agronomy

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# Alleviatory Effects of Silicon and 24-Epibrassinolide in Modulation of Growth, Osmolytes, Metabolites, Antioxidant Defense System, and Gene Expression in Lead-Exposed Fenugreek (*Trigonella foenum-graecum* L.) Plants

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**Abstract:** Amplified concentrations of lead (Pb) in cultivable soils, being a major environmental concern, bring about malicious consequences for plant and human health. *Trigonella foenum-graecum* (fenugreek) is a multipurpose herb used as a spice, tonic, leafy vegetable, and therapeutic agent. Earlier works have revealed the inhibitory effects of Pb toxicity in *Trigonella*, affecting its growth and productivity. Therefore, the current experimental work was planned with the purpose of evaluating the effects of exogenously supplemented silicon (Si; 2 mM) and 24-epibrassinolide (24-EBL; $10^{-7}$ M) (in both individual and combined form) on growth attributes, osmolytes, metabolite measures, and antioxidant defense mechanisms of *Trigonella foenum-graecum* plants in response to three discrete concentrations of Pb stress (0.5, 0.7, and 0.9 mM). The results revealed that Pb stress affected morphological parameters of fenugreek plants via the genesis of reactive oxygen species (ROS), as indicated by higher measures of oxidative damage indicators like malondialdehyde (MDA) and hydrogen peroxide ($H_2O_2$). Spraying foliage with Si together with a pretreatment of 24-EBL alone as well as in a combined form yielded better outcomes in terms of growth parameters in the Pb-stressed plants. Pb toxicity decreased osmolytes, proteins, and metabolites. Components of the antioxidative defense system, i.e., enzymes [ascorbate peroxidase (APX), guaiacol peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT), together with non-enzymes [ascorbic acid (AsA) and glutathione (GSH), were downregulated when subjected to Pb toxicity. Out of all, Pb III (0.9 mM) had a more adverse impact on various parameters in fenugreek compared to Pb I (0.5 mM) and Pb II (0.7 mM). However, external supplementation with Si and 24-EBL (individually and in combination) ameliorated the Pb-mediated oxidative stress in fenugreek plants by improving the content of different osmolytes and metabolites while upregulating the functioning of the antioxidative defense system. Downregulation in the expression of *SOD* and *CAT* genes was found in Pb-stressed plants, while their expression was upregulated by Si and 24-EBL both individually and in combination. The experimental study revealed that the combined application of Si and 24-EBL was significantly better at abating the Pb metal stress in fenugreek plants when compared with their individual applications.

**Keywords:** abiotic stress; food security; heavy metal toxicity; oxidative damage; stress physiology; plant growth regulators

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**Citation:** Sharma, D.; Bhardwaj, S.; Raza, A.; Singh, R.; Kapoor, D.; Sharma, N.R.; Prasad, P.V.V. Alleviatory Effects of Silicon and 24-Epibrassinolide in Modulation of Growth, Osmolytes, Metabolites, Antioxidant Defense System, and Gene Expression in Lead-Exposed Fenugreek (*Trigonella foenum-graecum* L.) Plants. *Agronomy* **2023**, *13*, 1884. https://doi.org/10.3390/agronomy13071884

Academic Editor: Hakim Manghwar

Received: 9 June 2023
Revised: 10 July 2023
Accepted: 10 July 2023
Published: 17 July 2023

## 1. Introduction

Naturally occurring heavy metals have increased in greater proportions in the entire biosphere due to anthropogenic interventions like urbanization and industrialization [1]. Their exceeding limits are toxic to the well-being of the environment and its biotic components, particularly plants, and humans [1–3]. Lead (Pb) finds its place amongst such heavy metals and is evaluated to be the second major hazardous metal on the "Substance Priority List" released by the US public health agency ATSDR (Agency for Toxic Substances and Disease Registry) in 2022 on account of its ubiquitous distribution and lethality. Once Pb enters the food chain, it causes various detrimental health consequences like anemia and brain and kidney damage, which may prove fatal in the long term [4]. Amplified measures of Pb in the soil negatively impact useful microbial flora and populations of earthworms (*Eisenia fetida*), resulting in decreased soil fertility [5,6]. Plants growing in such soils show intracellular deposition of Pb, mainly in roots, and (i) intensify the genesis of ROS, conferring oxidative damage; (ii) inhibit their enzyme action; and (iii) alter various vital physiological processes [7–9].

However, plants resort to a diverse range of defensive strategies to combat Pb-induced toxicity, such as (a) activating antioxidative enzymes to rectify oxidative damage; (b) detoxifying the Pb through sequestration in vacuoles or linking the Pb with amino acids, glutathione, and phytochelatins; (c) excluding Pb through its linkage with carboxyl groups of uronic acid followed by precipitation through oxalates or immobilizing it in the rhizosphere; (d) accumulation of osmolytes, free amino acids, and cell wall depositions such as callose and suberin; and (e) modulating the endogenous measures of regulators of plant growth, i.e., phytohormones [10–13]. However, these defensive strategies fall short of their efficacy under prolonged stress conditions. Therefore, a variety of other external procedures are needed. Out of them, extrinsic supplementation with phytoprotectants is preferred, as they are helpful in combating Pb-induced phytotoxicity.

Silicon (Si), termed a multifaceted, beneficial element, ranking second in its abundancy after oxygen in the earth's crust, exhibits versatility in its physiological roles in plants, which vary from better mechanical strength to enhancement of plant growth and productivity, along with conferring multiple stress tolerances [14–17]. Negative effects of heavy metal toxicity are mitigated by silicon either through escape mechanisms by decreasing the metal uptake and translocation; chelating metal ions with root exudates; or tolerance mechanisms by enhancing the activity of anti-oxidative enzymes; forming complexes with heavy metals and their confinement in cell walls or vacuoles [18]. For instance, adverse effects like reduced growth, biomass, and productivity in plants as a result of the presence of Pb are normalized by Si application in cotton (*Gossypium hirsutum*) plants by forming Pb-Si complexes or enhancing the deposition of Pb in cell walls so as to reduce its phytoavailability [19].

Brassinosteroids (BRs) are steroidal phytohormones that find their applicability in plants by influencing cell division and expansion, stomatal conductance, the synthesis of photosynthetic pigments, flowering, seed germination, and the mitigation of biotic and abiotic stresses [20,21]. Brassinosteroids can ameliorate heavy metal-induced stress such as oxidative damage by activating antioxidants (enzymatic and non-enzymatic ones) and by interacting with other phytohormones like IAA (indole acetic acid) and ABA (abscisic acid) to relieve stress and enhance metabolic activities in plants [22,23]. In cases of Pb toxicity, BRs (24-epibrassinolide form), when supplemented externally, decrease Pb uptake by 50% in beetroot (*Beta vulgaris*) compared to plants that are treated with this heavy metal alone [24].

Fenugreek (*Trigonella foenum-graecum* L.) is a small, aromatic herb of the Fabaceae family with huge utilitarian potential in terms of being used as a spice, stimulant, tonic, or leafy vegetable and in a variety of medicinal preparations. Its use in medicines to cure diabetes, hair loss, inflammations, liver ailments, skin eruptions, stomachaches, toothaches, bites from poisonous animals, and bacterial and fungal infections elevates its therapeutic value in herbal and traditional medicine [25]. However, this plant is reported to accumulate heavy metals, especially Pb, in its roots upon growing in contaminated soil in much

higher concentrations compared to other plants like *Aframomum corrorima* (Kororima), *Capsicum annuum* (red pepper), *Elettaria cardamomum* (cardamom), *Foeniculum vulgare* (fennel), *Thymus vulgaris* (thyme), and *Zingiber officinale* (ginger), so its consumption puts human health at risk [26].

Hence, the present research work was carried out to examine the ameliorative role of exogenous Si and 24-EBL (in individual and combination form) in improving Pb stress tolerance in fenugreek plants. To get insights into Si and 24-EBL-mediated Pb stress tolerance, we examined the morphological and biochemical attributes of plants supplemented with Si and 24-EBL. Moreover, expression levels of antioxidative genes were also analyzed to evaluate the key effects of Si and 24-EBL at the molecular level, which could be recognized as an efficient technique for understanding the Pb stress tolerance in fenugreek plants by the application of Si and 24-EBL.

## 2. Materials and Methods

### *2.1. Experimental Design*

Fenugreek seeds (var. Palam Somya) were procured from Chaudhary Sarwan Kumar, Himachal Pradesh Agricultural University, Palampur, India. Uniform size seeds were selected and surface sterilized by treating them with sodium hypochlorite (1%) for 5 min. Afterward, double-distilled water was used to thoroughly wash them. Subsequently, some seeds were administered a pre-treatment of 24-EBL ($10^{-7}$ M) for 8 h, and others were put in distilled water for the same duration. Sowing of seeds was performed afterward in grow bags (total 48 in number, 16 × 16 × 30 cm, @ 30 seeds per bag) containing soil and organic manure in 3:1 proportion. Lead nitrate ($PbNO_3$) was employed as a source for providing Pb to the soil. Preliminary studies while designing the experiment revealed that a 0.7 mM concentration of Pb resulted in reducing the growth by up to 50%; therefore, 0.5, 0.7, and 0.9 mM concentrations were selected for this research. Further, Si and 24-EBL were observed to bring about maximum growth at 2 mM and $10^{-7}$ M, respectively, so based on these preliminary results, Si and 24-EBL concentrations were chosen in this research (unpublished).

