

Contents lists available at ScienceDirect

Journal of Biotechnology



journal homepage: www.elsevier.com/locate/jbiotec

5-aminolevulinic acid regulates Krebs cycle, antioxidative system and gene expression in *Brassica juncea* L. to confer tolerance against lead toxicity



Ravinder Singh^a, Anup Kumar Kesavan^b, Marco Landi^{c,d}, Satwinderjeet Kaur^a, Sharad Thakur^b, Bingsong Zheng^e, Renu Bhardwaj^{a,*}, Anket Sharma^{a,e,*}

^a Plant Stress Physiology Laboratory, Department of Botanical and Environmental Science, Guru Nanak Dev University, Amritsar, 143005, Punjab, India

^b Department of Molecular Biology and Biochemistry, Guru Nanak Dev University, Amritsar, 143005, Punjab, India

^c Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, I-56124, Pisa, Italy

^d CIRSEC, Centre for Climatic Change Impact, University of Pisa, Via del Borghetto 80, I-56124, Pisa, Italy

^e State Key Laboratory of Subtropical Silviculture, Zhejiang A&F University, Hangzhou, 311300, China

ARTICLE INFO

Keywords: Antioxidant enzymes Brassica juncea Gene expression Oxidative stress Metal toxicity

ABSTRACT

Heavy metal pollution seriously impairs crop production and poses serious concerns for human health. Exogenous application of biomolecules has been efficiently tested for enhancing plant resistance to metal toxicity. Current study evaluates the possible effect of 5-aminolevulinic acid (ALA) in Brassica juncea L. seedlings subjected to lead (Pb) stress. Our results showed that shoot length, root length and chlorophyll contents were significantly recovered in Pb stressed seedlings after ALA application, accompanied by reduction in the Pb accumulation. Significant reduction in the contents of reactive oxygen species (ROS) like superoxide anion, hydrogen peroxide and malondialdehyde were also observed in ALA treated seedlings under Pb stress. Furthermore, we also noticed enhancement in the activities of antioxidative enzymes like superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), glutathione reductase (GR), glutathione-S-transferase (GST) and dehydroascorbate reductase (DHAR). We further noticed that ALA upregulated the expression of SOD (7.30 folds), POD (6.11 folds), CAT (3.52 folds), DHAR (6.42 folds), GR (6.04 folds), and GST (5.58 folds) under the Pb stress. However, RBOH1 (gene involved in ROS generation) and CHLASE (chlorophyllase) expressions were reduced in ALA treated seedlings grown under Pb stress (RBOH1 expression decreased to 3.44 from 6.50 fold and CHLASE expression decreased to 2.97 from 5.58 fold). Phenolic contents were increased in the presence of ALA and expression of genes like CHS (chalcone synthase; 7.50 fold) and PAL (phenylalanine ammonia lyase; 4.77 fold) was also stimulated by ALA under Pb stress. Furthermore, contents of the Krebs cycle metabolites (fumarate, succinate, malate and citrate) were also enhanced accompanied by upregulated expression of genes like CS (citrate Synthase; 8.13 fold), SUCLG1 (succinyl CoA ligase 1; 7.40 fold), SDH (succinate dehydrogenase; 5.10 fold) and FH (fumarate hydratase; 5.65 fold). In conclusion, current investigation revealed that ALA attenuated Pb toxicity by modulating the transcription patterns of key enzymes involved in plant defense system.

1. Introduction

Heavy metal contamination in soils is attributable to the rapid industrialization, use of chemical fertilizers, smelting of rocks and combustion of gasoline as well as other anthropogenic sources represent a global concern for modern agriculture (Kumar et al., 2019; Shahid et al., 2011). Lead (Pb) is one of the most impacting metal pollutants, which alters the eco-biome of soil ecosystem and the plant metabolisms (Andra et al., 2009; Punamiya et al., 2010; Zhou et al., 2019). Since last few decades, extensive work has been conducted in relation to the biogeochemical aspects of Pb in plant-soil framework because of its potentially hazardous effects to the ecosystem (Schreck et al., 2012). Lead toxicity alters plant performance and productivity by altering multiple biochemical and physiological pathways in plants (Kohli et al., 2019). For instance, Pb stress lowers nutrient (e.g. other bivalent cations) uptake due to cationic competition, alters the enzyme activities by binding to co-factor sites usually hosting other essential metals, and reduces the photosynthetic efficiency in plants (Kohli et al., 2017;

https://doi.org/10.1016/j.jbiotec.2020.09.004

Received 15 May 2020; Received in revised form 27 August 2020; Accepted 4 September 2020 Available online 22 September 2020 0168-1656/© 2020 Elsevier B.V. All rights reserved.

^{*} Corresponding authors at: Department of Botanical and Environmental Science, Guru Nanak Dev University, Amritsar, 143005, Punjab, India. *E-mail addresses:* renubharadwaj82@gmail.com (R. Bhardwaj), anketsharma@gmail.com (A. Sharma).

