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Glycine Betaine Mitigates Heavy Metal Toxicity in *Beta vulgaris* (L.): An Antioxidant-Driven Approach

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Abstract: Plants are often exposed to non-ideal conditions during their growth. The toxicity of heavy metals as abiotic stressors is a significant concern due to their harmful effects on plants. Glycine betaine (GB) is a potent compatible solute that helps plants resist abiotic stresses and plays a crucial role in alleviating them. This study aimed to determine the effective role of glycine betaine (0.5 and 1 mM) as a foliar treatment in sugar beet plants to cope with the toxicity of cadmium (50 mg/kg soil) and lead (100 mg/kg soil). The application of lead (Pb) and cadmium (Cd) in cultivation soil noticeably suppressed morphological growth attributes, such as chlorophylls, carotenoids, sugars, and proteins. At the same time, the aforementioned levels of heavy metals significantly increased the levels of non-enzymatic antioxidants (phenolics and proline) and enzymatic antioxidants (peroxidase, superoxide dismutase, polyphenol oxidase, and catalase) in the root and shoot tissues of sugar beet plants. In contrast, the use of glycine betaine as foliar treatment at 0.5 and 1 mM alleviated the adverse impacts of cadmium and lead by promoting the aforementioned attributes. Furthermore, the application of 1 mM GB was more effective in increasing the contents of phenolics in root by approximately 16% and 29%, phenolics in shoot by about 25% and 10%, peroxidase activity by about 82% and 116%, superoxide dismutase activity by about 56% and 47%, polyphenol oxidase activity by about 9% and 36%, catalase activity by about 19% and 25%, in cadmium- and lead-stressed plants, respectively. Additionally, it reduced the levels of proline in sugar beet tissues. Overall, the application of glycine betaine has the efficacy to counteract the adverse impacts of cadmium and lead toxicity on sugar beet plants by enhancing the metabolic indices as well as the non-enzymatic and enzymatic antioxidant activities.

Keywords: heavy metals; sugar beet growth; glycine betaine; antioxidant enzymes; photosynthesis; non-enzymatic antioxidants; sugars; proteins

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1. Introduction

Rapid population growth, urbanization, industrial drainage, agricultural fertilizer utilization, and other anthropogenic activities contribute to environmental pollution. Among these pollutants, heavy metals are recognized as one of the most significant threats to plant growth and development, plant-dependent animals, and ultimately human health due to their non-degradable nature [1,2]. The term "heavy metal" is a general collective term

used for metals and metalloids with atomic densities greater than 4 g/cm^3 or those that are five or more times denser than water [3]. Heavy metals encompass elements such as arsenic (As), lead (Pb), cadmium (Cd), nickel (Ni), cobalt (Co), zinc (Zn), chromium (Cr), iron (Fe), selenium (Se), silver (Ag), and platinum group elements. Some heavy metals, like copper and zinc, can serve as cofactors or activators in enzyme reactions, while others such as lead, arsenic, cadmium, and mercury, are highly toxic to metal-sensitive enzymes, resulting in growth retardation and apoptosis [4]. Heavy metals are widely distributed through activities such as metal mining, fossil fuel combustion, coal combustion, battery emissions, ore smelting, automobile exhaust, gasoline, paint, excessive use of pesticides, wastewater, and the application of fertilizers on agricultural lands [5–7].

Among the heavy metals that affect plants, lead (Pb) ranks second in terms of its toxicity to both humans and plants, as well as its distribution and widespread occurrence [8]. It is widely recognized that elevated levels of lead result in reduced root and shoot growth, protein and nitrogen metabolism disorders, and decreased concentrations of sugars and amino acids [9–11]. Furthermore, lead stress has negative effects on photosynthesis, respiration, enzymatic activities, water relationships, and nutrient distribution and absorption. Additionally, it induces oxidative stress by promoting the production of reactive oxygen species, which leads to metabolic dysfunction and the destruction of biomolecules [12–14]. In a recent investigation conducted by [15], it was found that the levels of leaf pigments and total carbohydrates in two barley varieties (BH-946 and BH-959) were adversely affected and significantly suppressed when these plants were cultivated in soil supplemented with lead. However, the activities of antioxidant enzymes such as peroxidase, catalase, and superoxide dismutase were markedly enhanced. Similarly, [16] it was demonstrated in another study that pakchoi (*Brassica chinensis* L.) plants exposed to lead stress exhibited significant inhibitions in morphological growth and biomass aspects, as well as effects on soluble proteins. On the other hand, proline accumulation was observed.

Cadmium (Cd), another phytotoxic metal, does not have any known biological activity in animals and plants. However, it does inhibit plant growth, biomass production, photosynthesis rate, and disrupts membrane permeability, leading to imbalances in water absorption and nutrient distribution within plants [17–19]. Additionally, it negatively affects enzymatic activities, the electron transport chain, and can cause alterations in DNA [20]. Despite not being a redox-active element, cadmium induces oxidative toxicity in plants through the formation of reactive oxygen species, resulting in physiological disorders that ultimately lead to reduced growth and biomass [21,22]. In a recent study conducted by [2], it was observed that levels of proline significantly accumulated in the roots, stems, and leaves of cucumber seedlings exposed to cadmium toxicity. Furthermore, [21] found that supplementation of cadmium in the cultivation soil led to a decrease in the morphological growth indices and protein contents of super black waxy maize. However, the activities of enzymatic antioxidants, such as peroxidase, glutathione reductase, and ascorbate peroxidase, were induced. Similarly, [23] reported an inhibition in total soluble sugars in pea plants, while [24] observed a decrease in chlorophyll contents in spinach, both as a result of exposure to cadmium toxicity.

To mitigate the toxicity of heavy metals in soil and plants, several treatment strategies can effectively reduce their phytotoxicity. One promising approach is the utilization of compatible solutes, which can potentially alleviate the adverse effects of heavy metals on plants [15,24]. Compatible solutes are non-toxic substances with low molecular weight and high solubility. They protect and aid in the recovery of plants from stress through various mechanisms; including scavenging reactive oxygen species, stabilizing proteins, regulating cell osmotic pressure, and maintaining membrane integrity [25].

Glycine betaine (GB) is one of the most abundant quaternary ammonium compounds used for mitigating and adapting to stressful environments [26,27]. It is a colorless, odorless, water-soluble, and metabolically stable compound, making it an excellent osmoprotectant [28]. GB acts as a potent compatible solute produced in chloroplasts through oxidation, helping to stabilize the structure and ensure the effectiveness of photosystem

II (PSII) under different stressful conditions [2,29]. Numerous studies demonstrated that GB can protect plants from various stresses, including temperature, drought, salinity, and metal toxicity. [30–33]. It was reported that GB scavenges reactive species by enhancing several enzymatic and non-enzymatic antioxidants, thereby protecting cells from oxidative damage [30,34]. Additionally, the external application of glycine betaine has been shown to be an effective method for alleviating the toxic effects of various abiotic stresses [15,35].

Sugar beet (*Beta vulgaris* L.), a member of the Amaranthaceae family, is considered a valuable staple crop, ranking second in sugar production after sugar cane. It contributes approximately 40% of global sugar production [36,37]. Apart from sugar production, the by-products of this crop are utilized in animal feed and the production of numerous economic, industrial, and biochemical compounds. Recently, countries worldwide have adopted policies aimed at achieving self-sufficiency by expanding both vertically and horizontally in sugar beet cultivation and production, with the goal of bridging the gap between consumption and production [4,38].

Based on the points mentioned above, the objective of this study was to assess the impact of cadmium and lead exposure on morphological growth indices, leaf pigments, sugars, proteins, enzymatic and non-enzymatic antioxidants in sugar beet plants. Additionally, the study aimed to investigate the potential effectiveness of glycine betaine application in mitigating the detrimental effects of cadmium and lead on growth, development, and metabolism.

2. Materials and Methods

2.1. Field Experiment Layout

The current experimental study was conducted at the Botanical Research Station of Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Cairo, Egypt. Seeds of the sugar beet variety MK4200 were provided by the Sugar Crops Research Institute (SCRI), Agricultural Research Centre (ARC), Giza, Egypt. Uniform sugar beet seeds were planted in forty-five (45) earthenware pots with a diameter of 50 cm, previously filled with 10 kg of sandy-loamy soil. These pots were divided into 3 groups: control (unstressed plants), cadmium-stressed plants (50 mg of cadmium nitrate/kg soil), and lead-stressed plants (100 mg of lead acetate/kg soil). Then, each group was divided into 3 sub-groups: control (untreated plants), foliar-treated plants with 0.5 mM glycine betaine, and foliar-treated plants with 1 mM glycine betaine. Five plants remained in each pot after thinning and were irrigated as needed with either water or water containing heavy metals. Glycine betaine treatments were applied to the plants as a foliar spray twice; on the 30th and 60th days of sowing. Then, randomized plant samples were harvested on the 70th day after sowing for analysis of morphological, biochemical, and physiological attributes in the roots (tubers) and shoots of the sugar beet plants.

