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Effect of Salicylic Acid on Proline Accumulation in Soybean Grown under Lead Stress

Siddhi Gupta

Department of Botany, S.B.D. Government College, Sardarshahar, 331403, Rajasthan, India
E-mail: siddhigupta24@gmail.com

Author for correspondence:

Siddhi Gupta
Department of Botany, S.B.D. Government College, Sardarshahar, 331403, Rajasthan, India
E-mail: siddhigupta24@gmail.com

Abstract

Salicylic Acid (SA) is a vital signaling molecule among naturally occurring plant hormones, recognized for its role in regulating various physiological processes. This study aimed to explore whether the exogenous application of SA could effectively enhance plant resilience in metal-contaminated environments. Various concentrations of lead acetate (200, 400, 600, 800, and 1000 mg/kg of soil) were administered to soybean seedlings alongside a 0.5 mM SA treatment and compared with control groups. Observations were conducted at three stages: pre-flowering (30 days), peak-flowering (45 days), and post-flowering (60 days), with the experiment replicated three times. Compared to the control group, there was a consistent increase in proline content corresponding to the rising metal concentrations in the soil. The highest proline levels were recorded at the 1000 mg/kg lead treatment, measuring 31.226, 32.594, and 35.604 μmoles at the pre-, peak-, and post-flowering stages, respectively. Proline accumulation is crucial for plants coping with heavy metal stress, as it functions as an osmolyte, reactive oxygen species (ROS) detoxifier, and protein stabilizer. Furthermore, the exogenous application of SA significantly enhanced proline accumulation under lead stress across all concentrations. For example, at the 1000 mg/kg lead treatment, proline levels reached 48.646±0.47 μmoles with SA, a significant increase compared to the untreated group ($p \leq 0.01$). These findings suggest that salicylic acid may act as a protective agent against heavy metal toxicity, potentially bolstering the internal immunity of soybean plants and increasing their resilience to environmental stressors, thereby providing valuable insights for agricultural practices.

Keywords: Heavy metal, Lead, Proline, Salicylic acid, Pre-flowering, Peak-flowering, Post-flowering

Introduction

Soybean (*Glycine max* L.) is a valuable crop recognized for its abundant content of proteins, oligosaccharides, edible fiber, isoflavones, and essential minerals (Mateos-Aparicio et al., 2008). However, various abiotic stresses often compromise its productivity, with heavy metal contamination being a significant concern. Lead (Pb), a toxic and nonessential metal, harms plants, even at low concentrations. The bioaccumulation of Pb in edible crops like soybean is of

particular concern due to its potential entry into the food chain, thus raising public health risks. Previous studies conducted by the authors demonstrated a direct relationship between increasing soil Pb concentrations and corresponding Pb accumulation in plant tissues, with the maximum uptake observed at 1000 mg/kg soil concentration, particularly during the post-flowering stage (Gupta & Meena, 2024). Exposure to Pb significantly disrupts vital physiological and biochemical processes in plants, including seed germination, seedling growth, photosynthesis, water relations, nutrient uptake, and protein synthesis. Observable symptoms of Pb toxicity include chlorosis, necrotic lesions on leaves, premature senescence, and overall stunted growth (Gupta & Meena, 2024). In response to such stress, plants activate a suite of defense mechanisms, including the synthesis and accumulation of compatible solutes such as proline. Proline plays a key role in osmotic adjustment, membrane stabilization, and the detoxification of reactive oxygen species, thereby serving as a reliable biochemical marker of abiotic stress.

Salicylic acid (SA), an endogenous phenolic compound, functions as a critical signaling molecule in modulating plant defense responses to both biotic and abiotic stressors, including heavy metal toxicity. Exogenous application of SA has been shown to mitigate the phytotoxic effects of heavy metals like Pb and Zn by enhancing germination percentage, root and shoot length, and biomass accumulation (Gupta et al., 2017). Nevertheless, the effects of exogenously applied SA on proline accumulation under lead (Pb) stress remain unclear and poorly documented. The primary objective of this study is to investigate how Pb stress affects proline accumulation in soybeans, given that proline is a known stress indicator, and to examine the impact of exogenous SA application on proline levels under such conditions. This research aims to provide insights into the role of SA in modulating the plant's response to stress.

