Exogenous application of salicylic acid improves tolerance of wheat plants to lead stress

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Abstract
Salicylic acid (SA) acts as a signaling molecule and plays an important role in various physiological and biochemical processes in plants. The aim of the present study was to evaluate the role of SA in the enhancement of lead (Pb) tolerance in wheat (Triticum aestivum) plants. When 2–3 true leaves had appeared, treatments were applied to the plants. The treatments were as follows: (i) no addition of SA and Pb (control), (ii) 2 µM SA + 0 mM Pb, (iii) 8 µM SA + 0 mM Pb, (iv) 0 µM SA + 2 mM Pb, (v) 2 µM SA + 2 mM Pb, and (vi) 8 µM SA + 2 mM Pb. One-way analysis of variances (ANOVA) was used to compare the means, and Duncan’s multiple-range test (DMRT) was used to determine significant (P < 0.05) differences among the individual means of treatments. Exposure of Pb severely affected wheat plants by reducing plant height, fresh and dry weight, photosynthetic pigments (Chl a and Chl b, Chl a:b) and carbonic anhydrase enzyme activity, and by enhancing Chl degradation, electrolyte leakage (EL), malondialdehyde accumulation. Also, Pb treatment increased the accumulations of proline and total soluble carbohydrates (TSC) and activities of antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD)]. However, application of SA induced biosynthesis of pigments by suppressing Chl degradation, and EL and malondialdehyde accumulation. Furthermore, SA treatments further enhanced the production of proline and TSC, and the activities of SOD, CAT, and POD. SA directly or indirectly improved physiological processes, which helped wheat plants to overcome the oxidative damage induced by Pb toxicity. Also, this study reveals that exogenous application of SA is beneficial for plant growth and development of wheat plants by suppressing ill effects of heavy metal stress. Therefore, this study opens up the hidden role of SA in tolerance of plants to heavy metal toxicity to explore its new regulatory role and defensive mechanism at physiological and molecular levels. Also, exogenous application of SA could be beneficial for sustainable agriculture.

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1. Introduction
Heavy metal toxicity has become a major threat to our environment globally. It affects plants, animals, and ultimately human health. Increasing urbanization and industrialization have led to the emission of heavy metals into the environment (Fan et al., 2016). Lead (Pb) is considered a major heavy metal pollutant, which causes toxicity in plants when its...
concentration in soil is greater than 30 ppm (ATSDR, 2005; Ruley et al., 2004; Tchounwou et al., 2012). Similar to other heavy metals, Pb causes toxicity to plants by impairing physiological and biochemical parameters that lead to reduced plant growth and development (Sharma and Dubey, 2005). Lead has no documented beneficial role in plants, and even when present in small amounts, it causes toxicity by altering many physiological processes (Sharma and Dubey, 2005). Plants grown in Pb-contaminated soil suffer disruption of many metabolic mechanisms, which leads to decreases in root and shoot growth, biomass production, water uptake, pigment synthesis and inhibition of photosynthesis (Gupta et al., 2013; Leal-Alvarado et al., 2016; Agami and Mohamed, 2013). In plants, Pb causes inhibition of many enzyme activities, disturbance in nutrients and water balance, and alteration in hormonal status and membrane permeability (Sharma and Dubey, 2005). Lead also affects a large number of plant species by inducing oxidative stress, via disruption of the antioxidant defense system and overproduction of reactive oxygen species (ROS). Tolerance of plants to stress depends on the activity of antioxidant enzymes that help to scavenge ROS and on the accumulation of solutes [proline (Pro), glycinebetaine (GB) and soluble carbohydrates] (Siddiqui et al., 2009). However, the defense mechanisms of plants and the mechanism of heavy metal toxicity in plants are complex phenomena (Nareshkumar et al., 2015). On the other hand, plants exhibit a great magnitude of intra- and inter-specific variations and also physiological and biochemical changes occur in response to heavy metals. Therefore, it is important to improve the tolerance of plant to heavy metal stress by utilizing new practices.