2.2. Plant Growth and Biomass

Five random samples of sugar beet plants were collected from each group to measure growth characteristics. The following parameters were recorded: shoot length (cm), root length (cm), number of leaves, fresh weight of shoot (g), dry weight of shoot (g), fresh weight of root (g), and dry weight of root (g).

2.3. Enzymatic Antioxidants

In the described method of [39], terminal buds of sugar beet seedlings were utilized for the extraction of peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), and polyphenol oxidase (PPO).

Superoxide dismutase (E.C.1.15.1.1) activity was estimated based on the described methods of [40]. Briefly, distilled water (3.6 mL), crude enzyme (0.1 mL), phosphate buffer (5.5 mL, 50 mM in concentration, 7.8 in pH), and pyrogallol (0.8 mL, 3 mM in concentration and solubilized in 10 mM hydrochloric acid) were added. The rate of pyrogallol reduction was measured at 325 nm within 60 s.

Peroxidase (E.C.1.11.1.7) activity was estimated according to the methods described by [41]. The assay mixture included phosphate buffer (5.8 mL, 50 mM in concentration, 7.0 in pH), enzyme extract (0.2 mL), pyrogallol (2 mL, 20 mM in concentration), and hydrogen peroxide (2 mL, 20 mM in concentration). The increase in pyrogallol absorbance was measured spectrophotometrically at 470 nm within 60 s.

For polyphenol oxidase (E.C.1.10.3.1) activity, the enzyme was estimated using the method illustrated in [42]. In this method, enzyme extract (1 mL) was mixed with phosphate buffer (1 mL, 0.2 M in concentration, pH 7), catechol (10 mL, 1 mM in concentration), and distilled water (3 mL). By a UV–Visible spectrophotometer, the change in catechol absorbance at 495 within 60 s was read.

Catalase (E.C.1.11.1.6) activity was assayed by measuring the cleavage of hydrogen peroxide according to the described method in [43]. The enzyme extract was added to the reaction mixture (3 mL) containing phosphate buffer (100 mM, pH 7) and hydrogen peroxide (75 mM). The catalase activity was spectrophotometrically measured at 240 nm within a 60-s time frame.

2.4. Photosynthetic Pigments

Chlorophylls a, b, total chlorophylls, and carotenoids were assayed in sugar beet leaves using acetone (85%), as illustrated by [44]. Briefly, fresh tissues from leaves (1 g) were homogenized with acetone 80% (100 mL). The mixture was filtered, then diluted with acetone 80% to 100 mm as total volume. To determine the levels of the mentioned pigments, the absorbance was measured spectrophotometrically at wavelengths of 470 nm, 649 nm, and 665 nm.

2.5. Total Soluble Sugars

Levels of soluble sugars in sugar beet tissues (shoot and root) were determined following the technique described in [45]. Briefly, 1 g of tissues was placed in phenol 2% (5 mL) and trichloroacetic acid 30% (10 mL). The homogenate was kept overnight and then clarified using charcoal. The cleared extract (2 mL) was reacted with anthrone reagent (4 mL). At 620 nm, the absorbance was measured spectrophotometrically.

2.6. Total Soluble Proteins

In dried samples of sugar beet tissues (shoot and root), contents of soluble proteins were assayed according to the technique of [46]. Plant sample (1 g) was mixed with phenol (5 mL, 2% in concentration) and distilled water (10 mL). The mixture was then filtered using charcoal. The filtrate (1 mL) was mixed with alkaline reagent (5 mL), previously prepared from sodium carbonate, sodium hydroxide, copper sulfate, and sodium potassium tartrate. Finally, Folin reagent (0.5 mL) diluted 1:3 (v/v) was added. After allowing the reaction to proceed for half an hour, the absorbance of the resulting color was measured at 750 nm.

2.7. Phenolic Compounds

The content of phenolics in dried root and shoot tissues of sugar beet plants was determined using the procedure described in [47]. Plant tissues (1 g) were soaked in ethanol 80% (5–10 mL) for 24 h. The mixture was filtered, and the remaining material was extracted two more times with the same solvent. All the extracts were combined and made up to a final volume of 50 mL with ethanol. The extract (0.5 mL) was mixed well with Folin reagent (0.5 mL) and shaken for 3 min. Then, saturated sodium carbonate solution (1 mL) and distilled water (3 mL) were added and mixed well. After 1 h, the absorbance was measured spectrophotometrically at 725 nm.

2.8. Free Proline

Determination of proline levels in sugar beet tissues were based on the employed method of [48]. In it, the dried plant samples (0.5 g) were reacted with sulfosalicylic acid 3% (10 mL). The homogenate was filtered and then placed (2 mL) with acid ninhydrin solution

(2 mL) which was freshly prepared and glacial acetic acid (2 mL) in a heated water bath for 1 h. The mixture was then put in an ice bath, and toluene (4 mL) was added to the reaction. The absorbance was recorded spectrophotometrically at 520 nm.

2.9. Statistical Analysis

Analysis of variance (One-way ANOVA) was performed to determine significant changes among treatments in the presence of *F*-values and *p*-values, as recommended by [49], using a statistical data Minitab 18 software. Tukey's test was applied at 5% probability to imply the differences between treatments. The data were presented as mean ± error of standard (5 replicates for morphological growth and plant biomass attributes while 3 replicates for biochemical constituents). Correlation analysis was carried out among control, Cd+Gb2 (glycine betaine at 1 mM), and Pb+Gb2 (Gb2 refers to glycine betaine at 1 mM) for morphological and biochemical parameters. The simple linear regression using scattered graphs represented the correlated results to different extents.

3. Results

3.1. Morphological Plant Growth

The impact of cadmium, lead, glycine betaine, and their interactions on shoot length, root length, and leaves number of *Beta vulgaris* plants is presented in Figure 1. Exposure of sugar beet plants to cadmium and lead resulted in significant inhibitions in length of shoot by 15.31% and 14.09%, length of root by 21.81 and 32.91%, and leaves number by 20 and 23.33%, respectively.

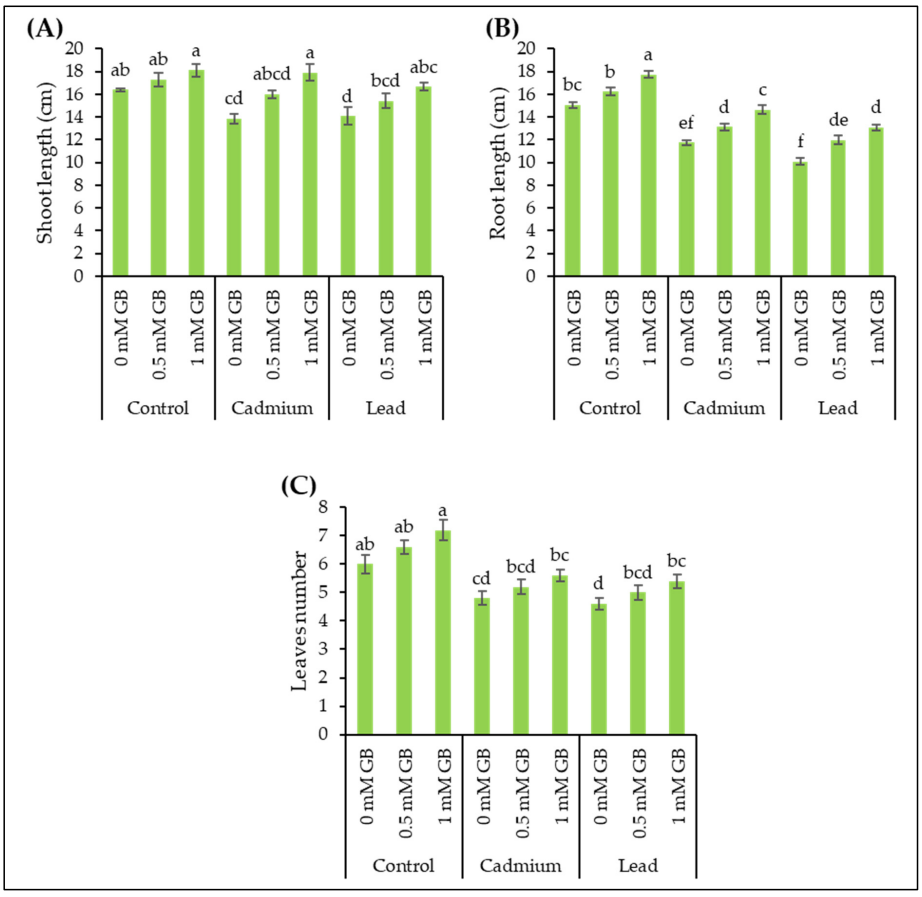

Figure 1. Effect of cadmium and lead exposure, glycine betaine treatment and their interaction on shoot length (**A**), root length (**B**) and number of leaves (**C**) of sugar beet plants. Vertical bars refer to five replicates mean ± standard error. The significant differences between means are that they do not share a letter. *p*-values were <0.05. The lowest *F*-value was 8.95 at shoot length while the highest *F*-value was 55.14 at root length.

