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Assessing the mitigation of Pb toxicity by the synergistic application of Oxalic acid and salicylic acid on maize plants for a duration of 15, 30 and 45 days

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Abstract

Lead (Pb) pollution poses significant threats to plant growth and overall ecosystem health. Phytohormones, such as Oxalic acid (OA) and Salicylic acid (SA) play an important role in the physiological processes of plants. In our investigation, we explored the potential protective effects of exogenous application of oxalic acid, salicylic acid and their combination on lead- induced stress in maize over a 45-day period, analysing the impact at 15-day intervals. Lead-exposed maize exhibited reduced growth parameters, chlorophyll pigments, anthocyanin, carotenoid, and xanthophyll pigments, along with compromised stomatal conductance and relative water content. However, pre-treatment of maize seeds with a combination of Oxalic acid and Salicylic acid mitigated these adverse effects, preserving growth metrics, pigment levels, and stomatal conductance. This combination enhanced antioxidant enzyme activity, forming metal ion complexes that hindered lead uptake. Additionally, combined application of OA+SA stimulated the synthesis of chlorophyll, anthocyanin, carotenoid, and xanthophyll pigments, while also improving stomatal conductance and relative water content. Our findings underscore the regulatory role of OA+SA in alleviating lead stress, promoting maize growth, and enhancing physiological resilience. This highlights the potential of OA+SA as a promising strategy for mitigating lead induced damage and fostering plant health and development in contaminated environments.

Keywords: Pb stress, Oxalic Acid, Salicylic Acid, photosynthetic pigments, plant growth parameters, stomatal conductance.

Introduction

The escalation of heavy metal pollution on a worldwide basis is primarily attributed to heightened usage in fertilizers, petrol, metal plating, as well as natural processes like rock weathering, volcanic eruptions, and industrial effluents, with smelting exacerbating soil pollution, particularly in agricultural lands (Talha *et al*.,2023). When Pb and Fe concentrations exceed the required levels, they negatively affect plants, animals, and humans (Viehweger*et al*.,2014). Pb, the most toxic heavy metal, adversely affects plant growth and development, leading to chlorosis, reduced growth, and impaired water translocation, which reduces nutrient uptake (Abd Elnabi*et al*.,2023; Wang *et al*.,2022). The reduced availability of water and nutrients also negatively impacts stomatal conductance and the biosynthesis of photosynthetic pigments, resulting in a diminished rate of photosynthesis and transpiration.

Among major crops like rice and wheat maize, holds significant importance as a staple food and feed crop for both humans and animals (Zhang *et al.*,2020). Given the increasing demand for maize in the food industry due to its high nutritional value, it becomes imperative to address the oxidative stress induced by heavy metals in maize plants (Talha *etal.*,2023). Maize plants exhibit hyperaccumulator traits, accumulating heavy metals in their leaves, with concentrations escalating alongside environmental metal levels (Atta *et al*.,2023).

Pb toxicity leads to oxidative stress in plants, which is manifested by a reduction in growth parameters, increased lipid peroxidation of cell membranes, altered permeability, and decreased water and nutrient translocation to aerial plant parts. As a result, relative water content decreases and mineral and nutrient uptake is reduced (He *et al.,*2023). Impaired absorption of Fe and Mg ions further impedes chlorophyll pigment biosynthesis, causing damage to the photosynthetic machinery and altering stomatal functioning (Zulfiqar *et al.,* 2019).

In the intricate regulatory network of plant physiology under abiotic stress, phytohormones such as Salicylic Acid (SA) and Jasmonic Acid (JA) play pivotal roles (Per *et al*.,2017). SA acts as a signalling molecule, activating antioxidant enzymes during oxidative stress induced by heavy metals, thereby proving instrumental in maintaining normal physiological functioning (Nazli *et al.,*2020). Plants grow and develop more efficiently when SA restores the photosynthetic apparatus, enhances water and nutrient transport, and promotes root and shoot growth (Khan *et al*.,2015). According to Zanganeh, Salicylic acid plays a significant role in maintaining normal plant function under Pb stress (Zanganeh *et al*.,2020).

OA, functioning as a metal chelator, dissolves or chelates metal ions, reducing the transport of heavy metal ions to various parts of the plants by reducing their availability in the soil (Rasool *et al*.,2021; Metanat*et al*.,2019). Triggered in the presence of heavy metal-induced stress, OA safeguards plants from adverse effects by initiating programmed cell death (Dageng*et al*.,2023).

In a study investigating the influence of different levels of OA on the growth of plants and Pb accumulation in intercropped *Arabis alpina* and *Zea mays*, it was found that a 5 mmol kg/L OA treatment improved maize plant growth and reduced Pb translocation. However, higher OA concentrations (25-50 mmol kg/L) decreased Pb accumulation in *Arabis alpina* (Dageng*et al.,* 2023). Consequently, it was concluded that OA effectively decreases Pb accumulation while enhancing the activity of antioxidant enzymes to protect plants from Pb stress (Tindanzor*etal*., 2023).

Despite an extensive literature search, the synergistic effect of Salicylic acid and Oxalic acid on Pb-stressed maize plants remains elusive. This study aims to explore the protective effect of combining both OA and SA on Pb-stressed maize plants over 15, 30, and 45 days. Parameters to be discussed include growth parameters, photosynthetic pigments (chlorophyll, xanthophyll, carotenoid, and anthocyanin), relative water content with stomatal conductance observed through SEM. The main goal is to discover an efficient approach to alleviate Pb stress in maize plants by employing a synergistic combination of Salicylic acid and Oxalic acid.

