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ORIGINAL RESEARCH PAPER

Foliar application of salicylic acid improves growth and yield attributes by upregulating the antioxidant defense system in *Brassica campestris* plants grown in lead-amended soils

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Abstract
Lead (Pb) toxicity causes a severe impact on plant growth and productivity. A protective role of salicylic acid (SA) is well known under different abiotic stress conditions. However, very little is known about the SA-induced Pb resistance mechanism. In this study, we investigated the effect of SA on mustard plants (*Brassica campestris* L.) under Pb-stress conditions. Plants were exposed to three levels of Pb amendment to the soil (0.25, 0.50, 1.00 mM), with or without SA (0.25 mM). Plant growth, yield attributes, and yield at harvest were reduced depending on the severity of the Pb stress. Exogenous application of SA improved plant growth and yield. Biochemical data revealed that Pb toxicity resulted in higher oxidative damage by reducing nonenzymatic antioxidants such as ascorbate and glutathione at the higher dose of Pb treatment. Antioxidant enzymes (ascorbate peroxidase – APX, monodehydroascorbate reductase – MDHAR, dehydroascorbate reductase – DHAR, glutathione reductase – GR, guaiacol peroxidase – POD, glutathione *S*-transferase – GST, and catalase – CAT) responses varied with the Pb doses. Both the nonenzymatic and enzymatic components of the antioxidant defense system were upregulated after application of SA, resulting in lower oxidative damage under Pb-stress conditions. Taken together, the results suggest that exogenous application of the SA mitigates Pb-induced oxidative damage and consequently results in better growth and yield in mustard plants.

Keywords
abiotic stress; phytohormones; reactive oxygen species; soil pollution; toxic metals

Introduction

In recent decades, heavy metal contamination of soils, air, and water has increased and posing threats to agricultural ecosystems and environments globally due to rapid urbanization and industrialization. These metals can be taken up by plants from contaminated soil and by atmospheric deposition reducing plant growth and productivity. Subsequent transfer to human beings through the food chain is a potential serious issue with consequences to human health. Among the toxic metals and metalloids, lead (Pb) is a hazardous element along with chromium (Cr), cadmium (Cd), arsenic (As), which are highly poisonous due to their toxic effects on animal and human health [1]. Lead contamination in the environment can result both from natural sources and

from anthropogenic activities. The main sources of Pb contamination in the environment are the weathering of Pb-containing rocks and minerals, industrial and refining operations such as the manufacturing of paints, batteries, gasoline, and petroleum. Other sources are from the use of industrial effluents for irrigation, application of biosolids to land, and the excessive use of pesticides and fertilizers. Both animals and plants are adversely affected by Pb toxicity. Lead is taken up by plants by roots under appropriate soil conditions, but due to its low mobility and a strong binding capacity on to the organic phases in soil and to plant roots, its uptake is generally limited [1,2]. However, when lead becomes mobilized it can impact on seed germination and cause malformation of cellular structure, chlorosis, and stunted plant growth. Furthermore, it blocks K^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Fe^{3+} to entry into the root system impacting on cellular functions and subsequently resulting in phytotoxicity to plants [3,4].

When plants are under Pb stress, several physiological processes are disrupted by the production of increased amounts of reactive oxygen species (ROS) leading to oxidative stress within the plant cells so inhibiting photosynthetic activity, chlorophyll (chl) biosynthesis, hormonal balance, ion homeostasis, membrane stability, ATP production, lipid peroxidation, DNA damage, and imbalances in nutrient and water relations resulting in cell abnormalities and death [5–7]. Plants deal with Pb toxicity by different physiological and biochemical processes. Amongst the latter, plants endogenously activate both enzymatic and nonenzymatic antioxidant defense systems to scavenge ROS. Nonenzymatic antioxidants such as ascorbate (AsA), reduced glutathione (GSH), oxidized glutathione (GSSG), α-tocopherol, phenolic compounds, alkaloids, and nonprotein amino acids. Enzymatic antioxidants involved either directly or indirectly in scavenging ROS under Pd-stressed conditions include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione peroxidase (GPX), glutathione *S*-transferase (GST), and guaiacol peroxidase (POD) [8–10]. Moreover, some plants can also accumulate different compatible solutes or osmolytes to maintain osmotic adjustment under heavy metal stress conditions. Exogenous application of different phytohormones, including salicylic acid (SA), jasmonic acid, abscisic acid, and gibberellins leads to an induction of signaling cascades which can increase plants growth and productivity under different environmental stresses by enhancing antioxidant activities and scavenging ROS [11,12]. Among these phytohormones, SA acts as an omni-present growth regulator which can regulate various physiological and metabolic processes and it plays an important role in the defense mechanism against lead toxicity [13–15]. It has been reported that SA triggers different gene expressions related to defense, either directly or by the H_2O_2-mediated signal transduction pathway which regulates mitogen-activated protein kinase (MAPK) [16]. Previous research has elucidated that foliar supplementation of SA increases resistance under different heavy metal stress conditions in different crops, such as Pb stress in rice and barley [17,18] and Cd stress in maize [19]. Exogenous application of SA can maintain the stability and integrity of the cell membrane [19], increase the antioxidant activity by reducing ROS [20,21], upregulate heme oxygenase [22], improve the photosynthetic capacity [23], and reduce heavy metal uptake [24,25]. *Brassica* species are notable accumulators of heavy metals and possess a strong antioxidant defense mechanism to deal with heavy metal toxicity. However, the effect of SA on the *B. campestris* under lead-stressed conditions has yet to be studied. The present study was carried out to understand the antioxidant mechanisms of Pb-induced heavy metal stress in *B. campestris* after foliar application of SA.