From sowing to 5 days of seedling emergence, silicic acid was foliar sprayed at a selected concentration of 2 mM. Careful uprooting of plants was performed on the 30th day after sowing to determine different morphological and biochemical attributes. Furthermore, evaluation was carried out with the plant leaves. The experiment involved 16 treatments ($T_1$ to $T_{16}$) where $T_1$—control (CN), $T_2$—Pb I (0.5 mM), $T_3$—Pb II (0.7 mM), $T_4$—Pb III (0.9 mM), $T_5$—Si, $T_6$—Si + Pb I, $T_7$—Si + Pb II, $T_8$—Si + Pb III, $T_9$—24-EBL, $T_{10}$—24-EBL + Pb I, $T_{11}$—24-EBL + Pb II, $T_{12}$—24-EBL + Pb III, $T_{13}$—Si + 24-EBL, $T_{14}$—Si + 24-EBL + Pb I, $T_{15}$—Si + 24-EBL+ Pb II, $T_{16}$—Si + 24-EBL+ Pb III.

### *2.2. Determination of Morphological Traits*

The uprooted, intact plants were cleaned of soil and dust particles by washing them with water. Growth attributes such as root and shoot length were ascertained using a ruler, while fresh plant weight was measured quickly after harvest. Further, for the estimation of dry weight, *Trigonella* plant samples from each treatment were packed in the respective labeled paper bags and oven dried (70 °C temperature; 72 h duration).

### *2.3. Oxidative Damage Indicators*

#### 2.3.1. Malondialdehyde (MDA)

Heath and Packer's [27] standard procedure was adopted to measure MDA concentration. Firstly, homogenization of leaf tissue (1 g) in trichloroacetic acid (TCA) (0.1% *w*/*v*) and subsequent centrifugation (5000 rpm). Next, the supernatant (1 mL) was blended with trichloroacetic acid (20% *w*/*v*) and thiobarbituric acid (TBA) (0.5% *w*/*v*), and the mixture was incubated at 95 °C for 30 min. The mixture was cooled in an ice bath, and optical density was recorded at a wavelength of 532 nm. Rectifications for unspecific turbidity were performed by deduction of the optical density at 600 nm. Lastly, for computing the

malondialdehyde concentration in the samples, we used an extinction coefficient with a value of 155 mM $cm^{-1}$.

#### 2.3.2. Hydrogen Peroxide ($H_2O_2$)

For determining $H_2O_2$ concentrations, the standard procedure of Velikova et al. [28] was used. Briefly, the leaf samples (100 mg) were subjected to homogenization in 0.1% trichloroacetic acid (5 mL) and subsequent centrifugation (12,000 rpm; 15 min). Further, the mixing of the supernatant (0.5 mL) was carried out in 10 mM potassium phosphate buffer (0.4 mL; pH 7.0) plus 1M potassium iodide (0.8 mL; pH 7.4). The optical density of this plant sample was noted at 390 nm. $H_2O_2$ concentrations were finally calibrated by using a standard curve with $H_2O_2$ as the standard.

### *2.4. Estimation of Osmolytes*

#### 2.4.1. Proline

The approximation of proline concentration was executed in accordance with the Bates et al. [29] standard protocol. Homogenization of plant samples was carried out in sulfosalicylic acid (3%) with subsequent centrifugation (10,000 rpm, 10 min). Afterward, the supernatant (2 mL) was blended with ninhydrin and glacial acetic acid (2 mL each). Next, the incubation of this mixture was performed at boiling temperature for 60 min. It was then extracted by using toluene, and the analysis of proline was performed by noting down the absorbance at 520 nm. Finally, the proline measures of the sample were calibrated from the graph plotted between absorbance (520 nm) and concentration for the standard solutions of L-proline.

#### 2.4.2. Glycine-Betaine (GB)

In order to ascertain the GB concentration, the method given by Grieve and Grattan [30] was adopted. Homogenization of plant material (1 g in dried form) was carried out in distilled water (10 mL), followed by its filtration. Afterward, filtrate (1 mL) was mixed with 2 M hydrochloric acid (1 mL). This mixture (0.5 mL) was then given an addition of potassium tri-iodide (0.2 mL). Thorough mixing was followed by putting it in an ice bath for 1.5 h. Subsequently, distilled water (2 mL; ice-cooled) and 1,2-dichloromethane (20 mL) were added to the mixture. The formation of two distinct layers was observed to take place, which were later made to mix by passing an uninterrupted stream of air. After discarding the top aqueous layer, the absorbance of the remaining organic part was noted at a wavelength of 365 nm. Then, the GB was approximated using the standard curve.

### *2.5. Estimation of Metabolites*

#### 2.5.1. Anthocyanin

Total measures of anthocyanin were estimated in accordance with the protocol given by Mancinelli [31]. Respective proportions of methanol, water, and HCl were kept at 79:20:1 to prepare the extraction mixture. Next, this extraction mixture (3 mL) was used as a medium for crushing fresh leaf tissue (0.5 g). This homogenate was then put through centrifugation (13,000 rpm; 20 min). The supernatant was then used to record its absorbance at two different wavelengths of 530 and 657 nm.

#### 2.5.2. Flavonoid

Kim et al. [32] protocol was followed for appraising the total measures of flavonoids in the plant samples. Absolute methanol (3 mL) was used as a medium for crushing fresh leaf tissue (500 mg). This homogenate was then centrifuged for 20 min at a speed of 13,000 rpm. Next, the supernatant (1 mL) was taken and given an addition of double-distilled water (4 mL), sodium nitrite (0.3 mL), and aluminum chloride (0.3 mL), followed by a subsequent incubation in a dark place for 5 min. The appearance of a pink color was observed after pouring sodium hydroxide (2 mL) into it. Finally, the addition of distilled water (2.4 mL) was performed. For recording optical density, a wavelength of 510 nm was

used. Total flavonoid concentrations were ascertained by using a standard curve with rutin as a standard.

#### 2.5.3. Phenolic

For analyzing total phenolic measures, the Malik and Singh [33] protocol was followed. Ethanol (80%) was used for crushing the leaves (0.5 g); the homogenate was then made to centrifuge (10,000 rpm; 20 min). Next, an addition of folin-ciocalteu reagent (0.5 mL) and sodium carbonate (20% $w/v$) was performed to the supernatant. The measurement of the optical density of the mixture was performed at a wavelength of 650 nm, and as a reference standard, gallic acid was used.

### *2.6. Evaluation of Protein and Antioxidative Defense System*

#### 2.6.1. Protein

For the appraisal of protein concentration, the protocol proposed by Lowry [34] was used. Phosphate buffer (3 mL) was used to extract leaf tissue (500 mg), and afterward, the mixture was put through centrifugation (10,000 rpm; 10 min). Pipetting of the plant sample and standard solutions (0.1 mL each) was performed next, and then, to make the final volume of solutions in each of the tubes 1 mL, distilled water was added to them. A test tube containing distilled water (1 mL) worked as a blank. Reagent A, i.e., sodium carbonate + sodium hydroxide, was mixed with reagent B, i.e., copper sulfate and potassium sodium tartarate, to prepare reagent C. This reagent C (0.5 mL) was then poured into all the test tubes, followed by thorough mixing and a standing time of 10 min. Next, reagent D (folin-ciocalteu reagent) in a 0.5 mL quantity was put into each test tube, and all were given an incubation at ambient temperature in the dark for half an hour. The appearance of blue was noticed. Absorbance was recorded at 660 nm. Protein measures (mg/g) in the plant samples were calculated from the graph plotted for the standard solutions of protein between the concentrations and absorbance values.

#### 2.6.2. Activities of Antioxidative Enzymes

Superoxide Dismutase (SOD)

An approximate estimate of the activity of this enzyme was performed as per the protocol given by [35]. Briefly, the test cuvettes were filled with 50 mM sodium carbonate buffer (1.3 m; pH 10.0), 96 µM nitroblue tetrazolium (NBT) (500 µL), and 0.6% Triton X-100 (100 µL). Then, the reaction was started by mixing hydroxylamine hydrochloride (100 µL) into the reaction mixture. The addition of the enzyme extract (70 µL) was followed by 2 min. Percent inhibition @ NBT was noted as an enhancement in optical density at 540 nm.

Catalase (CAT)

The standard method given by Aebi [36] was chosen for evaluating the functional aspects of the CAT enzyme. Enzyme extraction was performed by homogenizing leaf tissue (1 g) in 100 mM phosphate buffer (3 mL, pH 7.0) and subsequently putting it through centrifugation (5000 rpm; 20 min). A plant sample (50 µL) with an addition of 100 mM phosphate buffer (2.650 mL) and 100 mM $H_2O_2$ (300 µL) was used for estimating the change in absorbance at a wavelength of 240 nm. An extinction coefficient having a value of 43.6 $mM^{-1}$ $cm^{-1}$ was utilized for the final calculations.

Ascorbate Peroxidase (APX)

Evaluation of the functional aspect of this enzyme was carried out as per the standard protocol of Nakano and Asada [37]. Enzyme extraction was brought about by the homogenization of leaf tissue (1 g) in 100 mM phosphate buffer (3 mL; pH 7.0) with subsequent subjection of the homogenate to centrifugation (5000 rpm; 20 min). For recording the change in absorbance at 290 nm, a plant sample (50µL) was taken in a cuvette having 100 mM phosphate buffer (2.370 mL), ascorbate (0.3 mL), and 0.5 mM $H_2O_2$ (0.3 mL). An extinction coefficient with a value of 2.8 $mM^{-1}$ $cm^{-1}$ was used for the final computations.

Guaiacol Peroxidase (POD)

The estimation of the activity of this enzyme was carried out as per the standard protocol given by Putter and Becker [38]. In brief, the enzyme extraction was made by homogenizing the leaf tissue (1 g) in 100 mM phosphate buffer (3 mL; pH 7.0), and subsequently, centrifugation was carried out (5000 rpm; 20 min). Next, the difference in absorbance was recorded at 436 nm by taking a plant sample (50 µL) in a cuvette having 0.1 M phosphate buffer (2.370 mL), 20 mM guaiacol (0.3 mL), and 12.3 mM $H_2O_2$ (0.3 mL). An extinction coefficient with a value of 25.5 $mM^{-1}$ $cm^{-1}$ was used for computing the final values.