Salicylic acid (SA) is recognized as an important plant growth regulator that is present in plants in the form of a glycosylated or methylated glucose-ester, amino acid conjugate, or in the free state (Lee et al., 1995). SA is a secondary metabolite (small phenolic compound) synthesized by plants that plays a key role in the regulation of many physiological processes, and also in the induction of plant immune systems (Venkatesh and Park, 2014; Kovács et al., 2014; Rahmani et al., 2015). It regulates seed germination, plant growth and development, biomass production, photosynthesis, respiration, stomatal closure, senescence-associated gene expression, and enzyme activity under adverse environmental conditions (Klessig and Malamy, 1994; Sahu et al., 2002; Liu et al., 2016). Furthermore, SA is involved in signaling pathways that enhance the tolerance of plants to abiotic stress. However, to date, there have been few studies on the effect of SA on wheat under Pb stress. Therefore, in the present study we examined the role of SA in the regulation of the antioxidant system and biosynthesis of photosynthetic pigments and solutes. The results obtained could be helpful in exploring the role of SA in plant tolerance to Pb stress by enhancing plant growth and development.

Materials and Methods

Experimental material and plan

Seeds of wheat (Triticum aestivum var. Yecora Rojo) were obtained from a local market. The experiments were performed in a growth chamber (temperature 25 ± 3 °C, photoperiod 90 μmol of photons m⁻² s⁻¹; 16/8-h light/dark cycle and relative humidity 50–60%). After sterilization of seed in a sodium hypochlorite solution, the sterilized seeds were sown in plastic pots (6 in. diameter) filled with acid-washed sand. Before sowing, Raukura’s nutrient solution was supplied to keep the sand moist. The experiments were performed in a growth chamber (temperature 25 ± 3 °C, photoperiod 90 μmol of photons m⁻² s⁻¹; 16/8-h light/dark cycle and relative humidity 50–60%). After sterilization of seed in a sodium hypochlorite solution, the sterilized seeds were sown in plastic pots (6 in. diameter) filled with acid-washed sand. Before sowing, Raukura’s nutrient solution was supplied to keep the sand moist. Smith et al., (1983). When 2–3 true leaves had appeared, treatments were applied to the plants. The treatments were as follows: (i) 0 μM SA + 0 mM Pb (control), (ii) 2 μM SA + 0 mM Pb, (iii) 8 μM SA + 0 mM Pb, (iv) 0 μM SA + 2 mM Pb, (v) 2 μM SA + 2 mM Pb, and (vi) 8 μM SA + 2 mM Pb. The source of Pb was lead chloride. All treatments were applied as a basal dose. At 3-day intervals, double-distilled water (DDW) was used for irrigation to keep the sand moist.
Four replicates of each treatment were performed. Five weeks after sowing, samples of plants subjected to each treatment were taken to measure plant growth attributes (plant height plant\(^{-1}\), fresh weight plant\(^{-1}\) and dry weight plant\(^{-1}\)) and physiological and biochemical characteristics (chlorophyll (Chl) \(a\), Chl \(b\) and Chl \(a/b\); Chl degradation; proline (Pro), total soluble carbohydrate (TSC) concentration, malondialdehyde (MDA) concentration, electrolyte leakage (EL), and activity of carbonic anhydrase (CA), catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) enzyme).

**Measurement of growth characteristics**

Samples of all treated plants were taken for morphological analysis. The plant height was recorded directly using a meter scale. After determining the fresh weight (FW), samples were then placed in an oven run at 60°C for drying. After 48 h, the (dry weight) DW of samples was measured.

**Determination of physiological and biochemical parameters**

The concentration of pigments was measured in fully expanded leaves using the dimethyl sulfoxide (DMSO) methods of Barnes et al. (1992). The concentration of Chls was calculated using the following formula:

\[
\text{Chl } a = 14.85 A_{664.9} - 5.14 A_{648.2} \\
\text{Chl } b = 25.48 A_{648.2} - 7.36 A_{664.9}
\]

Chl degradation was measured spectrophotometrically (SPEKOL 1500; Analytik Jena AG, Jena, Germany) and expressed as the ratio between the absorbance at 435 and 415 nm (\(A_{435}/A_{415}\)), as suggested by Ronen and Galun (1984).

The concentration of Pro was determined colorimetrically according to the method of Bates et al. (1973) and measured at \(A_{520}\) using a standard curve of L-proline and calculated on a FW basis.

TSC concentration was determined according to the method of Dubois et al. (1956). TSC was expressed on the basis of DW (mg g\(^{-1}\) DW).

Electrolyte leakage was measured according to the method of Lutts et al. (1995).

Lipid peroxidation was assayed by measuring MDA content in samples according to the method of Heath and Packer (1968).

The activity of CA was determined by the method of Dwivedi and Randhawa (1974) and expressed as \(\mu\text{Mol (CO}_2\text{)} \text{kg}^{-1} \text{(FW)} \text{s}^{-1}\).

