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ORIGINAL ARTICLE

Role of salicylic acid and hydrogen sulfide in promoting lead stress tolerance and regulating free amino acid composition in *Zea mays* L.

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Abstract

Lead toxicity is a threat to growth and production of crop plants. In this study, effect of salicylic acid and sodium hydrosulfide pretreatments was investigated on the alteration in the Pb accumulation, protein content, nitrate reductase activity and metabolism of selected free amino acids in maize plants subjected to lead stress. Maize seeds were soaked in 0.5 mM salicylic acid and sodium hydrosulfide for 12 h and then exposed to 2.5 mM Pb $(NO_3)_2$ for 9 days. Results demonstrated that lead stress significantly reduced the plant growth, shoot glutathione content and protein content, nitrate reductase activity in the shoots and roots while increased glutathione content in roots of maize plants. We also observed accumulation of most free amino acids composition except tyrosine and tryptophan under lead stress condition. Plants pre-treated with salicylic acid and sodium hydrosulfide exhibited improvement of plant growth, decrease of Pb accumulation, increase of protein and glutathione contents and nitrate reductase activity. Salicylic acid and sodium hydrosulfide pretreatments decreased most amino acids content in shoot of lead stressed plants. In case of roots, salicylic acid and sodium hydrosulfide had different effects on the content of amino acids. Pretreatment of sodium hydrosulfide resulted in greater levels of free amino acids in roots. These results indicate that moderate lead stress can be attributed to effect of salicylic acid and sodium hydrosulfide by improvement of nitrate reductase activity and glutathione content and regulation of amino acids metabolism.

Keywords Amino acids · Hydrogen sulfide · Pb stress · Salicylic acid · *Zea mays* L.

Introduction

Due to the industrialization of cities and increased pollution, plants are exposed to a wide range of materials including heavy metals which cause pollution of water, soil and air. The deposition of heavy metals in the soil can impact on many parameters related to plant growth and inhibit the activity of many enzymes and metabolic reactions in plants (Sharma and Dubey 2005). Lead (Pb) is one of non-essential and highly toxic elements, causing adverse effects to all living organisms. Pb toxicity in plants is manifested as damage to plant growth, degradation of protein, metabolism of nitrogen, imbalance of water, disturbance of photosynthesis (Sharma and Dubey 2005).

Nitrate reductase (NR) (rate-limiting enzyme in nitrogen assimilation) is involved in the assimilation of nitrate into amino acids. The enzyme is sensitive to heavy metals toxicity including Pb (Gao et al. 2013). Pb after taken up in cells can be detoxified by some beneficial metabolites including amino acids. Amino acids directly or indirectly (compounds diverted from their metabolism) can play a crucial role in providing enhanced abiotic stress tolerance in plants (Zafari et al. 2016; Khan et al. 2014).

Salicylic acid (SA) and hydrogen sulfide (H_2S) are key signaling molecules that play a role in the physiological functions of the plant, as well as in the alleviation of abiotic stresses, including heavy metals (Khan et al. 2014; Chen et al. 2017). Singh and Usha (2003) and Singh et al. (2015) have shown that SA and NaHS supplementations improve nitrate reductase (NR) activity and protein content under water and arsenic stress in wheat and pea plans, respectively. There are several investigations about the effect of SA and

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NaHS on the free amino acids composition, but the number of these in case of NaHS is very limited (Hussein et al. 2007; Farhangi-Abriz and Ghassemi-Golezani 2016; Zhang et al. 2008). The aim of this study was to investigate Pb impact in plant growth, protein content, nitrate reductase activity, free amino acid composition and glutathione (GSH) content and the ameliorating capacity of SA and NaHS to improve Pb stress through modulating protein and glutathione (GSH) contents and NR enzyme activity and regulation of free amino acid composition.

Materials and methods

Maize (*Zea mays* cv. 704) seeds were disinfected with 10% (v/v) sodium hypochlorite for 5 min, washed thoroughly with distilled water and pretreated with SA (0.5 mM) and NaHS (0.5 mM) for 12 h (concentrations were optimized in preliminary experiments). Four concentrations (0.2, 0.5, 1 and 2 mM) of SA and NaHS were applied to measure seeds germination to determine the optimum concentration (supplementary data, Tables 1 and 2).

Then, the seeds were transferred to Petri-dishes for germination at 26 °C in dark. The uniformly seedlings were cultivated in pot containing sand and perlite in the ratio of 2:1. Plants were grown under long-day conditions with 16 h/8 h day/night cycle at a temperature between 20 and 29 °C. The humidity of growth chamber was set to 60–80%. The 6-day-old seedlings were divided in two groups: group 1 was exposed to lead stress (2.5 mM $Pb(NO_3)_2$) (lead nitrate was added to each pot at the time of sowing) and group 2 irrigated with tap water as control for 9 days. The stressed plants were supplied with sub-optimal nitrate concentrations (decreasing nitrates in nutrient solutions). Concentrations of Ca and K were balanced by $CaCl_2$ and KCl. Then, plants were harvested for further analyses. Plant height was measured with a ruler. The fresh weights of shoots and roots after separating were determined and for estimation of dry weight, plants were oven-dried for 48 h at 65–75 °C and then weighted.