Under normal conditions, foliar treatment with glycine betaine (0.5 and 1 mM) led to enhancements of the aforementioned morphological growth characteristics of sugar beet plants. Under cadmium stress, the application of glycine betaine, especially at 1 mM, resulted in various progressive enhancements including a 29.11% increase in length of shoot, a 24.66% increase in length of root, and a 16.67% increase in leaves number. Similarly, it caused enhancements in lead-stressed sugar beet plants, with a 18.39% increase in shoot length, a 29.53% increase in root length, and a 17.39% increase in leaves number.

3.2. Plant Biomass

The effectiveness of cadmium, lead, glycine betaine, and their interactions on the fresh weight of shoot, dry weight of shoot, fresh weight of root, and dry weight of root of *Beta vulgaris* plants is shown in Figure 2. The results demonstrated that cadmium and lead treatments significantly decreased the weights of fresh shoot by 30.06% and 32.31%, dry shoot by 40% and 41.25%, fresh root by 62.48% and 58.88%, and dry root by 46.28% and 53.72%, respectively.

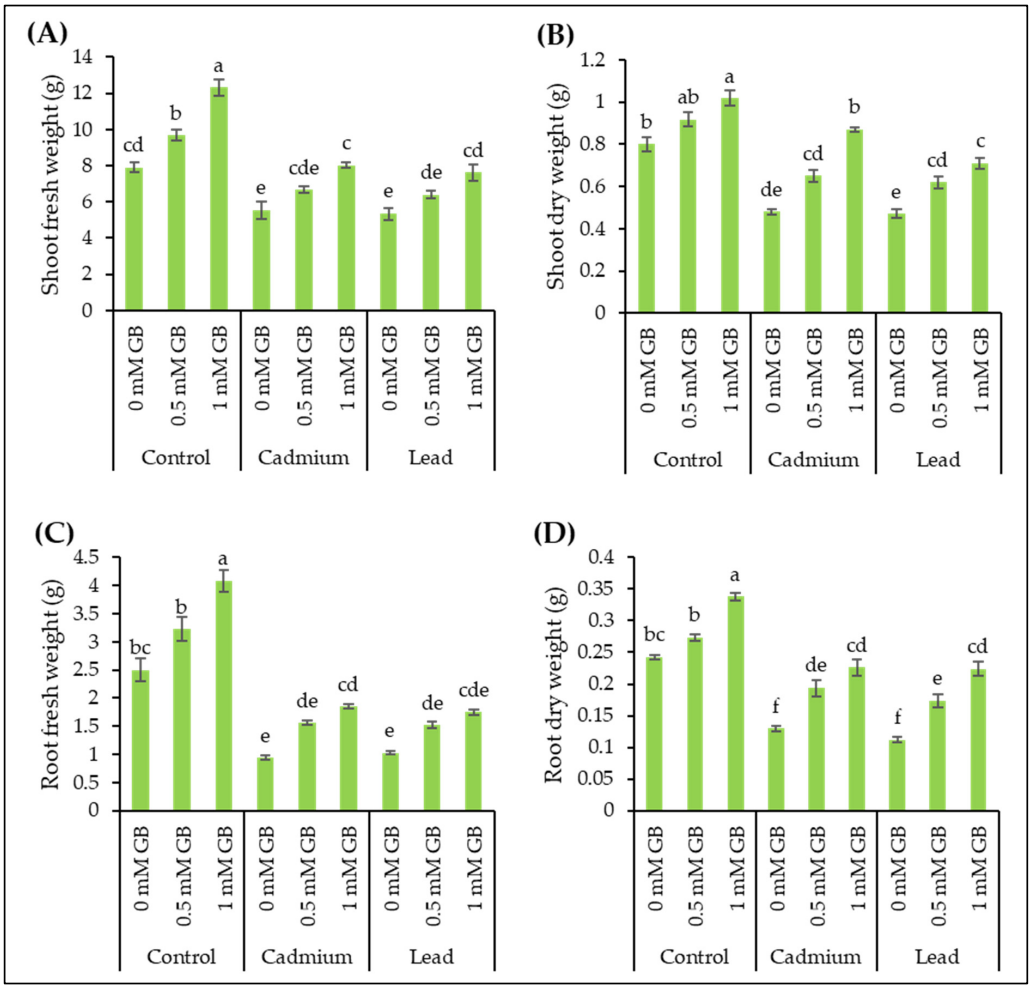

Figure 2. Effect of cadmium and lead exposure, glycine betaine treatment and their interaction on fresh weight of shoot (**A**), dry weight of shoot (**B**), fresh weight of root (**C**), and dry weight of root (**D**) of sugar beet plants. Vertical bars refer to five replicates mean ± standard error. The significant differences between means are that they do not share a letter. *p*-values were <0.05. The lowest *F*-value was 42.75 at shoot fresh weight while the highest *F*-value was 67.04 at root dry weight.

The application of glycine betaine at concentrations of 0.5 and 1 mM as foliar treatments promoted the fresh and dry weights of both roots and shoots of sugar beet plants under normal or heavy metal-stressed conditions, considering untreated plants. In the

case of cadmium-stressed plants, 1 mM of glycine betaine significantly increased shoot fresh weight, shoot dry weight, root fresh weight, and root dry weight by approximately 45.19%, 81.25%, 97.23%, and 73.85%, respectively. Additionally, in lead-stressed plants, glycine betaine at the same concentration was the significant treatment which increased the weights of fresh shoot, dried shoot, fresh root, and dried root by approximately 42.50%, 51.06%, 70.43%, and 100%, respectively, in comparison with untreated plants.

3.3. Antioxidant Enzymes

According to the data presented in Figure 3, the activities of several enzymatic antioxidants of sugar beet plants varied in response to cadmium and lead exposure as well as to glycine betaine treatments. Overall, cadmium and lead exposure led to a promotion of antioxidant enzyme activities. Activities of superoxide dismutase, polyphenol oxidase, and catalase were significantly enhanced by approximately 168%, 77%, 183%, respectively, in sugar beet plants grown under cadmium stress as well as activities of the abovementioned enzymes being significantly enhanced by approximately 381%, 132%, 212%, respectively, in sugar beet plants grown under lead stress. However, the activities of peroxidase were insignificantly affected by either cadmium or lead exposure on comparing with unstressed plants.