Pourrut et al., 2011; Sharma et al., 2020). Lead toxicity also disturbs regulation of gene expression involved in plant development (Lyu et al., 2020; Maestri et al., 2010) and its accumulation in plant tissue also interferes with cell redox status triggering the production of reactive oxygen species (ROS), species such as hydroxy radical anion (-OH), superoxide anion (O_2^-) , and hydrogen peroxide (H_2O_2) (Amari et al., 2017; Ashraf et al., 2017; Pinho and Ladeiro, 2012). Lead ions trigger the production of ROS through multiple mechanisms including: (i) the activation of NADPH oxidase, (ii) the displacement of the prosthetic groups of various enzymes, and (iii) the inhibition the activity of enzymes which contains -SH groups (Pourrut et al., 2011). However, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and glutathione-s-transferase (GST) represent a powerful first line of defence for preserving the cellular redox homeostasis in plants subjected to Pb stress (Gupta et al., 2009; Li et al., 2012; Venkatachalam et al., 2017; Wang et al., 2012). Non enzymatic antioxidants, such as glutathione (GSH), ascorbic acid and tocopherols also cooperate with enzymatic antioxidants to counteract excess of Pb-triggered ROS (Kohli et al., 2017).

5-aminolevulinic acid (ALA) is a non-protein amino acid present in complex organisms, including plants (Beale and Castelfranco, 1974; Gibson et al., 1958; Schmied et al., 2018). ALA plays an important role in the biosynthesis of tetrapyrrole, the precursor of pivotal molecules, i. e. chlorophylls (Anwar et al., 2018). Additionally, ALA also ameliorates plant growth and strengthens plant defense mechanisms under various abiotic stresses including heavy metal toxicity (Akram and Ashraf, 2013; Ali et al., 2018; Cai et al., 2020; Tian et al., 2014; Wu et al., 2019). ALA reduces the harmful effects of various abiotic stresses as well as improves the chlorophyll biosynthesis, photosynthetic rate and antioxidative system (Naeem et al., 2010; Sheteiwy et al., 2017). Keeping in mind the potential of ALA in plant stress defense, the present investigation was designed to study the effect of exogenous treatment of ALA on the level of oxidative stress generated in Brassica juncea seedlings under Pb stress, for which there is a general lack of knowledge. Based on the results obtained from the current investigation, it is hypothysed that ALA can be a potential biomolecule to raise new heavy metal tolerant varieties. However, this needs further studies by combining biotechnological and molecular breeding tools.

2. Materials and methods

2.1. Raising of study material

Certified *B. juncea* L. (RLC-1 var.) seeds provided by Punjab Agricultural University, Ludhiana, (India) were sterilized with sodium hypochlorite (5 % v/v) and then rinsed with distilled water. 5-aminolevulinic acid (ALA) was purchased by Sigma Aldrich (Mumbai, India). Seeds were then pre-sown in 10 mg/L, 20 mg/L of ALA solution for 8 h. ALA-treated seeds were then grown in Petri plates on Grade 1-Whatman filter paper and filled with Pb solution (IC-50 value 0.5 mM). Twenty-five seeds per Petri plates were seeded and three replicates of each treatment were investigated. Petri plates were then placed under controlled illumination, temperature and humidity conditions in the seed germinator (photoperiod at 16 h, luminous intensity at 175 µmol m⁻² s⁻¹, and temperature at 25 ± 0.5 °C). Ten days old seedling were harvested and analyzed for ROS production, antioxidative enzyme activities and other biochemical parameters including gene expression of key enzymes.

2.2. Root, shoot length measurement and estimation of Pb accumulation

Shoot and root length of *B. juncea* seedling was measured after 10 days of seed sowing. For Pb content estimation, 1 g of fresh plant material (seedlings) was digested in aquaregia (nitric acid and hydrochloric acid in 3:1) and the extract was purified through Whatman filter paper 1.

After filtration, distilled water was added to reach a 50 mL final volume. Atomic absorption spectrophotometer (Schimadzu 6200) was used to determine the lead content.

2.3. Chlorophyll, anthocyanin and total phenol estimation

Chlorophyll content was determined by following method mentioned by Arnon (1949). The content of total anthocyanins was estimated by following standard method given by Mancinelli (1984). Method mentioned by Singleton and Rossi (1965) was used to quantify total phenolic content.

2.4. Estimation of oxidative stress markers

Superoxide anion (O_2^-) content was determined by using method mentioned by Wu et al. (2010). Hydrogen peroxide (H₂O₂) was determined by following the method given by Patterson et al. (1984). Malondialdehyde (MDA) content was estimated as per protocol given in Heath and Packer (1968).

2.5. Estimation of the activities of antioxidative enzymes

The extract of the plant was prepared by homogenizing 1 g of seedlings in 50 mM potassium phosphate buffer (PPB) with pH-7.0, followed by centrifugation at 10,000 g (4 °C) for 20 min. The supernatant was used to analyze the activity of antioxidant enzymes. Standard protocols were followed to estimate enzyme activities as mentioned below:

Catalase (CAT) activity was analyzed using Aebi (1974) method. Guaiacol Peroxidase (POD) activity was determined using the Pütter (1974) methodology. Glutathione Reductase (GR) activity was estimated following method of Carlberg and Mannervik (1975). Glutathione-S-transferase (GST) activity was measured according to Habig and Jakoby (1981). Superoxide dismutase (SOD) activity was determined by the method given by Kono (1978). Dehydroascorbate reductase (DHAR) activity was estimated according to Dalton et al. (1986).

2.6. Estimation of the Krebs cycle intermediates using gas chromatography-mass spectrometry (GC-MS)

Gas chromatography-mass spectrometry (GC–MS) was used to quantify contents of organic acids including fumarate, succinate, malate and citrate. Samples were prepared and analysed using GC–MS as per original method given by Sharma et al. (2016).