Material and methods

Plant materials and treatments applied

Seed of *B. campestris* 'SAU Sarisha-3' was obtained from Sher-e-Bangla Agricultural University, Dhaka, Bangladesh. Before sowing, the soil was mixed with the recommended dose of fertilizer and 2-L uniform-sized pots were filled with the mixed soil. After germination, the mustard seedlings were thinned to three uniform and healthy

plants in each pot. Four Pb treatments including a control (no treatment) were applied on days 28, 35, 42, 49, and 56 after sowing. The treatments were C (control), $Pb_{0.25}$ (0.25 mM Pb), $Pb_{0.5}$ (0.5 mM Pb), and $Pb_{1.0}$ (1.0 mM Pb). $Pb(NO_3)_2$ was used as the source of Pb. Salicylic acid (SA) at 0.25 mM concentration was used as a protectant applied as spray solution (at 3-day interval) under the Pb-stressed conditions ($Pb_{0.25+SA}$, $Pb_{0.5+SA}$, $Pb_{1.0+SA}$). A randomized complete block design (RCBD) was used to set up the experiment with three replications. The experiment was carried out in a polythene shed house and allowed to grow until harvest and samplings were done at different stages based on the requirements. Two sets of plants were grown – one for growth and biochemical analyses and the other for yield attributes and measurement.

Measurement of relative water content

Relative water content (RWC) was measured using the method of Barrs and Weatherley [26]. Fresh weight (FW) of three leaf blades was taken from the randomly selected plant. Then, turgid weight (TW) was taken after soaking the leaf blades in a Petri dish with distilled water for 4 h in dark conditions. Finally, the leaf blades were placed in a drying oven at 80°C for 48 h and dry weight (DW) was recorded. RWC was then calculated using the following formula: $RWC\ \% = FW - DW\ /\ TW - DW \times 100$.

Measurement of chlorophyll content (as SPAD)

Leaf chl content was measured as a soil plant analysis development (SPAD) index – an indirect method of chlorophyll measurement with the Minolta SPAD-502 (Konica Minolta Sensing, Tokyo, Japan). Measurements were taken from the middle of the lamina of the second leaf from the upper for each Pb-treated and control conditions.

Assaying lipid peroxidation

MDA estimation was used as the main measure of lipid peroxidation and was assessed using a slight modification of the methods of Heath and Packer [27], employing thiobarbituric acid (TBA) as a reactive substance. Leaf samples (0.5 g) were homogenized in 5% trichloroacetic acid and centrifuged it at 11,500 *g* for 15 min and then mixed with 4 mL of the reaction mixture (0.5% TBA dissolved in 20% TCA). The mixture was then boiled for 30 min at 95°C and cooled quickly in an ice bath and then again centrifuged as before. The absorbance of the solution was measured at 532 and 600 nm using spectrophotometer. The absorbance at 600 nm was used as the nonspecific and was deducted from reading at 532 nm to get the actual absorbance. The MDA concentration was then determined and expressed as nmol g^{-1} fresh weight.

Estimation of H_2O_2

The method of Yu et al. [28] was used to assess H_2O_2. Half gram of leaf samples were homogenized in 3 mL potassium-phosphate buffer (50 mM) at 6.5 pH and centrifuged at 11,500 *g* for 15 min. The supernatant was then mixed with 0.1% $TiCl_4$ dissolved in 1 mL of 20% H_2SO_4 and then the mixture was left at room temperature for 10 min, and centrifuged again as before. The absorbance was measured at 410 nm, and the concentration of H_2O_2 expressed as mol g^{-1} fresh weight.

Ascorbate and glutathione extraction and assay

Three mL of aliquots of acidic extraction buffer (1 mM EDTA in 5% metaphosphoric acid) were used in a cold condition to homogenize 0.5 g of fresh leaves with a pestle and mortar and then centrifuged for 15 min at 11,500 *g* and 4°C. After centrifugation, the ascorbate and glutathione concentrations were analyzed in the supernatant solution.

After a slight modification of the methods of Huang et al. [29], the ascorbate concentration was measured. 0.5 M K-P buffer was used to neutralize the supernatant solution and then AsA was determined spectrophotometrically at 265 nm in 100 mM K-P buffer (pH 7.0). Ascorbate concentrations were then calculated from a standard calibration curve.

The glutathione pool was determined with slight modification of the method of Paradiso et al. [30], where 0.5 M K-P buffer (pH 7.0) was used to neutralize the supernatant. Then, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) was used to oxidize GSH which was then reduced by NADPH in the presence of GR. After reduction of DTNB, 2-nitro-5-thiobenzoic acid (NTB) was generated and its absorption was measured at 412 nm. 2-Vinylpyridine was used to remove GSH for measuring GSSG. The final calculation of GSH and GSSG was from standard curves from known concentrations. GSH concentration was calculated by GSSG subtraction from the total GSH concentration.

Enzyme extraction and measurement

One mL of 50 mM K-P buffer (pH 7.0) containing 100 mM KCl, 1 mM ascorbate, 5 mM β-mercaptoethanol and 10% glycerol was used to homogenize 0.5 g of fresh leaves and then centrifuged for not more than 10 min at 11,500 *g*. After centrifugation, the supernatant was collected to determine enzyme activity. The same supernatant was also used to determine protein concentration using the method of Bradford [31].

Ascorbate peroxidase (APX; EC: 1.11.1.11) activity. The activity of APX was measured following Nakano and Asada's [32] procedure. The reaction buffer contained 50 mM K-P buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2, 0.1 mM EDTA, and enzyme extract. When reactions started after the addition of H_2O_2, the color was changed and the APX activity was assayed spectrometrically at 290 nm absorbance.

Monodehydroascorbate reductase (MDHAR; EC: 1.6.5.4). The MDHAR activity was determined according to the method of Hossain et al. [33] and measured by preparing a reaction mixture which contained 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADPH, 2.5 mM AsA, and 0.5 unit of ascorbate oxidase (AO; EC 1.10.3.3) and enzyme solution. After the addition of AO, the reaction started and then the MDHAR activity was determined at 340 nm absorbance.

Dehydroascorbate reductase (DHAR; EC: 1.8.5.1) activity. The DHAR activity was assayed at 265 nm absorbance following the procedure of Nakano and Asada [32] using a reaction buffer where the enzyme solution was mixed with 50 mM K-P buffer (pH 7.0), 2.5 mM GSH, and 0.1 mM dehydroascorbate.

Glutathione reductase (GR; EC: 1.6.4.2) activity. The procedure of Hossain et al. [34] was employed. The reaction mixture contained 0.1 M K-P buffer (pH 7.8), 1 mM EDTA, 1 mM GSSG, 0.2 mM NADPH mixed with extracting enzyme. GR was determined with measuring the absorbance at 340 nm which was initiated with GSSG.