#### 2.6.3. Non-Enzymatic Antioxidants

Ascorbic Acid (AsA)

The standard protocol proposed by Roe and Kuether [39] was put to use for estimating the measures of AsA. Briefly, 50 mM tris buffer having a pH of 10 was used as a crushing medium for leaf tissue (0.5 g), followed by centrifugation of this homogenate (13,000 rpm; 20 min). Next, the supernatant (0.5 mL) was given an addition of double-distilled water (4 mL), charcoal (100 mg), and 50% $w/v$ TCA (0.5 mL). Whatman filter paper 1 was used for filtering this mixture. To the filtrate, 2,4-dintrophenyl hydrazine (0.4 mL) and 65% $w/v$ cold $H_2SO_4$ (1.6 mL) were carefully added one after another. An hour-long incubation of this mixture at 37 °C was performed first, followed by keeping it at ambient temperature for the next 30 min. Ascorbic acid in a quantity of 1 mg 100 $mL^{-1}$ was utilized as the standard. The measurement of optical density was performed at a wavelength of 520 nm.

Glutathione (GSH)

Sedlak and Lindsay's [40] protocol was utilized for measuring the levels of GSH. 50 mM tris buffer (3 mL) having a pH of 10 was used as a crushing medium for leaf tissue (1 g), followed by the centrifugation of this homogenate (13,000 rpm; 20 min). The extract of the plant (100 µL) was then subjected to the addition of 0.2 M of tris buffer (1 mL; pH 8.2), 0.01 M of 5,5′-dithiobis-(2-nitrobenzoic acid) (50 µL), and absolute methanol (4 mL). The next step was a 15 min incubation of this mixture, followed by its centrifugation (3000 rpm, 15 min). Glutathione concentration was finally measured by taking 1 mg 100 $mL^{-1}$ of glutathione as a standard. The measurement of optical density was performed at a wavelength of 412 nm.

### *2.7. Gene Expression*

The TRIzol method was followed for RNA isolation, which was then quantified by a nanodrop spectrophotometer and then subjected to a quality check on 2% agarose gel electrophoresis. The housekeeping gene used was actin, and the $2^{-\Delta\Delta ct}$ method was used by calculating the Ct values by Livak and Schmittgen [41].

### *2.8. Statistical Evaluation*

The analysis of the data was brought about by means of SPSS 16.0 software (SPSS Inc., Chicago, II, USA). Evaluation of the statistical significance was made with ANOVA (one-way analysis of variance) and Tukey's test, with a significance level of $p < 0.05$ among all the treatments. Bar graphs with means having dissimilar letters possess statistical significance at a $p$ value of less than 0.05. The experimental design involved a completely randomized triplicate setup for all sixteen treatments, which were performed twice separately. Means ± SEM values are used to represent data in Figures. For the purpose of Principal component analysis (PCA), the fviz-pca function of the factoextra R package Ver. 1.0.7 in RStudio was used. Further, Pearson's correlation analysis was carried out by using the mcor function, and then the corrplots were processed with the help of the corrplot package Ver. 0.89 in RStudio.

## 3. Results

### *3.1. Growth and Biomass*

Growth attributes (length of root and shoot; weight in fresh and dry form) of fenugreek plants that were subjected to Pb stress (0.5, 0.7, and 0.9 mM) and exposed to the individual or combined influence of Si and 24-EBL are depicted in Figure 1A–D. A 17, 35, and 56% decrease was observed in Pb I, II, and III stressed plants in comparison to CN plants (Figure 1A). The Pb + Si treatment increased root length by 34% in Pb I +Si, 48% in Pb II + Si, and 56% in Pb III + Si, relative to their respective control plants. Only a decrease of 29, 17, and 21% was noticed in the case of EBL-treated stressed plants at Pb I, II, and III concentrations, respectively. The combined application of Si and EBL showed an elevation of 28% at Pb I, 19% at Pb II, and 16% at Pb III in root length when compared with control plants.

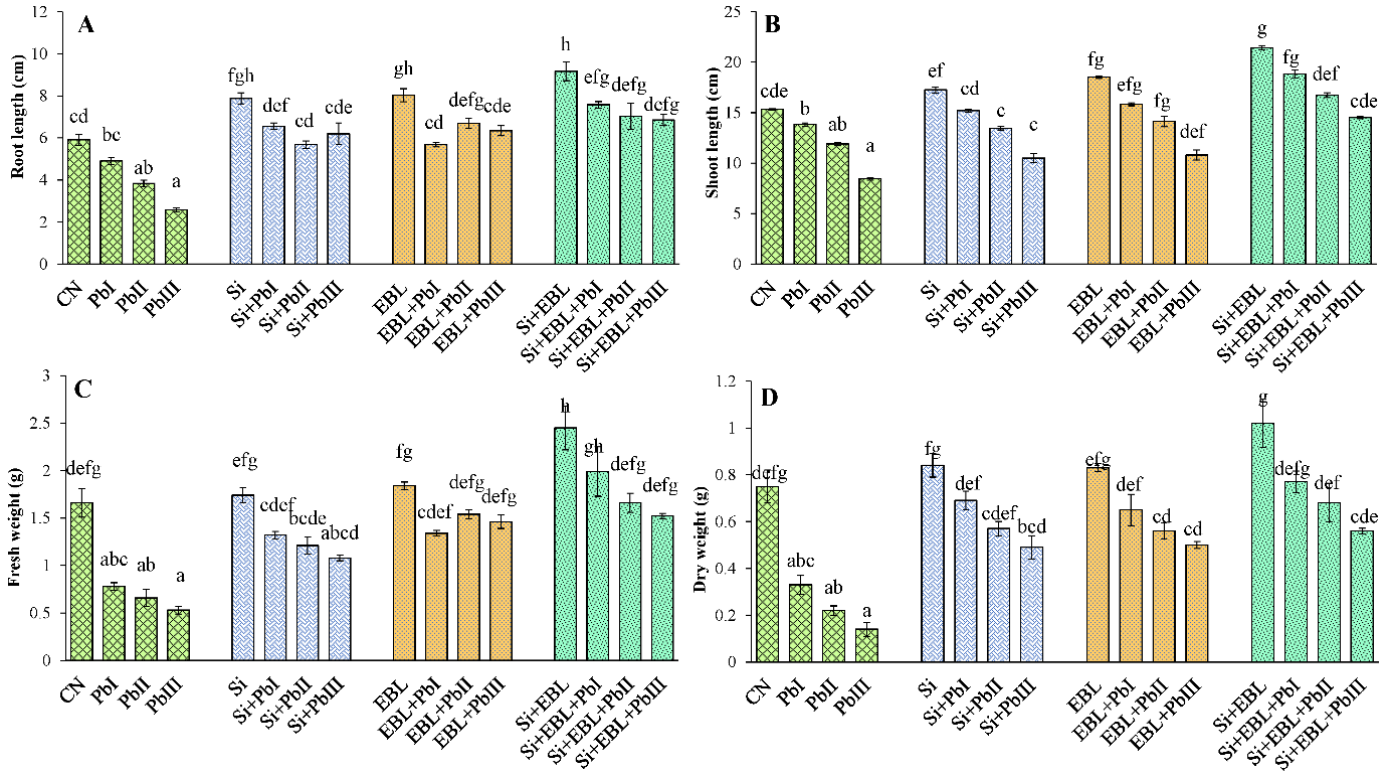


**Figure 1.** Depiction of effect of Si and EBL on root length (**A**), shoot length (**B**), fresh weight (**C**), and dry weight (**D**) in 30 days old Pb-stressed *Trigonella foenum-graecum* plants. Each value stands for the mean of three replicates in relation to every treatment level, together with standard error of mean (SEM). Letters above a column followed by dissimilar letters above other columns express significant differences at $p < 0.05$. CN—control; Si—silicon; EBL—24-epibrassinolide; Pb I—0.5 mM; Pb II—0.7 mM; Pb III—0.9 mM.

Minimum shoot length was found at Pb III concentration, with a decrease of 45% as compared to CN plants (Figure 1B). Application of Si reduced this percentage to 12% at Pb I, 22% at Pb II, and 39% at Pb III concentrations, relative to the Pb alone-treated plants. The Pb + EBL application also improved the shoot length, with the highest Pb I concentration showing a 14% increase as compared to the respective control plants. The highest shoot length among all treatments was noticed in Si + EBL treated plants. Under stressed conditions, Si + EBL applied plants showed a maximum increase of 23% at Pb I concentration when compared with CN plants.

Another growth attribute, i.e., fresh weight (FW), was found to be curtailed with the increase in the Pb concentration (Figure 1C). A decrease of 53, 60, and 68% was found at Pb I, II, and III concentrations, respectively, as compared to CN plants. The Pb + Si and

Pb + EBL treatments showed the highest FW at Pb I and II concentrations, with a decrease of 24 and 16%, respectively, relative to their control plants. The combination of Pb + Si + EBL showed maximum FW at Pb I concentration with a decrease of only 19% as compared to Si + EBL treated control plants.

Dry weight was recorded to be reduced in Pb-treated plants vis-a-vis untreated CN plants, showing a reduction of 56% at Pb I, 71% at Pb II, and 85% at Pb III (Figure 1D). Applying Si and EBL individually to the stressed plants refined this reduction in dry weight, which was highest at Pb I (18%) in Si and at Pb III (33%) in EBL. The combined application of Si and EBL resulted in a respective decrease of 25% at Pb I, 33% at Pb II, and 45% at Pb III concentrations when compared to the untreated control plants.

### 3.2. Reduction in $H_2O_2$ and MDA

Levels of MDA were increased upon enhancing the Pb concentration (Figure 2A). The highest MDA level was at Pb III concentration, which was 72% higher than CN plants. The application of Si and EBL decreased the MDA level when applied individually as well as in combination. The Pb + Si, Pb + EBL, and Pb + Si + EBL treatments lowered the levels of MDA by 45, 41, and 15%, respectively, in the case of the highest concentration of Pb, i.e., Pb III, when correlated with the plants kept under control.