Materials and Methods

Experimental set-up

This research study was carried out in the Department of Biotechnology, University Institute of Biotechnology, Chandigarh University, India. The procurement of disease-resistant and certified Suvarna maize (*Zea mays L.*) seeds sourced from a commercial supplier in Chandigarh, India. A pot experiment was conducted during the summer season of 2021- 2022 to evaluate the impact of Pb on maize crop. Oxalic Acid and Salicylic Acid were procured from Sigma-Aldrich (Sigma Aldrich, St. Louis, MO, USA) followed by the preparation of stock solution of SA (25mg/L), OA (25mg/L) and OA + SA (25 mg/L each) in distilled water (Thermo fisher scientific, Waltham, MA). Lead nitrate [$Pb(NO_3)_2$] was purchased from Sigma-Aldrich (Sigma Aldrich, St. Louis, MO, USA) and used as a source of Pb for in vivo experimentation. For experimentation, both a control group (Pb 0 mM) and the Pb treated group for Pb (0.50 mM) were selected. The standalone & thecombination of Pb, OA, SA and OA + SAfor in vivo studies in Zea mays, as shown in Table 1.

Table 1: Combinations of Lead (Pb) and OA, SA and OA + SA selected for in vivo studies.

Control	0
Pb	0.5mM
Pb + SA	0.5mM +25mg/L
Pb + OA	0.5mM +25mg/L
Pb + SA + OA	0.5mM +25mg/L+25mg/L

Pot preparation and fertilizer application

Soil from the Gharuan, Kharar tehsil, Mohali district, Punjab was used in this pot experiment. The soil was sandy loam in texture with a pH of 7.8, organic carbon concentration 0.81 %, total nitrogen concentration of 0.119 %, an available Phosphorus (P) amount of 20.4 mg/kg soil, exchangeable Potassium (K) amount of 0.294 meq/100 g soil. The salinity of soil measured was 0.43 dS/m. The concentrations of Zn in the soil sample was measured to be of 9.6 mg/Kg, Fe was 7.19 mg/kg, Mn was 12.8 mg/kg and Cu was 1.32 mg/kg. The size of the pot was 15.5 cm (diameter) × 13.5 cm (height). Each pot was filled with 2.228 kg of soil mixed with manure (3:1 ratio). Uniform distance was maintained between each pot.

In vivo cultivation and seed priming for enhanced plant tolerance

For optimizing plant growth and stress resilience, a comprehensive experimental protocol was employed for the in vivo cultivation of *Zea mays*. To ensure seed surface sterility, *Zea mays* seeds surface sterilised by soaking them in 10% sodium hypochlorite solution for 15 minutes, followed by thorough rinsing with distilled water. Subsequently, seed priming, a pivotal pre-germination treatment, was conducted for 12 hours, employing various solutions: distilled water (Control), SA at 25 mg/L, OA at 25 mg/L, and a combination of SA (25 mg/L) and OA(25 mg/L). Post-priming, seeds were initiated in a germination process on filter paper that had been moistened over a three-day period. The experiment incorporated three biological replicates, each comprising five pots representing distinct treatment groups:

Control (Pb 0 mM), Pb (0.5 mM), Pb + OA (0.5 mM + 25 mg/L), Pb + SA (0.5 mM + 25 mg/L), and Pb + OA + SA (0.5 mM + 25 mg/L + 25 mg/L). Each pot accommodated two plants, constituting a single experimental replicate, thereby providing a robust foundation for assessing the impact of priming treatments on subsequent plant responses to Pb exposure.

Pb-stress induction and growth conditions for maize plants:

In order to investigate the impact of Pb stress on *Zea mays*, a systematic approach was adopted for stress induction and subsequent cultivation. The maize plants were bifurcated into two groups, with one group serving as the control [0 mM of Pb $(NO_3)_2$], and the other subjected to Pb stress [0.5 mM of Pb $(NO_3)_2$]. The growing conditions were meticulously controlled, with each replicate consisting of five pots containing a soil and manure mixture in a 3:1 ratio. The Pb-treated plants were subjected to a 1-litre solution of Pb $(NO_3)_2$ with a concentration of 0.5 mM applied to the soil before sowing. In contrast, the control group pots were irrigated with regular water prior to seed planting. Following the transplantation of seedlings into their respective pots, all plants were positioned outdoors under natural sunlight conditions. For a duration of 15 days, 30 days, and 45 days, the plants were subjected to regular watering to maintain optimal growth conditions. At the culmination of each time interval, maize plants were harvested, and their leaves were meticulously preserved at -80°C for subsequent analyses.

Determination of physiological parameters of *Zea mays*

Physiological parameters

The following are the physiological parameters used in the experiment.

Root and Shoot length

Root and shoot lengths of maize *(Zea mays)* were measured after 15, 30 and 45 days.

Relative water content

The protocol of (Wolf *et al.*,1997) was used to estimate the relative water content. The fresh plant samples weight was measured, after that the weight of the evaporating dish. The samples were taken in the evaporating plate and heated in the oven for 16 hours at 102–105°C. Lastly, the dried sample weight was measured.

Chlorophyll content

For the estimation of chlorophyll content (Arnon,1949) method was used. At 40°C, 4 mL of 80% acetone was blended with 1 gram of fresh plant tissue. The homogenised mixture was then centrifuged for 20 min. at 4^0C at 20,000 rpm. The optical density of the sample was measured at 645 nm and 663 nm to determine the chloroplast concentration of plant material.

Carotenoid test

For the determination of total carotenoid content (Maclachlan and Zalik, 1963) method was used.By using motor and pestle 1gram of fresh plant leaves were crushed by adding 4mL of 80% acetone at 4^0C. Now the crushed sample was centrifuged at 20,000 rpm for 20 min by maintaining the temperature 4^0C. After Centrifugation the supernatant`s absorbance was taken at 480 and 510 nm.

Total Anthocyanin Content

The anthocyanin content is analysed by using (Mancinelli,1984) protocol. Prepare a mixture of 3 mL methanol, water and HCL of ratio 79:20:21 and homogenize this mixture with 1 gram of fresh leaves and then centrifuge the mixture for 20 min. at 20,000 rpm at 4^0C. By using absorbance of supernatant, the anthocyanin content was estimated at 530 nm and 657 nm.