Glutathione *S*-transferase (GST; EC: 2.5.1.18) activity. After a slight modification of the method of Hossain et al. [35], GST was assayed using a reaction mixture containing 100 mM Tris-HCl buffer (pH 6.5), 1.5 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and extracted enzyme. The activity of GST was measured at 340 nm with increasing absorbance which was initiated by CDNB addition.

Guaiacol peroxidase (POD; EC: 1.11.1.7) activity. Phosphate buffer (0.1 M) (pH 7.0), 0.04 mL of 0.1 M H_2O_2, 0.04 mL of 0.2% *O*-dianisidine, and 0.02 mL of enzyme extract was mixed to prepare a reaction mixture and then POD activity was measured at 470 nm following the method of Shannon et al. [36].

Catalase (CAT; EC: 1.11.1.6) activity. The enzyme solution was mixed with reaction buffer [50 mM K-P buffer (pH 7.0), 15 mM H_2O_2] to estimate CAT activity and then absorbance was determined at 240 nm following the method of Hossain et al. [34].

Assaying yield and yield-contributing attributes

Plant heights were measured in each pot, from soil level to leaf apex. Primary and secondary branches of plants were also recorded as the number per plant and as a determinant of plant yield. Similarly, the number of pods per plant and seeds per pod were recorded from randomly selected plants from each pot. One hundred clean, sun-dried grains were weighed after random collection from the seed stock and then converted into the weight per 1,000 seeds. After the complete sun-drying process, the biomass from each pot was threshed manually, and the seed yield per pot was recorded and then converted it into kg ha^{-1}. The straw was also weighed to determine biological yield. The biological yield was calculated by using the following formula: *Biological yield = Grain yield + Straw yield*.

Finally, a harvest index (HI) was calculated by using the following formula of Gardner et al. [37]: $HI = Grain\ yield / Biological\ yield \times 100$.

Statistical analysis

The software package XLSTAT 2017 [38] was used to analyze the all data using analysis of variance (ANOVA) for all parameters and the significance of differences between treatment means tested by LSD at the 5% level of significance.

Results

Growth and biomass

Mustard plants exposed to increasing doses of Pb showed a reduction in plant height, FW, and DW at 30 days after sowing (DAS), 45 DAS, and at harvest. The greatest growth and biomass reductions (37% in height, 49% in FW, 24% in DW, 22% in primary branch number, and 36% in secondary branch number) compared to the control were observed at 45 DAS for plants treated with 1 mM Pb (Tab. 1 and Fig. 1). It was notable that in most cases, application of SA improved plant growth and biomass suffering Pb toxicity compared to Pb-treated plants alone. Under severe Pb-induced stress, exogenous SA could not significantly improve growth and biomass (Tab. 1 and Fig. 1).

Relative water content

RWC decreased with increasing Pb in a dose-dependent manner. The reduction was by 20%, 30%, and 31% compared to the control at 0.25, 0.5, and 1 mM Pb, respectively (Fig. 2). However, SA application increased the RWC by 12%, 17%, and 10% in 0.25, 0.5, and 1 mM Pb-affected mustard plants, respectively, compared to only Pb-treated plants.

Chlorophyll content

Lead stress decreased the photosynthetic pigment content as indicated by SPAD values. The chlorophyll reduction was detected in a dose-dependent manner, where the greatest reduction (27%) was observed in the 1 mM Pb treatment. By contrast, exogenous SA improved chlorophyll contents compared to the Pb-stressed plants but remained lower than that of the control plants (Fig. 2).

Yield components

Lead stress greatly impacted on the yield contributing components and the overall yield of the mustard plants in a dose-dependent manner. Reductions in these parameters

Tab. 1 Salicylic acid-induced changes in plant height and biomass of mustard plants exposed to lead toxicity.

	Plant height (cm)			Fresh weight (g)		Dry weight (g)	
Treatment	**30 DAS**	**45 DAS**	**At harvest**	**30 DAS**	**45 DAS**	**30 DAS**	**45 DAS**
Control	15.08 ±0.96 ^{a}	55.33 ±3.78 ^{a}	96.25 ±6.38 ^{a}	2.65 ±0.33 ^{a}	19.75 ±1.85 ^{a}	0.384 ±0.037 ^{a}	3.39 ±0.22 ^{a}
$Pb_{0.25}$	17.50 ±0.79 ^{bc}	40.67 ±3.41 ^{bc}	86.00 ±6.42 ^{b}	2.09 ±0.18 ^{bcd}	14.00 ±1.47 ^{cd}	0.340 ±0.026 ^{ab}	2.88 ±0.27 ^{b}
$Pb_{0.25+SA}$	15.08 ±0.83 ^{a}	45.50 ±3.18 ^{b}	89.50 ±5.67 ^{ab}	2.37 ±0.16 ^{ab}	16.00 ±1.00 ^{b}	0.358 ±0.025 ^{ab}	3.08 ±0.25 ^{ab}
$Pb_{0.5}$	18.83 ±1.97 ^{c}	36.17 ±3.28 ^{cd}	73.25 ±6.30 ^{c}	2.03 ±0.17 ^{cd}	11.75 ±1.32 ^{ef}	0.315 ±0.024 ^{bc}	2.51 ±0.21 ^{cd}
$Pb_{0.5+SA}$	16.92 ±1.29 ^{ab}	41.92 ±2.89 ^{b}	75.63 ±5.11 ^{c}	2.19 ±0.14 ^{bc}	15.08 ±1.49 ^{bc}	0.318 ±0.023 ^{bc}	2.81 ±0.22 ^{bc}
$Pb_{1.0}$	19.00 ±1.56 ^{c}	35.00 ±3.62 ^{d}	71.08 ±4.47 ^{c}	1.85 ±0.14 ^{d}	10.00 ±0.71 ^{f}	0.291 ±0.041 ^{c}	2.33 ±0.18 ^{d}
$Pb_{1.0+SA}$	17.42 ±1.26 ^{ab}	35.67 ±3.66 ^{cd}	70.38 ±5.68 ^{c}	1.96 ±0.19 ^{cd}	12.25 ±1.04 ^{de}	0.313 ±0.034 ^{bc}	2.30 ±0.29 ^{d}

Here, $Pb_{0.25}$, $Pb_{0.5}$, $Pb_{1.0}$, and SA indicates 0.25 mM $Pb(NO_3)_2$, 0.5 mM $Pb(NO_3)_2$, 1.0 mM $Pb(NO_3)_2$, and 0.5 mM salicylic acid, respectively. Means ±*SD* are calculated from four replicates. In the same column, letter(s) indicates no statistical difference according to Tukey's honest significant difference test at $p \leq 0.05$.