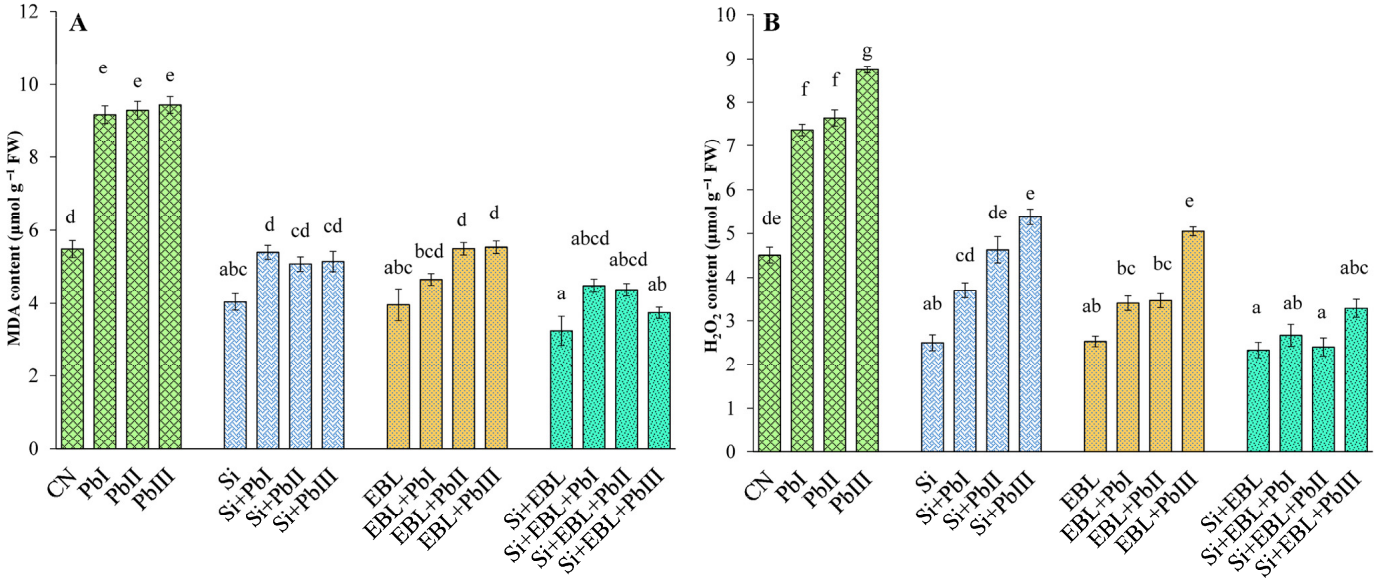


**Figure 2.** Depiction of the effect of Si and EBL on MDA (**A**) and $H_2O_2$ (**B**) levels in 30 days old plants of *Trigonella foenum-graecum* under Pb stress. Each value stands for the mean of three replicates in relation to every treatment level, together with standard error of mean (SEM). Letters above a column followed by dissimilar letters above other columns express significant differences at $p < 0.05$. CN—control; Si—silicon; EBL—24-epibrassinolide; Pb I—0.5 mM; Pb II—0.7 mM; Pb III—0.9 mM.

Maximum $H_2O_2$ was noted at Pb III concentrations that were 48% greater than those in CN plants (Figure 2B). The Pb + Si treatment alleviated the Pb-induced toxicity by 50% at Pb I, 39% at Pb II, and 38% at Pb III concentrations, relative to CN plants. Further, in Pb + EBL application, minimum $H_2O_2$ was noticed at Pb I concentration, with only a 35% increase relative to control plants. In Pb + Si + EBL-applied plants, the lowest $H_2O_2$ concentration was noticed at Pb II concentration, which was only 3% greater than their respective control plants.

### 3.3. Improvement in Osmolytes

A reduction in proline concentration was recorded in Pb-treated plants in comparison to the CN plants (Figure 3A). A 25, 35, and 46% decrease was observed in Pb I, II, and III stressed plants as contrasted with CN plants. This reduction was normalized and even

enhanced further by Si, with an increase of 19% at Pb I, 23% at Pb II, and 44% at Pb III concentrations when contrasted against Pb-alone applied plants. The EBL-applied Pb-stressed plants exhibited a maximum decline of 44% at Pb III concentrations, relative to the respective control plants. The Pb + Si + EBL application showed a 22, 26, and 39% decrease in proline concentration at Pb I, II, and III concentrations, respectively, when correlated to the control plants.

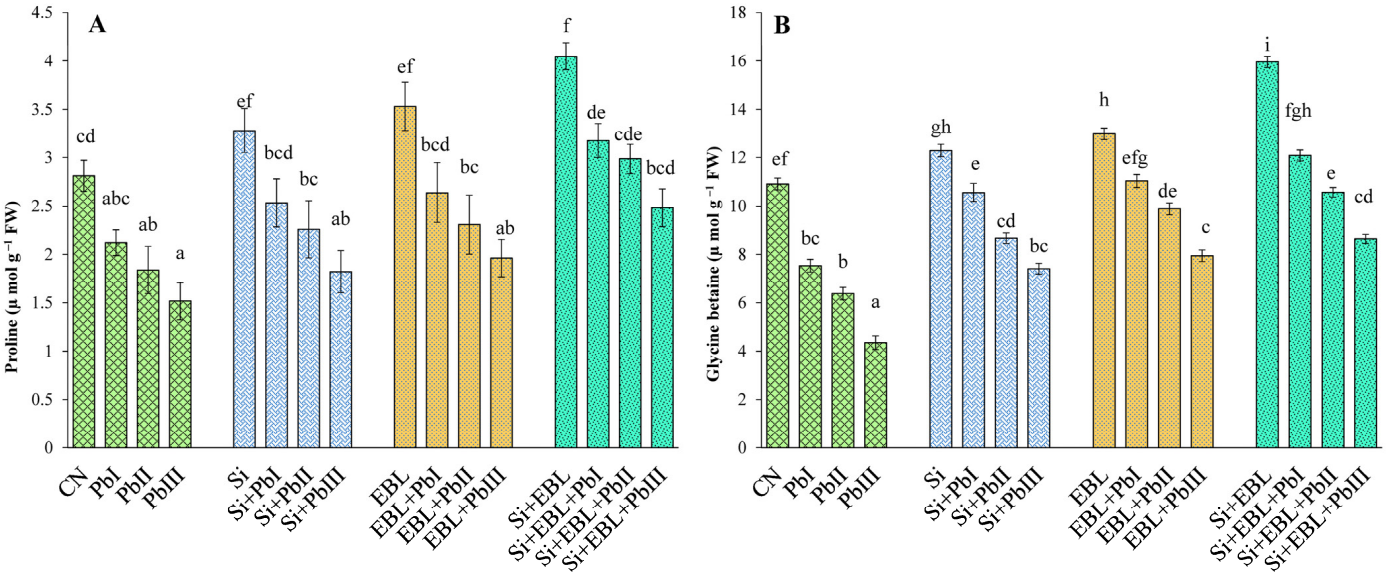


**Figure 3.** Depiction of effect of Si and EBL on proline (**A**) and glycine betaine (**B**) content in 30 days old plants of *Trigonella foenum-graecum* under Pb stress. Each value stands for the mean of three replicates in relation to every treatment level, together with standard error of mean (SEM). Letters above a column followed by dissimilar letters above other columns express significant differences at $p < 0.05$. CN—control; Si—silicon; EBL—24-epibrassinolide; Pb I—0.5 mM; Pb II—0.7 mM; Pb III—0.9 mM.

The highest GB concentration among Pb alone-treated plants was observed at Pb I concentration, which was 31% lower than CN plants (Figure 3B). The maximum improvement of 70% in GB concentration was brought about by Si alone treatment, relative to CN conditions. On similar lines, EBL-treated stressed plants also exhibited the highest GB measures at Pb I concentrations, being only 15% lower than the respective control plants. In the Pb + Si + EBL combined treatments, the highest GB concentration was noticed at the Pb I concentration, which was only 24% lower than their respective control plants.

### *3.4. Metabolites*

Anthocyanin concentration was reduced under Pb stress conditions, with the minimum content at Pb III concentration, which was 61% lower than CN plants (Figure 4A). In Si + Pb I treated plants, only a decrease of 22% was noticed relative to their respective control plants. The maximum anthocyanin concentration was in individual EBL-treated plants under Pb II stress. In Si + EBL applied plants, a decrease of 19, 27, and 35% was found at Pb I, II, and III concentrations when compared to the respective plants kept under control conditions, i.e., Si + EBL.

Flavonoid concentration was recorded to be reduced in Pb-treated plants compared to CN plants (Figure 4B). A decrease of 21, 33, and 46% was observed in Pb I, II, and III stressed plants in comparison to CN plants. The Pb + Si and Pb + EBL applications resulted in an increase in flavanoid measures, which were highest in the case of Pb I concentrations, i.e., 10% and 17%, respectively, when contrasted against Pb-alone treated plants. The combined application of Si and EBL lowered the reduction in flavanoid measures to 16% at Pb I, 21% at Pb II, and 28% at Pb III concentrations when correlated with the respective control plants.

