an important role in the separation and homeostasis of various metals through sulfur assimilation (Nazar et al. 2011, 2015).

The results indicate that Pb treatment significantly ( $p < 0.05$ ) elevated CAT in both shoot and root and APX activities of the root in maize plants as a defense response to Pb toxicity, revealing more involvement of these two enzymes in scavenging of free radicals (Table 3). Oxidative damage that occurs during Pb stress is due to the imbalance between the production of ROS and its scavenging (Sharma and Dubey 2005). SA pretreatment stimulated all studied antioxidant enzymes under Pb stress (Table 3). There are several reports demonstrating an increase in antioxidant enzymes activity under SA treatment exposing different stresses (Zhou et al. 2009; Nazar et al. 2011). Therefore, the protective effects of SA pretreatment can be attributed to the activation of antioxidative responses. GSH and AsA play a vital role in sustaining stable redox state by removing excess ROS from different cellular compartments, and subsequently detoxifying the cell (Shan et al. 2011). The  $H_2O_2$  reduction in SA primed plants could be linked to AsA-GSH pathway (Shan et al. 2011). The elevation in GSH content of SA pretreated plants could be explained by an increase in GR activity in these plants. Pretreatment of SA improved GSH and AsA contents, and GR and APX activities in the maize plants under Pb stress (Tables 2 and 3) which show the possible role of SA in inducing AsA-GSH cycle during Pb stress.

$H_2S$  is an important signaling molecule and a sulphur-containing compound, which plays a role in physiological functions and the acquisition of stress tolerance in higher plants (Ali et al. 2014). The results demonstrated that Pb stress decreased ( $p < 0.05$ )  $H_2S$  level. However, SA pretreatment increased  $H_2S$  by stimulating L-DES activity, which is evident from the data presented in this study (Figures 2 and 3). SA-induced  $H_2S$  accumulation has also been reported in maize and Arabidopsis plants under heat and cadmium stresses, respectively (Li et al. 2015; Qiao et al. 2015). In our previous study, we documented that SA and  $H_2S$  can regulate methionine and arginine metabolic pathways to relieve Pb toxicity (Zanganeh et al. 2018). Hence, our data emphasize the possible cross talk between SA and  $H_2S$ . In addition, considering the similar roles of  $H_2S$  in alleviating stress in previous studies (sulfur assimilation and improvement of oxidative stress) (Ali et al. 2014; Cui et al. 2014), the possibility of cross talk increases between  $H_2S$  and SA. In this research, the acquisition of Pb tolerance was coupled with higher  $H_2S$  content in SA-pretreated Pb-stressed plants. Thus, SA's role in the

improvement of Pb stress could be attributed to  $H_2S$  accumulation. These findings are in accordance with the results of Li (2015), reporting that  $H_2S$  acts as a downstream signal molecule in SA-induced heat tolerance in maize.

## Conclusion

This study provided an insight into the role of SA in ameliorating injurious damages of Pb stress in maize plant and its possible interaction with  $H_2S$ . Therefore,  $H_2S$  can act as a downstream signaling molecule in SA-induced Pb tolerance. Here, we emphasized the possible cross talk between SA and  $H_2S$  through a different style and method. However, further studies are still needed in this regard.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

## Notes on contributors

*Roya Zanganeh*, PhD student in Plant Physiology.

*Rashid Jamei*, Associate Professor in Plant Physiology.

*Fatemeh Rahmani*, Associate Professor in Biotechnology.

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