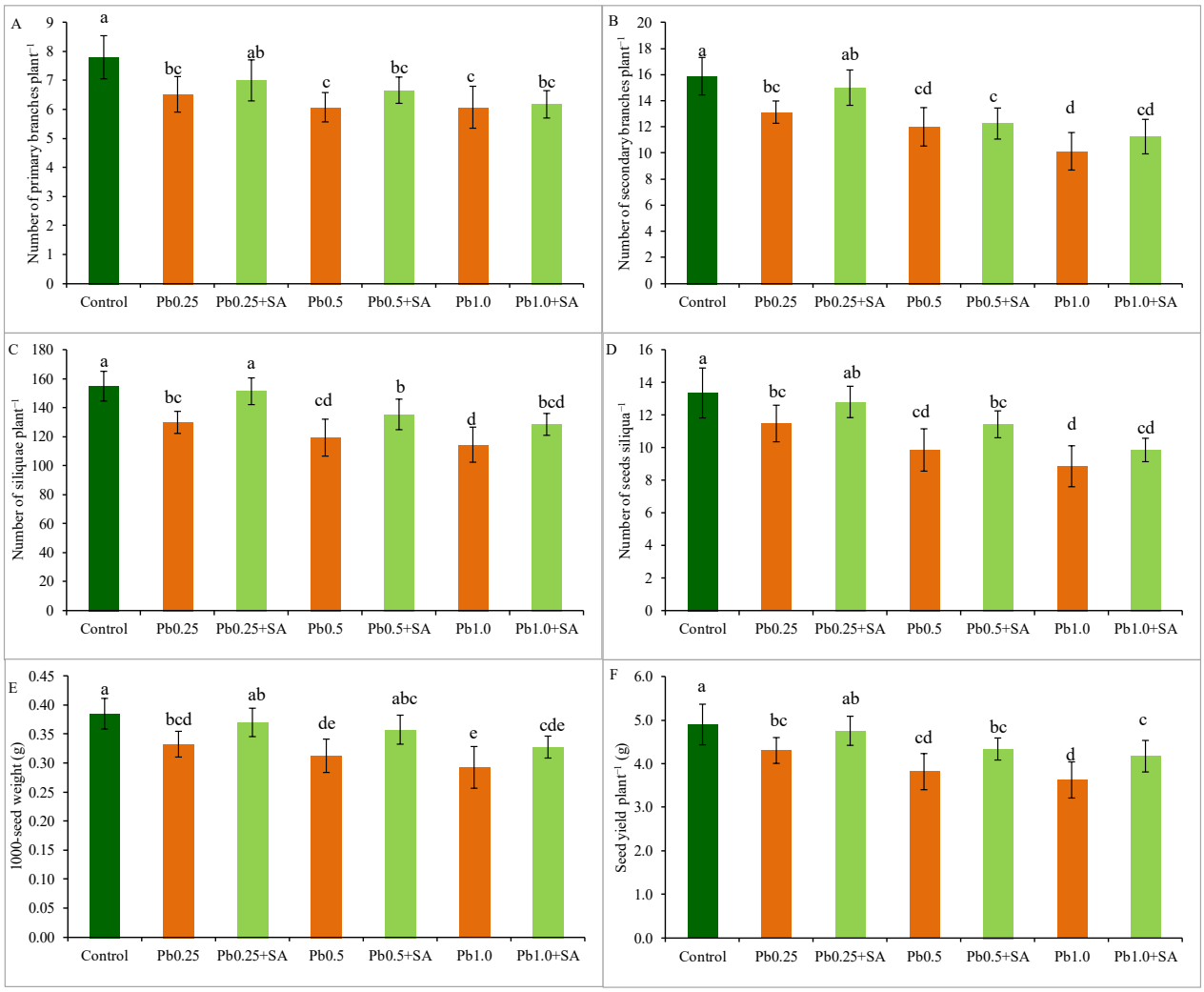

Fig. 1 Salicylic acid-induced changes in yield contributing characters and seed yield of mustard plants exposed to lead toxicity. Here, $Pb_{0.25}$, $Pb_{0.5}$, $Pb_{1.0}$, and SA indicates 0.25 mM $Pb(NO_3)_2$, 0.5 mM $Pb(NO_3)_2$, 1.0 mM $Pb(NO_3)_2$, and 0.5 mM salicylic acid, respectively. Means ±*SD* are calculated from four replicates. Bars with different letters are significantly different according to Tukey's honest significant difference test at $p \leq 0.05$.

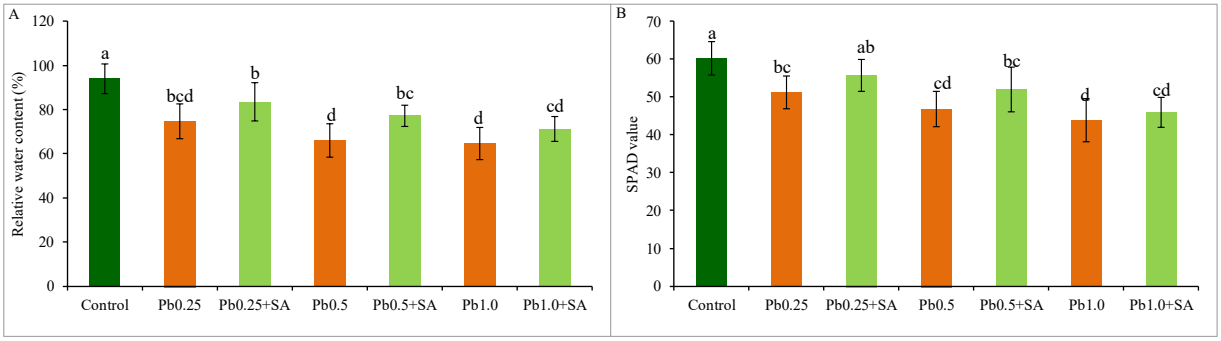

Fig. 2 Salicylic acid-induced changes in leaf relative water content and SPAD value of mustard plants exposed to lead toxicity. Other notes are the same as in Fig. 1.

increased with the increasing Pb concentrations in the soil. However, maximum yield reductions were found from at the highest dosage of Pb (1 mM). The 1 mM Pb treatment caused a reduction of 26% in the number of siliquae per plant, 34% in the number of seeds per siliqua, 26% in 1,000 seed weight, and 26% in seed yield per plant, compared to controls (Fig. 1). However, foliar spray of SA increased all the above yield attributes under Pb stress. Seed yield was enhanced by 15% after application of SA under 1 mM Pb stress compared to the Pb treatment only (Fig. 1).