To analyze the activities of antioxidant enzymes, fresh leaf samples were homogenized using a chilled mortar and pestle in an extraction buffer (0.5% Triton X-100 and 1% polyvinylpyrrolidone in 100 mM potassium phosphate buffer, pH 7.0). After filtration with muslin cloth, the homogenate was centrifuged at 15,000 \(\times\) g for 20 min at 4°C, and the resulting supernatant was stored at -20°C for the enzymatic assays. The activity of SOD was measured using the method of Giannopolitis and Ries (1977); that of catalase (CAT), using the method of Aebi (1984); that of peroxidase (POD), using the method of Chance and Maehly (1955), and that of glutathione reductase (GR), using the method of Foyer and Halliwell (1976). Enzyme activities were expressed as units of enzyme activity mg\(^{-1}\) protein, which was assayed according to Bradford (1976).

**Statistical analysis**

Statistical analysis was performed for the results obtained from four replicates of each treatment and analyzed using SPSS statistical software version 22.0 for MAC. One-way analysis of variances (ANOVA) was used to compare the means, and Duncan’s multiple-range test (DMRT) was used to determine significant \((P<0.05)\) differences among the individual treatments.
Results

Table 1 shows that application of both levels of SA increased growth characteristics (plant height, fresh weight and dry weight) of wheat plants under non-stress condition. Application of 8 µM SA gave the highest values for these growth attributes as compared to 2 µM SA. However, under Pb toxicity condition, the growth characteristics of plant were decreased. Under Pb toxicity condition, application of both levels SA improved the growth parameters, while maximum improvement was observed with the application of 8 µM SA. Under Pb stress, applications of 2 µM and 8 µM SA improved plant height by 74.25 and 95.26%, fresh weight by 87.02 and 161.95%, and dry weight by 56.17 and 99.70%, respectively, as compared to Pb application.

Exogenous applications of SA enhanced the biosynthesis of Chl a and b under controlled conditions (Fig. 1A). The highest levels of Chl b were recorded with the application of 8 µM SA, compared to 2 µM SA, whereas the levels of Chl a were not significantly different in plants subjected to both these SA treatments. Application of 2 µM and 8 µM SA notably increased Chl a by 17.24% and 26.72%, and Chl b by 141.18% and 213.79%, respectively, compared to the respective controls. However, the synthesis of both pigments, Chl a:b, and Chl degradation in functional leaves of wheat plants were affected by Pb toxicity (Fig. 1A, B, and C). Pb toxicity in plants reduced both photosynthetic pigments and increased Chl a:b and Chl degradation. However, application of SA significantly increased Chl a and b concentration and inhibited the increase in the Chl a and b ratio and Chl degradation, although the effect was most notable with 8 µM SA under Pb toxicity.

In the present study, the accumulation of MDA was used to represent the level of lipid peroxidation, and EL indicated the extent of membrane damage and alteration (Fig. 2A and B). Plants showed maximum MDA accumulation and EL under Pb toxicity. However, application of SA (2 and 8 µM) suppressed the accumulation of MDA and EL, although the effects were more significant with 8 µM SA than with 2 µM SA. Application of 2 µM SA and 8 µM SA decreased EL by 38.15% and 51.19% and MDA by 38.89% and 50.49%, respectively, compared to Pb application.

Under non-toxic conditions, the accumulation of Pro and TSC was increased according to the levels of SA (Fig. 2C and D). Application of 8 µM SA was more effective than 2 µM SA for both parameters. Application of 2 µM and 8 µM SA increased Pro by 48.99% and 85.83% and TSC by 27.65% and 46.36%, respectively, compared to the control. Under conditions of Pb toxicity, plants showed higher contents of Pro and TSC than the control. However, application of SA further increased Pro and TSC. Application of 2 µM and 8 µM SA enhanced the levels of Pro by 2.28% and 19.09% and TSC by 3.77% and 16.34%, respectively, compared to Pb application.