Xanthophyll Content

For the estimation of xanthophyll content AOAC method (Lawrence *et al*., 1990) was used. 1 g of the dried plant leaf was blended with 30 mL of dissolution medium containing 7 mL toluene, 6 mL absolute alcohol, 7 mL acetone, and 10 mL hexane, and gently shaken for 10 minutes. After adding 2 mL of 40% methanolic potassium hydroxide (KOH) to the blended samples, they were hot saponified. After that, the assay combination was kept in the dark for an hour. After that, 30 mL of hexane and 10 % sodium sulphate solution were added to the conical flask to make up a 100 mL volume. The analytic mixture was agitated and incubated in the dark for 1 hour. In a 50 mL volumetric flask, the upper layer of the reaction mixture was collected. At 474 nm, optical densities were measured.

Scanning Electron Microscopy

The preparation of samples for studying stomatal conductance followed a protocol from (Müller & Zechmann,2018).

Statistical analysis

Descriptive data will be presented as median & range. Normality distribution of data will be determined using Shapiro–Wilk test/ Kolmogorov Smirnov test. Student's t-test will be usedfor continuous variables and uniformly distributed data between 2 groups. Mann-Whitney U/Kruskal Wallis will be performed for non-uniformly distributed data. 2-wayAnova followed by Post-Hoc Tukey test will be performed to rule the statistical significance. Values of p less than 0.05 will be considered statistically significant and represented *$p< 0.05$, **$p< 0.01$, ***$p< 0.001$, ****$p< 0.0001$. All statistical tests will be two-tailed, with a significance level of $p<0.05$. All the statistical analysis will be performed using licensed GraphPad Prism (v9.2) & SPSS 22.0 (IBM SPSS Inc. NY, US).

Results

Root Length

Figure 1(a) depicts the influence of 0.5 mM Pb exposure on maize plant's root length. Plants treated exclusively with Pb displayed significantly shorter root lengths compared to the control group across all observed durations (15, 30, and 45 days) ($p<0.0001$). Conversely, the combined application of OA and SA (25 mg/L each) resulted in significantly longer root lengths compared to all other treated groups ($p<0.0001$) at the specified time points. These findings suggest that Pb exposure inhibited maize root growth, whereas the combined addition of OA and SA alleviated this inhibition and fostered improved root growth compared to Pb treatment alone.

Shoot Length

InFigure 1(b) the shoot length resultsof maize plants treated with a 0.5 mM dose of Pb demonstrated significantly shorter shoot lengths compared to the control group across all time points (15, 30, and 45 days) (p<0.0001). However, the combination of OA and SA (25 mg/L) produced significantly longer shoot lengths compared to all other treated groups (p<0.0001), indicating that the combined addition of OA and SA mitigated the adverse effects of Pb exposure and promoted greater shoot growth.

Relative water content

Figure 1(c) depicts the relative water content of maize plants treated with Pb at a dose of 0.5 mM was significantly lower compared to the control group across all time points (15, 30, and 45 days) (p<0.0001). However, when OA + SA (25 mg/L each) were applied to Pb-stressed plants, the mean relative water content increased significantly (p<0.0001) compared to all other treated groups, suggesting that the combination treatment mitigated the decline induced by Pb exposure and preserved a higher relative water content.

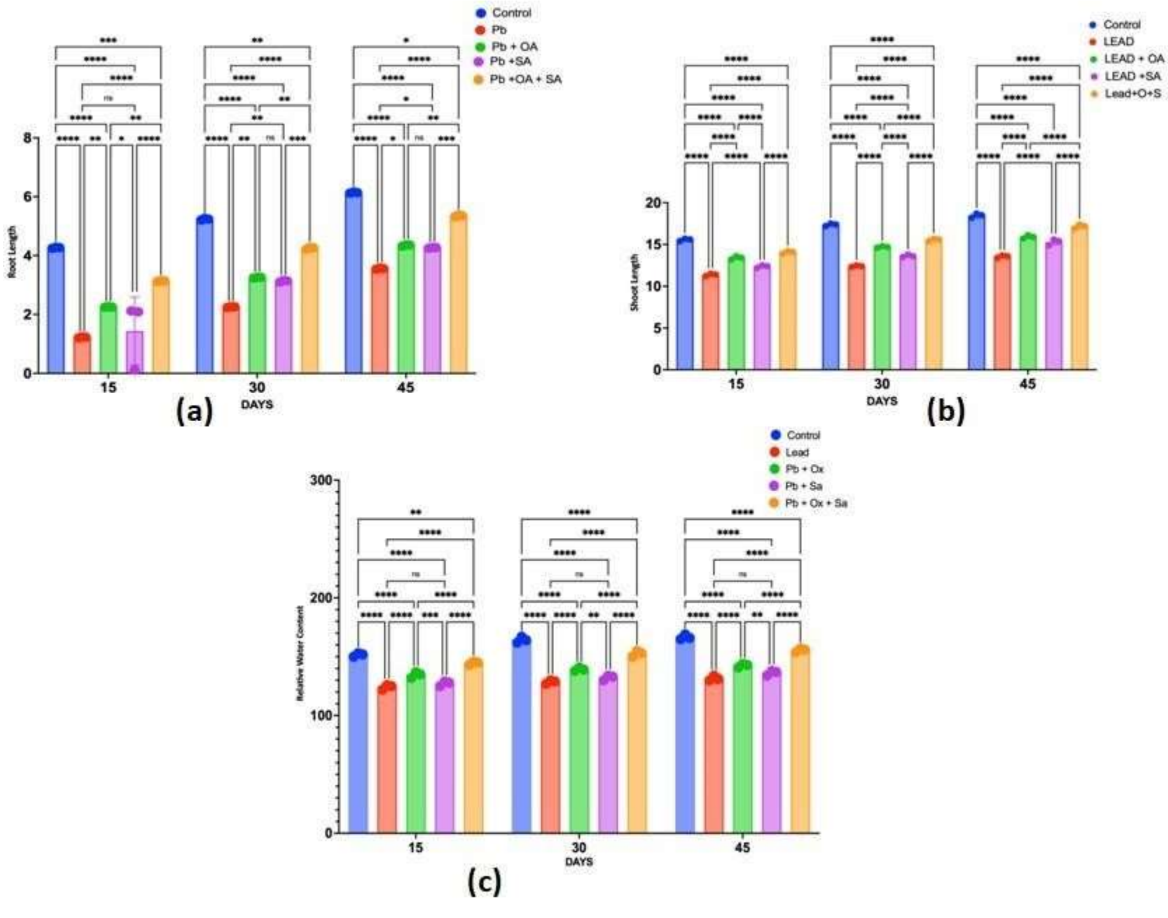

Figure 1(a, b, c):*Demonstrating the effect of Pb (0.5 µm) onroot length (cm.), shoot length (cm.), relative water content (%) of plants treated with Oxalic acid (OA), Salicylic acid (SA), and the combination of both (OA and SA) over a duration of 15 days, 30 days, and 45 days.*