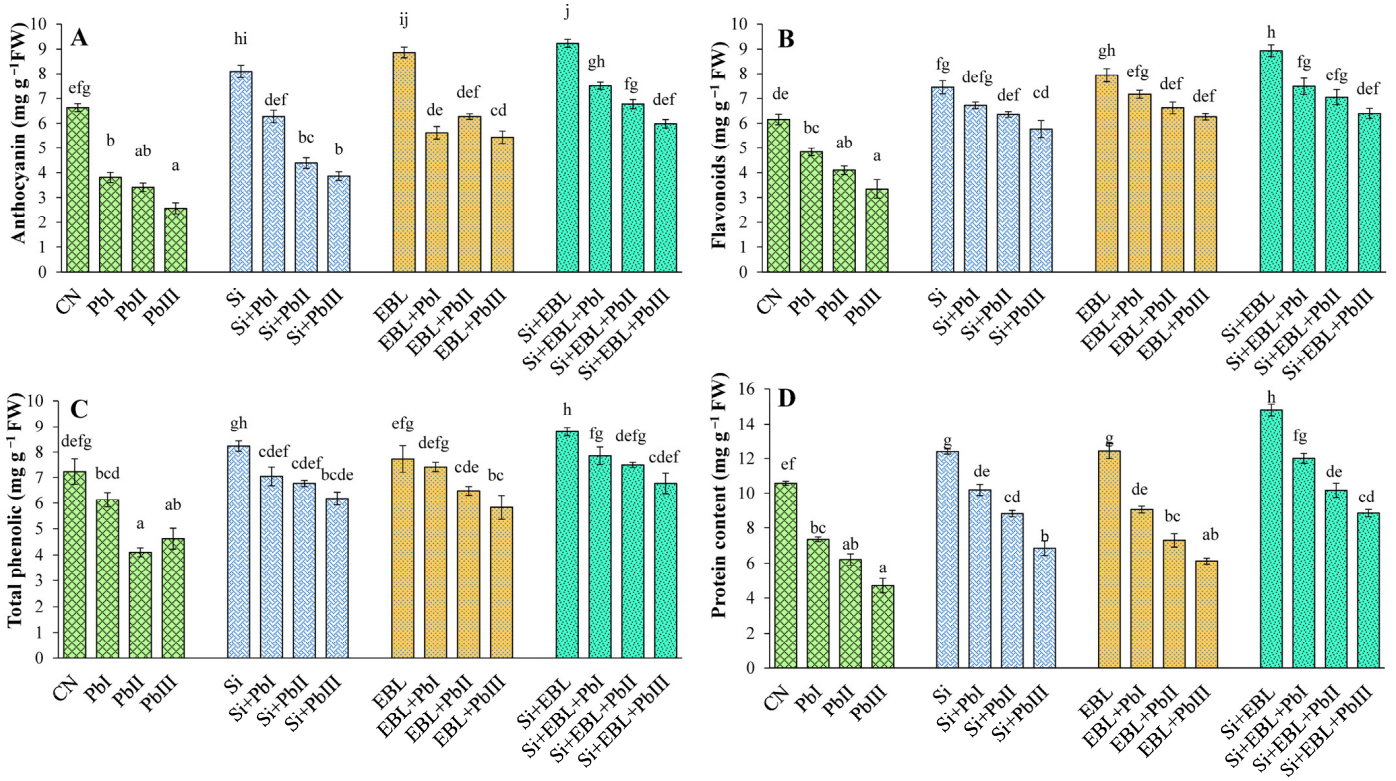


**Figure 4.** Depiction of effect of Si and EBL on anthocyanin (**A**), flavonoid (**B**), phenolic (**C**), and protein (**D**) content in 30 days old plants of *Trigonella foenum-graecum* under Pb stress. Each value stands for the mean of three replicates in relation to every treatment level, together with standard error of mean (SEM). Letters above a column followed by dissimilar letters above other columns express significant differences at $p < 0.05$. CN—control; Si—silicon; EBL—24-epibrassinolide; Pb I—0.5 mM; Pb II—0.7 mM; Pb III—0.9 mM.

The minimum phenolic concentration was found at Pb II concentration, with a decrease of 43% as compared to CN plants (Figure 4C). The Pb + Si application recorded a decline of 14% at Pb I, 18% at Pb II, and 25% at Pb III in phenolic concentration as per their comparison to the respective control plants, i.e., Si alone treated plants. Supplication of EBL to Pb-stressed conditions also improved the phenolic concentration, with the highest Pb I concentration showing an enhancement of 21% relative to Pb I alone-treated plants. Under stressed conditions, Si + EBL applied plants showed a maximum increase of 9% at Pb I concentration when compared to the respective plants kept under control conditions.

### *3.5. Modulation of Protein and Antioxidative Defense Mechanisms*

#### 3.5.1. Protein

Protein concentration was decreased in Pb-treated plants with regard to their CN plants (Figure 4D). The Pb-alone-treated plants resulted in a respective decrease of 30, 41, and 55% at Pb I, II, and III concentrations, relative to CN plants. Individual application of Si augmented the protein concentration under stressed conditions, with the highest increase of 46% at Pb III concentration when contrasted to their respective Pb-alone treated plants. In the case of Pb + EBL treatment, a decrease of 27% at Pb I, 41% at Pb II, and 51% at Pb III was noticed relative to their respective control plants. The combined application of Si and EBL showed a 19, 31, and 40% decrease in protein content at Pb I, II, and III concentrations, respectively, when correlated with untreated control plants.

### 3.5.2. Antioxidant Defense System

#### Antioxidative Enzymes

A reduction in the action of the SOD enzyme was recorded with an increase in the Pb concentration (Figure 5A). The highest SOD activity was at Pb I concentrations, which were 25% lower than in CN plants. The Pb + Si and Pb + EBL treated plants exhibited optimal enhancement in the enzymatic action of SOD by 65 and 72%, respectively, at Pb III concentration when correlated with the Pb III alone-stressed plants. In Pb + Si+ EBL combination treatments, the highest SOD activity was noticed at Pb I concentration, which was only 11% lower than the respective control plants.

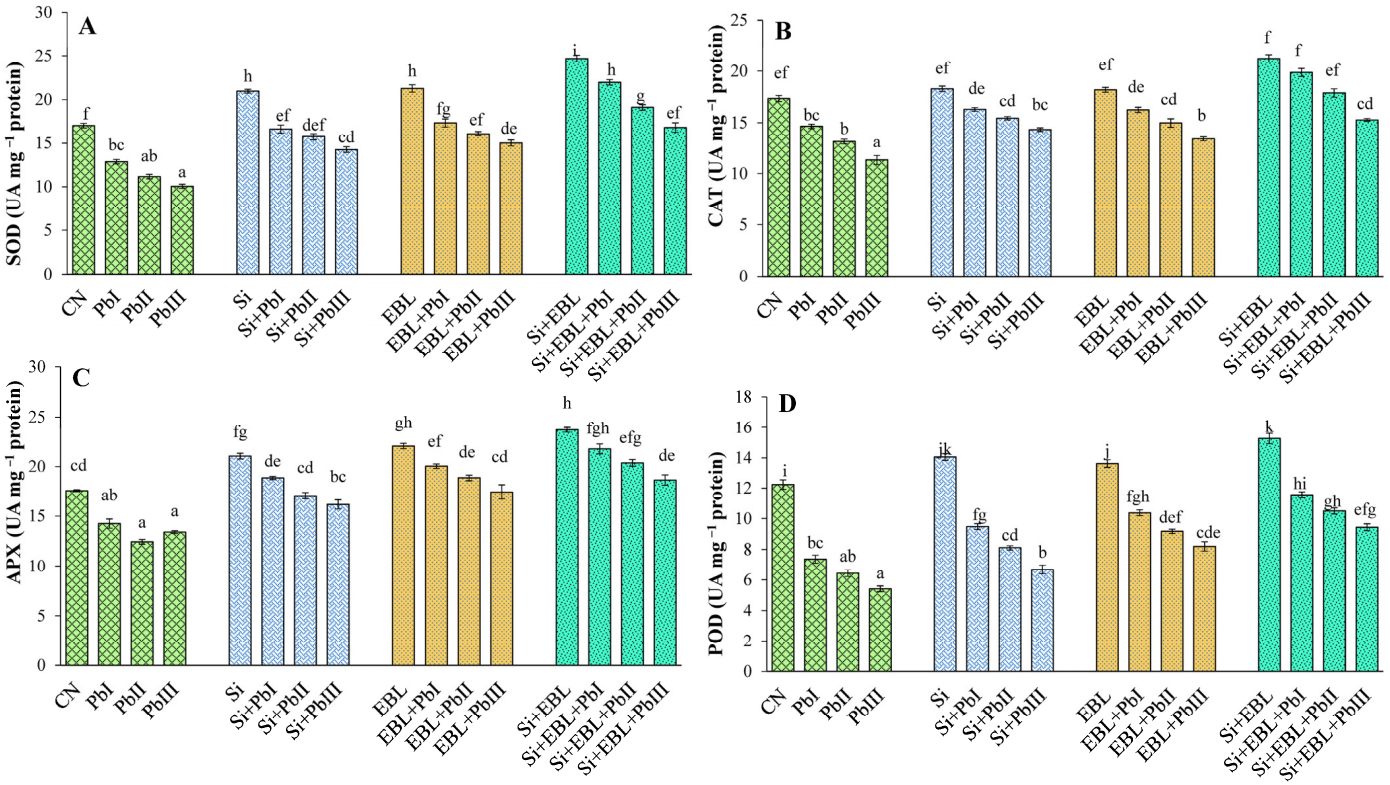


**Figure 5.** Depiction of effect of Si and EBL on SOD (**A**), CAT (**B**), APX (**C**), and POD (**D**) enzyme activities in 30 days old plants of *Trigonella foenum-graecum* under Pb stress. Each value stands for the mean of three replicates in relation to every treatment level, together with standard error of mean (SEM). Letters above a column followed by dissimilar letters above other columns express significant differences at $p < 0.05$. CN—control; Si—silicon; EBL—24-epibrassinolide; Pb I—0.5 mM; Pb II—0.7 mM; Pb III—0.9 mM.

A reduction in the functional pursuit of the CAT enzyme was recorded in Pb treated plants when they were compared with CN plants (Figure 5B). Lead toxicity reduced CAT activity by 16% at Pb I, 24% at Pb II, and 35% at Pb III concentrations, relative to their respective control plants. The Pb + Si and Pb + EBL treated plants exhibited optimal enhancement in the enzymatic action of CAT by 27 and 26%, respectively, at Pb III concentration when correlated with the Pb III alone stressed plants. In Pb + Si+ EBL combination treatments, there was a curtailment of 6, 16, and 28% in CAT activity at Pb I, II, and III concentrations, respectively, when contrasted against control plants.