2.7. Gene expression analysis

Trizol (Invitrogen) method was followed to extract total RNA content from fresh seedlings of *B. juncea*. The total RNA was used for the synthesis of cDNA by the reverse transcription process using MuLv enzyme (RT). Genbank and EMBL Database were used to develop gene specific primers (Supplementary Table 1). qRT-PCR was performed following reaction amplification conditions mentioned by Sharma et al. (2019b). *Actin* was used as housekeeping gene. Method by Livak and Schmittgen (2001) was referred to find fold change in gene expression.

2.8. Statistical analysis

Experiments were carried out in three replicates. Data are analyzed using Two-way ANOVA followed by Tukey's HSD, and multiple linear regression analysis (MLR) using self-coded software in MS-Excel 2010.



Fig. 1. Effect of ALA application on growth parameters, chlorophyll content and lead (Pb) accumulation in 10 days old *B. juncea* seedlings grown under Pb stress. Data presented here as mean \pm standard deviation. HSD (honestly significant difference, $p \le 0.05$), MLR (multiple linear regression), CN (control), ALA1 and ALA2 (10 mg/L and 20 mg/L 5-aminolevulinic acid), Pb (0.5 mM/L), X1 (Pb), X2 (ALA), β_{Pb} (β -regression coefficient for Pb), β_{ALA} (β -regression coefficient for ALA), r (correlation coefficient), *** indicates significant at $p \le 0.001$.

3. Results and discussion

3.1. Impact of ALA application on seedling growth, chlorophyll content and Pb uptake

The shoot and root lengths were reduced under Pb stress as compared to untreated seedlings. Moreover, exogenous supplementation of ALA enhanced the shoot length and root length under Pb stress (Fig. 1A,B). Chlorophyll degradation was also recovered significantly in Pb stressed seedling after ALA application (Fig. 1C). MLR analysis also predicted positive β -regression for shoot length, root length and chlorophyll content after ALA application in Pb stressed seedlings (Fig. 1). In the current investigation, we also noticed that ALA application significantly reduced the accumulation of Pb content and statistical analysis using MLR further proved the role of ALA in reduction of Pb residues in the seedlings as indicated by negative β -regression value (Fig. 1D).

A consistent effect exerted by Pb toxicity is the restriction of plant growth, though Pb toxicity can act as a multi-target affecter in plant metabolism (Ali et al., 2014). As other metal ions, Pb competes with the uptake of other bivalent ions (e.g. Mg and Fe), which are essential for chlorophyll biosynthesis (Burzynski, 1987). In addition, Pb toxicity induces a decline in photosynthetic rate due to altered chlorophyll

ultrastructure, restrained synthesis plastoquinones and carotenoids, constrained electron transport, inhibited activities of Calvin cycle enzymes, as well as reduction of CO_2 uptake as a result of stomatal closure (Stefanov et al., 1995). Moreover, in current investigation, we also noticed that ALA application reduced the expression of *CHLASE* expression (chlorophyllase enzyme which is responsible for degradation of pigment chlorophyll).

The outcomes acquired in the present investigation reveal that shoot and root lengths were severely constrained in plants subjected to Pb treatment, as also observed by other researches (Sharma and Dubey, 2005). The decrease in shoot and root lengths might be due to the restraint in mitotic cell separation under Pb toxicity (Bhatti et al., 2013). Reduction in the growth might also be in response to hindered uptake of essential minerals which may disturb the biochemical metabolism, eventually leading to reduced cell growth as well as cell separation (Boroumand et al., 2011). The present study further revealed that seeds treated with ALA improved the growth of root and shoot lengths of mustard seedlings under Pb stress. The increase in growth parameters might be due to the positive action of ALA in the regulation of multiple metabolic systems, thereby increasing the growth and productivity of various plant physiological processes under abiotic-stressed plants, including heavy metals (Ahmad et al., 2017; Ali et al., 2018).



Fig. 2. Effect of ALA application on oxidative stress markers in 10 days old *B. juncea* seedlings grown under Pb stress. Data presented here as mean \pm standard deviation. HSD (honestly significant difference, $p \le 0.05$), MLR (multiple linear regression), CN (control), ALA1 and ALA2 (10 mg/L and 20 mg/L 5-aminolevulinic acid), Pb (0.5 mM/L), X1 (Pb), X2 (ALA), β_{Pb} (β -regression coefficient for Pb), β_{ALA} (β -regression coefficient for ALA), r (correlation coefficient), *** indicates significant at $p \le 0.001$.

One of the most deleterious effects of Pb in plant metabolism is the imbalance of photosynthesis, which is principally dependent to the impairment of chlorophyll biosynthesis due to reduce uptake of Mg and Fe (Burzynski, 1987). In our experiments, ALA stimulated the accumulation of chlorophylls in control plants and in Pb-treated plants the highest dose of ALA restored the level of chlorophyll content. Among other possible positive effect exerted by ALA, the reduction of Pb accumulation could be one of the main ameliorative roles offered by this compound. Indeed, ALA application reduced the accumulation of Pb ions, for which, however, our dataset does not allow finding a proper explanation. A lower Pb accumulation may have limited the Pb-triggered redox homeostasis imbalance, which is a common reaction of plants subjected to Pb toxicity (Kohli et al., 2019).