Pb accumulation

The samples were oven-dried at 105 °C for 24 h. Powdered samples of each plant material (500 mg) were digested for 12 h with (7.5 mL) 65% nitric acid and (2.5 mL) 36% hydrochloric acid at 25 °C, then heated for 2 h at 105 °C. The Pb content was determined by atomic absorption spectroscopy (SHIMADZU AA-6300, Japan) (Nóvoa-Muñoz et al. 2008).

Protein content

Protein content was measured by the Lowry method (Lowry et al. 1951). For this purpose, fresh segments weighing 0.2 g were homogenized in an ice bath in 4 mL of 0.2 M Tris–HCl buffer (pH 8.0) containing 17.2 g sucrose and 0.1 g ascorbic acid, and the homogenate was centrifuged at 4000*g* for 20 min. The supernatant was used for protein content assay. The amount of protein was calculated using a standard curve prepared with known concentration of bovine serum albumin.

Assay of NR (E.C. 1.6.6.1) activity

NR activity was assayed by the method of Jaworski (1971). Frozen samples were homogenized in 50 mM phosphate buffer (pH 7.5), 10 mM KNO_3 and 0.5% n-propanol. The samples were incubated in the dark at 30 °C for 30 min. The reaction was stopped by placing the tubes in boiling water for 5 min. Subsequently, the formed nitrite was calorimetrically determined at 540 nm after azo coupling with sulfanilamide and naphthylene diamine dihydrochloride. After 20 min, absorbance was measured at 540 nm. The calibration curve was used for calculation of NR activity.

Table 1 Effect of SA and NaHS on Pb content, protein content, NR activity and GSH content in shoots and roots of maize under Pb stress

Parameters	Tissue	Control	SA (0.5 mM)	NaHS (0.5 mM)	Pb (2.5 mM)	Pb + SA	Pb + NaHS
Pb content (mg/g DW)	Shoot	Nd*	Nd	Nd	3.29 ± 0.61^{a}	1.36 ± 0.50^{b}	1.11 ± 0.44^{b}
	Root				7.16 ± 0.87^{a}	2.59 ± 0.70^{b}	2.12 ± 0.89^{b}
Protein content (mg/g FW)	Shoot	11.13 ± 0.41^{b}	11.91 ± 0.24^{ab}	13.11 ± 0.31^{a}	9.51 ± 0.76^{c}	10.97 ± 0.22^{b}	11.23 ± 0.20^{b}
	Root	9.65 ± 0.50^{b}	10.91 ± 0.07^{a}	10.42 ± 0.31^{ab}	7.63 ± 0.33^{c}	11.30 ± 0.52^{a}	10.55 ± 0.33^{ab}
NR activity (μmol NO_2^-/g FW.h)	Shoot	3.19 ± 0.22^{ab}	3.30 ± 0.22^{ab}	3.59 ± 0.11^{a}	1.69 ± 0.16^{c}	2.49 ± 0.18^{b}	3.03 ± 0.44^{ab}
	Root	1.66 ± 0.06^{b}	2.20 ± 0.15^{a}	2.47 ± 0.31^{a}	0.72 ± 0.07^{c}	1.40 ± 0.11^{b}	1.64 ± 0.07^{b}
GSH content (μmol/g FW)	Shoot	0.20 ± 0.008^{c}	0.25 ± 0.03^{bc}	0.22 ± 0.01^{c}	0.11 ± 0.01^{d}	0.32 ± 0.03^{ab}	0.34 ± 0.02^{a}
	Root	0.09 ± 0.02^{d}	0.20 ± 0.02^{c}	0.25 ± 0.01^{bc}	0.19 ± 0.03^{c}	0.30 ± 0.03^{ab}	0.33 ± 0.01^{a}

Values are mean ± SE of three independent replications. Different superscripted letters (a–d) within the row indicate statistically significant difference between treatments at $P \leq 0.05$, according to Duncan's multiple range test

*Not detected

Table 2 Effect of SA and NaHS on amino acids content in shoots and roots of maize under Pb stress