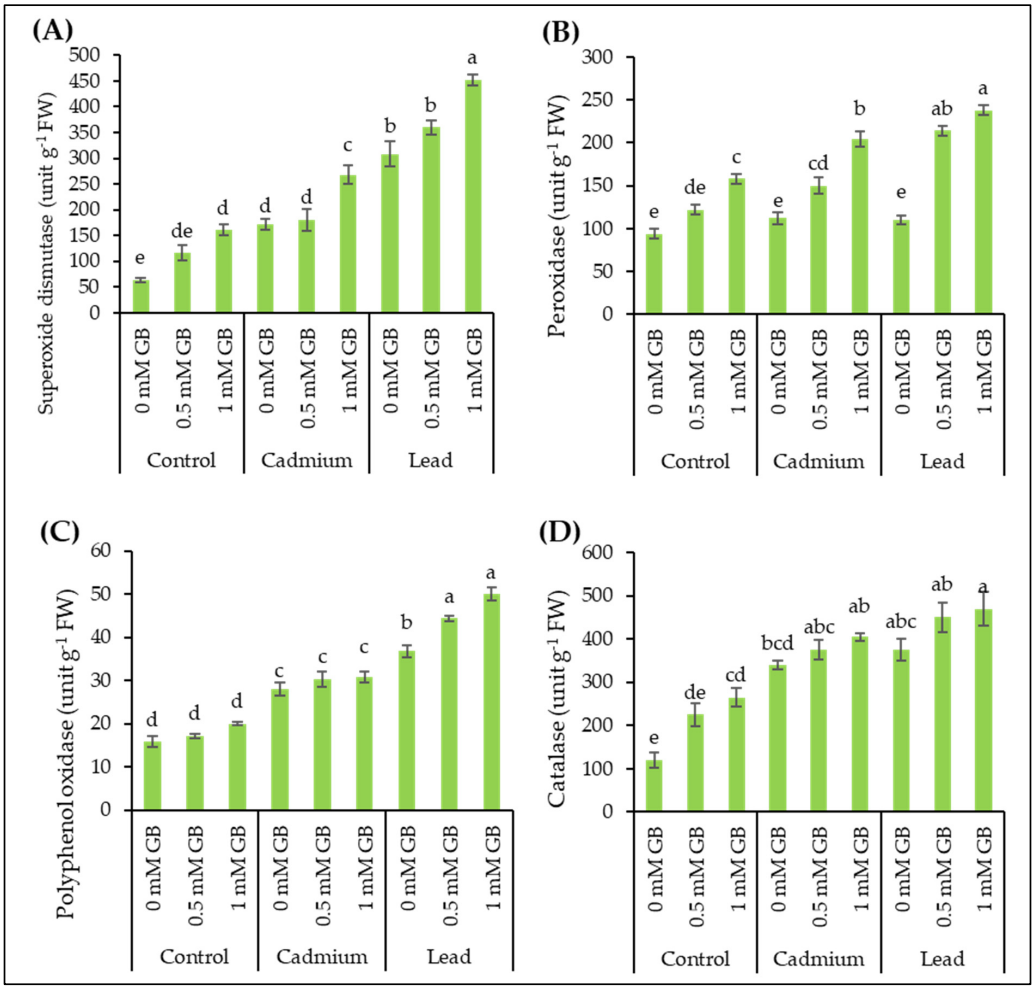

Figure 3. Effect of cadmium and lead exposure, glycine betaine treatment and their interaction on (**A**) superoxide dismutase, (**B**) peroxidase, (**C**) polyphenol oxidase, and (**D**) catalase of sugar beet (*Beta vulgaris* L.) plants. Vertical bars refer to five replicates mean ± standard error. The significant differences between means are that they do not share a letter. *p*-values were <0.05. The lowest *F*-value was 20.56 for catalase while the highest *F*-value was 95.89 for polyphenol oxidase.

In terms of glycine betaine treatment, when applied individually at a concentration of 0.5 mM, there were insignificant increases in the levels of the tested enzymatic antioxidant (SOD, PPO, CAT, and POD) compared to untreated plants. However, treatment with 0.1 mM of glycine betaine resulted in a significant induction of SOD, POD, and CAT by 150%, 68.09%, and 120%, respectively, with insignificant enhancements in PPO. Furthermore, application of glycine betaine, particularly at 1 mM, on cadmium-stressed plants significantly induced SOD and POD activities by about 55% and 82%, respectively, while insignificantly induced PPO and CAT in comparison with unstressed plants. Regarding the interaction between glycine betaine treatment and lead toxicity, concentrations of 0.5 and 1 mM boosted the activities of SOD by 16.88% and 46.75%, POD by 94.55% and 116.4%, PPO by 20.6% and 35.87%, and CAT by 20% and 25.33%, respectively, in lead-stressed sugar beet plants on comparing with unstressed plants.

3.4. Leaf Pigments

Leaf pigments in sugar beet plants were altered in response to cadmium and lead exposure as well as glycine betaine treatment according to the presented data in Figure 4. Significant inhibitions were observed in the contents of chlorophyll a (32.71% and 26.98%), total chlorophylls (25.21% and 20.42%), and carotenoids (29.80% and 30.68%) in sugar beet plants grown in cadmium- and lead-amended soil, respectively, in comparison with those of unstressed plants. However, there was an insignificant inhibition in chlorophyll b contents in both cases.

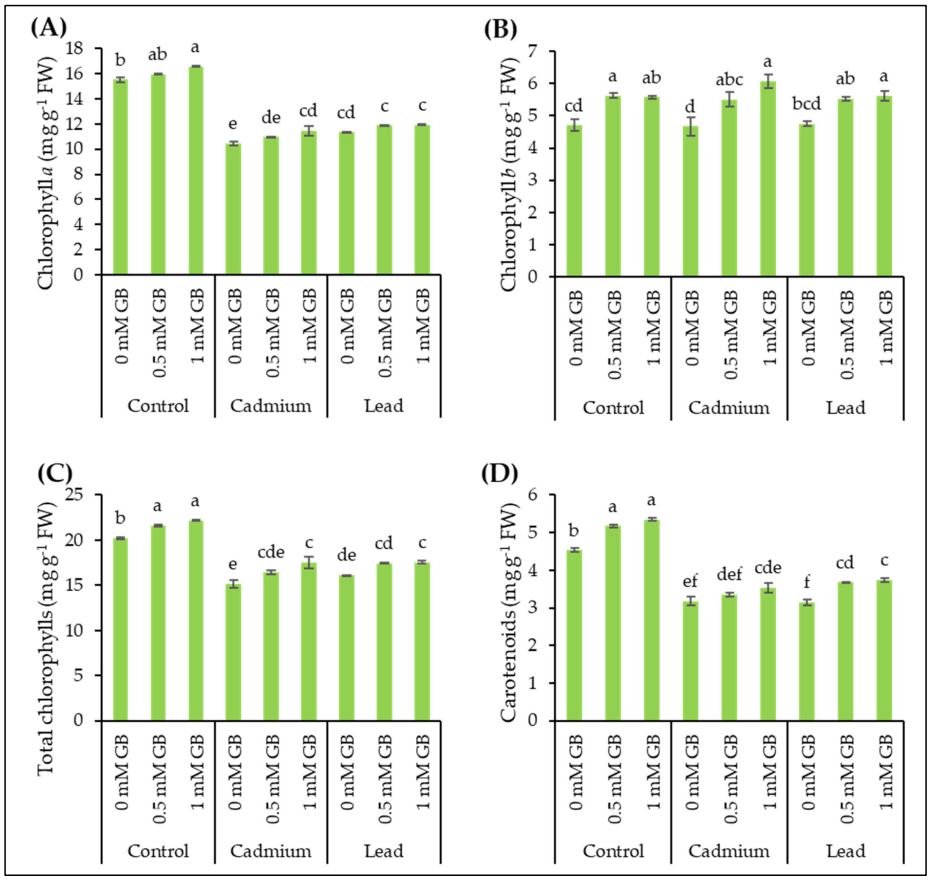

Figure 4. Effect of cadmium and lead exposure, glycine betaine treatment and their interaction on chlorophyll *a* (**A**), chlorophyll *b* (**B**), total chlorophylls (**C**), and carotenoids (**D**) of sugar beet (*Beta vulgaris* L.) plants. Vertical bars refer to five replicates mean ± standard error. The significant differences between means are that they do not share a letter. *p*-values were <0.05. The lowest *F*-value was 9.09 at chlorophyll *b* while the highest *F*-value was 223.2 at chlorophyll *a*.

It is worth noting that foliar treatment with glycine betaine at 0.5 and 1 mM gradually enhanced the amounts of chlorophylls and carotenoids in sugar beet leaves that were grown under normal condition with respect to untreated plants. On top of that, glycine betaine application relieved the harmful effects of heavy metals on photosynthetic pigments. The most significant treatment was observed with glycine betaine at 1 mM, which markedly promoted chlorophyll a about 9.57%, chlorophyll b about 29.98%, and total chlorophylls about 15.80% in cadmium-stressed sugar beet plants. In the case of lead-stressed sugar beet plants, it promoted chlorophyll b by 18.11%, total chlorophylls by 8.94%, and carotenoids by 19.11%, with respect to untreated plants.

3.5. Soluble Sugars

The impact of cadmium and lead exposure and glycine betaine treatment on soluble sugar contents in roots and shoots of sugar beet plants is illustrated in Figure 5. Both the mentioned heavy metals significantly decreased the sugar contents in sugar beet plants in comparison with un-exposed plants. Cadmium decreased sugar contents about 27.91% and 34.24%, while lead decreased the same contents by about 33.74% and 32.50% in roots and shoots, respectively.

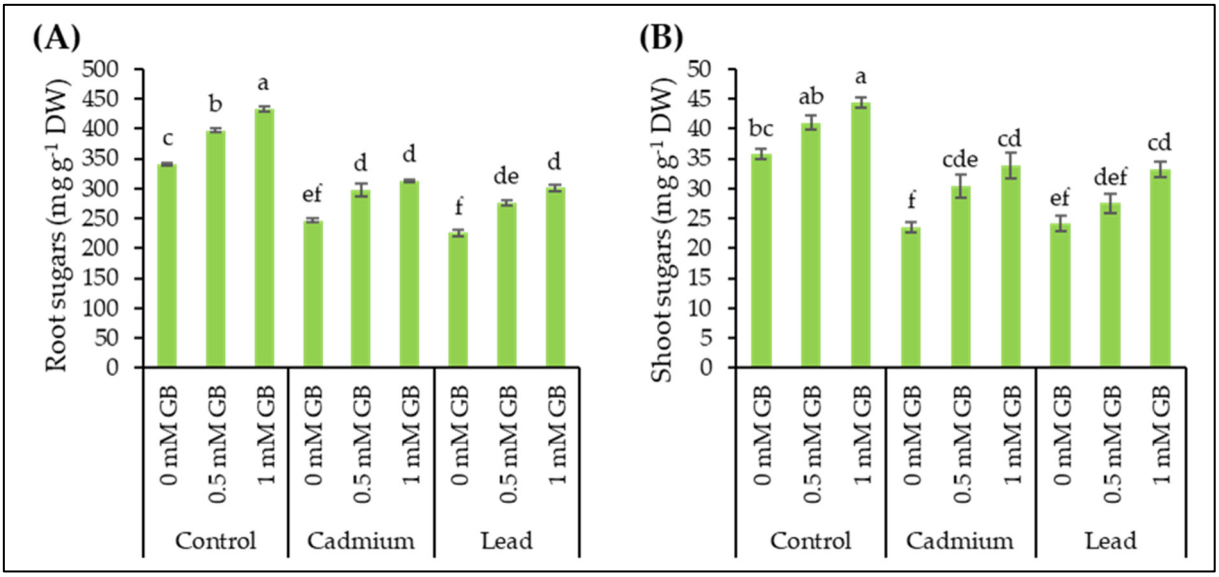

Figure 5. Effect of cadmium and lead exposure, glycine betaine treatment and their interaction on total soluble sugars contents in root (**A**) and shoot (**B**) of sugar beet (*Beta vulgaris* L.) plants. Vertical bars refer to five replicates mean ± standard error. The significant differences between means are that they do not share a letter. *p*-values were <0.05. *F*-value for root sugars was 168.31 while for shoot sugars was 26.13.