The activity of the APX enzyme was found to be decreased under Pb stress (Figure 5C). Lead stress alone reduced the enzymatic activity by 19, 29, and 24% at Pb I, II, and III concentrations, respectively, as compared to CN plants. The Pb + Si, Pb + EBL, and Pb + Si + EBL treatments exhibited the highest activity of this enzyme at Pb I concentration, with a decrease of 11, 16, and 9%, respectively, relative to their control plants.

Lead stress alone enhanced the enzymatic action of POD, which was maximal at a Pb I concentration that was 40% lower than in CN plants (Figure 5D). The Pb + Si and Pb + EBL treatments exhibited improvement in POD activity, which was maximum in the case of Pb I, i.e., 28 and 24%, respectively, relative to their respective control plants. In Pb + Si + EBL applied plants, the highest POD activity was also noticed at Pb I concentration, which was reduced by 25% compared to their respective control plants.

Non-Enzymatic Antioxidants

The Pb-alone application decreased glutathione concentration, which was lowest at Pb III concentration, which was 58% less than CN plants (Figure 6A). In Si + Pb I as well as EBL + Pb I treated plants, only a decrease of 25% was recorded with respect to control plants. In Si + EBL applied plants, a decrease of 28, 36, and 48% was found at Pb I, II, and III concentrations, relative to their respective plants kept under control conditions, i.e., Si + EBL.

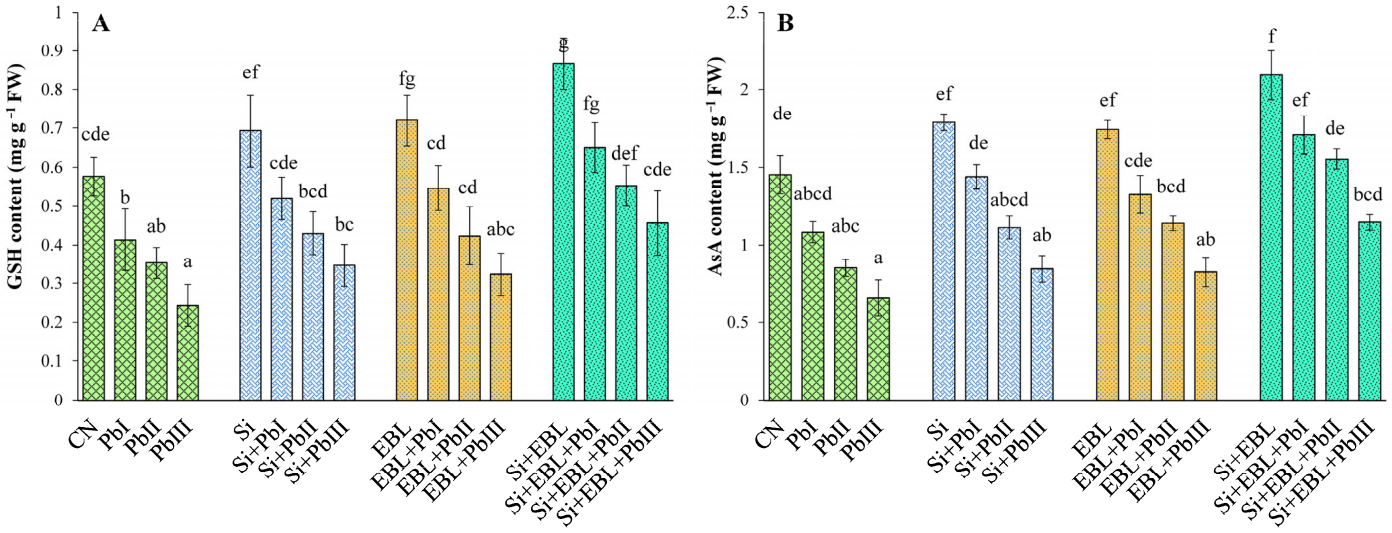


**Figure 6.** Depiction of effect of Si and EBL on GSH (**A**) and AsA (**B**) content in 30 days old plants of *Trigonella foenum-graecum* under Pb stress. Each value stands for the mean of three replicates in relation to every treatment level, together with standard error of mean (SEM). Letters above a column ensued by dissimilar letters above other columns express significant difference at $p < 0.05$. CN—control; Si—silicon; EBL—24-epibrassinolide; Pb I—0.5 mM; Pb II—0.7 mM; Pb III—0.9 mM.

The ascorbic acid concentration was decreased in Pb-treated plants with regard to their CN plants (Figure 6B). A decrease of 26, 41, and 55% was observed in Pb I, II, and III stressed plants in contrast to CN plants. Individual application of Si and EBL enhanced the AsA concentration under stressed conditions, with the highest concentration at Pb I, which was reduced only by 19% and 24%, respectively, relative to untreated plants of control. Combined Si and EBL decreased AsA concentrations by 19, 26, and 45% at Pb I, II, and III concentrations, respectively, in comparison to their respective plants kept under control conditions.

### *3.6. Regulation of Gene Expression*

Expressions of *SOD* and *CAT* genes were downregulated under Pb toxicity (Figure 7). It was diminished by 61 and 52% in *SOD* and *CAT* gene expression, respectively, as compared to CN plants. Treatment with Si under stressed conditions increased the expression of *SOD* and *CAT* genes by 33 and 51%, respectively, when contrasted against CN plants. Similar results were obtained by EBL supplementation as well, which got improved further by the combined application of Si + EBL under stressed conditions.

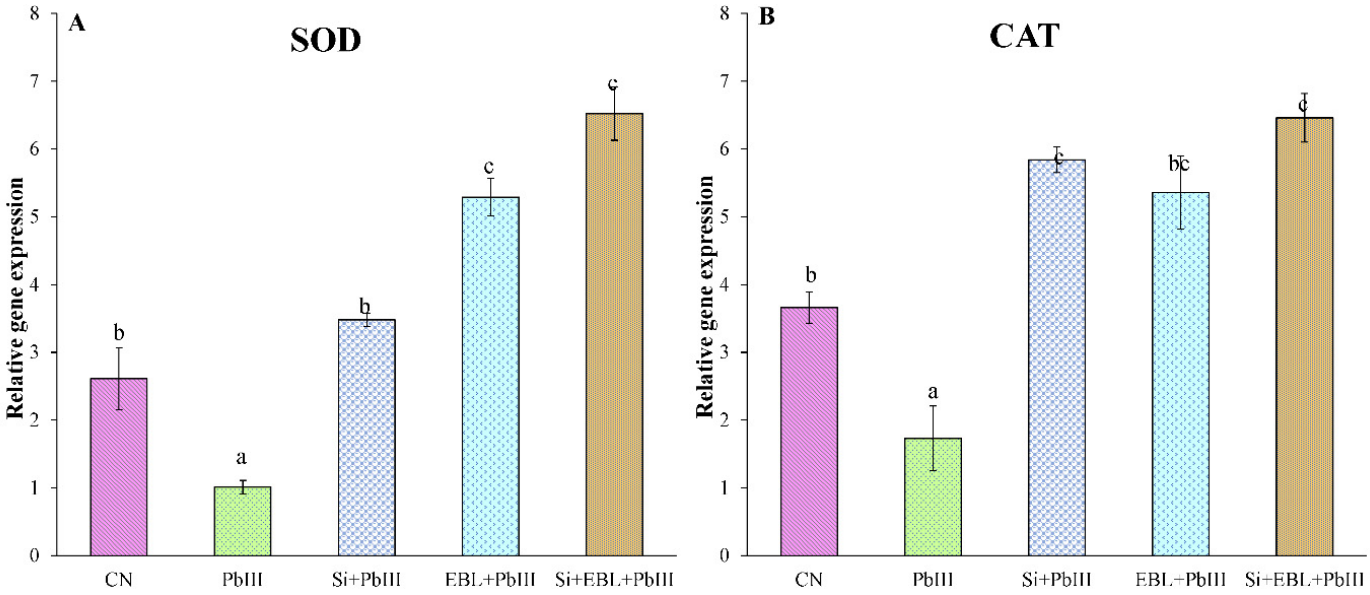


**Figure 7.** Depiction of effect of Si and EBL on *SOD* (**A**) and *CAT* (**B**) gene expression in 30 days old plants of *Trigonella foenum-graecum* under Pb stress. Each value stands for the mean of three replicates in relation to every treatment level, together with standard error of mean (SEM). Letters above a column followed by dissimilar letters above other columns express significant difference at $p < 0.05$. CN—control; Si—silicon; EBL—24-epibrassinolide; Pb I—0.5 mM; Pb II—0.7 mM; Pb III—0.9 mM.

### *3.7. Principal Component and Correlation Analysis*

To ascertain the influence of Si and EBL treatments on the investigated characters of fenugreek plants, the implementation of score and loading diagrams was carried out (Figure 8). Two of the initial components, i.e., Dim1 (PC1, 91.2%) and Dim2 (PC2, 4.8%), displayed the maximum participation and constituted 96% of the overall variance. With respect to Si, EBL, and Pb treatment, similar applications at diverse levels/combinations were clustered nearby. Furthermore, the different treatments were well split up into the initial two components (Figure 8A). This splitting up of the different treatments clearly hinted at the strong ameliorative effect of the Si and EBL applications on the various investigated traits of fenugreek plants under Pb stress with respect to CN. A positive correlation was exhibited between the first set of the variables of PCA (PC1) and most of the variables, namely the root length, shoot length, FW, DW, proline, GB, anthocyanin, flavonoid, phenolic, protein, SOD, CAT, APX, POD, GSH, and AsA (Figure 7B). On the contrary, a significant negative correlation of PC1 variables (MDA and $H_2O_2$) was noticed to be in alignment with PC2 (Figure 8B).