Amino acids (μmol/g FW)	Tissue	Control	SA (0.5 mM)	NaHS (0.5 mM)	Pb (2.5 mM)	Pb + SA	Pb + NaHS
Aspartate	Shoot	43.02 ± 2.64^{e}	86.83 ± 2.89^{d}	114.18 ± 2.88^{c}	261.26 ± 3.39^{a}	131.58 ± 3.55^{b}	79.68 ± 3.68^{d}
	Root	9.31 ± 1.19^{d}	34.19 ± 1.74^{b}	14.73 ± 2.65^{cd}	38.11 ± 1.73^{b}	21.46 ± 3.27^{c}	159.13 ± 3.64^{a}
Glutamate	Shoot	217.76 ± 3.37^{f}	258.49 ± 3.37^{e}	410.87 ± 2.40^{d}	958.04 ± 5.80^{a}	826.34 ± 5.74^{c}	866.57 ± 12.53^{b}
	Root	102.29 ± 4.05^{e}	294.23 ± 4.62^{c}	147.46 ± 4.64^{d}	328.48 ± 5.79^{b}	343.30 ± 7.13^{b}	1595.75 ± 9.03^{a}
Asparagine	Shoot	21.00 ± 1.45^{d}	31.18 ± 2.45^{d}	235.18 ± 4.13^{c}	790.54 ± 3.74^{a}	224.35 ± 5.16^{c}	457.31 ± 5.29^{b}
	Root	6.01 ± 0.57^{d}	10.16 ± 0.60^{d}	4.63 ± 0.88^{d}	88.37 ± 4.41^{b}	24.71 ± 3.69^{c}	468.59 ± 4.88^{a}
Serine	Shoot	132.53 ± 4.81^{d}	139.33 ± 3.37^{d}	114.13 ± 5.47^{e}	470.85 ± 3.36^{a}	174.26 ± 5.51^{c}	212.16 ± 5.29^{b}
	Root	60.05 ± 2.88^{c}	80.02 ± 2.88^{b}	60.81 ± 3.81^{c}	84.13 ± 2.31^{b}	78.50 ± 3.49^{b}	138.02 ± 4.61^{a}
Histidine	Shoot	8.39 ± 0.48^{cd}	9.17 ± 0.48^{cd}	6.82 ± 0.51^{d}	25.16 ± 1.45^{a}	17.92 ± 2.70^{b}	12.85 ± 1.95^{bc}
	Root	4.08 ± 0.58^{e}	7.02 ± 0.57^{c}	4.49 ± 0.63d^{e}	11.05 ± 0.58^{b}	6.57 ± 0.80^{cd}	22.22 ± 1.17^{a}
Glycine	Shoot	11.92 ± 0.99^{b}	13.44 ± 0.96^{ab}	11.87 ± 0.98^{b}	17.53 ± 0.96^{a}	11.23 ± 1.92^{b}	12.49 ± 2.09^{b}
	Root	6.19 ± 0.60^{b}	7.08 ± 0.58^{b}	6.56 ± 0.29^{b}	9.80 ± 0.43^{a}	10.15 ± 0.59^{a}	9.73 ± 0.43^{a}
Threonine	Shoot	224.39 ± 4.33^{b}	166.20 ± 4.83^{d}	117.79 ± 3.73^{e}	380.14 ± 4.81^{a}	210.05 ± 3.62^{c}	168.98 ± 3.00^{d}
	Root	79.14 ± 2.89^{c}	89.19 ± 4.04^{c}	30.66 ± 3.71^{d}	113.23 ± 3.47^{b}	120.39 ± 2.82^{b}	149.10 ± 4.61^{a}
Arginine	Shoot	154.27 ± 2.89^{c}	187.02 ± 4.04^{b}	112.83 ± 4.38^{e}	413.21 ± 5.77^{a}	406.30 ± 3.75^{a}	139.07 ± 4.61^{d}
	Root	24.10 ± 1.15^{d}	40.31 ± 1.76^{c}	24.47 ± 1.44^{d}	52.30 ± 1.75^{b}	35.38 ± 2.36^{c}	95.12 ± 2.88^{a}
Alanine	Shoot	9.06 ± 0.58^{b}	0.83 ± 0.06^{d}	5.21 ± 0.61^{c}	23.05 ± 1.15^{a}	6.94 ± 1.15^{bc}	1.88 ± 0.55^{d}
	Root	3.40 ± 0.73^{cd}	5.21 ± 0.64^{bc}	1.83 ± 0.58^{d}	3.76 ± 0.39^{cd}	6.44 ± 0.73^{b}	10.18 ± 0.60^{a}
Tyrosine	Shoot	18.15 ± 0.59^{ab}	16.37 ± 0.73^{bc}	17.07 ± 0.58^{bc}	6.40 ± 0.30^{d}	20.31 ± 1.19^{a}	15.31 ± 0.67^{c}
	Root	10.23 ± 0.62^{a}	9.35 ± 0.72^{ab}	8.45 ± 0.74^{ab}	3.28 ± 0.72^{c}	10.04 ± 0.57^{a}	7.45 ± 0.76^{b}
Cysteine	Shoot	62.54 ± 2.67^{d}	76.42 ± 3.39^{c}	133.84 ± 2.16^{a}	76.27 ± 3.30^{c}	75.05 ± 3.19^{c}	93.83 ± 1.62^{b}
	Root	44.86 ± 2.85^{b}	40.24 ± 3.00^{b}	86.82 ± 3.56^{a}	42.70 ± 2.75^{b}	38.40 ± 2.04^{b}	46.37 ± 3.50^{b}
Valine	Shoot	4.04 ± 0.57^{ab}	3.32 ± 0.33^{b}	3.03 ± 0.55^{b}	5.73 ± 0.72^{a}	4.89 ± 0.49^{ab}	4.06 ± 0.58^{ab}
	Root	1.42 ± 0.05^{f}	1.87 ± 0.04^{d}	2.52 ± 0.05^{b}	1.69 ± 0.05^{e}	2.73 ± 0.06^{a}	2.07 ± 0.04^{c}
Tryptophan	Shoot	382.05 ± 5.77^{c}	2057.18 ± 11.54^{a}	202.93 ± 4.60^{d}	107.20 ± 2.89^{e}	190.29 ± 5.20^{d}	703.21 ± 7.50^{b}
	Root	126.20 ± 4.05^{a}	108.03 ± 4.04^{b}	72.41 ± 3.90^{d}	95.20 ± 3.47^{c}	111.31 ± 3.47^{b}	55.67 ± 3.17^{e}
Leucine	Shoot	17.11 ± 1.16^{bc}	24.30 ± 1.75^{a}	12.28 ± 1.69^{c}	18.89 ± 1.38^{b}	14.67 ± 1.19^{bc}	16.91 ± 1.73^{bc}
	Root	8.24 ± 0.62^{ab}	7.31 ± 0.65^{b}	10.08 ± 0.58^{a}	7.34 ± 0.67^{b}	10.25 ± 0.63^{a}	10.06 ± 0.58^{a}
Isoleucine	Shoot	29.02 ± 2.88^{d}	28.06 ± 2.63^{d}	66.70 ± 3.53^{a}	49.18 ± 4.62^{b}	33.47 ± 3.33^{cd}	42.90 ± 2.66^{bc}
	Root	66.14 ± 2.89^{d}	76.01 ± 3.46^{cd}	191.07 ± 4.61^{a}	39.04 ± 2.30^{e}	92.21 ± 4.04^{c}	78.30 ± 3.47^{b}