The activity of CA and antioxidant enzymes (SOD, CAT, and POD) was enhanced with increasing levels of SA under non-Pb toxicity (Fig. 3A-D). Application of 8 µM SA was found to be more effective than 2 µM SA for enhancing the activities of all these enzymes, with the exception of CAT. Application of 2 µM and 8 µM SA increased the activity of CA by 11.50% and 17.75%, SOD by 25.33% and 52.65%, CAT by 20.77% and 34.03% and POD by 7.72% and 18.32%, respectively, compared to the respective controls. Pb toxicity in plants enhanced the activity of antioxidant enzymes and decreased CA activity. However, application of SA enhanced antioxidant enzymes activity further, although the effects were most notable with 8 µM SA for the activities of all enzymes. The activity of CA, SOD, POD, and CAT was increased 120% and 140.71%, 5.57% and 42.47%, 11.88% and 23.60%, and 5.67% by and 10.70%, respectively, in plants treated with 2 µM and 8 µM SA, compared to plants treated with Pb.
**Table 1.** Exogenous application of salicylic acid (SA) affects plant height, fresh weight and dry weight of wheat plants under Pb toxicity.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Plant height (cm)</th>
<th>Plant fresh weight (mg)</th>
<th>Plant dry weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.57±0.33d</td>
<td>249.51±09.89c</td>
<td>48.89±3.45cd</td>
</tr>
<tr>
<td>2 µM SA + 0 mM Pb</td>
<td>13.07±0.33b</td>
<td>324.67±12.40b</td>
<td>62.47±2.19b</td>
</tr>
<tr>
<td>8 µM SA + 0 mM Pb</td>
<td>15.37±0.52a</td>
<td>557.90±09.75a</td>
<td>74.47±2.58a</td>
</tr>
<tr>
<td>0 µM SA + 2 mM Pb</td>
<td>6.33±0.69c</td>
<td>117.69±09.81d</td>
<td>26.58±1.82c</td>
</tr>
<tr>
<td>2 µM SA + 2 mM Pb</td>
<td>11.03±0.42c</td>
<td>220.10±10.90c</td>
<td>41.51±2.54d</td>
</tr>
<tr>
<td>8 µM SA + 2 mM Pb</td>
<td>12.36±0.25b</td>
<td>308.29±10.33b</td>
<td>53.08±2.73c</td>
</tr>
</tbody>
</table>

Average of four determinations are presented with standard error. Different letters show statistical difference at \( p < 0.05 \) (Duncan’s multiple-range test).

**Figure 1.** Exogenous application of salicylic acid affects (A) Chlorophyll \( (Chl) \) a and b, (B) \( Chl \) a and b ratio and (C) \( Chl \) degradation in wheat plant under Pb toxicity. Vertical bars represent standard error. Average of four determinations are presented with bars labeled with the different letters show statistical difference at \( p < 0.05 \) (Duncan’s multiple-range test).
Discussion

Lead toxicity inhibits plant growth by affecting plant metabolisms. In the present experiment, application of Pb decreased growth characteristics (plant height, fresh and dry weight) (Table 1). Under Pb toxicity, application of SA improved growth parameters by mitigating adverse effects of Pb. It may be due to the role of SA in plant growth and development (Sahu et al., 2002; Venkatesh and Park, 2014; Rahmani et al., 2015; Liu et al., 2016). SA induces cell division within the apical meristem of seedling resulting in increased plant growth (Shakirova et al., 2013). Also, application of SA might be beneficial in improving plant growth or in alleviating the toxic effects of Pb by restoring of hydraulic conductance and stimulating of antioxidant response.

The photosynthetic pigments in plants are key components for photosynthesis, and play important roles in plant growth and development and dry matter yield. In the present study, Pb toxicity in plants caused a substantial reduction in Chl a and b and enhanced Chl a:b and Chl degradation (Fig. 1A-C). A decrease in photosynthetic pigment accumulation in Pb-treated plants might be attributable to the destruction of chloroplast membranes by enhanced levels of MDA and EL (Fig. 2A and B) and decreases in the related enzymes [δ-aminolevulinic acid (ALA) synthase, ALA dehydratase, and porphobilinogenase], and uptake of water and nutrients, which lead to Chl degradation (Fig. 1C) (Mourato et al., 2015; Siddiqi et al., 2012). Furthermore, heavy metals cause over-production of ROS that damage cell membranes and chloroplast pigments (Siddiqi et al., 2012). Under conditions of Pb toxicity, the ratio of Chl a to b was increased, which is one of the stress indicators. This result is consistent with the findings of Zengin and Munzuroglu (2005). However, on the basis of obtained results, it is clear that application of SA enhanced the accumulation of photosynthetic pigments and decreased Chl degradation and Chl a:b under both stress and non-stress conditions. This may be attributable to the protective role of SA in decreasing Chl degradation, MDA accumulation, and EL in plants under stress (Figs. 1A and 2A and B). It has been reported that the concentration of pigments increased in rapeseed (Baghai et al., 2002) and wheat (Siddiqui et al., 2012) with the application of SA. Therefore, it is postulated that the increased photosynthetic pigments and decreased Chl degradation and Chl a:b in response to SA confirms the role of SA in the tolerance of wheat plants to Pb toxicity.