Oxidative damage

Lead stress enhanced oxidative damage as indicated by MDA and H_2O_2 contents in a dose-dependent manner. The greatest oxidative stress was detected in the 1 mM Pb treatment. MDA and H_2O_2 increased threefold and twofold, respectively, at 1 mM Pb compared to controls. Foliar application of SA reduced the MDA and H_2O_2 contents significantly in all cases compared to their corresponding Pb-only treatments (Fig. 3).

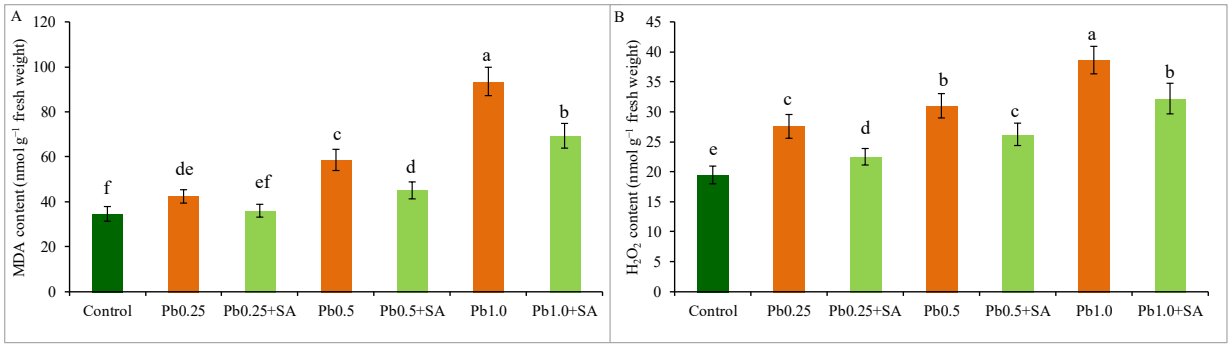

Fig. 3 Salicylic acid-induced changes in oxidative stress markers of mustard plants exposed to lead toxicity. Other notes are the same as in Fig. 1.

Nonenzymatic antioxidants

The activity of two important antioxidants, AsA and GSH, altered greatly under Pb stress. Lead stress reduced the AsA content in a dose-dependent manner, whereas the GSH content increased except at the highest dose (1 mM) of Pb (Fig. 4). The ascorbate content decreased by 14%, 28%, and 35% at 0.25, 0.5, and 1 mM Pb stress, respectively, compared to the equivalent Pb-only treatments. However, exogenous SA increased both the AsA and GSH contents under Pb toxicity. The mustard plants had improved GSSG contents upon Pb exposure dose-dependently along with a decrease of the GSH/GSSG balance in control treatments. Moreover, SA application improved this ratio significantly along with a decrease in GSSG content in all cases under Pb stress compared to Pb stress alone (Fig. 4).

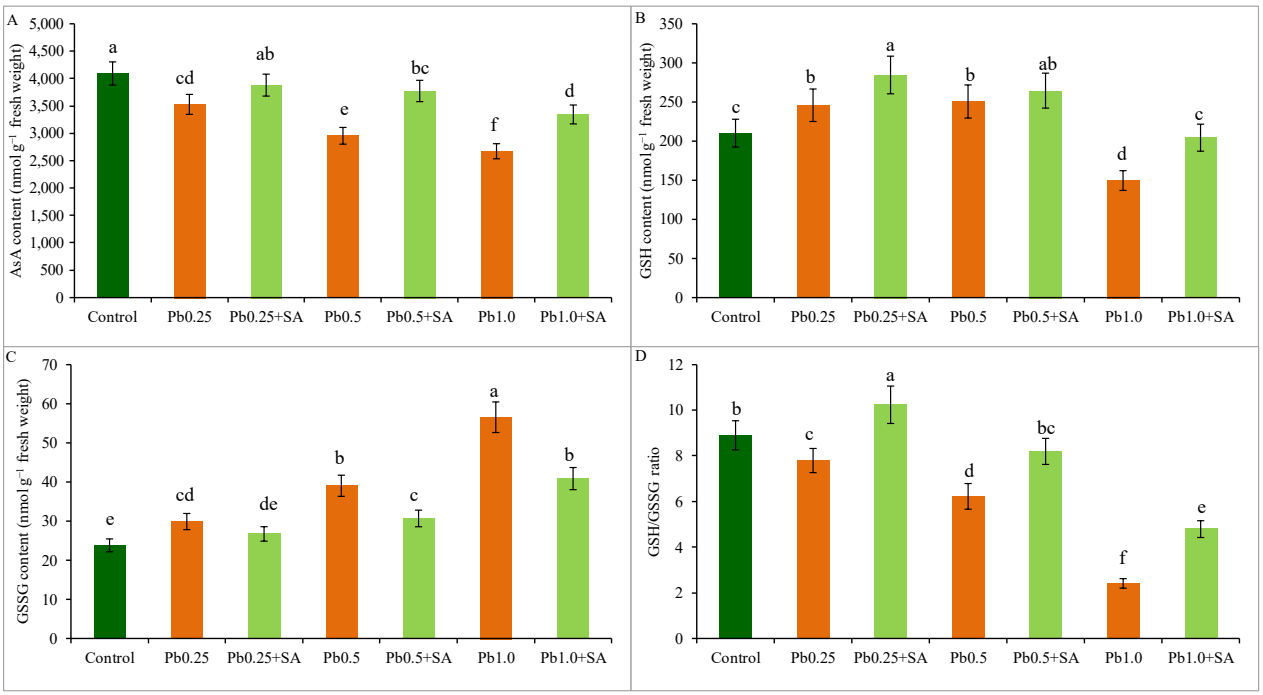

Fig. 4 Salicylic acid-induced changes in ascorbate–glutathione pool of mustard plants exposed to lead toxicity. Other notes are the same as in Fig. 1.