Values are mean ± SE of three independent replications. Different superscripted letters (a–f) within the row indicate statistically significant differences between treatments at $P \leq 0.05$, according to Duncan's multiple range test

GSH content

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

Chromatographic separation of amino acids

The Stines et al. (1999) method was followed for the estimation of amino acids. The plant material (0.2 g of fresh weight) was homogenized in water, chloroform, methanol (HPLC grade) in ratio of 3:5:12 (v/v). The extract was centrifuged at 12,000*g* for 5 min; the supernatant was diluted 1:5 (v/v) with 0.25 M borate buffer, pH 8.5. In this study, *O*-phthalaldehyde (OPA) was used as pre-column derivatizing reagent. OPA-derivatized amino acids were injected to HPLC system (Shimadzu, Japan (for separation on Eurospher 100-5-C-18 column (25 cm × 4.6 mm; 5 µm). The mobile phase was a mixture of 50 mM sodium acetate, pH 7.02 (79%) and methanol (21%) (buffer A) and 50 mM sodium acetate, pH 7.02 (25%) and methanol (75%) (buffer B). Resolution of amino acid derivatives were monitored through fluorescence detector with excitation and emission set at 330 nm and 450 nm, respectively. Data were

acquired and processed by ChromGate chromatography management system.

Statistical analysis

All the assays were carried out in triplicate. The results are expressed as mean values and standard error (SE) of the mean. Data analyses were performed using SPSS software version 19 and the means were compared using Duncan's multiple range (DMRT) test at $p < 0.05$ following analysis of variance (ANOVA).

Results

Growth parameters

The plants grown in medium containing excess Pb exhibited reduced growth parameters (length 32 and 39%, fresh weight 36 and 48% and dry weight 14 and 16%) in shoot and root, respectively, but application of SA and NaHS improved growth parameters of shoots and roots (Fig. 1).