Chlorophyll content

Data presented in Figure 2(a) show that the total chlorophyllcontent of Pb-treated maize plants was significantly lowercomparedto the controls. Pre-sowing treatment of maize seeds withSA and OA increased total chlorophyll content in plants under Pbstresswhile OA+SAtreatmenthadsignificantimpact onchlorophyllcontents in Pb stressed plants

Chl-a

In maize plants treated with 0.5 mM Pb, chlorophyll-a content markedly decreased ($p < 0.0001$) compared to controls Figure2(b). Application of OA or SA individually significantly increased chlorophyll-a levels ($p < 0.0001$). Remarkably, the combined OA and SA treatment resulted in the highest chlorophyll-a content ($p < 0.0001$), indicating a synergistic effect in alleviating Pb-induced chlorophyll-a reduction. These findings highlight the potential of OA and SA co-application in preserving chlorophyll-a content in Pb-stressed maize plants.

Chl-b

In maize plants exposed to 0.5 mM Pb, chlorophyll-b content significantly decreased ($p < 0.0001$) compared to controls, Figure 2(c). Treatment with OA or SA individually led to notable increases in chlorophyll-b levels ($p < 0.004$, $p < 0.0001$, $p < 0.0001$). Remarkably, the combined application of OA and SA resulted in the highest chlorophyll-b content ($p < 0.0001$, $p < 0.0001$, $p < 0.002$), demonstrating a synergistic effect in mitigating Pb-induced chlorophyll-b reduction.

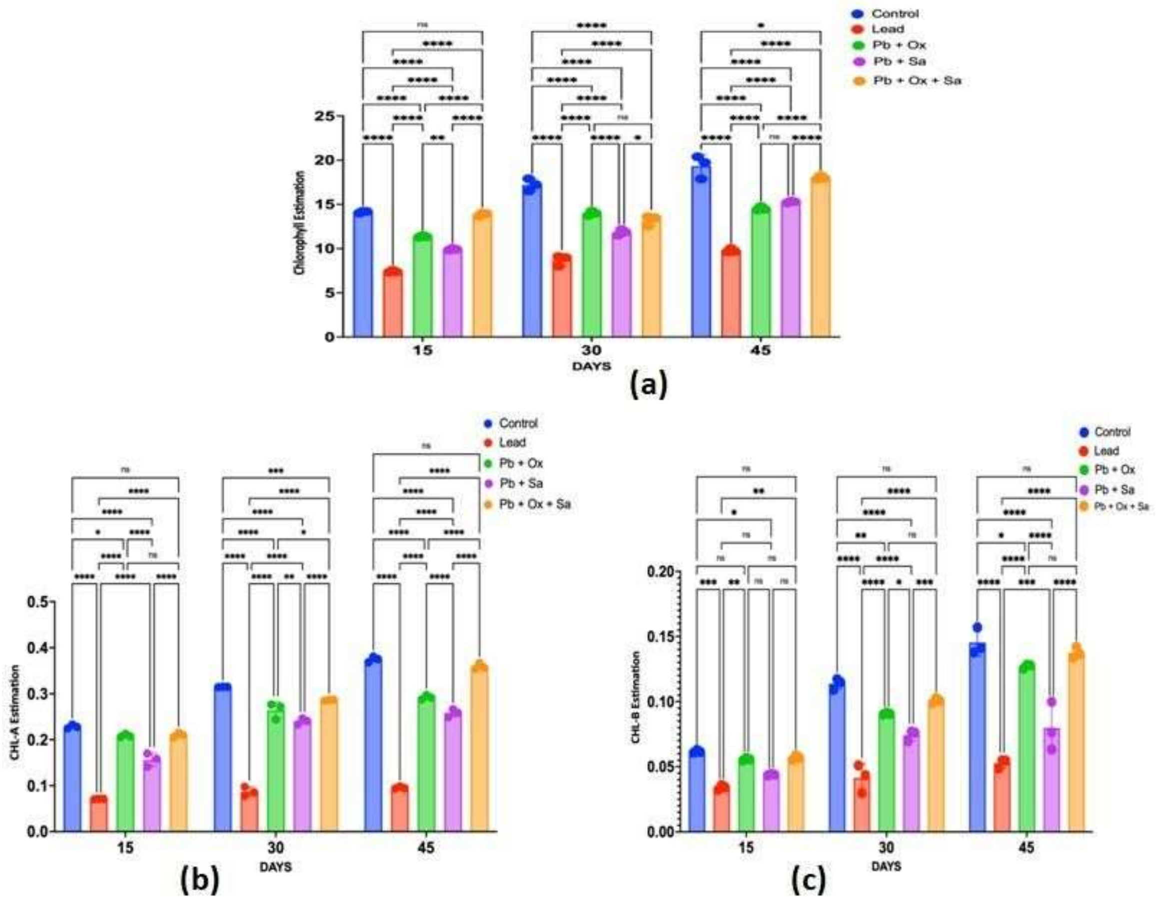

Figure 2(a, b, c):*Demonstrating the effect of Pb (0.5 µM) on chlorophyll (µg/g FW), chl-a (µg/g FW), chl-b (µg/g FW) in plants treated with Oxalic Acid (OA), Salicylic Acid (SA), and the combination of both (OA and SA) over a duration of 15 days, 30 days, and 45 days. FW=Fresh weight*

Anthocyanin content

In maize plants exposed to 0.5 mM Pb, significant reduction in anthocyanin content occurred ($p<0.0001$) compared to the control. Treatment with OA (25 mg/L) and SA (25 mg/L) individually resulted in a significant increase in anthocyanin content ($p<0.0001$) compared to Pb-treated plants. Additionally, simultaneous application of OA and SA significantly increased anthocyanin content ($p<0.0001$) compared to all other treatments, indicating a synergistic effect, Figure 3(a).