Enzymatic antioxidants

Enzymes involved in antioxidant defense responded differently to Pb stress alone and Pb stress along with SA in the mustard plants. APX and GR activity increased under mild stress (0.25 mM), but the activity of these enzymes decreased with an increase in Pb doses (Fig. 5). Under mild stress, the APX activity increased by 12% and the GR activity increased by 24%. With a high dose, APX and GR activity reduced by 27% and 12%, respectively. CAT, MDHAR, and DHAR activities reduced under Pb stress in a dose-dependent manner (Fig. 5, Fig. 6). GST activity increased at 0.25 and 1.0 mM Pb stress but did not change at 0.5 mM Pb, whereas POD activity upregulated under mild stress but did not alter at 1.0 mM Pb compared to the control (Fig. 6). Importantly, exogenous application of SA upregulated all the enzymes, which were downregulated under Pb stress and further upregulated the enzymes which showed enhanced activity under Pb stress (Fig. 5, Fig. 6).

Discussion

The present study was conducted to evaluate the effect of exogenous application of SA on Pb-induced stress on mustard plants and observe any improvements in Pb resistance as apparent in plant growth, physiology, and yield-contributing attributes and to the antioxidant defense system. The results revealed the overall detrimental effect of Pb with a severe reduction in plant height, branch number, and fresh and dry weights of plants of *B. campestris* compared to the plants grown in the control conditions. This reduction might be due to a higher accumulation of Pb within plants but this was not measured. Lead treatment induced a reduction in leaf water status, chlorophyll content, and disturbed the cell membrane permeability. Lead toxicity inhibits photosynthesis, resulting in reduced plant height and growth and lower dry matter accumulation and yield. Moreover, Pb toxicity reduced the activities of enzymes involved in photosynthesis and protein metabolism. Lead-induced reductions in plant growth, chlorophyll biosynthesis, and yield have also been found by others in different crop plants such as *Oryza sativa* [39], *Talinum fruticosum* [40], *B. juncea* [41], and *Zea mays* [42]. In our study, yield-contributing characters such as the number of siliquae, seeds per siliqua,

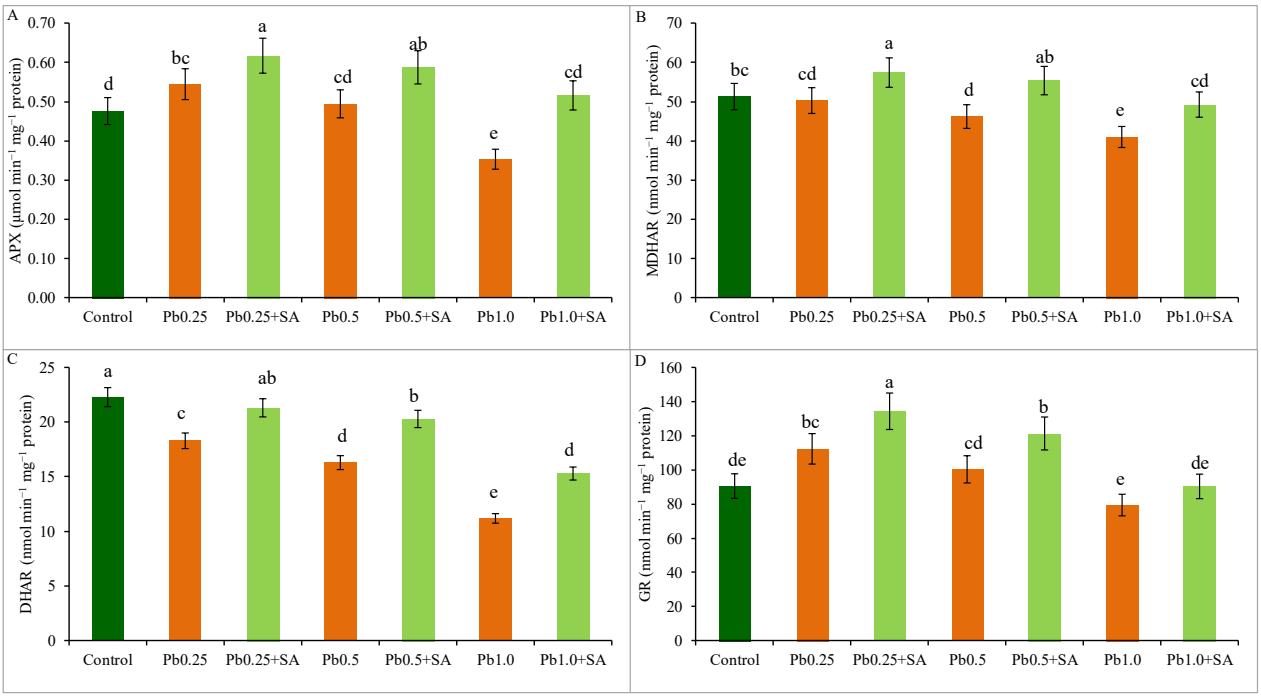

Fig. 5 Salicylic acid-induced changes in AsA–GSH cycle enzymes of mustard plants exposed to lead toxicity. Other notes are the same as in Fig. 1.

