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Research Article

Physio-Biochemical and Growth Attributes of Cadmium Stressed Soybean [*Glycine Max* (L.) Merr.] With 24-Epibrassinolide

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Abstract

The hyper accumulation of Cadmium (Cd) in soil rhizosphere due to anthropogenic activities have detrimental effects on the growth and development of plants. The study was aimed at investigating the efficacy of 24-epibrassinolide (24-EBL) in enhancing growth and physio-biochemical attributes of Cd-stressed soybean. Soybean seedlings at 20 DAS (Days After Sowing) were subjected to Cd treatments alone and in supplementation of 24-$EBL_{1.0,\ 3.0\mu M}$. A notable reduction of growth attributes, photosynthetic pigments, and Relative Leaf Water Content (RLWC) accompanied by an increased leakage of ions, MDA (Malondialdehyde), H_2O_2 (Hydrogen Peroxide), and proline content resulted with Cd. 24-EBL application improved the morphological and physiological attributes including photosynthetic pigments, RLWC, biomass accumulation, and root-shoot length of plants. Largely the restoration of MDA, H_2O_2 and EL (Electrolyte Leakage) was accompanied by pooling up of the ascorbic acid and proline content in the leaves. Hence, up regulated content of osmolytes and antioxidants along with a significant improvement in the level of stress indicators provided an enhanced stress tolerance against Cd in soybean possibly through the osmoregulation and ROS (Reactive Oxygen Species) scavenging. Thus, our findings prove the efficacy of exogenously supplied 24-EBL in ameliorating Cd toxicity with improved photosynthesis, osmoregulation, membrane stabilization and regulation of heavy metal stress indicators.

Keywords: *Heavy metals, Brassinosteroids, Osmolytes, Rhizosphere, ROS, Osmoregulation*

Introduction

The presence of heavy metals in cropland areas is hazardous to productivity compromising the yield and quality of food crops. Various factors such as soil characteristics and agricultural practices using sewage sludge in farmland leads to excessive accumulation of heavy metals ions in the soil [1]. Cadmium (^{48}Cd) from group II B of the periodic table is noxious heavy metal among others. Although present naturally in the soil, Cd tends to accumulate to toxic levels due to anthropogenic activities such as mining, smelting, use of phosphate fertilizers and sewage sludge application in agriculture. Its entry in the agricultural food chain has raised serious health concerns both for human and animals [2]. Cd accumulation results in the several stress related plant symptoms like reduced growth, mineral nutrition, altered carbohydrate metabolism [3], absorption, transport, and utilization of nutrients [4], inhibited photosynthesis [5], altered enzyme activity [6], and injury signs like necrosis, chlorosis, root tip browning [7]. As a non-redox metal, Cd lacks the ability to directly produce ROS like superoxide anion ($O_2^{\cdot-}$), Singlet Oxygen ($_1O^2$), Hydrogen Peroxide (H_2O_2), and Hydroxyl Radical (OH.), however, induced oxidative the stress by disrupting antioxidant defence system [8].

Brassinosteroids (BRs), a group of polyhydroxylated steroidal hormones widely spread throughout plant kingdom plays pivotal role in physiological responses such as stem elongation, pollen tube expansion, leaf curvature and epinasty, inhibition of root growth, ethylene biosynthesis, activation of proton pumps, initiation of vascular differentiation, nucleic acid and protein synthesis, and the process of photosynthesis [9]. BRs also bolster plant resilience against biotic and abiotic stresses [10]. BRs have capacity to ameliorate the impact of extreme temperatures [11], moisture [12], drought [13], salinity stress [14], and heavy metal [15] stress. 24-epibrassinolide (EBL) has a multifaceted role in metabolic processes like seed germination [16], cell division [17], cell elongation [18], root establishment [19], reproductive development [20], senescence, abscission, and maturation [21] including the gene regulation [22]. Apart from that EBL has anti-stress properties against drought, cold, salt, and heavy metals [23].

Glycine max (L.) Merr. (Soybean) in legumes is a valuable crop for its oil (13-22%) and protein (30-48%) content. The productivity and quality of soybeans is highly impacted with Cd contaminated soil. In light of the stress-alleviating properties associated with BRs especially with EBL, present study was undertaken to assess its role in regulating growth characteristics, physio-biochemical responses in Cd-stressed soybean.