Carotenoid content

Carotenoid content significantly decreased ($p<0.0001$) under Pb stress compared with untreated-Pb plants, Figure 3(b). Both OA and SA treatments individually led to notable increases in carotenoid content ($p<0.0001$) in Pb-stressed maize plants. Furthermore, a significant elevation in carotenoid levels ($p<0.0001$) was observed with the combined application of OA+SA, indicating a synergistic effect in mitigating the Pb-induced decline.

Xanthophyll content

For xanthophyll content, a significant reduction ($p<0.0001$) was evident in Pb-stressed maize plants compared to controls, Figure3(c). However, treatment with OA, SA, or their combination markedly enhanced xanthophyll levels ($p<0.0001$), partially counteracting the Pb-induced decrease. These results suggest that the combined application of OA and SA effectively ameliorates the adverse effects of Pb toxicity on xanthophyll content in maize plants.

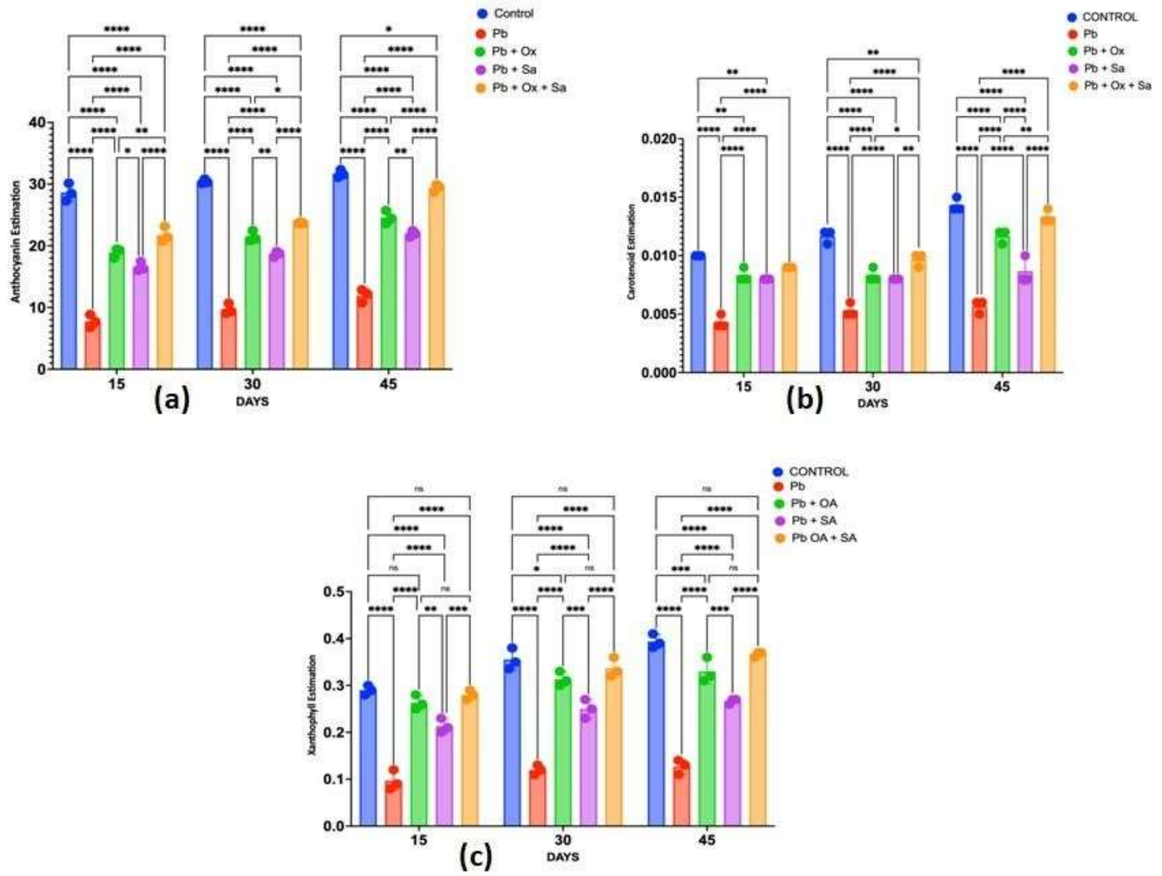

Figure 3(a, b, c):*Demonstrating the effect of Pb (0.5 µM) on anthocyanin content (µg/g FW), carotenoid content (µg/g FW), xanthophyll content (µg/g FW) in plants treated with Oxalic Acid (OA), Salicylic Acid (SA), and the combination of both (OA and SA) over a duration of*

15 days, 30 days, and 45 days. FW=Fresh weight

Stomatal Conductance

In the investigation of stomatal conductance in maize plants subjected to Pb stress, scanning electron microscope (SEM) images were analysed over a 45-day period. In Pb-treated maize plants, SEM images revealed a consistent decrease in stomatal conductance, with stomatal pores appearing partially closed at day 15, further closure at day 30, and nearly closed at day 45, indicating a progressive negative impact on gas exchange, Figure 4 (a, b, c). In contrast, maize plants exposed to Pb stress but treated with a combination of OA and SA exhibited a notable improvement in stomatal conductance. SEM images illustrated a widening of stomatal pores after 15 days, continued widening at day 30, and significant enlargement at day 45, suggesting enhanced gas exchange capabilities with the combined treatment, Figure 5 (a, b, c). This visual representation emphasizes the contrasting effects on stomatal morphology and conductance under Pb stress and the ameliorative influence of OA + SA treatment.