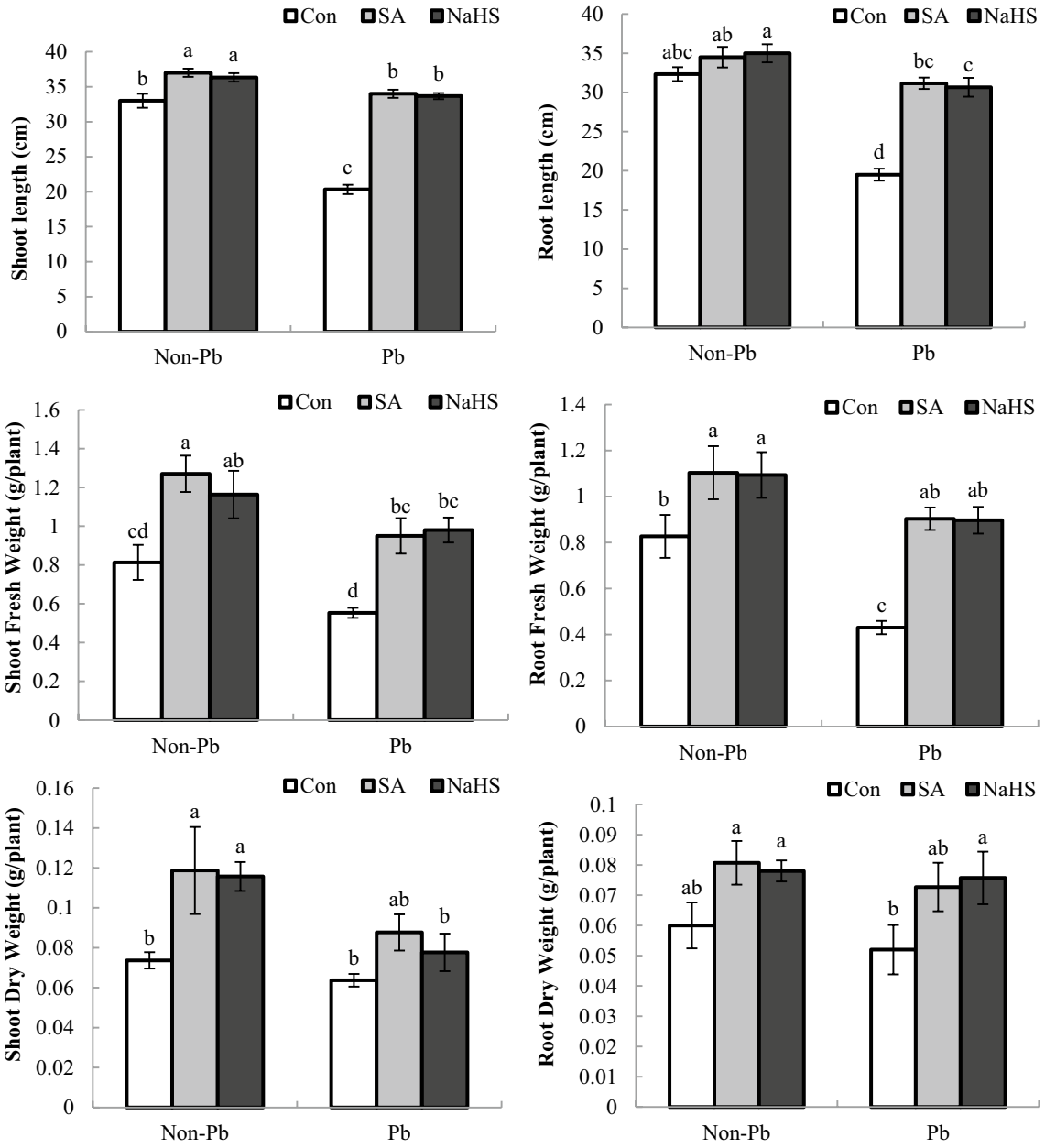

Fig. 1 Effect of SA and NaHS on length and fresh and dry weight of shoot and root of maize under Pb stress. Bars represent standard errors (SE) of the mean ($n = 3$). Different letters indicate significant difference between treatments at $P \leq 0.05$, according to Duncan's multiple range test

Pb accumulation

Under Pb stress, Pb accumulated in both shoots and roots. Seed pretreatment with SA and NaHS, however, markedly reduced the Pb content in shoot and root of maize.

Content of soluble proteins and NR activity

Exposure of maize plants to Pb led to decrease of protein content and activity of NR, however, protein content and NR activity increased in the shoots and roots when seeds were pretreated with SA and NaHS. Marked increase in NR activity was observed in both shoot and root in SA- and NaHS-pretreated plants under normal condition. Moreover, in non-stressed plants, NaHS and SA pretreatments significantly increased shoot and root protein contents, respectively (Table 1).

GSH content

Our observation showed that Pb treatment increased GSH content by 52% in roots while decreased it by 45% in shoots of maize plants. Compared to Pb stress alone, pretreatment with both SA and NaHS resulted in increase in GSH content. GSH content showed significant rise in roots of SA- and NaHS-pretreated plants under non-stress condition (Table 1).

Changes in free amino acids content

The effects of Pb stress and SA and NaHS supplementations on the free amino acid profile are presented in Table 2. Among 15 detected free amino acids, Pb treatment markedly increased the aspartate, glutamate, asparagine, serine, histidine, glycine, threonine, arginine and isoleucine contents in both shoot and root of maize plants. Pb stress also increased alanine and cysteine content in shoot and valine content in root significantly. Two amino acids, tyrosine and tryptophan showed decline compared to control in both shoot and root.

In shoots, both SA and NaHS pretreatments decreased 8 amino acids including aspartate, glutamate, asparagine, serine, histidine, glycine, threonine and alanine contents in Pb-contaminated plants. SA pretreatment also decreased isoleucine and NaHS decreased cysteine content in shoot, significantly. However, tyrosine and tryptophan contents were enhanced in shoot of both SA- and NaHS-pretreated plants. SA and NaHS pretreatments had no significant effect on arginine, valine and leucine contents in shoot of Pb stressed maize plants (Table 2).

In case of roots, SA pretreatment accumulated 6 amino acids (alanine, tyrosine, valine, tryptophan, leucine and isoleucine) content in stressed plants. However, SA priming decreased aspartate, asparagine, histidine and arginine content and had no significant effect on other amino acids content in roots of Pb contaminated plants. Pre-sowing seed treatment with NaHS increased 12 amino acids content in roots of stressed plants. However, NaHS pretreatment decreased tryptophan content and had no significant effect on glycine and cysteine contents in roots of Pb-treated plants (Table 2).