Material and Methods

Certified seeds of soybean [*Glycine max* (L.) Merr. cv. SL688] were procured from Punjab Agricultural University, Ludhiana, Punjab, India. Uniform sized healthy-looking seeds were surface sterilized with 0.01% aq. $HgCl_2$ solution followed by repeated distilled water washing and soaked overnight for inoculating with specific rhizobial strain making thick slurry of activated charcoal. Seeds were sown in earthen pots lined with perforated polyethylene bags filled using washed river sand and kept in dome-shaped outhouses under natural conditions. At the seedling stage thinning was done to maintain three healthy plants per pot and irrigated with tap water following fortnight application of nutrient medium [24].

Experimental Design and Treatments

24-EBL stock solution (10μM) was prepared in ethanol and final volume prepared with distilled water containing 0.5% tween-20 as surfactant. Following 10 combination treatments were given: Control; $Cd_{0.2mM}$, $Cd_{0.2m⊠}$ + $EBL_{1μ⊠}$, $Cd_{0.2m⊠}$ + $EBL_{3μ⊠}$; $Cd_{0.4m⊠}$, $Cd_{0.4m⊠}$ + $EBL_{1μ⊠}$, $Cd_{0.4m⊠}$ + $EBL_{3μ⊠}$; $Cd_{0.6m⊠}$, $Cd_{0.6m⊠}$ + $EBL_{1μ⊠}$, $Cd_{0.6m⊠}$ + $EBL_{3μ⊠}$. Two Cd treatments were given with a 15d gap along with nutrient medium. Seedling were allowed to grow and at day 20 stage, foliage was sprayed with 24-EBL for 3 consecutive days. Controls were supplied with nutrient medium only and foliage was sprayed with distilled water containing tween-20 without EBL. Fresh leaves were harvested at the reproductive stage (90 DAS) and assessed for various physiological and biochemical parameters. Growth parameters were analysed at the end of experiment.

Physiological and Biochemical Analysis

Standardized protocols were followed to determine the total chlorophyll [25] and carotenoid content [26], electrolyte leakage [27] and relative leaf water content (RLWC) [28], proline content [29], Hydrogen Peroxide (H_2O_2) content [30], Ascorbic Acid (AsA) content [31], MDA content [32]. Observations were made by measuring Optical Density (O.D.) with thermo-scientific Evolution-201 UV-Visible spectrophotometer.

Growth Analysis

Growth attributes such as the root-shoot length (cm), fresh and dry weight (g) were analysed at harvest. Fresh weight was recorded immediately after removing plant carefully, followed by oven drying at 60°C till weight measurement become stable.

Statistical Analysis

The data was subject to One-Way Analysis of Variance (ANOVA) using SPSS-16 software with probability level of 5%. Mean differences obtained by performing Fisher's LSD (Least Significant Difference) post-hoc test was compared by calculating LSD value. Mean values were significant at $P \leq 0.05$. All values were in triplicates (n=3) and represented as mean ± SE (Standard Error).

Results

Physiological Attributes

Chlorophyll Content: Leaf chlorophyll content of Cd-treated plants dropped upto 30.77% ($Cd_{0.2mM}$), 53.85% ($Cd_{0.4mM}$), and 69.23% ($Cd_{0.6mM}$) in comparison to control. The green pigment loss was comparatively lesser with 24-$EBL_{1.0μM}$ foliar application *i.e.*, 11.54% ($Cd_{0.2mM}$) and 30.77% ($Cd_{0.4mM}$), and gaining +7.69% ($Cd_{0.6mM}$) to that of control. The corresponding values in 24-$EBL_{3.0μM}$ treatments were 38.46% ($Cd_{0.2–0.4mM}$) and gain of +26.92% ($Cd_{0.6mM}$) reflecting the effectiveness of EBL at 1.0 and 3.0μM concentrations against higher doses of cadmium. A notable decline in the green pigment was noticed when comparing control in low $Cd_{0.2mM}$ + $EBL_{3.0μM}$ treatment (Figure 1a).

Carotenoid Content: Leaf carotenoid content also dropped upto 38.46% ($Cd_{0.2mM}$), 53.85% ($Cd_{0.4mM}$), and 69.23% ($Cd_{0.6mM}$) in comparison to control. $EBL_{1.0μM}$ application checked the depletion of contents in Cd treatments upto +30.77% ($Cd_{0.2mM}$), 7.69% ($C_{d0.4mM}$), and 23.08% ($Cd_{0.6mM}$) when comparing control. Further, these losses in $EBL_{3.0μM}$ were 46.15% ($Cd_{0.2mM}$), 23.08% ($Cd_{0.4mM}$), and +7.69% ($Cd_{0.6mM}$). The rise of carotenoid pigment with EBL application was significant in response to Cd treatments (Figure 1b).