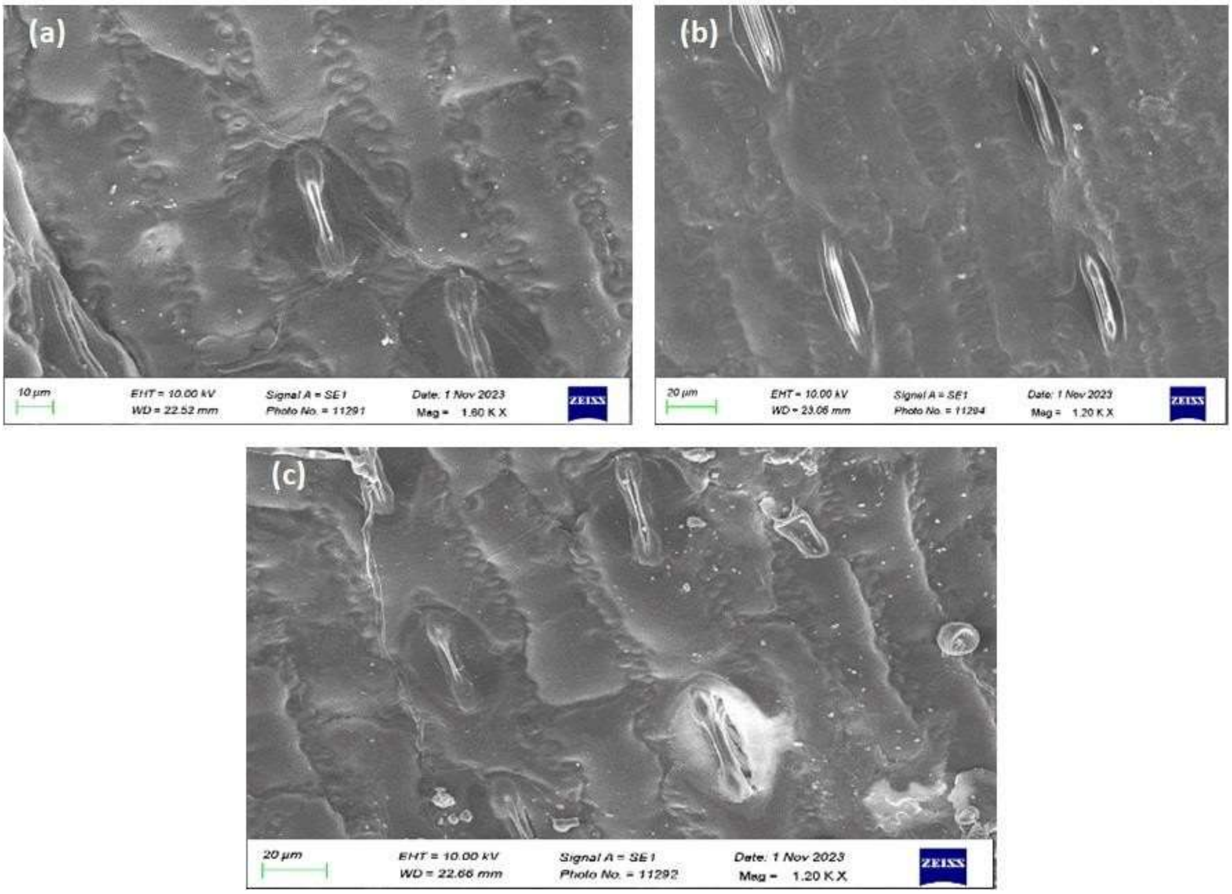

Figure 4 (a, b, c):*Demonstrating the impact of Pb (0.5 µM) on stomatal conductance in maize plants (Duration: 15 days, 30 days, 45 days), through Scanning Electron Microscope (SEM) images.*