3.2. Impact of ALA application of oxidative stress and antioxidative defense system

In current study, Pb application to *B. juncea* seedlings promoted the accumulation of superoxide anion and hydrogen peroxide, which were accompanied by an enhanced lipid peroxidation (Fig. 2), which is a well-known indicator of membrane damage due to oxidative stress (Kohli et al., 2019). However, ALA application resulted in significant reduction in the contents of superoxide anion (Fig. 2A), hydrogen peroxide (Fig. 2B) and MDA (Fig. 2C).

We also noticed that ALA application significantly enhanced the activities of antioxidative enzymes under Pb stress (Fig. 3). It has been clearly demonstrated that a powerful antioxidant system and a rapid activation of the antioxidant apparatus by ALA (Ali et al., 2014) is essential for plants to counteract the Pb-triggered oxidative stress, which can otherwise damage cell membrane (Verma and Dubey, 2003). For this reasons, besides biometric analyses, emphasis is given in the present investigation to validate biochemical response (antioxidative defense system) by studying the gene expression pattern of key enzymes. Moreover, genes responsible for respiratory burst oxidases also get upregulated under abiotic stresses, resulting in over accumulation of ROS (Sharma et al., 2017). In the current investigation, we also noticed that expression of *RBOH1* gene was upregulated by Pb toxicity (Fig. 3G), supporting above mentioned facts.

Antioxidant enzymes are key defenses in plants subjected to Pb toxicity (Ali et al., 2014; Kohli et al., 2019) and a prompt and massive activation of the activity and the expression of antioxidant enzymes is essential for plants to counteract the effect of Pb-triggered ROS. Our dataset revealed that Pb toxicity enhanced the activities of enzymatic antioxidants (SOD, CAT, POD, GR, GST and DHAR) as well as upregulated gene expression (SOD, CAT, POD, GST, GR and DHAR). Notably, the application of ALA in our experiment induced a further significant stimulation in the activity of most antioxidant enzymes (Fig. 3A-F) as well as in their gene expression (Fig. 4A-F). This highlights the capacity of ALA-treated plants to respond better to Pb-triggered oxidative stress and to contain the damages related to ROS spread. This role of ALA is not simply dependent to the lower level of Pb accumulated in ALA-treated plants, given that ALA stimulated directly the activity and the expression of most antioxidant enzymes, even when applied in control plants. This is supportive for a causal effect between ALA application and antioxidant enzyme activity and expression activation, which contributes in making ALA application a promising strategy to increase Pb tolerance in plants.

3.3. Impact on contents of anthocyanins, total phenolics and Krebs cycle

Besides the positive effect to antioxidant enzymes in Pb-treated plants, ALA also stimulates the accumulation of antioxidant compounds such as total polyphenols, and in particular the flavonoid group of anthocyanins. In the current investigation, we noticed that Pb stress as well as ALA application enhanced the contents of total anthocyanins (Fig. 5A) and total phenolics (Fig. 5B). Analysis of data using MLR also revealed the promontory role of ALA in the biosynthesis of these phenolics (Fig. 5).

The accumulation of polyphenols is a common response of plants against a plethora of abiotic stresses, including heavy metals, due to their high antioxidant power (Sharma et al., 2019a). Besides their antioxidant prerogative, anthocyanins may also assist the photosynthetic apparatus by acting as sunscreen (Lo Piccolo et al., 2020), thereby reducing the excess of light attributable to chloroplast impairment in Pb-stressed plants (Hadi and Aziz, 2015). In our experiments, Pb promoted the accumulation of total polyphenols and anthocyanins, but the stimulation of the polyphenol metabolism was greater in Pb-ALA treated plants. Accordingly, ALA significantly stimulated the expression of key genes of the phenylpropanoid metabolism, including *CHS* (Fig. 6B) and *PAL* (Fig. 6C) which are indeed responsible for the stronger



Fig. 3. Effect of ALA application on antioxidative enzymes in 10 days old *B. juncea* seedlings grown under lead (Pb) stress. SOD (superoxide dismutase), CAT (catalase), POD (peroxidase), GR (glutathione reductase), GST (glutathione-s-transferase), DHAR (dehydroascorbate reductase), Data presented here as mean \pm standard deviation. HSD (honestly significant difference, $p \le 0.05$), MLR (multiple linear regression), CN (control), ALA1 and ALA2 (10 mg/L and 20 mg/L 5-aminolevulinic acid), Pb (0.5 mM/L), X1 (Pb), X2 (ALA), β_{Pb} (β -regression coefficient for Pb), β_{ALA} (β -regression coefficient for ALA), r (correlation coefficient), *** indicates significant at $p \le 0.001$.

accumulation of total phenols and anthocyanins in both ALA- and Pb-ALA-treated plants.

Another positive role exerted by ALA in our experiment is the stimulation of the Krebs cycle as demonstrated by increased level of some intermediates like fumaric acid, succinic acid, malic acid and citric acid (Fig. 5C-F) as well as the upregulation of genes encoding key enzymes involved in this pathway including *CS*, *SUCLG1*, *SDH* and *FH* (Fig. 6D–G). It has been clearly demonstrated that these organic acids can act as metal chelators (Naidu and Harter, 1998). In particular, malic and citric acid (the two which were much more strongly accumulated in our experiments in Pb-ALA-treated plants) can form stable complexes with divalent cations (Cieśliński et al., 1998), thereby sequestrating Pb ions and reducing the possibility of Pb ions to affect plant metabolism. Accordingly, citric acid improves lead (Pb) phytoextraction in *Brassica napus* L. by mitigating pb-induced morphological and biochemical damages (Cieśliński et al., 1998).