Regarding glycine betaine treatment, either 0.5 or 1 mM, it promoted the accumulation of sugar contents in root and shoot of sugar beet plants under normal conditions with respect to untreated plants. Moreover, glycine betaine at 0.5 mM caused significant enhancements in soluble sugars by about 20.95% and 29.59% in roots and shoots, respectively, of cadmium-stressed plants as well as significant increases by 22.25% in lead-stressed plants in roots only. The most significant treatment was observed with 1 mM of glycine betaine, which alleviated the deleterious effects of heavy metals and increased sugar contents in root and shoot by about 27.32% and 44.36% in cadmium-stressed plants, and about 33.07% and 37.62%, respectively, in lead-stressed plants with respect to unstressed plants.

3.6. Soluble Proteins

The presented data in Figure 6 portray the influence of cadmium and lead exposure and glycine betaine treatment on soluble protein contents in roots and shoots of sugar beet

plants. It was observed that the contents of soluble proteins were significantly suppressed in roots and shoots by about 37.81% and 31.30%, respectively, in sugar beet plants which had been cultivated in soil amended with cadmium. Similarly, in sugar beet plants which had been cultivated in soil amended with lead, the contents of soluble proteins were significantly decreased by approximately 52.32% and 37.11% in the roots and shoots, respectively.

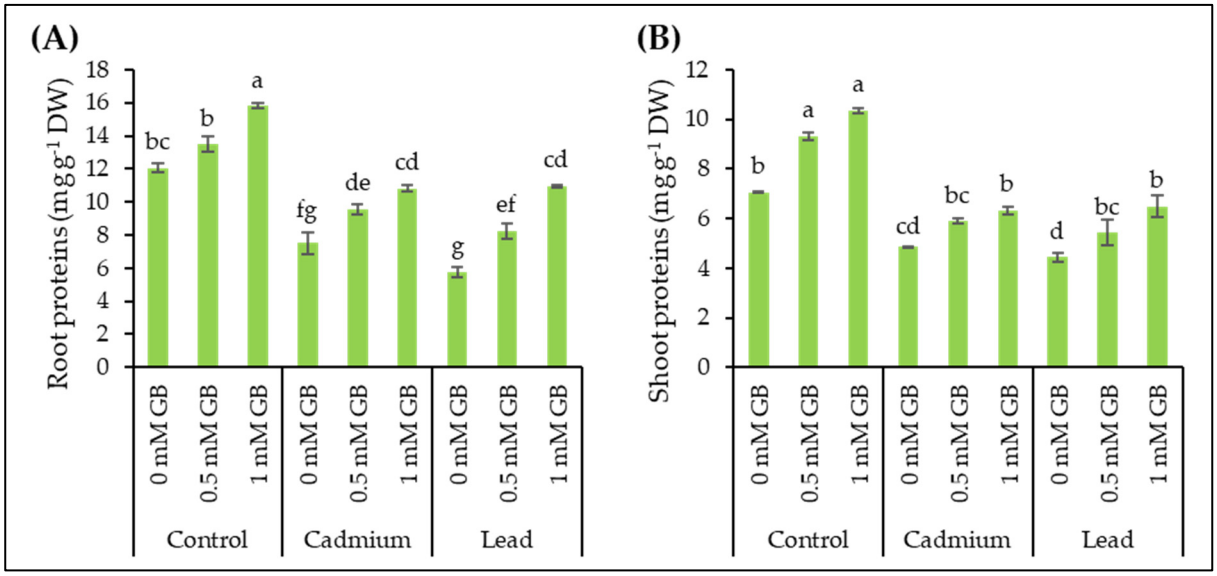

Figure 6. Effect of cadmium and lead exposure, glycine betaine treatment and their interaction on total soluble protein contents in root (**A**) and shoot (**B**) of sugar beet (*Beta vulgaris* L.) plants. Vertical bars refer to five replicates mean ± standard error. The significant differences between means are that they do not share a letter. *p*-values were <0.05. *F*-value for root proteins was 71.37 while for shoot proteins was 62.01.

On the other hand, exogenous treatment of glycine betaine at 0.5 and 1 mM was found to promote the accumulation of soluble proteins in sugar beet plants grown under normal conditions. The levels of soluble proteins gradually increased by 17.77% and 31.01% in roots, and by 32.01% and 46.46% in shoots due to 0.5 and 1 mM of glycine betaine treatment compared to untreated plants, respectively. Furthermore, the tested concentrations of glycine betaine mitigated the negative effects of the heavy metals. At 0.5 mM of glycine betaine, soluble protein contents were obviously increased in roots and shoots by 27.33% and 21.44%, respectively, in cadmium-stressed sugar beet plants, and by 43.48% and 22.07%, respectively, in lead-stressed sugar beet plants compared to unstressed plants. At 1 mM of glycine betaine, soluble protein contents were significantly boosted in roots and shoots by 44.27% and 30.31%, respectively, in cadmium-stressed sugar beet plants, and by 90.43% and 45.95%, respectively, in lead-stressed sugar beet plants compared to unstressed plants.

3.7. Phenolic Compounds

As shown in Figure 7, levels of phenolic compounds in sugar beet plants responded to cadmium, lead, glycine betaine treatments, and their interactions. There were insignificant enhancements in phenolic contents in sugar beet roots by 27.59% and 17.24% in response to cadmium- and lead-amended soils, respectively. However, significant augmentations were recorded in phenolic contents in sugar beet shoots by 56.45% and 87.10% in response to cadmium- and lead-amended soils, respectively, compared to the control (un-stressed plants).

On the other hand, application of glycine betaine as foliar treatment at 0.5 and 1 mM led to marked enhancements in phenolic levels, of about 37.93% and 55.17% in sugar beet roots, and about 19.35% and 35.48% in sugar beet shoots, respectively. In terms of the interaction between glycine betaine treatment and the exposure to cadmium and lead, glycine betaine

at 0.5 and 1 mM gradually increased the accumulation of phenolic compounds in roots and shoots of sugar beet that were grown under cadmium or lead conditions.

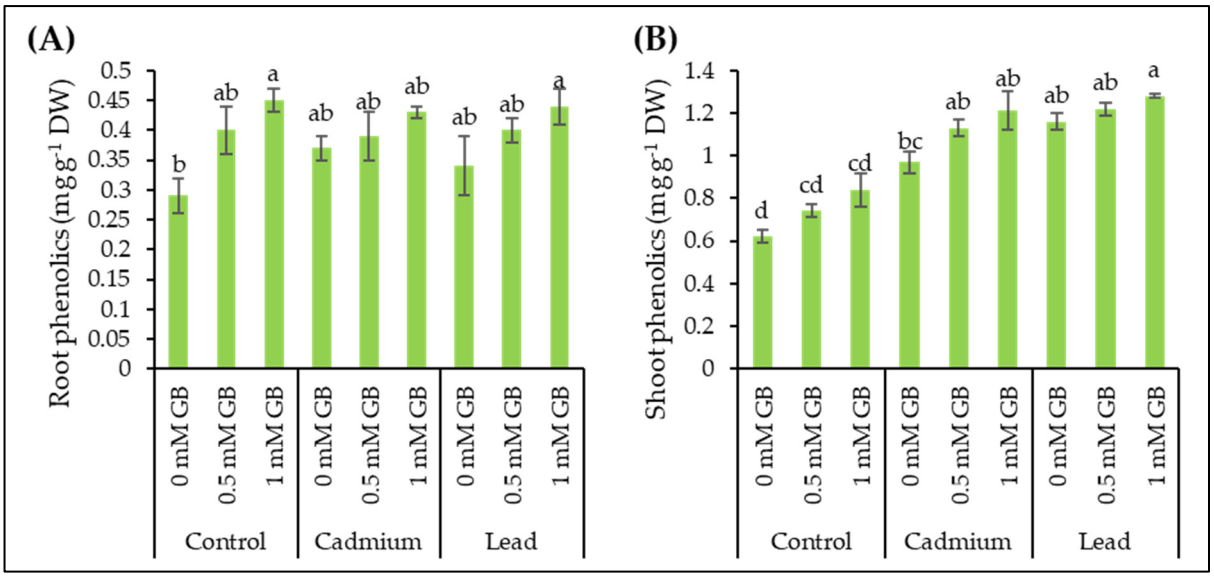

Figure 7. Effect of cadmium and lead exposure, glycine betaine treatment and their interaction on phenolic compound contents in root (**A**) and shoot (**B**) of sugar beet (*Beta vulgaris* L.) plants. Vertical bars refer to five replicates mean ± standard error. The significant differences between means are that they do not share a letter. *p*-values were <0.05. *F*-value for root phenolics was 2.75 while for shoot phenolics was 22.28.