Material and methods

Experimental setup and sampling

The experiment was carried out in April at the greenhouse of the University of Rajasthan's Botany Department. Pots, measuring 30 cm in height and 25 cm in diameter, were filled with 4 kg of garden soil and randomly arranged to minimize environmental variations. Lead acetate was applied at concentrations of 200, 400, 600, 800, and 1000 mg/kg of soil, along with 0.5 mM salicylic acid (SA), without adding additional nutrients. Control pots did not contain lead or 0.5 mM SA. Soybean seeds were surface sterilized using 0.1% mercuric chloride ($HgCl_2$) and were sown to a depth of 2 cm, with ten seeds planted per pot. Consistency in plant count was maintained throughout the experiment, and pots were watered every other day. Each treatment included three replicates at each stage: pre-flowering (30 days), peak-flowering (45 days), and post-flowering (60 days), allowing for comprehensive data collection for proline estimation.

Proline estimation

Proline content was evaluated using the acid ninhydrin method (Bates et al., 1973). A fresh leaf samples weighing 0.5 g was homogenized in 10 ml of 3% sulfosalicylic acid, followed by filtration. This was then mixed with 2 ml each of acid ninhydrin and glacial acetic acid. The mixture was subsequently heated, cooled, and extracted with toluene.

The absorbance of the toluene phase was measured at 520 nm. Proline concentration was determined using a standard curve of L-proline and expressed in mg/g of proline.

Statistical analysis

Statistical analysis was done using SPSS ver. 25.0 and Microsoft Office Excel 2016. All parameters were expressed as mean ± standard error (S.E.) The data was analyzed for the statistical significance of the differences between means of treatments by analysis of variance (ANOVA).

Results

The data demonstrates a strong positive correlation between lead concentration and proline accumulation, with the highest lead levels consistently associated with significantly elevated proline levels at all growth stages ($p \leq 0.01$). The findings suggest salicylic acid (SA) notably enhances lead-induced proline accumulation (Table 1). These results underscore salicylic acid's potential as a therapeutic agent for improving plant tolerance to heavy metal toxicity.

Pre-flowering stage

In the pre-flowering stage, proline accumulation in plants exposed to lead stress was significantly greater than in control plants. At a lead concentration of 200 mg/kg, proline levels rose to 15.175±0.39 µmoles/g, representing a significant increase from the control level of 5.144±0.42 µmoles/g ($p \leq 0.01$). As lead concentrations increased, proline accumulation continued rising, reaching 31.226±0.12 µmoles/g at 1000 mg/kg, significantly higher than the control ($p \leq 0.01$). Applying salicylic acid (0.5 mM) positively influenced all concentrations' proline accumulation under lead stress. For example, at 200 mg/kg lead treatment, proline levels with salicylic acid were 18.002±0.47 µmoles/g, significantly higher than those in untreated plants ($p \leq 0.01$). This trend was consistently observed at elevated lead concentrations, where proline levels with salicylic acid remained higher than those without.

Peak-flowering stage

During the peak-flowering stage, the proline accumulation exhibited a pattern similar to that observed during the pre-flowering stage. Lead

Table1. Impact of lead on Proline (µmoles/g) accumulation in *Glycine max* with and without SA at different stages of plant growth

Treatment	Pre-flowering stage		Peak-flowering stage		Post-flowering stage	
	Without SA	SA (0.5 mM)	Without SA	SA (0.5 mM)	Without SA	SA (0.5 mM)
Control	5.144±0.42	5.768±0.27	7.879±0.47	8.191±0.31	10.159±0.69	10.005±0.43
200 mg/kg	15.175±0.39^{c}	18.002±0.47^{a}	17.455±0.16^{c}	21.559±0.61^{c}	18.276±0.19^{c}	22.92±0.54^{c}
400 mg/kg	16.543±0.27^{c}	21.742±0.35^{b}	21.012±0.25^{c}	24.022±0.48^{c}	21.286±0.22^{c}	24.569±0.42^{c}
600 mg/kg	21.742±0.15^{b}	28.217±0.48^{c}	22.562±0.34^{b}	28.946±0.42^{c}	24.751±0.17^{c}	32.959±0.63^{c}
800 mg/kg	26.575±0.21^{c}	34.783±0.47^{c}	28.217±0.22^{b}	39.343±0.45^{c}	30.041±0.21^{b}	41.623±0.54^{c}
1000 mg/kg	31.226±0.12^{c}	41.248±0.41^{c}	32.594±0.12^{c}	46.457±0.61^{c}	35.604±0.38^{c}	48.646±0.47^{c}