4. Conclusion

The present investigation offers the evidence that ALA treatment exerted ameliorative effects in Pb-stressed plants in different ways. ALA treatment increased the performances of *B. juncea* plants by: i) reducing the level of Pb accumulated; ii) promoting the activity and the expression of pivotal antioxidant enzymes; iii) stimulating the biosynthesis of key non-enzymatic antioxidant compounds (total polyphenols and anthocyanins); and iv) promoting the biosynthesis of organic acids (Krebs cycle) which can act as metal chelator. It is conceivable that all these mechanisms contribute in ameliorating *B. juncea* performances and accommodating Pb-triggered stress, thereby suggesting the use of ALA as a possible strategy to increase plant tolerance against Pb toxicity, for example in case of phytoremediation. Moreover, exploring in-depth molecular mechanisms can be of special interest for crop biotechnologists and molecular plant breeders to raise new heavy metal tolerant varieties.



Fig. 4. Effect of ALA application on gene expression of antioxidative defense system in 10 days old *B. juncea* seedlings grown under lead (Pb) stress. Data presented here as mean \pm standard deviation. *SOD* (superoxide dismutase), *CAT* (catalase), *POD* (peroxidase), *GR* (glutathione reductase), *GST* (glutathione-s-transferase), *DHAR* (dehydroascorbate reductase), *RBOH1* (respiratory burst oxidase homologue 1). HSD (honestly significant difference, $p \le 0.05$), MLR (multiple linear regression), CN (control), ALA1 and ALA2 (10 mg/L and 20 mg/L 5-aminolevulinic acid), Pb (0.5 mM/L), X1 (Pb), X2 (ALA), β_{Pb} (β -regression coefficient for Pb), β_{ALA} (β -regression coefficient for ALA), r (correlation coefficient), *, ** and *** indicates significant at $p \le 0.05$, $p \le 0.01$ and $p \le 0.001$ respectively.

CRediT authorship contribution statement

Ravinder Singh: Methodology, Investigation, Formal analysis, Writing - original draft. Anup Kumar Kesavan: Methodology, Investigation, Formal analysis. Marco Landi: Writing - review & editing. Satwinderjeet Kaur: Resources. Sharad Thakur: Methodology, Investigation, Formal analysis. Bingsong Zheng: Writing - review & editing. **Renu Bhardwaj:** Conceptualization, Resources. **Anket Sharma:** Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.



Fig. 5. Effect of ALA application on the contents of total anthocyanin, total phenols and organic acids in 10 days old *B. juncea* seedlings grown under lead (Pb) stress. Data presented here as mean \pm standard deviation. HSD (honestly significant difference, $p \le 0.05$), MLR (multiple linear regression), CN (control), ALA1 and ALA2 (10 mg/L and 20 mg/L 5-aminolevulinic acid), Pb (0.5 mM/L), X1 (Pb), X2 (ALA), β_{Pb} (β -regression coefficient for Pb), β_{ALA} (β -regression coefficient for ALA), r (correlation coefficient), *** indicates significant at $p \le 0.001$.



Fig. 6. Effect of ALA application on the gene expression of 10 days old *B. juncea* seedlings grown under Pb stress. Data presented here as mean \pm standard deviation. HSD (honestly significant difference, $p \le 0.05$), MLR (multiple linear regression), CN (control), ALA (20 mg/L 5-aminolevulinic acid), Pb (0.5 mM/L), β_{Pb} (β -regression coefficient for Pb), X1 (Pb), X2 (ALA) β_{ALA} (β -regression coefficient for ALA), r (correlation coefficient), *CHLASE (Chlorophyllase), CHS (Chalcone synthase), PAL* (Phenylalanine ammonia lyase), *CS (Citrate Synthase), SUCLG1* (Succinyl CoA ligase 1), *SDH (Succinate dehydrogenase), FH (Fumarate hydratase).* ** and *** indicates significant at $p \le 0.01$ and $p \le 0.001$ respectively.

Acknowledgement

UGC-BSR Fellowship from the University Grant Commission (UGC), New Delhi, India.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2020.09.004.

References

- Aebi, H., 1974. Catalase. In: Methods of Enzymatic Analysis, Vol. 2, pp. 673–684. https://doi.org/10.1016/b978-0-12-091302-2.50032-3.
- Ahmad, R., Ali, S., Hannan, F., Rizwan, M., Iqbal, M., Hassan, Z., Abbas, F., 2017. Promotive role of 5-aminolevulinic acid on chromium-induced morphological, photosynthetic, and oxidative changes in cauliflower (*Brassica oleracea* botrytis L.). Environ. Sci. Pollut. Res. 24 (9), 8814–8824. https://doi.org/10.1007/s11356-017-8603-7.