Discussion

In present study under Pb treatment a significant amount of Pb was accumulated by the maize plant, especially in the roots (Table 1). Therefore, the role of root is very important, because the roots can act as the main site for the deposition and inactivation of metals (Sharma and Dubey 2005). Pb accumulation is associated with a reduction in growth in this study (Fig. 1). In many studies on plants exposed to abiotic stresses, accumulation of amino acids has been observed (Pavlíková et al. 2014; Zemanová et al. 2017). Our study has also revealed increase in the content of most amino acids in Pb-treated plants. Amino acids play an important role in tolerance of stressed plants through osmotic regulation, intracellular pH adjustment, ion transfer regulation, cell homeostasis, ROS detoxification, xenobiotics and heavy metals chelation (Zemanová et al. 2017; Dixit et al. 2015). In addition, it has been reported that increase in content of amino acids may be due to accumulation of several compounds of Krebs cycle such as pyruvate and 2-oxoglutarate caused by the malfunctioning of respiratory activity, which may increase synthesis of certain amino acids, and may also be due to decreased protein synthesis and the change in the balance between soluble amino acids and proteins or increased protein degradation under stress condition (Sujatha and Priyadarshini 2009). In this investigation, growth and protein content decreased in shoot and root of maize plants under the Pb stress (Table 1).

In this research, aspartate content was increased under stress in shoot and root of maize plants. Similar to our results, Zemanová et al. (2017) reported an increase in aspartate in *Noccaea praecox* under cadmium stress. Increase of aspartate content may be due to a decrease in protein synthesis which was observed in this study (Table 1). Seed pretreatment with SA reduced aspartate content of shoot and root of maize under stress conditions, and seed pre-treatment with NaHS reduced shoot aspartate content and increased content of root aspartate. Induction in synthesis of some stress proteins in response to abiotic stress in SA- and NaHS-pretreated plants (Khan et al. 2015; Li et al. 2016b), can be a reason for reduction of amino acids content in maize plants.

Glutamate is an acidic amino acid and a central molecule in amino acid metabolism in plants. In this study, glutamate content increased under Pb stress in both shoots and roots compared with the control plants (Table 2). Similar to our

results, increase in glutamate content was reported in pigeon pea plant under lead and cadmium stresses (Sujatha and Priyadarshini 2009). Skopelitis et al. (2006) have reported that protease enzyme activity increases under abiotic stresses. Increasing glutamate content in this study can be due to increased activity of protease under stress. Increasing the activity of protease enhances the amount of ammonium which is toxic to the cell. Glutamate synthase enzyme uses this ammonium to synthesize glutamate (Skopelitis et al. 2006). High demand for glutamate under stress conditions has been reported to be due to its use for the synthesis of proline, polyamines and GSH. Glutamate is one of the three amino acids (cysteine, glutamate and glycine) involved in the GSH structure (Forde and Lea 2007). Seed pre-treatment with SA and NaHS reduced glutamate content in shoot under stress conditions. However, NaHS pretreatment increased root glutamate content in stressed plants. A slight decrease in this amino acid, as mentioned earlier, is probably due to its use for the production of GSH which increased with SA and NaHS pretreatments, in this study (Table 1). Similar to the results of this study, pretreatments of SA and NaHS have been shown to increase GSH content in mustard and pea under salt and arsenate stresses, respectively (Nazar et al. 2015; Singh et al. 2015). GSH is responsible for binding of metal ions, and also for protection against oxidative damage (Singh et al. 2015). Therefore, alleviating the Pb toxicity by this protective compound probably affects the metabolism of amino acids.

Among the 21 amino acids of proteinogenic, arginine has the highest ratio of nitrogen to carbon (4 N/6C), which makes it an appropriate form of organic nitrogen storage (Winter et al. 2015). In this study, arginine content increased under Pb stress (Table 2). Increasing the amount of arginine may be due to its osmolyte role (Azevedo Neto et al. 2009). Azevedo Neto et al. (2009) reported that arginine accumulation in maize plant during salinity stress is probably the result of de novo synthesis that prevents the accumulation of toxic ammonium at the time of growth retardation caused by salt stress. Seed pre-treatment with SA reduced arginine content in shoot and root of maize under stress conditions whereas seed pre-treatment with NaHS decreased arginine content in only shoot of maize plants under Pb stress. This amino acid is a precursor of the synthesis of polyamines, proline and also the nitric oxide signaling molecule with important physiological functions in plant (Winter et al. 2015). Therefore, it is likely that the reduction of arginine content by pretreatment of SA and NaHS may be due to the positive effect of these compounds on the catabolism of this amino acid for biosynthesis of products derived from its metabolism, which plays an important role in improving the stress.

The asparagine amino acid, like arginine, has a high N/C ratio. There is evidence that asparagine can be bound to cadmium, zinc and Pb (Lea et al. 2007). In this study, asparagine content increased under Pb stress (Table 2). Chaffei et al. (2004) reported that cadmium stress increases the concentration of asparagine in roots and leaves of tomato which was consistent with our results. They observed increased activity of asparagine synthase under cadmium stress. Increasing asparagine under stress in this study may be either directly due to its contribution to maintaining osmotic pressure or indirectly due to the restriction of protein synthesis under stress conditions (Lea et al. 2007). Pretreatment of seed with SA reduced asparagine content in shoot and root tissues under stress conditions. Seed pretreatment with NaHS decreased asparagine content in shoot of plants under Pb stress. Reducing asparagine content in SA- and NaHS-pretreated plants is probably due to stress relief and therefore a decrease in the need for high levels of asparagine. However, asparagine content increased in roots of NaHS-pretreated plants under stress conditions.