3.8. Free Proline

Efficacy of cadmium and lead exposure and glycine betaine treatment on proline levels in sugar beet tissues is shown in Figure 8. It was noticed that free proline levels significantly accumulated in sugar beet roots by 55.95% and in shoots by 26.75% in response to cadmium exposure, and by 107.14% in roots and 33.44% in shoots in response to lead exposure, in comparison with plants grown under normal conditions.

Individual application of glycine betaine at 0.5 and 1 mM on sugar beet plants under normal conditions resulted in marked enhancements of 17.86% and 28.57% in proline levels in roots, and obvious suppressions of 9.24% and 23.25% in proline levels in shoots, respectively, when compared with untreated plants. Regarding glycine betaine treatments on heavy metal-stressed plants, the 1 mM concentration of glycine betaine was found to be the most effective in suppressing the accumulation of proline contents in roots by 29.01% in cadmium-stressed plants, and by 22.99% in lead-stressed plants. In sugar beet shoots, both concentrations of glycine betaine (0.5 and 1 mM) had an insignificant effect on reducing proline levels in cadmium- or lead-stressed plants.

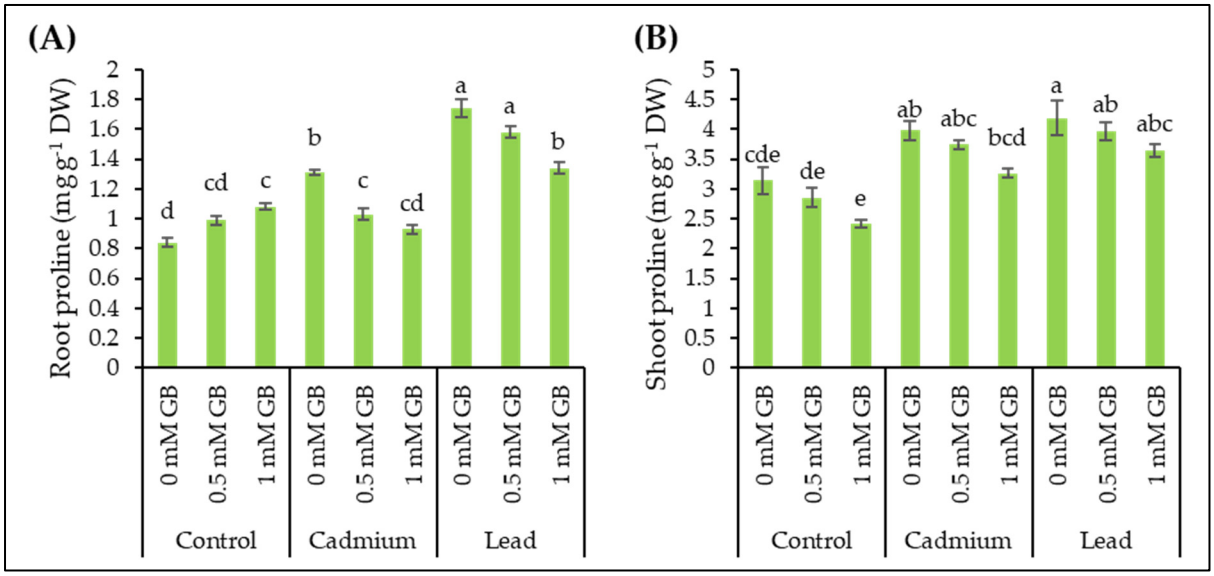

Figure 8. Effect of cadmium and lead exposure, glycine betaine treatment and their interaction on free proline contents in root (**A**) and shoot (**B**) of sugar beet (*Beta vulgaris* L.) plants. Vertical bars refer to five replicates mean ± standard error. The significant differences between means are that they do not share a letter. *p*-values were <0.05. *F*-value for root proline was 68.16 while for shoot proline was 13.97.

3.9. Correlation Analysis

All the correlated data of morphological analysis recorded a high value near to 1, Cd+Gb2 (glycine betaine at 1 mM) versus Pb+Gb2 recorded the highest correlated value while control versus Pb+Gb2 recorded the lowest one for biochemical analysis (Table 1).

Table 1. Correlation of control, Cd+Gb2, and Pb+Gb2 for morphological and biochemical parameters. GB2 refers to glycine betaine at 1 mM.

	Morphological Parameters		
	Control	**Cd+Gb2**	**Pb+Gb2**
Control	-	0.989	0.990
Cd+Gb2	0.989	-	0.993
Pb+Gb2	0.990	0.993	-
	Biochemical Parameters		
	Control	**Cd+Gb2**	**Pb+Gb2**
Control	-	0.753	0.634
Cd+Gb2	0.753	-	0.964
Pb+Gb2	0.634	0.964	-

The simple linear regression (SLR) curve as in Figure 9 represented the highest positive with the highest scattered similarity for control versus Cd+Gb2 in morphological analysis; on the other hand, Cd+Gb2 versus Pb+Gb2 scored the highest positive one while other chemical analyses scored the lowest positive ones.