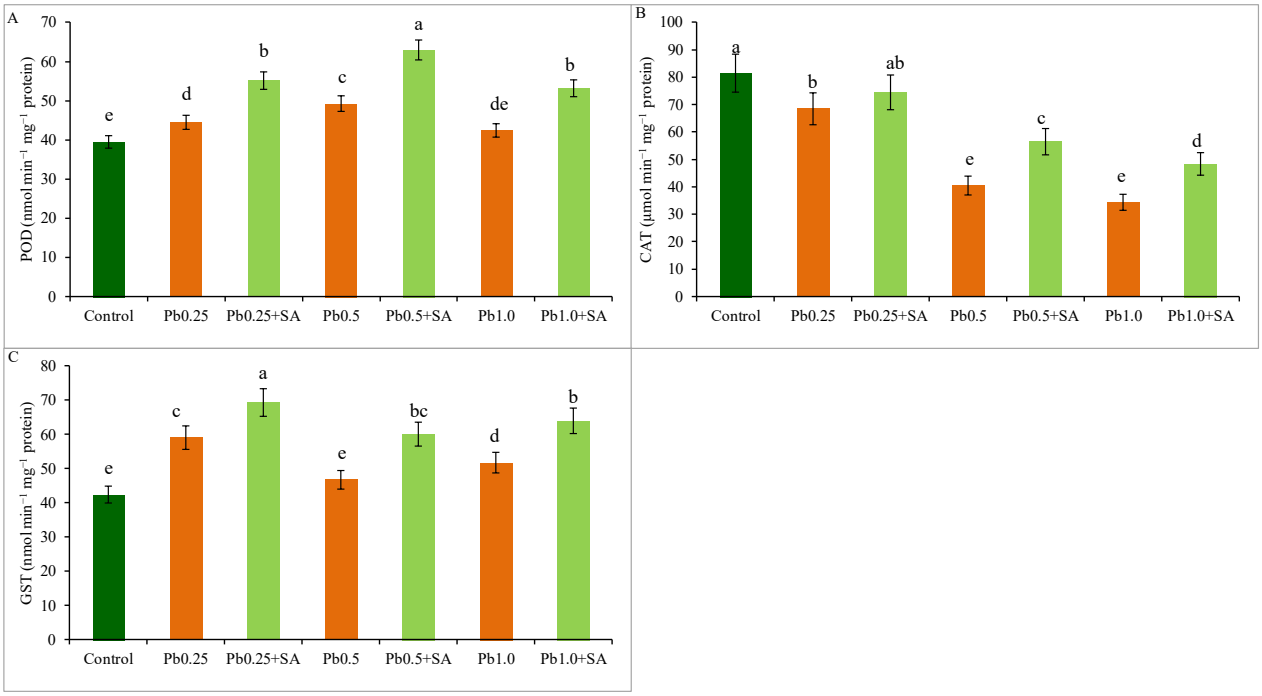

Fig. 6 Salicylic acid-induced changes in the activities of POD (**A**), CAT (**B**), and GST (**C**) of mustard plants exposed to lead toxicity. Other notes are the same as in Fig. 1.

and the 1,000 seed weight all declined under Pb stress conditions and the impact gradually increased with increasing stress levels. Moreover, our plants grown in Pb-contaminated soil showed a reduction in yield potential and severe yield loss in 1 mM Pb-treated plants. This reduction of yield could be due to the cumulative effects of a decrease in all yield contributing parameters in plants under Pb-stressed conditions. Previously, Ashraf and Tang [39] reported that a deterioration of yield-contributing characters is responsible for yield reduction under heavy metal stress conditions in their experiments.

Supplementation with SA to Pb-induced plants increased growth, biomass, and yield, which might be due to increasing water content and chlorophyll biosynthesis. SA significantly improved water content and biosynthesis of photosynthetic pigments, which have been shown to enhance plant growth, biomass accumulation, and net photosynthetic rate in mung bean [43]. The present overall results showed a very close relationship between growth parameters and yield with SA application along with an increased plant resistance to Pb toxicity.

Under heavy metal stressed conditions, plants take up greater amounts of metal ions through the root resulting in toxicity and a severe reduction in growth and productivity [44]. Lead toxicity initiates oxidative stress and induces cellular damage in plants. In this study, Pb-stressed mustard plants showed a greater accumulation of ROS (H_2O_2) content and thus suffered from oxidative damage. Consequently, increased H_2O_2 generation caused greater lipid peroxidation indicated by higher MDA contents. Lead-induced oxidative stress changes in morphology and structural properties of roots in different crops, resulting in a reduction of resistance, has also been demonstrated in other crop plants, e.g., *Vigna unguiculata* [45], *O. sativa* [39], *B. napus* [46], *Zea mays* [47], *Vicia faba* [48], *Zygophyllum fabago* [49], *Triticum aestivum* [50], and *Medicago sativa* [51]. Although some plants have developed an efficient antioxidant defense system to resist Pb-induced oxidative stress [51,52], excessive ROS generation results in disruption of the antioxidant defense mechanism, which is the major consequence of Pb toxicity [45].

Plants require a redox balance for resistance to heavy metal toxicity. Ascorbate and GSH are two major nonenzymatic components of the redox pool, where AsA prevents toxic H_2O_2 accumulation by converting H_2O_2 to H_2O assisted by the enzyme APX [53]. Ascorbate peroxidase is the first enzyme which detoxifies H_2O_2 to H_2O with peroxidation of AsA to produce MDHA and DHA by nonenzymatic disproportion, where both MDHAR and DHAR are involved in the regeneration of AsA. Another nonenzymatic antioxidant, GSH which improves defense against abiotic stress, acts a substrate for GPX, restricting protein oxidation and thus reducing ROS generation [54]. In this process, GSH is converted to GSSG by the action of DHAR, which is further rejuvenated by a NADPH-dependent enzyme, GR [55]. Thus, AsA and GSH are directly involved in ROS metabolism, regulated by four vital enzymes, viz. APX, MDHAR, DHAR, and GR. The AsA–GSH cycle therefore acts as a contributor to AsA and GSH production as well as ROS detoxification.

Salicylic acid is an hydroxyl radical scavenger in plants under various abiotic and biotic stresses [56,57]. In this study, it was observed that exogenous application of SA reduced H_2O_2 accumulation and MDA contents in plants suffering from the effects of Pb toxicity. Our observations are consistent with previous studies which have revealed that SA can restrict the entry of heavy metals or metalloids into plants such as As in rice [58], Cr in *Zea mays*, Cd in *Glycine max* [59], and Cd in *Triticum aestivum* [60]. SA enhances the antioxidant defense mechanism, which ultimately improves plant growth and resistance to heavy metal stress [57,61]. SA-induced reduction in ROS production might be due to a restriction in Pb accumulation in plants and so a decreased oxidative stress. Exogenous application of SA may upregulate the antioxidant defense system through a reduction in MDA and H_2O_2 concentrations. In this study, the increasing pattern of both nonenzymatic (AsA, GSH) and enzymatic (APX, GR, CAT, POD) antioxidant components with SA treatment was observed in Pb-stressed mustard plants, which limited ROS production as well as reducing oxidative damage. Higher activity of APX was found with the decreases in both MDHAR and DHAR activity under Pb-stressed conditions, which ultimately lower the AsA concentrations and thus decrease the H_2O_2 conversion in the Pb-stressed mustard plant. A similar result was found in various Cd-affected crop plant species such as *B. juncea* [53], *Vigna radiata* [62], and *B. napus* [63]. An increase in GSH was found under only moderate Pb stress, keeping in step with GR-dependent GSH increases and upregulation of GR activity. Under severe Pb stress, GSH was lower compared to the controls (where less activity of GR was found), which may be due to the affinity of GSH conversion to GSSG during metal detoxification, and thus increased GSSG content [44]. This increase in GSSG further contributed in the lower GSH/GSSG balance under Pb stress, which disrupted the redox balance, an observation which is also supported by other reports [53,62,64].