Pearson's correlation analysis was used to evaluate various traits of fenugreek plants (Figure 9). A negative correlation between MDA and $H_2O_2$ and other traits (root length, shoot length, FW, DW, proline, GB, anthocyanin, flavonoid, phenolic, protein, SOD, CAT, APX, POD, GSH, and AsA) was observed in the correlation analysis. As opposed to this, all other characters exhibited an intense positive correlation with one another (Figure 9). An underlying close bond among the growth parameters, osmolytes, and metabolites, together with antioxidant defense systems in fenugreek plants, could be characterized with the help of this correlation.

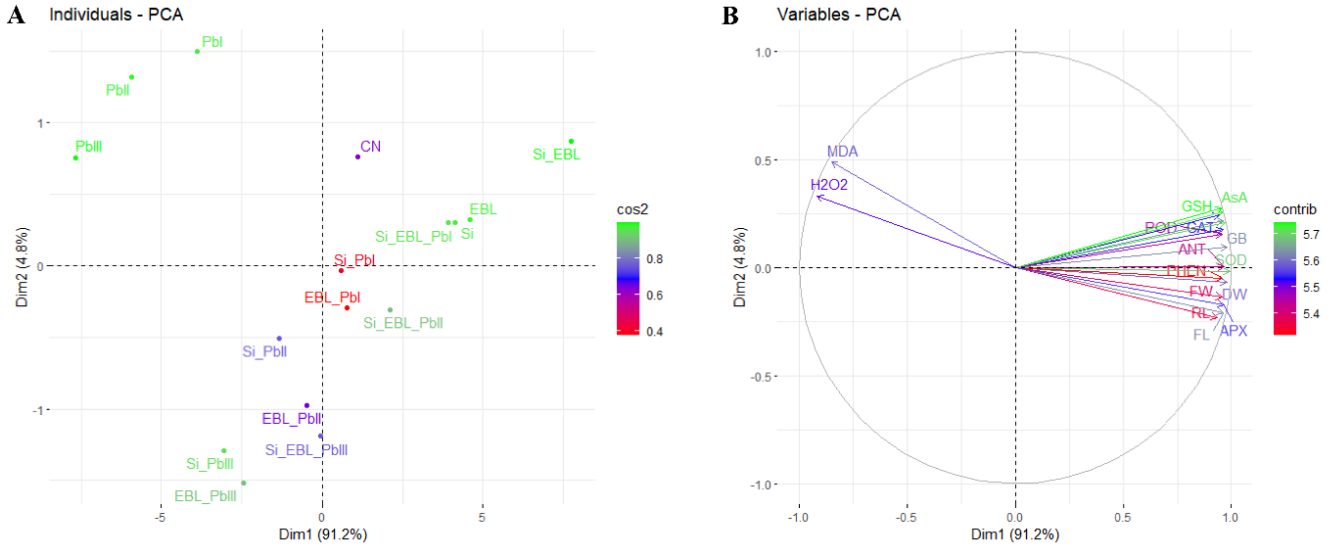


**Figure 8.** Principal component analysis (PCA) of (**A**) individual applications by PCA and (**B**) diverse analyzed parameters of fenugreek plants under Pb stress. (**A**) The score plot indicates the separation of treatments of Si, Pb, EBL, Si + EBL, Si + Pb, EBL + Pb, and Si + EBL + Pb. Abbreviations: Si—Silicon; EBL—24-epibrassinolide; Pb—lead; RL—root length; SL—shoot length; FW-fresh weight; DW—dry weight; MDA—malondialdehyde; $H_2O_2$—hydrogen peroxide; PROL—proline; GB—glycine betaine; ANT—anthocyanin; FL—flavonoid; PHEN—phenolics; PROT—protein; APX—ascorbate peroxidase; CAT—catalase; POD—guaiacol peroxidase; SOD—superoxide dismutase; GSH—glutathione; and AsA—ascorbic acid.

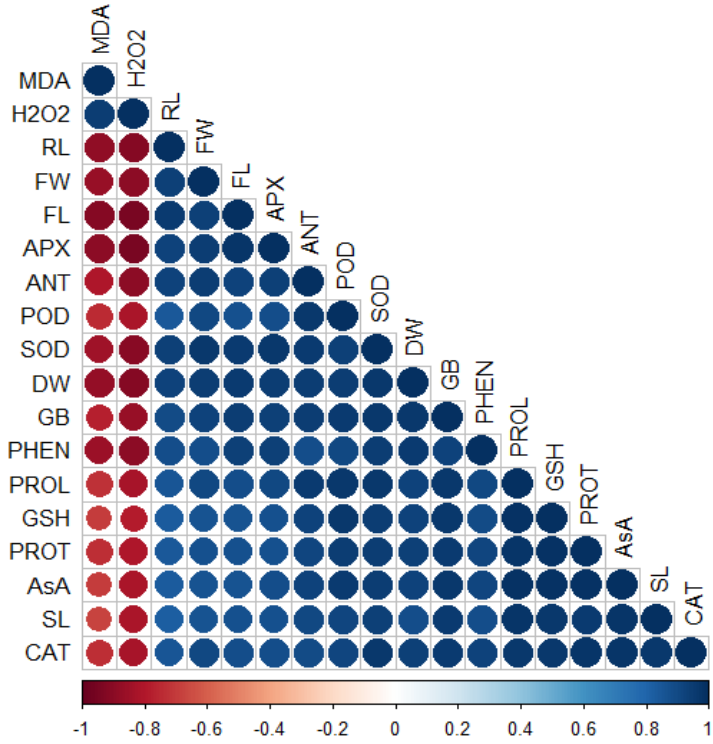


**Figure 9.** Pearson's correlation analysis between diverse analyzed parameters of fenugreek plants under Pb stress. Positive and negative correlations depicted by blue and brownish colors, respectively. Abbreviations: RL—Root length; SL—shoot length; FW—fresh weight; DW—dry weight; MDA—malondialdehyde; $H_2O_2$—hydrogen peroxide; PROL—proline; GB—glycine betaine; ANT—anthocyanin; FL—flavonoid; PHEN—phenolics; PROT—protein; APX—ascorbate peroxidase; CAT—catalase; POD—guaiacol peroxidase; SOD—superoxide dismutase; GSH—glutathione; and AsA—ascorbic acid.

## 4. Discussion

Negative effects of the heavy metal Pb on various growth attributes of plants were recorded in many research findings [7,8]. Lead-stressed *Solanum melongena* recorded reductions in root length, shoot length, and fresh plus dry weight [42]. The current study showed an inhibitory influence of Pb stress on the growth and biomass of fenugreek plants, which could be likely due to the stoppage of cell division by Pb exposure. However, the outcomes of the present work revealed that the damaging effects of Pb metal can be ameliorated by the external supplementation of stressed fenugreek plants with individual and combined applications of Si and 24-EBL. Previous studies revealed that the applications of 24-EBL and Si, both in an individual and coupled manner, mitigated the deleterious impact of Cd by enhancing growth, nutrient uptake, and biochemical variables in seedlings of *Pisum sativum* [43].

Strengthening of root architecture and betterment in mineral uptake have been reported to be linked with Si application in Cu- and Ni-stressed seedlings of *Triticum turgidum* and *Gossypium* sp., respectively [44,45]. Further, biochemical and molecular processes were stimulated in plants upon supplementation with the soluble form of Si, i.e., silicic acid, through enhanced biomass production [46]. Supplementation of 24-EBL elevated the endurance potential of seeds of *Brassica juncea* under Pb stress by improving their germination and growth rates [47]. This negation of Pb toxicity and enhancement in growth attributes might be taking place on account of the role of Si in reducing metal uptake and escalating the rate of mineral absorption, whereby photosynthetic potential increases, or simply due to the dominance of 24-EBL over cell division and cell expansion.

Overproduction of ROS results in the peroxidation of biomembrane lipids, leading to their disruption and elevation in MDA levels [48]. Similar observations were made in the current experimentation, where enhancement in ROS measures expressed in the form of MDA and $H_2O_2$ occurred in response to Pb stress. This could be related to the Pb-mediated generation of free radicals, which would change the stability of bio-membranes and enhance their permeability. However, these elevated oxidative stress markers were lowered by the individual as well as coupled supplementation of fenugreek plants with Si and 24-EBL. This resonates with the earlier findings where measures of ROS have been reduced by the individual application of 24-EBL in *Brassica juncea* under the influence of Cd stress and by the application of Si in *Brassica juncea* when exposed to As stress [49,50]. Though the cumulative application of Si with 24-EBL has been reported to be more efficient in regulating lipid peroxidation, decreasing MDA measures, and stabilizing membranes as compared to their individual applications in *Pisum sativum* plants that were subjected to Cd stress [43]. This scavenging of ROS by the coupled application of 24-EBL and Si brought about the same results as in the present work. Silicon is linked with the moderation of MDA and thereby with the maintenance of integrity and permeability of membranes [51], whereas 24-EBL is considered to reduce lipid peroxidation and strengthen the biological membranes via enhancing the endogenous production of phytohormones like ethylene and salicylic acid, which ameliorate metal stress through their intricate cross-talks inside plants [52]. This reduction in peroxidation of membranal lipids and thereby the generation of MDA has been possible only due to the effectiveness of Si and EBL as ROS scavengers.

Plants exposed to different heavy metals accumulated osmoprotectants like proline and GB in their defense to combat oxidative stress [53]. Further, several studies have substantiated the vital role of individual applications of Si and 24-EBL in enhancing the measures of these osmolytes to mitigate heavy metal stress. For instance, external supplementation with silicon in *Capsicum annuum* elevated the proline levels under Cd stress [54]. Similar findings have also been recorded for Pb stress in *Brassica juncea,* where enhanced production of proline and GB took place upon providing 24-EBL treatment [55]. The present work yielded results akin to these outcomes, where Si and 24-EBL alone as well as in coupled form exhibited accumulation of these osmoprotectants under Pb stress in fenugreek plants. However, the effectiveness of Si + EBL together under Pb stress was far better than their individual applications. This could likely be associated with the synergistic

or cumulative effects generated by their coupled application on the activation of genes carrying out the biosynthesis of osmolytes.