In this study, Pb stress increased the amount of histidine. Azevedo Neto et al. (2009) also reported an increase in content of histidine in maize plants under salt stress due to the synthesis of de novo and nitrogen storage to prevent toxicity of ammonium (due to reduced growth and protein degradation) resulting in increased contents of histidine and arginine amino acids. These two amino acids are suitable for storing nitrogen due to presence of two amides in their structure. Seed pre-treatment with SA reduced level of histidine in shoots and roots under stress conditions, but seed pre-treatment with NaHS reduced the amount of roots histidine and increased content of histidine in shoots.

Cysteine is involved in the production of several important cellular compounds such as glutathione, metallothionein, phytochelatin and hydrogen sulfide, and plays an important role in enhancing tolerance to stress (Erika et al. 2004). In this study, the cysteine content increased in shoots of maize. The increase of cysteine can be due to demand for biosynthesis of sulfur-containing compounds for stress adaptation. Similar to our results, Cui et al. (2014) reported an increase in cysteine content in Arabidopsis plants under cadmium stress. However, pretreatment of seeds with SA had no obvious effect on cysteine content in both shoots and roots under stress condition. However, NaHS pretreatment increased shoot cysteine content in Pb-stressed plants. Nazar et al. (2015) and Cui et al. (2014) observed that cysteine content increased in SA- and NaHS-treated plants under salt and Cd stresses, respectively. The contribution of SA and H_2S in sulfur assimilation and stress alleviation has been recorded (Nazar et al. 2015; Cui et al. 2014).

Tyrosine is an aromatic amino acid, produced from shikimate pathway. In addition to being used for protein biosynthesis, it is considered as secondary metabolite precursor such as flavonoids, phenolic acids and phytotoxins (Zafari et al. 2016). In this study, content of tyrosine in shoot and

root of maize plants decreased under Pb stress. Lehmann and Pollmann (2009) reported an increase in the activity of the tyrosine decarboxylase enzyme (converting tyrosine to tyramine) in Arabidopsis plants under drought stress. They reported the role of this enzyme in increasing plant defense. In addition, in the pathway of biosynthesis of phenolic compounds, two phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL) enzymes are known as the most important enzymes. However, TAL enzyme is an auxiliary enzyme in comparison with PAL. There are several reports that the TAL activity is increased under stress in plants (Nemat Alla et al. 2002). Therefore, the observed decrease in tyrosine content in the maize plant under Pb stress is possibly due to elevated activity of the tyrosine decarboxylase and TAL enzymes. Pretreatment of seeds with SA and NaHS enhanced the amount of tyrosine in the shoot and root of maize plants under Pb stress conditions. In accordance to our results, Hussein et al. (2007) reported that SA increases the amount of tyrosine in maize grown under salt stress.

Photorespiration is the main source of serine and glycine amino acids in photosynthetic tissues. In this study, glycine and serine contents increased under Pb stress (Table 2). Considering increase of glycine and serine (products of photorespiration) in maize as a C4 plant, possibly rate of photorespiration under Pb stress in maize is increased. Serine and glycine form the tryptophan side chain; this amino acid is involved in the synthesis of auxin (Pavlíková et al. 2014). The tryptophan amino acid, like tyrosine, is an aromatic amino acid derived from the shikimate pathway (Zafari et al. 2016). In this study, Pb stress reduced the tryptophan content in both shoot and root of maize plants (Table 2). The accumulation of glycine and serine in Pb stressed plant may be due to decreased in production of tryptophan. Seed pretreatment with SA elevated the content of tryptophan in shoot and root of maize plants and pretreatment with NaHS increased the content of tryptophan only in shoots. One reason for growth induction in SA- and NaHS-pretreated maize plants could probably be the positive effect of these compounds on the synthesis of auxin by tryptophan. Seed pretreatment with SA and NaHS reduced the content of glycine and serine in shoot of maize plants under stress conditions. In case of serine content of root under stress conditions, NaHS pretreatment increased it. Pavlíková et al. (2014) reported that glycine and serine contents reduced in transformed tobacco plants with a construct consisting of SAG12 promoter fused with the *ipt* gene for cytokinin synthesis under Zn toxicity stress which can affect the production of tryptophan and subsequently auxin and thus improve the growth. In addition, it has been reported that GSH biosynthesis and phytochelatin are related to the amino acids of glycine, serine and glutamate which are important as markers in heavy metal stress (Zemanová et al. 2017). Therefore, the reduction of serine and glycine amino acids can be due to their use for the production of these compounds.

The branched-chain amino acids, such as leucine, isoleucine and valine play an important role as osmolyte (Zemanová et al. 2017). The breakdown products of these amino acids, such as acetyl coenzyme A, propionyl coenzyme A, and acetoacetate are potential sources of energy for plant (Zemanová et al. 2017). In this study, isoleucine, leucine and valine contents increased in shoot (Table 2). Lea et al. (2007) reported that the cause of branched-chain amino acids accumulation is result of disruption of acetyl coenzyme A or glycolysis under cadmium stress. The accumulation of these amino acids may be a substrate for the synthesis of stress-induced protein and regulation of gene expression as signaling molecules (Zemanová et al. 2017). Similar to our results, increase in isoleucine content has been reported in response to cadmium in *Noccaea caerulescens* (Zemanová et al. 2017). Planchet et al. (2015) reported that two amino acids of methionine and threonine are precursors of isoleucine. They showed that increase of isoleucine is associated with gene expression induction of threonine deaminase and methionine γ-lyase. Seed pre-treatment with SA and NaHS enhanced these three amino acids in maize root under stress conditions (Table 2). Hussein et al. (2007) also reported that exogenous SA increases leucine, isoleucine and valine contents in maize under salt stress. The use of normal sulfur increased leucine and isoleucine content in rice plant under arsenic stress but decreased valine content (Dixit et al. 2015). Jasmonate hormone plays an important role in stress signaling. The isoleucine can be conjugated to this hormone and play a role in regulation of hormone signaling (Li et al. 2016a). Possibly, increase of isoleucine in the roots of pretreated plants with SA and NaHS will positively affect the regulation of jasmonate signaling.