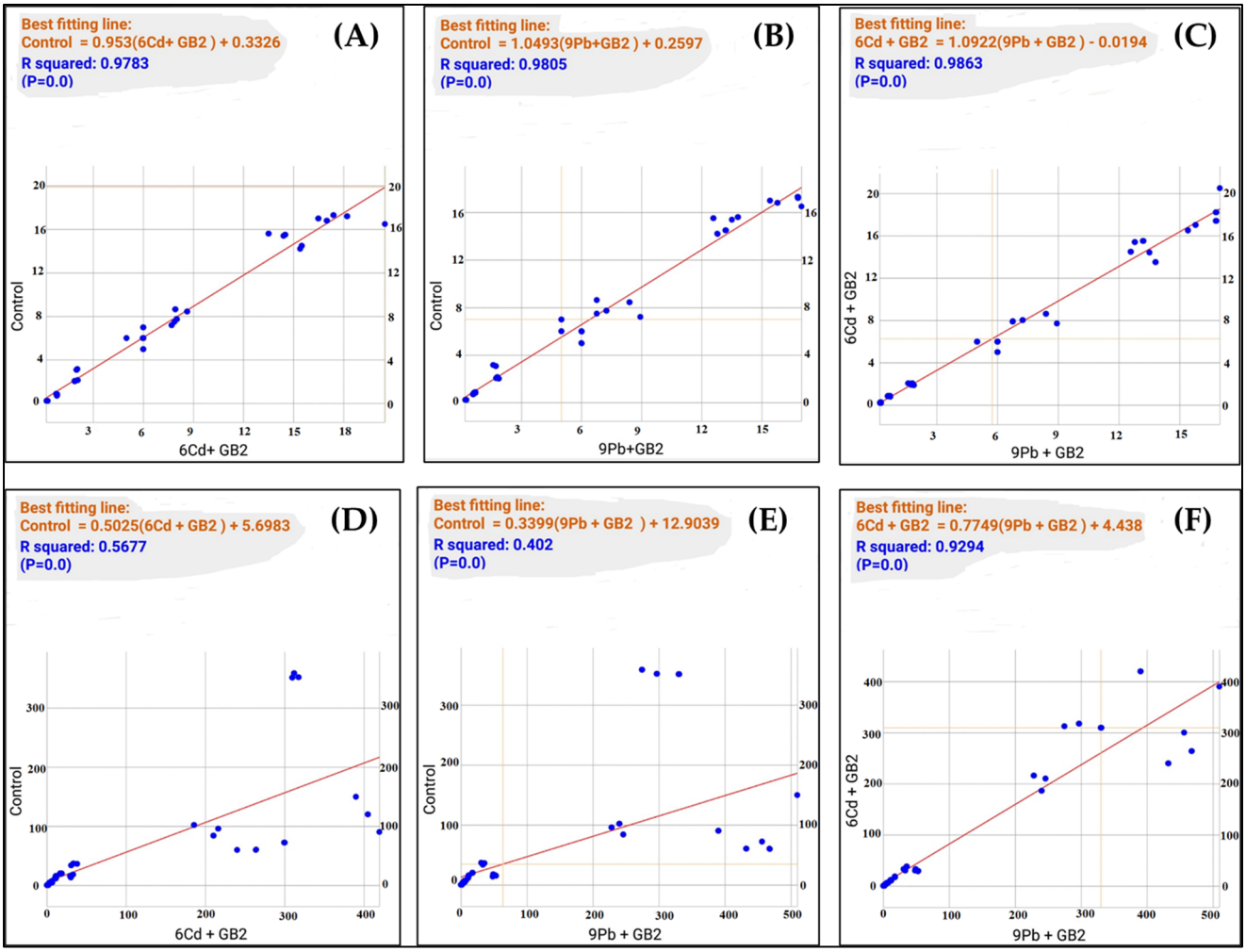

Figure 9. Simple linear regression curve for control versus Cd+GB2 in morphological parameters (**A**), control versus Pb+GB2 in morphological parameters (**B**), Cd+GB2 versus Pb+GB2 in morphological parameters (**C**), control versus Cd+GB2 in biochemical parameters (**D**), control versus Pb+GB2 in biochemical parameters (**E**), and Cd+GB2 versus Pb+GB2 in biochemical parameters (**F**). GB2 refers to glycine betaine at 1 mM.

4. Discussion

Plants often grow under unfavorable conditions such as drought, salinity, heat, cold, heavy metals, and with pathogens that negatively affect plant growth, development, and production [50–52]. Heavy metals, as abiotic stressors, can interfere with biomolecules, causing an accumulation of reactive oxygen species, thus limiting plant growth and productivity. So, it is essential to explore sustainable solutions to improve plant tolerance to these stresses and their ability to survive under stress conditions [53,54]. Application of various bio-stimulants or osmo-protectants such as glycine betaine is an alternative strategy to alleviate the adverse impacts of heavy metals on plant life.

In our present study, we observed a similar inhibition in morphological plant growth and biomass patterns due to heavy metal stress, consistent with previous findings in various crops [55–58]. It was documented that different concentrations of cadmium, applied on cotton plants, caused progressive inhibitions in certain morphological growth attributes [59]. Additionally, lead stress caused significant suppression in growth parameters (root depth, plant height, leaves number, and leaf area) and plant biomass (fresh and dry weights of root, stem, and leaf) of cotton plants [11]. The decrease in plant growth and biomass could be attributed to oxidative damage, reduction in antioxidant enzyme activities [60], reduced mineral nutrient uptake by plants [61], or reduced cell expansion [62]. Around

the ameliorative role of glycine betaine, its application has been found to promote the morphological growth characteristics of cotton plants [59] and super black waxy maize plants [21], either under normal or cadmium-stressed conditions. Additionally, the utilization of glycine betaine at different concentrations, either individually or in interaction with lead toxicity, exhibited marked enhancements in the different growth indices of cotton plants [11] and pakchoi [16]. Moreover, [63] and [64] observed that heavy metal stress tolerance in rice (*Oryza sativa*) and wheat (*Triticum aestivum*) was significantly improved after foliar spray with glycine betaine, respectively. The application of glycine betaine may enhance mineral nutrient uptake and photosynthetic characteristics, and promote growth in stressed plants [21].

The induction of enzymatic antioxidants plays a crucial role in relieving and alleviating stress [65]. Our findings revealed increases in the activities of certain enzymes. In another study, the activities of catalase, superoxide dismutase, peroxidase, and ascorbate peroxidase in cotton plants, either in leaves or root, were obviously induced in response to cadmium stress [59]. Similarly, the investigation of [16] exhibited marked inductions in peroxidase activities in shoots and roots of pakchoi plants in response to the exposure to lead stress. It is worthy of note that our results are in agreement with those of [11] which documented promotions in the activities of some antioxidant enzymes (superoxide dismutase, peroxidase, catalase, and ascorbate peroxidase) in cotton plants in response to lead toxicity. The increase in antioxidant enzyme activities under heavy metal stress may be attributed to the activation of the plant's defense mechanism [66]. Regarding glycine betaine and its impact, it has been demonstrated that application of glycine betaine at 1 mM significantly boosted the levels of peroxidase, ascorbate peroxidase, catalase, and superoxide dismutase in leaves and roots of cotton plants grown under normal and cadmium-stressed conditions [59]. Significant inductions were also observed in the activities of ascorbate peroxidase, superoxide dismutase, glutathione reductase, catalase, and peroxidase due to foliar application of glycine betaine on cadmium-stressed maize plants [21]. Moreover, the application of glycine betaine at 2 mM caused progressive inductions in superoxide dismutase, peroxidase, and catalase activities in leaves and roots of two barley varieties (BH-946 and BH-959), particularly when these plants were grown in soil amended with various lead concentrations [15]. Enhancements in ascorbate peroxidase, catalase, peroxidase, and superoxide dismutase were found in the shoots and roots of lead-stressed pakchoi plants as a result of foliar treatment with glycine betaine at 0.5, 1, 2, and 5 mM [16]. Furthermore, our results align with those of [11], which reported progressive increases in peroxidase, ascorbate peroxidase, superoxide dismutase, and catalase activities in lead-stressed cotton plants following exogenous glycine betaine application. Glycine betaine triggers the antioxidant system of plants by activating the activities of various antioxidant enzymes, ultimately protecting the plant from oxidative damage [34].

Photosynthesis is a vital metabolic process that utilizes light photons to biosynthesize several organic compounds, thereby promoting plant growth [67]. In the present study, the contents of photosynthetic pigments were influenced by exposure to heavy metal and glycine betaine treatments. In a previous study, cadmium treatments markedly reduced chlorophyll and total carotenoid contents in cotton plants [59]. Additionally, chlorophylls and pigments of pakchoi plants were significantly decreased when the plants were exposed to lead toxicity [16]. The decrease in photosynthesis could be due to the inhibition of the activities of key enzymes of the Calvin cycle and the photosynthetic electron-transport chain, as well as the impairment of the gas exchange characteristics of the plants [59]. With respect to glycine betaine application, treating cotton plants with 1 mM of glycine betaine, either in normal or under cadmium-stressed conditions, resulted in obvious increases in chlorophyll and total carotenoid contents [59]. Under normal as well as cadmium-stressed conditions, glycine betaine as exogenous treatment increased the levels of chlorophylls *a* and *b* in spinach plants [24]. In a recent study, application of glycine betaine at 2 mM relieved the harmful effects resulting from lead stress via promoting the contents of chlorophylls and carotenoids in two barley varieties (BH-946 and BH-959) either under normal or lead-

stressed conditions [15]. In another study, exogenous treatment of glycine betaine at 0.5, 1, 2, and 5 mM induced the amounts of chlorophylls and total pigments of pakchoi plants grown under lead stress condition [16]. Glycine betaine is known to enhance the efficiency of the photosynthetic machinery. In addition, the increase in pigment levels may be attributed to the ability of glycine betaine to maintain the integrity of the photosynthetic machinery, the Rubisco structures, and membranes in stressful environments [27,68].

One of the mechanisms employed in plants to counteract and mitigate the adverse impacts of stress is the utilization of osmo-protectants, which decreases oxidative damage and improves plant tolerance [69]. Soluble sugar contents were progressively reduced in super black waxy maize plants grown in cadmium-amended soil compared to unstressed plants [21]. In cotton plants, different levels of lead caused inhibitions in soluble sugar levels in both the root and leaf tissues [11]. Additionally, heavy metals disrupt the ultrastructure of chloroplasts and photosynthetic pigments, leading to decreased carbon assimilation efficiency and carbohydrate production [70]. On the relieving role of glycine betaine, it has been recorded that exogenous application of glycine betaine alleviated the harmful impact of cadmium by significantly increasing the levels of soluble sugar in cadmium-stressed maize plants [21]. The carbohydrate contents of two barley varieties (BH-946 and BH-959) were enhanced as a result of a foliar supply of 2 mM glycine betaine, whether the plants were grown under normal or lead-stressed conditions [15]. Also, soluble sugar levels were higher in both the roots and shoots of lead-stressed pakchoi plants following glycine betaine application, particularly at concentrations of 1 and 2 mM [16]. In cotton plants, the application of 1 mM glycine betaine was documented to increase levels of soluble sugars either in unstressed or lead-stressed plants in both leaf and root tissues [11]. The increase in sugar contents can be attributed to the ability of glycine betaine to maintain the photosynthetic capacity by preserving the chloroplast ultrastructure and enhancing the photochemical activity of photosystem II under heavy metal stress, thereby facilitating carbohydrate accumulation [31].