It is well established that plants under different environmental stresses suffer from oxidative damage, as measured by lipid peroxidation and EL. Wheat plants had maximum MDA accumulation and EL under Pb toxicity (Fig. 2A and B), which results in cellular dysfunction. These results substantiate previous findings that Pb toxicity in plants enhances EL and MDA accumulation (Dugar and Bafna, 2013). However, application of SA inhibited lipid peroxidation by suppressing MDA accumulation and EL, which may be attributable to the increased accumulation of Pro and TSC, and increased antioxidant enzyme activities (Fig. 2A and B and 3B-D), as these inhibit the over-production of ROS.

To cope with abiotic stress, plants produce organic solutes to regulate many physiological processes. Under Pb toxicity, plants exhibited higher levels of Pro and TSC (Fig. 2C and D). However, levels of Pro and TSC increased further when SA was supplied to the plants. An increase in the level of Pro in plants might be attributable to the up-regulation of pyrroline-5-carboxylate reductase and γ-glutamyl kinase activity and down-regulation of the activity of Pro oxidase in plants receiving SA (Misra and Saxena, 2009). Enhanced Pro accumulation in plants may have been responsible for countering Pb-induced lipid peroxidation and membrane alteration (Fig. 2 A and B) (Mehta and Gaur, 1999). Furthermore, in the present study, SA application increased TSC, which are highly sensitive to different environmental stresses and regulate various genes associated with growth and
metabolism, thereby providing carbon and energy resources (Rosa et al., 2009).

The enzyme CA plays key role in ion exchange and acid and base balance. It is required for PSII maximum photosynthetic efficiency (Shitov et al., 2018). Lead toxicity in wheat plants inhibited CA enzyme activity (Fig. 3A). A decrease in CA activity might be attributable to the enhanced lipid peroxidation and membrane damage. However, application of SA increased CA activity. This result is consistent with the findings of Al-Whaibi et al. (2012). Enhanced CA activity could explain the increases in physiological processes by maintaining ion balance and providing a constant supply of CO₂ to Rubisco. The improved activity of CA may help in improving maximum efficiency of the PSII photosynthetic electron transport in plants (Shitov et al., 2018). Therefore, we conclude that SA is not only involve in plant growth, it also reduces adverse effects of Pb by improving photosynthetic enzymes (Rubisco) and maximum electron transport (Siddiqui et al., 2016; Shitov et al., 2018).

It is well established that the antioxidant system in plants constitutes a defensive mechanism against ROS. In our study, the activity of antioxidant
enzymes, such as SOD, CAT, and POD, increased when wheat plants were exposed to Pb (Fig. 3B, C, and D). Interestingly, application of SA further increased activity of these enzymes under Pb toxicity. These results are consistent with the earlier findings of Al-Whaibi et al. (2012). The increased activity of these enzymes enhanced the tolerance of wheat plant to Pb toxicity by modulating the relative amounts of ROS. The activity of SOD provides a first-line defense system against ROS in coordination with CAT and POD. During Pb-toxicity, altered physiological functions may also be restored by SA because it acts as signaling molecule which induces secondary messengers (ROS) that regulate defense related genes associated with systematic acquired resistance in different plants (Rivas-San Vicente and Plasencia, 2011; Siddiqui et al., 2013).

Conclusions

We conclude that exposure of Pb (2 mM) induced toxicity in wheat plant. Under Pb toxicity, plant exhibited reduced pant growth parameters and decreased photosynthetic pigments Chl a and b, Chl a:b, and CA enzyme activity. Lead toxicity in wheat plants enhanced Chl degradation; the accumulation of Pro, TSC, EL, and MDA; and antioxidant enzyme activities. However, application of SA (both 2 and 8 µM of SA) increased the biosynthesis of pigments by inhibiting Chl degradation, EL, and MDA.
Furthermore, SA treatments enhanced Pro, TSC, and antioxidant enzymes (SOD, CAT and POD) further. Therefore, it is postulated that SA directly or indirectly increases physiological processes, which help wheat plants to tolerate Pb toxicity.

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