Alanine regulates intracellular pH (Zemanová et al. 2017). In this study, alanine content increased under Pb stress in shoot and root of maize plants. An increase in alanine content may be due to a decrease in protein synthesis and interruption in reaction of alanine aminotransferase (Zemanová et al. 2017). Alanine plays a role in photosynthesis of C4 plants (Azevedo Neto et al. 2009). Azevedo Neto et al. (2009) have suggested that increase in alanine content in maize plant under salt stress is due to induction of glycolysis, and thus increasing the amount of pyruvate and respiration for energy production under stress conditions and provision of carbon skeleton for photorespiration cycle. Seed pretreatment with SA and NaHS reduced alanine content in maize shoot under Pb stress. Similar to our results, Hussein et al. (2007) reported an increase in the amount of alanine in SA-treated maize plants under salt stress conditions. However, alanine content increased in roots of NaHS-pretreated plants under stress conditions.

In this study, Pb stress has led to an increase in the content of threonine in shoot and root of maize plants. These results were consistent with results of Zemanová et al. (2017), which showed an increase in content of threonine in *Noccaea caerulescens* under cadmium stress. As noted earlier, heavy metal stress induces water status imbalance in plants. Azevedo Neto et al. (2009) reported that threonine is divided into amino acids that contribute to reduction of osmotic potential of cell, hence, is important in heavy metal stress-induced water status imbalance alleviation. Seed pretreatment with SA and NaHS reduced threonine levels in maize shoot under stress condition; however, NaHS pre-treatment increased it in root. Improvement of Pb-induced water regime in SA- and NaHS-pretreated plants has been reported in many investigations (Kohli et al. 2018; Mostofa et al. 2015) under stress conditions, reducing the need to accumulation of free amino acid composition.

In the present work, pretreatment with SA and NaHS attenuated the level of shoot and root Pb accumulation (Table 1). According to our results, there are studies on the effect of exogenous SA and NaHS on reducing heavy metal uptake (Kohli et al. 2018; Mostofa et al. 2015). The significant increase in NR activity and protein content was also observed in plants treated with SA and NaHS (Table 1). Aftab et al. (2011) reported that SA application promotes NR activity in *Artemisia annua* under salt stress. They attributed this observation to the role of SA in stabilizing the plasma membrane and uptake of nutrients including nitrate. Increasing the membrane stability has also been revealed in NaHS-treated pea plant under arsenate stress (Singh et al. 2015). Increase in the activation of NR in SA- and NaHS-pretreated plants caused the increment of protein content. There is close relationship between nitrate assimilation and amino acid biosynthesis. Generally, effects of pretreatment of SA and NaHS on the amino acids content was different in roots of maize plants under Pb stress. NaHS has been shown to increase root amino acids content (glutamate, aspartate, asparagine, serine, histidine, threonine, arginine, alanine, tyrosine, cysteine, leucine and isoleucine) in maize under stress conditions, which may be part of major change in metabolism and amino acid transport into root to cope with stress and activation of defense pathways. Zhang et al. (2008) reported that seed priming with NaHS promoted accumulation of free amino acids in wheat seeds subjected to copper stress. However, SA's effect on increase in content of amino acids was lower, it would probably be protective role of SA in a different way than H_2S. In case of shoot, the content of most amino acids was reduced in SA- and NaHS-pretreated plants under Pb stress. Reduction of amino acids content by these two compounds pretreatment may be seen as assistance to the activation of adaptation processes to the toxic effect of Pb. Since amino acids are precursors for biosynthesis of stress-induced protein and protective metabolites against plant stress (Zemanová et al. 2017).

In conclusion, maize plants exposed to Pb stress displayed the inhibition of growth and decrease of NR activity and protein content. Pb stress also resulted in increase of most free amino acids content in maize plants which can be a defensive response to stress. SA and NaHS pretreatments in addition to reduction of Pb accumulation through triggering defense mechanism such as regulating amino acids content have also been involved in alleviating Pb-induced damage. Probably, amino acids directly or indirectly (synthesis of stress-induced proteins and compounds diverted from their metabolism like GSH which was investigated in this study) can play an important role in alleviating Pb stress. Effect of pretreatments of SA and NaHS on the amino acids content in roots was different. This shows the complex regulation of amino acids levels by these two compounds.

Author contribution statement RZ designed the study, performed the experiments, analyzed data and wrote the manuscript. RJ and FR supervised the study.

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