Regarding proteins, our findings are in agreement with those of [59] which reported a significant decrease in soluble protein levels in the leaves and roots of cotton plants under cadmium stress. A noticeable reduction was also recorded in the total proteins of barley plants (BH-946 and BH-959 varieties) due to application of different lead stress levels [15]. The inhibition of soluble protein contents may be attributed to increased oxidative stress during metal stress, as oxidative stress can damage vital biomolecules and disrupt cellular metabolism [71]. Meanwhile, utilizing glycine betaine at 1 mM on cadmium-stressed cotton plants was found to enhance protein contents in both the leaves and roots [59]. Additionally, foliar treatment of glycine betaine when applied on cadmium-stressed maize plants significantly increased soluble protein contents [21]. In a recent study on the impact of glycine betaine, an induction in the total protein contents was observed in two barley varieties (BH-946 and BH-959) cultivated in either normal or lead-amended soils after treatment with 2 mM glycine betaine [15]. Moreover, significant increases in soluble protein contents were documented in both shoots or roots in lead-stressed pakchoi plants following treatment with different concentration of glycine betaine, particularly at 1 mM [16]. Glycine betaine not only functions as an osmoregulator but also helps maintain enzyme structure and activity, protein complexes, cell membrane integrity, and provides resistance against the harmful impacts of stressful conditions [72].

Non-enzymatic antioxidant, such as phenolic compounds, are secondary metabolites that are believed to play a role in preventing lipid peroxidation, DNA damage, and protein denaturation as well as scavenging ROS [73,74]. They are also involved in plant protection against various stresses. Phenolics are considered effective electron donors due to their hydroxyl groups, which directly contribute to their antioxidant activity [56]. Our present results observed enhancements in phenolic compounds in sugar beet plants in response to heavy metals, glycine betaine, and their interactions as a defense mechanism. In a recent investigation, levels of phenolics were conspicuously increased in the roots, stems, and leaves of cucumber plants after exposure to cadmium [2]. Furthermore, the content

of phenolic compounds was significantly accumulated in two barley varieties (BH-946 and BH-959) grown in a soil amended with lead levels [15]. Regarding application of glycine betaine, using it as an exogenous treatment was found to enhance total phenolics of stressed-maize plants [72]. Also, application of glycine betaine as foliar treatment was documented to boost phenolic compounds in stressful conditions in wheat and cotton crops [75,76]. Enhancing of non-enzymatic antioxidants in several plant species under stressful conditions by glycine betaine has been extensively investigated and documented in various studies [77]. The defensive role of glycine betaine may have a positive impact on enzymes and membrane integrity acting as an osmo-protectant, indirectly protecting against environmental stress through the mechanism of signal transduction [11].

Proline is a low molecular weight osmoregulatory molecule that modulates redox potential, reduces oxidative damage, scavenges hydroxyl radicals, and maintains cell membranes under stressful conditions. It has been documented that proline levels significantly increase in super black waxy maize plants in response to cadmium supplementation in cultivation soil [21]. Recently, proline amounts were progressively accumulated in two barley varieties (BH-946 and BH-959) grown under lead-stress conditions [15]. Consistent with our findings, a study by [2] exhibited enhancements in proline levels in cucumber roots, stems, and leaves in response to 0.5 mM glycine betaine treatments in unstressed as well as cadmium-stressed conditions. It was recorded in a recent study that glycine betaine supplementation reduced the accumulation of free proline in two barley cultivars (BH-946 and BH-959) that had been cultivated in lead-amended soil [15]. It may be suggested that the addition of glycine betaine can significantly enhance the capacity of defense against oxidative damage induced by metal toxicity [11].

The highest value of *F*-test indicated that the data showed high homogeneity. According to correlation analysis, the morphological parameters expressed as being more compatible than the chemical ones while SLR showed that Cd and Pb besides Gb2 (glycine betaine at 1 mM) described co-variable integrated regression with highly significant results compared to the control.

Basically, plants continuously produce excess reactive oxygen species (ROS) under heavy metals stress. Ions of heavy metals can bind to proteins found in a variety of plants, displacing specific cations at binding sites, leading to the generation of ROS and inactivation enzymes. As a result, enzymes are inhibited, DNA and RNA are damaged, amino acids are oxidized, and proteins are degraded. Plants produce some enzymatic and non-enzymatic antioxidants as reducing agents to scavenge free radicals and prevent oxidative reactions. A rapid increase in the production of these molecules is associated with a decrease in the oxidative burst [15]. Glycine betaine does not directly attenuate ROS but can reduce its harmful effects by activating or stabilizing ROS-inhibiting enzymes or reducing ROS formation [27]. As summarized in Figure 10, reports indicate that glycine betaine may act as an osmotic agent and enhance the plant's antioxidant system to mitigate the harmful effects caused by heavy metals [78].

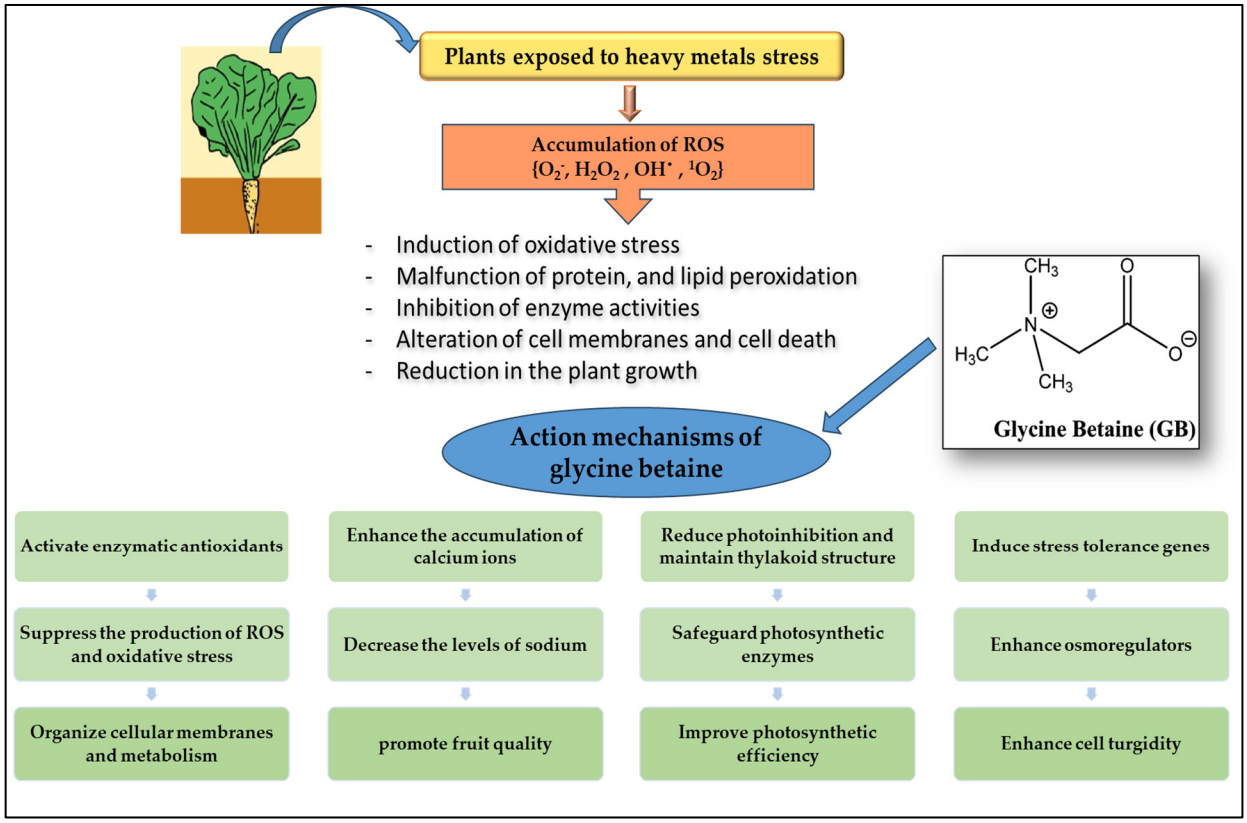

Figure 10. Overview of action mechanisms of glycine betaine to improve the plants' tolerance under heavy metal stress.

5. Conclusions

The current study was carried out to assess the efficacy of glycine betaine as a compatible solute in mitigating the adverse influence of cadmium and lead in sugar beet plants. From the obtained results, we can conclude that both cadmium and lead levels are considered toxic and negatively affect its characteristics as represented in plant growth morphology, biochemical constituents, and enzymatic activity of the tested plants. On the other hand, utilization of glycine betaine acts as a vital role in abolishing the negative effects of cadmium and lead stress via minimizing oxidative damage and enhancing plant growth and biomass, antioxidant enzymes, photosynthesis, and osmolytes in sugar beet plants. One of the distinguished points in the current study, is the reduction of the severity of cadmium and lead toxicity in sugar beet plants by using lower concentrations of glycine betaine, in contrast with some previous studies. This makes this study worthy to be included in sustainable development strategies, using modern methods and alternatives that are economical and environmentally friendly.

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