Current work also recorded an upsurge in the measures of metabolites, i.e., anthocyanin, flavonoids, and phenolics, by the coupled supplication of Si and 24-EBL under Pb toxicity. Similar outcomes in terms of elevated metabolite measures were recorded by Bhardwaj et al. [56] in As stressed *Raphanus sativus*. The bioactivity and physiological aspects of these metabolites in combating different stresses of abiotic nature, heavy metal toxicity in particular, have been well documented [53,57–60]. These compounds scavenge free radicals responsible for the disruption of biological membranes via peroxidation of their constituent lipids [61]. Silicon is observed to cause the chelation of metallic ions via enhanced synthesis of these metabolites for the mitigation of heavy metal-generated stress [62,63]. Further, Al-stressed *Hordeum vulgare* was reported to have improved phenolic compound levels when supplemented with Si [64]. Similarly, 24-EBL promoted the biosynthesis of both phenolics and flavonoids in *Tinospora cordifolia,* which subsequently ameliorated oxidative damage [65].

The antioxidative defense mechanisms, including both antioxidative enzymes (CAT, APX, SOD, GR, POD, etc.) and non-enzymatic antioxidants (AsA and GSH), also become more pronounced under heavy metal stress and efficiently battle out the oxidative harm conferred by the exceedingly high levels of ROS [11,66,67]. A drastic reduction in protein levels inside the heavy metal-stressed plants is also an integral part of inciting the plant's antioxidative defense response. An appraisal of the antioxidative defense system in the current experimental study revealed that individual and coupled applications of silicon and 24-EBL enhanced protein measures and the production and physiology of antioxidative enzymes in Pb-stressed fenugreek plants. This can be corroborated with the findings of earlier works where Si application mitigated Cu, Cd, Mn, Pb, and Zn toxicity by upregulating the functioning of antioxidative enzymes [19]. Moreover, to battle out the ROS levels efficiently, Si upregulated the antioxidative defense system, compartmentalized heavy metal inside tissues, and modulated various plant parts at the structural and molecular level [68]. Enhancement in the expression of enzymes of antioxidative nature in fenugreek plants under Pb stress through Si supplementation could possibly be linked to processes of similar nature.

Stressed plants with 24-EBL optimally regulated the ROS measures and accelerated the activities of enzymatic antioxidants, thereby modulating the physiological status of plants. 24-EBL mitigates the Pb and Zn-generated oxidative damage on the roots of *Brassica juncea* and *Citrullus lanatus* by activating the antioxidative defense structure via enhanced expression of genes encoding for antioxidative activities [69]. Out of all of them, the gene encoding for BSK 1 (BR signaling kinase 1) is majorly upregulated to mitigate oxidative stress through the enhancement of endogenous measures of salicylic acid [70].

Furthermore, the role of non-enzymatic antioxidants is equally important in ameliorating heavy metal phytotoxicity. The antioxidant GSH protects heavy metal-stressed plants by producing non-toxic complexes [71]. Additionally, glutathione acts as a buffer inside cell organelles to regulate redox balance for the mitigation of various stresses of an abiotic nature [72]. In the current experimental work, individual assays, together with the coupled application of Si and 24-EBL, have resulted in the enhancement of endogenous measures of AsA and GSH under Pb stress. This can be corroborated with the alleviation of Si-mediated Cd and As stress through elevated expression of non-enzymatic antioxidants in *Brassica rapa* subspecies *chinensis, Oryza sativa,* and *Triticum aestivum,* respectively [73–75]. This significant enhancement in the antioxidant pool by Si supplementation might be linked with distinct alterations in the measures of cysteine and methionine (sulphur-containing amino acids), which are responsible for higher levels of reduced GSH as found in Si-augmented *Pisum sativum* seedlings [76]. Further, EBL mitigated the negative influence of Cd in the seedlings of *Raphanus sativus* [77] and Pb on the plants of *Acutodesmus obliquus* [78] by elevating the measures of antioxidants of non-enzymatic nature, especially AsA and GSH. Combined supplementation of Si and EBL could be operational behind the up-regulated

expression of biosynthetic genes involved in the stimulation of antioxidative components of the AsA-GSH cycle.

The outcomes of the current study have been briefly summarized in Figure 10, which depicts the toxic influence of Pb metal stress in *Trigonella* plants along with its amelioration by applying mitigants, i.e., Si and 24-EBL.

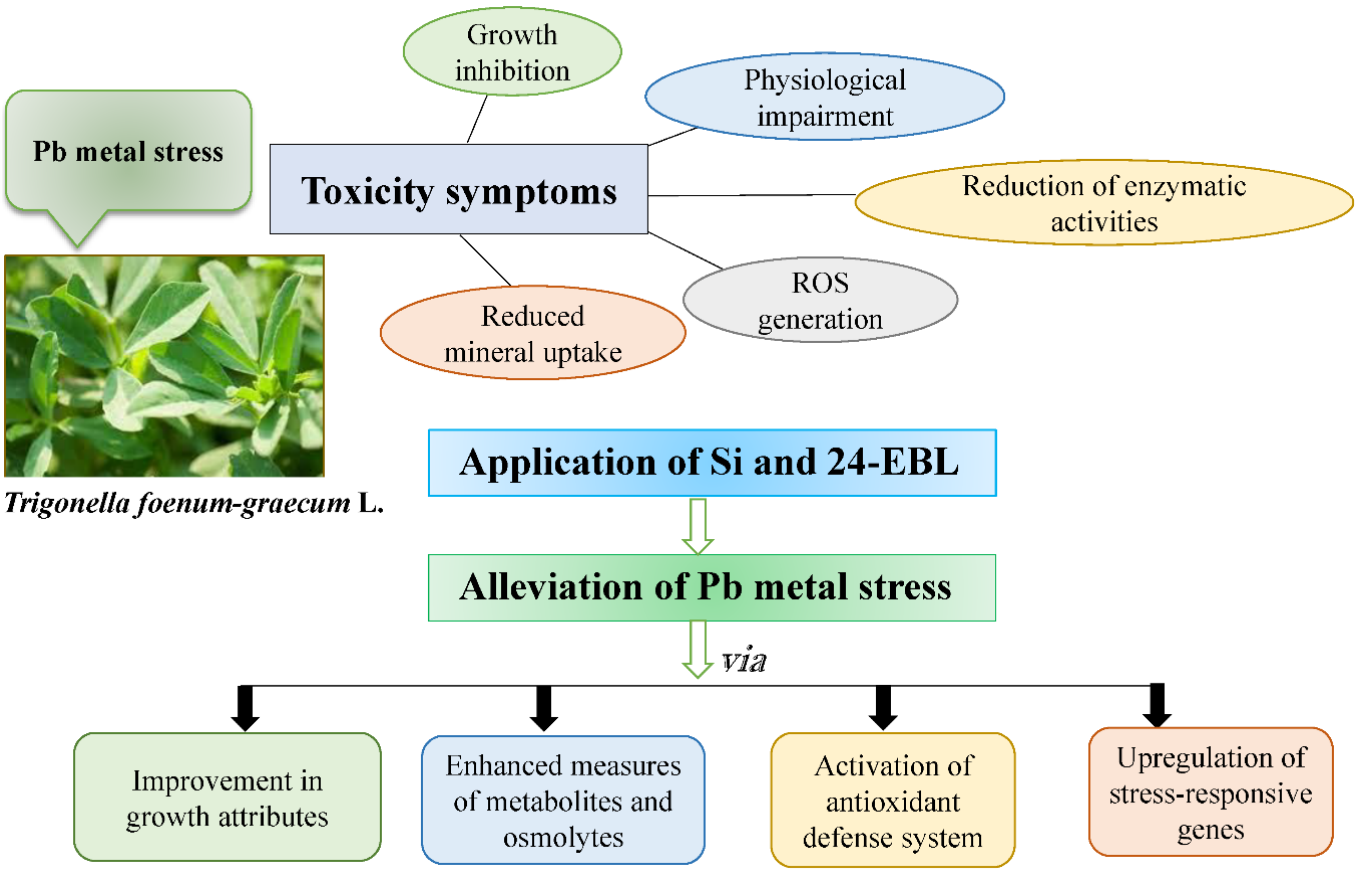


**Figure 10.** Depiction of mitigation of Pb metal stress by the application of Si and 24-EBL in fenugreek plants.

## 5. Conclusions

Current work exhibited severe oxidative damage to the fenugreek plants upon their subjection to Pb metal toxicity, which was expressed in the form of elevated measures of oxidative stress markers and a reduction in growth attributes. However, the stressed plants were recorded to have enhanced potential in combating lead toxicity under individual plus combined application of Si and 24-EBL. This was made possible by scavenging the ROS and minimizing its oxidative harm. Improvement in morphological and physiological parameters along with pronounced anti-oxidative defense mechanisms indicate the effectiveness of the ameliorates chosen for the present study, especially in their combined form. Moreover, gene expression of *SOD* and *CAT* genes was up-regulated by Si and 24-EBL under stressed conditions. Similar research under field conditions in the future would provide deep insight into the defense mechanisms operational at molecular levels for mitigating Pb metal toxicity with regard to the inter-mutual talks between Si and 24-EBL.

**Author Contributions:** Conceptualization, D.S., D.K. and N.R.S.; methodology, D.S.; software, D.S.; formal analysis, D.S., D.K. and N.R.S.; writing—original draft preparation, D.S., S.B., D.K. and N.R.S.; writing—review and editing, D.S., S.B., A.R., R.S., D.K., N.R.S. and P.V.V.P.; supervision, D.K. and N.R.S. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Data Availability Statement:** The data presented in this study will be available from the corresponding author.

**Acknowledgments:** The authors are thankful to Lovely Professional University, Punjab, for providing all the laboratory facilities necessary to carry out the present study.

**Conflicts of Interest:** The authors declare no conflict of interest.

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