- Akram, N.A., Ashraf, M., 2013. Regulation in plant stress tolerance by a potential plant growth regulator, 5-Aminolevulinic acid. Plant Growth Regul. 32 (3), 663–679. https://doi.org/10.1007/s00344-013-9325-9.
- Ali, B., Xu, X., Gill, R.A., Yang, S., Ali, S., Tahir, M., Zhou, W., 2014. Promotive role of 5aminolevulinic acid on mineral nutrients and antioxidative defense system under lead toxicity in *Brassica napus*. Ind. Crops Prod. 52, 617–626. https://doi.org/ 10.1016/j.indcron.2013.11.033.
- Ali, S., Rizwan, M., Zaid, A., Arif, M.S., Yasmeen, T., Hussain, A., Abbasi, G.H., 2018. 5-Aminolevulinic acid-induced heavy metal stress tolerance and underlying mechanisms in plants. J. Plant Growth Regul. 37, 1423–1436. https://doi.org/ 10.1007/s00344-018-9875-y.
- Amari, T., Ghnaya, T., Abdelly, C., 2017. Nickel, cadmium and lead phytotoxicity and potential of halophytic plants in heavy metal extraction. S. Afr. J. Bot. 111, 99–110. https://doi.org/10.1016/j.sajb.2017.03.011.
- Andra, S.S., Datta, R., Sarkar, D., Saminathan, S.K., Mullens, C.P., Bach, S.B., 2009. Analysis of phytochelatin complexes in the lead tolerant vetiver grass [*Vetiveria zizanioides* (L.)] using liquid chromatography and mass spectrometry. Environ. Pollut. 157, 2173–2183. https://doi.org/10.1016/j.envpol.2009.02.014.
- Anwar, A., Yan, Y., Liu, Y., Li, Y., Yu, X., 2018. 5-Aminolevulinic acid improves nutrient uptake and endogenous hormone accumulation, enhancing low-temperature stress tolerance in cucumbers. Int. J. Mol. Sci. 19, 3379. https://doi.org/10.3390/ ijms19113379.
- Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24, 1–15. https://doi.org/10.1104/pp.24.1.1.
- Ashraf, U., Kanu, A.S., Deng, Q., Mo, Z., Pan, S., Tian, H., Tang, X., 2017. Lead (PB) toxicity: physio-biochemical mechanisms, grain yield, quality, and PB distribution proportions in scented rice. Front. Plant Sci. https://doi.org/10.3389/ fbls.2017.00259.
- Beale, S.I., Castelfranco, P.A., 1974. The biosynthesis of δ-aminolevulinic acid in higher plants. Plant Physiol. 53, 297–303. https://doi.org/10.1104/pp.53.2.297.
- Bhatti, K.H., Anwar, S., Nawaz, K., Hussain, K., Siddiqi, E.H., Sharif, R.U., Talat, A., Khalid, A., 2013. Effect of heavy metal Lead (Pb) stress of different concentration on wheat (Triticum aestivum L.). Middle East J. Sci. Res. 14, 148–154.
- Boroumand, J.S., Lari, Y.H., Ranjbar, M., 2011. Effect of salicylic acid on some plant growth parameters under lead stress in *Brassica napus* var. Okapi. Iran. J. Plant Physiol. 1, 177–185.
- Burzynski, M., 1987. Influence of lead and cadmium on the absorption and distribution of potassium, calcium, magnesium and iron in cucumber seedlings. Acta Physiol. Plant. 9, 229–238.
- Cai, C., He, S., An, Y., Wang, L., 2020. Exogenous 5-aminolevulinic acid improves strawberry tolerance to osmotic stress and its possible mechanisms. Physiol. Planta. 168, 948–962.
- Carlberg, I., Mannervik, B., 1975. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J. Biol. Chem. 250, 5475–5480.
- Cieśliński, G., Van Rees, K., Szmigielska, A., Krishnamurti, G., Huang, P., 1998. Lowmolecular-weight organic acids in rhizosphere soils of durum wheat and their effect on cadmium bioaccumulation. Plant Soil 203, 109–117. https://doi.org/10.1023/a: 1004325817420.
- Dalton, D.A., Russell, S.A., Hanus, F.J., Pascoe, G.A., Evans, H.J., 1986. Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules. Proc. Natl. Acad. Sci. 83, 3811–3815. https://doi.org/10.1073/ pnas.83.11.3811.
- Gibson, K.D., Laver, W.G., Neuberger, A., 1958. Initial stages in the biosynthesis of porphyrins. 2. The formation of δ-aminolaevulic acid from glycine and succinylcoenzyme a by particlesfrom chicken erythrocytes. Biochem. J. 70, 71–81. https:// doi.org/10.1042/bi0700071.
- Gupta, D., Nicoloso, F., Schetinger, M., Rossato, L., Pereira, L., Castro, G., Srivastava, S., Tripathi, R., 2009. Antioxidant defense mechanism in hydroponically grown Zea mays seedlings under moderate lead stress. J. Hazard. Mater. 172, 479–484. https:// doi.org/10.1016/j.jhazmat.2009.06.141.
- Habig, W.H., Jakoby, W.B., 1981. Assays for differentiation of glutathione S-transferases.
- Methods Enzymol. 77, 398–405. https://doi.org/10.1016/s0076-6879(81)77053-8. Hadi, F., Aziz, T., 2015. A mini review on lead (PB) toxicity in plants. J. Biol. Life Sci. 6, 91. https://doi.org/10.5296/jbls.v6i2.7152.
- Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts. Arch. Biochem. Bionhys. 125, 189–198. https://doi.org/10.1016/0003-9861(68)90654-1.
- Biophys. 125, 189–198. https://doi.org/10.1016/0003-9861(68)90654-1.
 Kohli, S.K., Handa, N., Sharma, A., Gautam, V., Arora, S., Bhardwaj, R., Alyemeni, M.N.,
 Wijaya, L., Ahmad, P., 2017. Combined effect of 24-epibrassinolide and salicylic acid mitigates lead (PB) toxicity by modulating various metabolites in *Brassica juncea* L. seedlings. Protoplasma 255, 11–24. https://doi.org/10.1007/s00709-017-1124-x.
- Kohli, S.K., Handa, N., Bali, S., Khanna, K., Arora, S., Sharma, A., Bhardwaj, R., 2019. Current scenario of PB toxicity in plants: unraveling plethora of physiological responses. Rev. Environ. Contam. Toxicol. 249, 153–197. https://doi.org/10.1007/ 398 2019 25.
- Kono, Y., 1978. Generation of superoxide radical during autoxidation of hydroxylamine and an assay for superoxide dismutase. Arch. Biochem. Biophys. 186, 189–195. https://doi.org/10.1016/0003-9861(78)90479-4.
- Kumar, V., Sharma, A., Kaur, P., Sidhu, G.P.S., Bali, A.S., Bhardwaj, R., Thukral, A.K., Cerda, A., 2019. Pollution assessment of heavy metals in soils of India and ecological risk assessment: a state-of-the-art. Chemosphere 216, 449–462.
- Li, X., Bu, N., Li, Y., Ma, L., Xin, S., Zhang, L., 2012. Growth, photosynthesis and antioxidant responses of endophyte infected and non-infected rice under lead stress conditions. J. Hazard. Mater. 213-214, 55–61. https://doi.org/10.1016/j. ihazmat.2012.01.052.

- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta$ CT method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.
- Lo Piccolo, E., Landi, M., Massai, R., Remorini, D., Guidi, L., 2020. Girled-induced anthocyanin accumulation in red-leafed *Printus cerasifera*: effect on photosynthesis, photoprotection and sugar metabolism. Plant Sci. 294, 110456 https://doi.org/ 10.1016/j.plantsci.2020.110456.
- Lyu, G., Li, D., Li, S., Ning, C., Qin, R., 2020. Genotoxic effects and proteomic analysis on Allium cepa var. agrogarum L. root cells under Pb stress. Ecotoxicology 29, 959–972.
- Maestri, E., Marmiroli, M., Visioli, G., Marmiroli, N., 2010. Metal tolerance and hyperaccumulation: costs and trade-offs between traits and environment. Environ. Exp. Bot. 68, 1–13. https://doi.org/10.1016/j.envexpbot.2009.10.011.
- Mancinelli, A.L., 1984. Photoregulation of anthocyanin synthesis. Plant Physiol. 75, 447–453. https://doi.org/10.1104/pp.75.2.447.
- Naeem, M.S., Jin, Z.L., Wan, G.L., Liu, D., Liu, H.B., Yoneyama, K., Zhou, W.J., 2010. 5-Aminolevulinic acid improves photosynthetic gas exchange capacity and ion uptake under salinity stress in oilseed rape (*Brassica napus* L.). Plant Soil 332, 405–415. https://doi.org/10.1007/s11104-010-0306-5.
- Naidu, R., Harter, R.D., 1998. Effect of different organic ligands on cadmium sorption by and extractability from soils. Soil Sci. Soc. Am. J. 62, 644–650. https://doi.org/ 10.2136/sssaj1998.03615995006200030014x.
- Patterson, B.D., MacRae, E.A., Ferguson, I.B., 1984. Estimation of hydrogen peroxide in plant extracts using titanium(IV). Biochem. Anal. Biochem. 139, 487–492. https:// doi.org/10.1016/0003-2697(84)90039-3.
- Pinho, S., Ladeiro, B., 2012. Phytotoxicity by lead as heavy metal focus on oxidative stress. J. Bot. 1–10. https://doi.org/10.1155/2012/369572.
- Pourrut, B., Shahid, M., Dumat, C., Winterton, P., Pinelli, E., 2011. Lead uptake, toxicity, and detoxification in plants. Rev. Environ. Contam. Toxicol. 213, 113–136. https:// doi.org/10.1007/978-1-4419-9860-6 4.
- Punamiya, P., Datta, R., Sarkar, D., Barber, S., Patel, M., Das, P., 2010. Symbiotic role of Glomus mosseae in phytoextraction of lead in vetiver grass [*Chrysopogon zizanioides* (L.)]. J. Hazard. Mater. 177, 465–474. https://doi.org/10.1016/j. ihazmat.2009.12.056.
- Pütter, J., 1974. Peroxidases. In: Methods of Enzymatic Analysis, Vol. 2, pp. 685–690. https://doi.org/10.1016/b978-0-12-091302-2.50033-5.
- Schmied, J., Hou, Z., Hedtke, B., Grimm, B., 2018. Controlled partitioning of glutamyltRNA reductase in stroma-and membrane-associated fractions affects the synthesis of 5-aminolevulinic acid. Plant Cell Physiol. 59, 2204–2213.
- Schreck, E., Foucault, Y., Sarret, G., Sobanska, S., Cécillon, L., Castrec-Rouelle, M., Uzu, G., Dumat, C., 2012. Metal and metalloid foliar uptake by various plant species exposed to atmospheric industrial fallout: mechanisms involved for lead. Sci. Total Environ. 427-428, 253–262. https://doi.org/10.1016/j.scitotenv.2012.03.051.
- Shahid, M., Pinelli, E., Pourrut, B., Silvestre, J., Dumat, C., 2011. Lead-induced genotoxicity to *Vicia faba* L. roots in relation with metal cell uptake and initial speciation. Ecotoxicol. Environ. Saf. 74, 78–84. https://doi.org/10.1016/j. ecoept. 2010.08.037.
- Sharma, P., Dubey, R.S., 2005. Lead toxicity in plants. Braz. J. Plant Physiol. 17, 35–52. https://doi.org/10.1590/s1677-04202005000100004.
- Sharma, A., Thakur, S., Kumar, V., Kanwar, M.K., Kesavan, A.K., Thukral, A.K., Bhardwaj, R., Alam, P., Ahmad, P., 2016. Pre-sowing seed treatment with 24-Epibrassinolide ameliorates pesticide stress in *Brassica juncea* L. through the modulation of stress markers. Front. Plant Sci. 7, 1569. https://doi.org/10.3389/ fpls.2016.01569.
- Sharma, A., Thakur, S., Kumar, V., Kesavan, A.K., Thukral, A.K., Bhardwaj, R., 2017. 24epibrassinolide stimulates imidacloprid detoxification by modulating the gene expression of *Brassica juncea* L. BMC Plant Biol. 17, 56. https://doi.org/10.1186/ s12870-017-1003-9.
- Sharma, A., Shahzad, B., Rehman, A., Bhardwaj, R., Landi, M., Zheng, B., 2019a. Response of phenylpropanoid pathway and the role of polyphenols in plants under abiotic stress. Molecules 24, 2452. https://doi.org/10.3390/molecules24132452.
- Sharma, A., Yuan, H., Kumar, V., Ramakrishnan, M., Kohli, S.K., Kaur, R., Thukral, A.K., Bhardwaj, R., Zheng, B., 2019b. Castasterone attenuates insecticide induced phytotoxicity in mustard. Ecotoxicol. Environ. Saf. 179, 50–61. https://doi.org/ 10.1016/j.ecoenv.2019.03.120.
- Sharma, A., Kumar, V., Shahzad, B., Ramakrishnan, M., Sidhu, G.P.S., Bali, A.S., Handa, N., et al., 2020. Photosynthetic response of plants under different abiotic stresses: a review. J. Plant Growth Regul. 39, 509–531. https://doi.org/10.1007/ s00344-019-10018-x.
- Sheteiwy, M., Shen, H., Xu, J., Guan, Y., Song, W., Hu, J., 2017. Seed polyamines metabolism induced by seed priming with spermidine and 5-aminolevulinic acid for chilling tolerance improvement in rice (*Oryza sativa* L.) seedlings. Environ. Exp. Bot. 137, 58–72. https://doi.org/10.1016/j.envexpbot.2017.02.007.
- Singleton, V.L., Rossi, J.A., 1965. Colorimetry of total phenolics with phosphomolybdicphosphotungstic acid reagents. Am. J. Enol. Viticul. 16, 144–158. https://www.aj evonline.org/content/16/3/144.
- Stefanov, K., Seizova, K., Popova, I., Petkov, V., Kimenov, G., Popov, S., 1995. Effect of lead ions on the phospholipid composition in leaves of zea mays and *Phaseolus vulgaris*. J. Plant Physiol. 147, 243–246. https://doi.org/10.1016/s0176-1617(11) 81511-7.
- Tian, T., Ali, B., Qin, Y., Malik, Z., Gill, R.A., Ali, S., Zhou, W., 2014. Alleviation of lead toxicity by 5-Aminolevulinic acid is related to elevated growth, photosynthesis, and suppressed ultrastructural damages in oilseed rape. Biomed. Res. Int. 1–11. https:// doi.org/10.1155/2014/530642.
- Venkatachalam, P., Jayalakshmi, N., Geetha, N., Sahi, S.V., Sharma, N.C., Rene, E.R., Sarkar, S.K., Favas, P.J., 2017. Accumulation efficiency, genotoxicity and antioxidant defense mechanisms in medicinal plantAcalypha indica L. under lead