Exogenous supplementation of SA to Pb-stressed mustard plants enhanced the upregulation of enzymatic antioxidants in the AsA–GSH pool. Our mustard plants showed stimulated activities of APX, MDHAR, DHAR, and GR under Pb stress by the application of SA. The increase of all these enzymes resulted in greater concentrations of both AsA and GSH as well as enhancing the ROS detoxification rate. Consequently, the plants were released from Pb-induced oxidative stress. Thus, AsA and GSH concentrations and their balance were also improved under stress conditions with SA, as well as reduced ROS generation which ultimately increased Pb resistance. The results were consistent with previous studies for SA-induced plant resistance against metal stress in other plants species such as *Vigna radiata* [43], *Triticum aestivum* [60], and *Cucumis melo* [15]. Furthermore, the enzymatic antioxidants POD, GST, and CAT are actively involved in antioxidant defense mechanism through scavenging of excess ROS, where the activities of these enzymes are upregulated when plants are challenged by abiotic stress conditions [44]. In the present study, Pb-treated mustard plants showed greater activities of POD but CAT activity was reduced. A similar result has been reported in *Vicia faba* roots [48]. Lead contamination significantly increased POD activity (compared to control plants) which is responsible for the detoxification of H_2O_2 to H_2O and O_2 [65]. In our study, foliar application of SA showed an increasing trend of POD activity above the control as well as in the Pb-treated plants. We can state that our mustard plants showed increased POD activity to decompose H_2O_2 to water under Pb-stress, and also that they became more protected when sprayed with SA.

GST is an important group of enzymes, which actively participate in heavy metal detoxification and hence significantly increased in Pb-stressed mustard plants. The results were consistent with previous studies [66,67] stating that the defensive role of GST enzymes changed due to Pb-induced toxicity and increased over the control treatment. SA supplementation again improved GST activity in Pb-treated plants and thus increased plant resistance further. Exogenous SA application increased GSH contents which further stimulated the activity of GST to scavenge H_2O_2 along with its xenobiotic activities.

CAT activity may vary depending on the plant species and severity of a stress. It has been documented that CAT activity elevated in leguminous crops, while it was reduced in rice under heavy metal stress conditions [45,68,69]. Moreover, CAT activity increased with a minor stress but decreased in severe conditions [50]. In our case, CAT activity reduced with increasing Pb concentrations but supplementation of SA enhanced the CAT activity (Fig. 6B). This increased CAT activity by SA caused lower concentrations of H_2O_2 under the stressed conditions. SA can therefore scavenge excess ROS during Pb-stress induced oxidative damage by upregulating antioxidant defense systems even at higher doses of Pb (1 mM), thus making plants more resistant to this form of abiotic stress.

Conclusion

Our study concluded that Pb toxicity might have a severe consequence on the growth of mustard plants and subsequent yield by changing internal physiological mechanisms and inhibiting biosynthesis of photosynthesis pigments. However, exogenous application of SA improved the growth, physiology, antioxidant activities, yield attributes, and final yield of mustard plants under Pb-stress conditions. The results suggest that foliar application of SA increase Pb-induced stress resistance in mustard plants by mitigating oxidative damage through upregulation of the antioxidant defense system, so improving growth and productivity.

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Dolistna aplikacja kwasu salicylowego wpływa korzystnie na parametry wzrostu i plonowania roślin *Brassica campestris* rosnących w glebie zanieczyszczonej ołowiem poprzez pozytywną regulację systemu obrony antyoksydacyjnej

Streszczenie

Toksyczność ołowiu (Pb) wywiera silny wpływ na wzrost i produktywność roślin. Ochronna rola kwasu salicylowego (SA) jest dobrze znana w warunkach różnych stresów abiotycznych. Jednak niewiele wiadomo na temat indukowanego SA mechanizmu oporności na Pb. W przeprowadzonych badaniach określiliśmy wpływ SA na rośliny gorczycy (*Brassica campestris* L.) w warunkach stresu wywołanego Pb. Rośliny poddano działaniu trzech poziomów Pb wprowadzonych do gleby (0,25, 0,50, 1,00 mM), z lub bez SA (0,25 mM). Wzrost, wskaźniki plonowania oraz plon roślin uległy zmniejszeniu w zależności od nasilenia stresu indukowanego Pb. Egzogenna aplikacja SA wpłynęła na poprawę wzrostu i plonowania roślin. Wyniki analiz biochemicznych wykazały, że toksyczność Pb powodowała większe uszkodzenia oksydacyjne związane z obniżeniem zawartości nieenzymatycznych antyoksydantów, takich jak askorbinian i glutation, w obecności wyższych stężeń tego metalu. Aktywność enzymów antyoksydacyjnych (peroksydazy askorbinianowej – APX, reduktazy monodehydroaskorbinianowej – MDHAR, reduktazy dehydroaskorbinianowej – DHAR, reduktazy glutationowej – GR, peroksydazy gwajakolowej – POD, *S*-transferazy glutationowej – GST i katalazy – CAT) była uzależniona od stężenia Pb. Zarówno nieenzymatyczne, jak i enzymatyczne elementy systemu obrony antyoksydacyjnej były pozytywnie regulowane po zastosowaniu SA, co powodowało mniejsze uszkodzenia oksydacyjne występujące w warunkach stresu Pb. Podsumowując, uzyskane wyniki sugerują, że egzogenna aplikacja SA łagodzi uszkodzenia oksydacyjne wywołane obecnością Pb, a w konsekwencji prowadzi do lepszego wzrostu i plonowania gorczycy w warunkach ekspozycji na różne poziomy stresu indukowanego Pb.