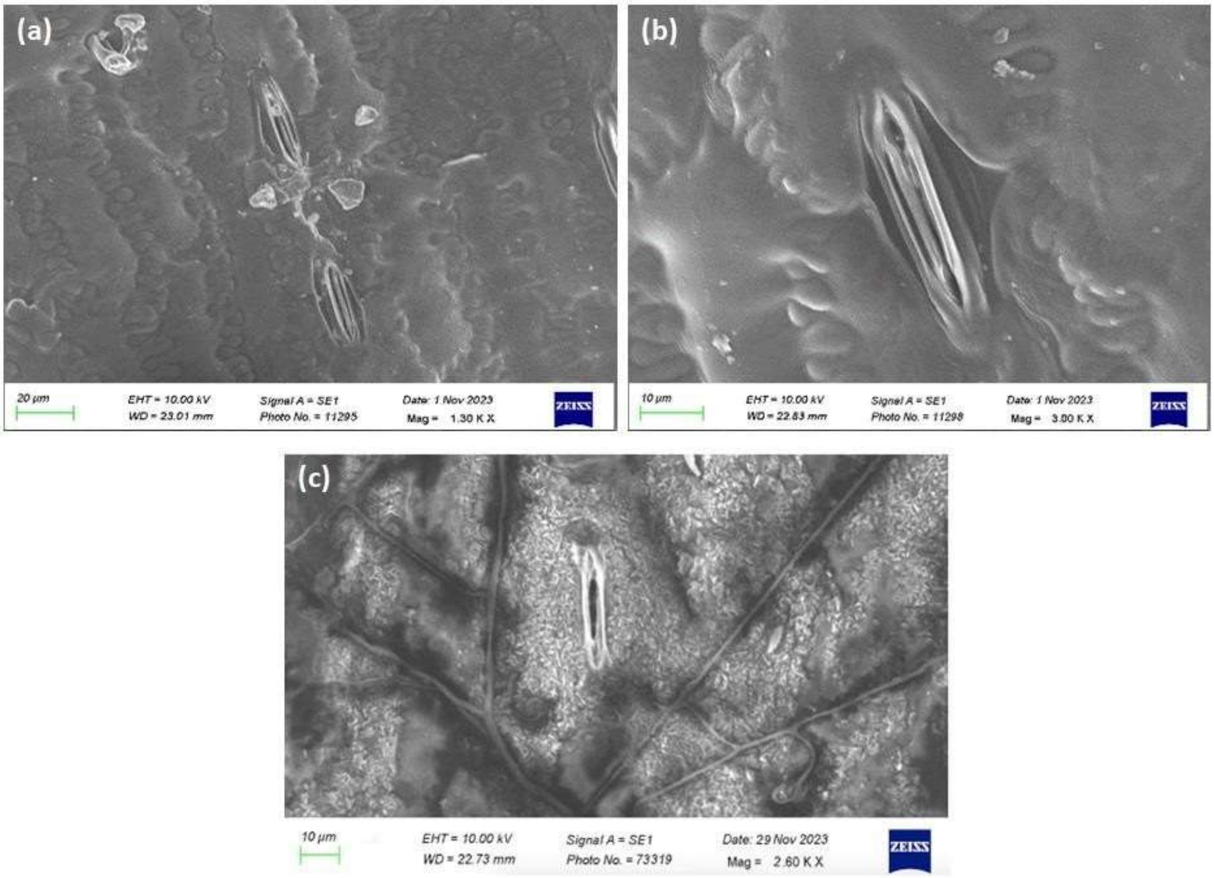

Figure 5 (a, b, c): *Demonstratingthe alleviating effects of a combination treatment with Oxalic Acid (OA) and Salicylic Acid (SA) under Pb Stress (Duration: 15 days, 30 days, 45 days), through Scanning Electron Microscope (SEM) images.*

Discussion

The combined application of exogenous phytohormones, Oxalic acid (OA), and Salicylic acid (SA) offers a potent strategy for shielding maize plants against lead toxicity. This study reveals their synergistic efficacy in mitigating effects of lead toxicity in plants, enhancing physiological markers like shoot and root growth, relative water content and photosynthetic pigments including anthocyanin, carotenoid and xanthophyll levels, indicating antioxidative action. These findings highlight potential role of synergistic application of these phytohormones for eco-friendly solutions in agriculture, thus promoting growth and productivity.

Due to lead stress, various physiological and biochemical functions of plants are adversely affected such as respiration, transpiration, nutrient absorption and ATP production (Li *et al*.,2014; Han *et al*.,2013; Hu *et al*.,2012). Current study observed that Lead toxicity in plants caused decline in the growth of the roots and shoots of maize plants compared to the control group. This decrease in the growth parameter can be attributed to the decreased water uptake and increased consumption of plant energy in overcoming the oxidative stress due to Pb toxicity resulting in the decrease of the plant biomass (Li *et al*.,2014; Hu *et al*.,2012). To overcome this issue, in current study, synergistic application of OA & SA was done in lead treated plants which significantly improved their growth parameters. Other studies have reported improvement in growth parameters with individual application of either SA or OA

(Zanganeh *et al*.,2019; Kohli *et al*.,2018). SA acts as a signaling molecule and functions to activate the antioxidant enzymes under stress conditions. OA acts as a heavy metal ion chelator thus reducing the availability of metal ions to the plants, thereby reducing the oxidative stress and thus helping in the improvement of growth parameters of plants (Song *et al*.,2018).

Chlorophyll pigments play a pivotal role in facilitating photosynthesis and promoting overall plant growth and development. The current investigation found a discernible reduction in the levels of photosynthetic pigments, including chl-a, chl-b, xanthophyll, carotenoid, and anthocyanin, in plants subjected to Pb treatment in comparison to the control group. Corroborating our findings, (Anjum *et al.*, 2016c) reported analogous decreases in chlorophyll content in maize plants exposed to metals such as Al and Cr. (Souahiet*et al*.,2021) also reported decrease in chlorophyll content, chl-a and carotenoid content in the leaves of Pb stressed *Triticum durum* and *T. aestivum, Hordeum vulgare* and *Avena sativaplants*. It is postulated that Pb may inhibit uptake of Mg^{2+} leading to the disruption of the chlorophyll structure and consequent reductions in chlorophyll pigments within the chloroplast (Hou *et al*.,2018). Additionally, heightened chlorophyllase activities induced by metal toxicity contribute to chlorophyll and carotenoid degradation or trigger photo-oxidation (Hou *et al*.,2018). Further, the reduction in photosynthetic pigments in Pb-treated plants can also be attributed to degradation of chloroplast membranes caused by increased malondialdehyde (MDA) levels, diminished water and nutrient absorption, leading to subsequent chlorophyll breakdown and altered chlorophyll a to b ratio (Hou *et al*.,2018).

In the present study, treatment of Pb-stressed plants with synergistic application of phytohormones OA and SA resulted in significant elevation in levels of photosynthetic and carotenoid pigments, accompanied by a reduction in the chl a/b ratio. These outcomes align with previous studies of (Alamri *et al*.,2018; Ullah *et al*.,2012) who also observed increase in concentration of photosynthetic and carotenoid pigments when Pb-stressed plants were treated with SA. Furthermore, in a study it was revealed that OA enhances the concentration of photosynthetic pigments in arsenic-stressed *Hydrilla verticillata* seedlings (Zheng *et al*.,2018). The positive impact of OA on mitigating metal toxicity in maize plants can be attributed to its role as a metal ion chelator, making the metal ion less available to plants, thereby reducing absorption and translocation of heavy metals in the plants and consequently, alleviating oxidative stress. However, the results obtained with combined treatment of OA and SA exhibited significantly increased efficacy in reducing Pb toxicity in maize plants, evidenced by increased accumulation of photosynthetic pigments and carotenoids, reduced chlorophyll degradation, and a lower chlorophyll a to b ratio. To the best of our knowledge, none of the studies has reported the effects of synergistic application of OA and SA on levels of chlorophyll a & b and their ratio.