R. Singh et al.

stress. Chemosphere 171, 544–553. https://doi.org/10.1016/j. chemosphere.2016.12.092.

- Verma, S., Dubey, R., 2003. Lead toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants. Plant Sci. 164, 645–655. https://doi.org/10.1016/s0168-9452(03)00022-0.
- Wang, P., Zhang, S., Wang, C., Lu, J., 2012. Effects of PB on the oxidative stress and antioxidant response in a PB bioaccumulator plant *Vallisneria natans*. Ecotoxicol. Environ. Saf. 78, 28–34. https://doi.org/10.1016/j.ecoenv.2011.11.008.
- Wu, Q., Zou, Y., Liu, W., Ye, X., Zai, H., Zhao, L., 2010. Alleviation of salt stress in citrus seedlings inoculated with mycorrhiza: changes in leaf antioxidant defense systems. Plant Soil Environ. 56, 470–475. https://doi.org/10.17221/54/2010-pse.
- Wu, Y., Hu, L., Liao, W., Mujitaba Dawuda, M., Lyu, J., Xie, J., Feng, Z., Calderón-Urrea, A., Yu, J., 2019. Foliar application of 5-aminolevulinic acid (ALA) alleviates NaCl stress in cucumber (*Cucumis sativus* L.) seedlings through the enhancement of ascorbate-glutathione cycle. Sci. Hortic. 257, 108761 https://doi.org/10.1016/j. scienta.2019.108761.
- Zhou, J., Du, B., Wang, Z., Zhang, W., Xu, L., Fan, X., Liu, X., Zhou, J., 2019. Distributions and pools of lead (Pb) in a terrestrial forest ecosystem with highly elevated atmospheric Pb deposition and ecological risks to insects. Sci. Tot. Environ. 647, 932–941.