Values were expressed as mean± SEM, Significance level: $^{a}p \leq 0.1$, $^{b}p \leq 0.05$, $^{c}p \leq 0.01$

stress increased proline levels, with the highest concentration recorded at 1000 mg/kg (32.594±0.12 µmoles/g), which was significantly higher than the control value of 7.879±0.47 µmoles/g ($p \leq 0.01$). Applying salicylic acid (SA) also led to elevated proline levels across all lead concentrations. The enhancement in proline accumulation with SA was particularly pronounced at higher lead concentrations, such as at 800 mg/kg, where proline levels reached 39.343±0.45 µmoles/g with SA compared to 28.217±0.22 µmoles/g without SA ($p \leq 0.01$).

Post-flowering stage

During the post-flowering stage, the pattern of proline accumulation exhibited an apparent dose-dependent increase in response to lead stress. The highest proline concentration recorded was at 1000 mg/kg, reaching 35.604±0.38 µmoles/g, which was significantly greater than the control value of 10.159±0.69 µmoles/g ($p \leq 0.01$). Furthermore, treatment with salicylic acid (SA) enhanced proline accumulation under lead stress at all concentrations. For example, the 1000 mg/kg proline level with SA was measured at 48.646±0.47 µmoles/g, markedly higher than the untreated group ($p \leq 0.01$).

Discussion

Proline is a vital indicator of environmental stress, particularly in response to heavy metal contamination. The results of this study indicate a progressive increase in proline content as lead concentrations in the soil rise compared to the control group. This suggests that proline accumulation is crucial in mitigating the adverse effects of lead-induced stress on plant growth, helping maintain normal physiological functions. Our findings are consistent with previous research, demonstrating that metal stress elevates proline levels across various plant species. For instance, Kalaikandhan et al. (2018) reported increased proline concentrations in *Sesuvium portulacastrum*, and similar outcomes were noted for tomato seedlings (Kisa, 2019), and *Zea mays* (Rizvi & Khan, 2018). Additionally, Chandrakar et al. (2018) emphasized the protective role of proline in promoting enhanced growth under stress conditions.

Proline serves as an osmolyte, helping to regulate the osmotic potential of plant cells under abiotic stress conditions (Yadu et al., 2016; Chandrakar et al., 2017; Wiesenthal et al., 2019; Alyemeni et al., 2018). This function is vital for maintaining cellular stability and promoting plant survival during stressful periods. Additionally, proline has been demonstrated to detoxify reactive oxygen species (ROS) and reduce oxidative damage triggered by metal toxicity (de Cassia Alves et al., 2018). It also functions as a molecular chaperone, stabilizing protein structures and protecting against lipid peroxidation (Per et al., 2017; Arroussi et al., 2018). The enzyme P5CS (Δ1-pyrroline-5-carboxylate synthetase) plays a crucial role in proline biosynthesis, and its overexpression has been associated with increased proline levels and enhanced tolerance to lead stress (Aslam et al., 2017).

Moreover, salicylic acid (SA) has significantly enhanced proline accumulation under metal stress. Our findings align with those of Faraz et al. (2019) and Alamri et al. (2018), where the application of SA notably increased proline levels in lead-stressed soybean

and *Triticum aestivum* L., respectively. Additionally, Parashar et al. (2012) demonstrated that SA alleviates heavy metal stress by promoting proline accumulation in *Brassica juncea*, likely through the increased activity of enzymes involved in proline synthesis. Beyond its role in osmotic regulation, the elevated proline content also improves water retention and provides cellular protection, strengthening the plant's defense mechanisms against environmental stressors (Zengin et al., 2005; Mishra & Mishra, 2012).

Conclusion

Proline accumulation is vital for plants in managing heavy metal stress, serving as an osmolyte, reactive oxygen species (ROS) detoxifier, and protein stabilizer. Furthermore, the exogenous application of SA amplifies proline accumulation, presenting a potential strategy for enhancing plant resilience in metal-contaminated environments. This study highlights the critical role of proline in plant stress physiology and emphasizes the potential for both genetic and chemical methods to bolster plant tolerance to environmental challenges.

Conflict of interest: Author declare no conflict of interest.

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