Xanthophylls and anthocyanins, another distinct classes of pigments in plants also play diverse roles in plant physiology. Xanthophylls, yellow or brown pigments primarily located in the thylakoid membranes of chloroplasts, contribute to light harvesting, photoprotection, and the dissipation of excess light energy as heat (Samanta *et al.*,2022). On the other hand, anthocyanins, responsible for red, purple, or blue colors in plants, serve functions such as attracting pollinators, protecting against UV radiation, and potentially acting as antioxidants, mainly localized in the vacuoles (Landi *et al*.,2014). While these pigments have separate roles and locations, they share a connection in contributing to the overall pigmentation and appearance of plants. Additionally, both are involved in photoprotection, albeit through different mechanisms (Samanta *et al.*,2022; Landi *et al.*,2014). Notably, the biosynthetic pathways for xanthophylls and anthocyanins are distinct, governed by different enzymes and precursors, responding differently to environmental stimuli, including heavy metal toxicity like lead stress. The observed decrease in xanthophyll and anthocyanin content in Pb stress plants in current study is in line with the findings of (Samanta *et al*.,2022). This decrease is a consequence of various physiological and biochemical responses influenced by Pb toxicity. Pb-induced oxidative stress overwhelms the antioxidant defense system, leading to pigment degradation (Foyer *et al*.,2011). Disruption of the photosynthetic apparatus affects xanthophyll stability, and lead interference with enzymatic processes hampers pigment synthesis (Sharma *et al.*,2012). Chloroplast damage, altered gene expression, and nutrient imbalances further contribute to the decline. In the current study, 0.5 mM Pb maize plants were treated with a combination of Oxalic acid (OA) and Salicylic acid (SA) and the plants exhibited elevated levels of anthocyanin and xanthophyll compared to plants treated solely with Pb. This finding aligns with prior studies demonstrating the capacity of externally applied SA to increase anthocyanin levels in wheat (Horva´th*et al.*,2007a), application of melatonin to As stressed rice cultivars increased anthocyanin and xanthophyll content (Samantha *et al*.,2022) and enhance anthocyanin content in rice seedlings subjected to saline stress (Khan *et al*.,2019). Our results suggest that the concurrent application of OA and SA demonstrated enhanced efficacy in ameliorating Pb toxicity in maize plants, evident through amplified accumulation of anthocyanin and xanthophyll content. These outcomes underscore the synergistic effectiveness of the OA + SA combined treatment in reinforcing maize plant tolerance to Pb toxicity.

Another crucial parameter for gas exchange in plants is stomatal conductance. It faces intricate regulation influenced by factors such as lead (Pb) toxicity. In the current investigation, authors observed significant reduction in stomatal conductance in maize plants treated with Pb (0.5mM). This finding aligns with the results reported by (Xavier *et al*., 2023), who observed the same results on Cd stressed *Brassica juncea* plants. The Pb-induced reduction in stomatal conductance results from oxidative stress, water balance disruption, membrane damage, and disruptions in ion transport (Sharma *et al*.,2012). Calcium, acting as a secondary messenger, plays a key role in stomatal movements, with changes influencing aperture and closure (McAinsh*et al*., 2000). Pb interference with essential ion uptake and translocation, particularly calcium and potassium, further disrupts stomatal regulation. Potassium, regulating guard cell turgor pressure, impacts stomatal opening and closure (Kashtoh*et al*.,2021; Bharath *et al*.,2021). Pb also induces oxidative stress by generating reactive oxygen species (ROS), impacting guard cells and diminishing stomatal conductance (Bharath *et al*.,2021). Disruptions in water balance and membrane damage, including those in the stomatal complex, compromise stomatal aperture control, thus altering conductance (Xavier *et al*.,2023). In contrast, the combined application of OA and SA showed substantial efficacy in mitigating Pb stress effects and significantly increased stomatal conductance in plants. This might be due to their antioxidant properties, which helped in protection of cell membranes by mitigating oxidative stress and maintaining ion homeostasis, thus ensuring optimal stomatal conductance. Some of the previous studies (Bharath *et al.*,2021; Ding and

Ding,2020; Alamri *et al.*,2018) have reported increased stomatal conductance with individual use of SA or OA, but the effects observed with synergistic application of SA and OA are quite better, thereby enhancing conductance. Therefore, it can be inferred that treatment of Pb-stressed plants with the combined application of OA + SA exhibits multifaceted actions, including increasing antioxidant activity, membrane protection, and ion homeostasis, highlighting its potential as a strategic approach to alleviate the adverse impact of lead stress on plant physiology.

Current study observed substantial decrease in relative water content in Pb stressed plants when compared to the control group. This finding has been reported in previous research on Pb-stressed wheat by (Sharma and Dubey,2005) and *Viciafaba L.* by (Bouziani *et al.*,2023), both of which found a decrease in relative water content. The decreased relative water content can be related to a reduction in leaf area, which leads to the inhibition of transpiration to reduce the translocation of Pb from roots to aerial plant parts of the plants as an adaptive measure to overcome the Pb stress. When Pb stressed plants were treated with combined application of SA and OA for various durations, an increase in relative water content was observed in Pb stressed maize plants compared to the control. This proves that synergistic application of SA and OA is effective in increasing the water transport to the aerial parts of the Pb stressed plants. Similar results were reported by (Dugogi*et al.*,2012), who also found an enhancement in the area of the Indian mustard (*Brassica juncea*) leaves after the application of SA and (Sadak *et al.*,2015) who observed the increase in relative water content after the application of OA in heat stressed wheat plants.

Conclusion

The current study supports the efficacy of exogenous organic acids, particularly the combined application of oxalic acid (OA) and salicylic acid (SA), in protecting plants against Pb toxicity, surpassing individual OA or SA treatments. Significant improvements were observed in various physiological markers, including root and shoot height, and levels of photosynthetic pigments such as chlorophyll, carotenoid, xanthophyll, and anthocyanin, as well as relative water content. Application of OA + SA reduced Pb accumulation and enhanced growth, along with increasing photosynthetic pigment accumulation, indicating protection against chlorophyll degradation.

In summary, the combined application of OA + SA offers substantial protection against Pb toxicity, enhancing physiological parameters and promoting the accumulation of photosynthetic pigments and other vital compounds. These findings highlight the potential of OA + SA as effective strategies for mitigating Pb stress in agriculture, thus improving plant growth and productivity. Further research is needed to elucidate the underlying mechanisms and optimize application methods across different crops and environmental conditions.

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