Relative Leaf Water Content: The percentage loss in RLWC noticed was upto 7.73% (Cd$_{0.2m⊠}$), 13.16% ($Cd_{0.4m⊠}$), and 17.23% ($Cd_{0.6m⊠}$) with Cd treatment. This loss was comparatively lesser with $EBL_{1.0μM}$ *i.e.*, upto 2.31% ($Cd_{0.2m⊠}$), 4.61% ($Cd_{0.4m⊠}$), and 12.21% ($Cd_{0.6m⊠}$); and with $EBL_{3.0μM}$ upto 10.04% ($Cd_{0.2m⊠}$), 8.14% ($Cd_{0.4m⊠}$), and 10.45% ($Cd_{0.6mM}$) in comparison to control. Thus, the application of EBL has significantly improved the RLWC under Cd stress (Figure 1c).

Electrolyte Leakage: Cadmium treatments have raised the electrolyte leakage of membranes up to 18.97% ($Cd_{0.2m⊠}$), 40.21%

(Cd$_{0.4m\boxtimes}$), and 59.38% (Cd$_{0.6m\boxtimes}$) in comparison to control. Such rise in EBL supplementation was comparatively lesser *i.e.*, upto 10.31% (Cd$_{0.2m\boxtimes}$), +8.04% (Cd$_{0.4m\boxtimes}$), and 39.79% (Cd$_{0.6m\boxtimes}$) with EBL$_{1.0\mu M}$ **and upto 32.78% (Cd$_{0.2m\boxtimes}$)**, 1.22% (Cd$_{0.4m}$), and 13.20% (Cd$_{0.6m\boxtimes}$) **with EBL$_{3.0\mu M}$ (Figure 1d).**

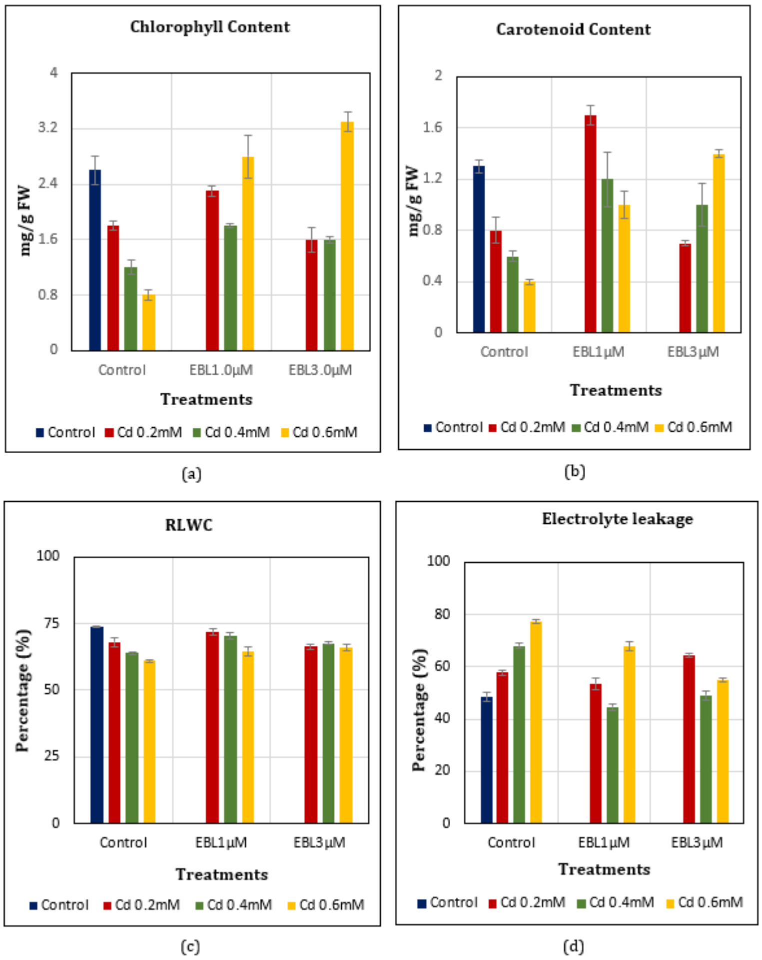

Figure 1 (a-d): Effect of Cd alone and in combination with 24-EBL on (a) total chlorophyll content (LSD$_{0.05}$=0.44) (b) Total carotenoid content (LSD$_{0.05}$=0.29) (c) RLWC (LSD$_{0.05}$=3.13) (d) Electrolyte leakage (LSD$_{0.05}$=4.06) in soybean plants. Each value represents the mean ± SE of three replicates (n=3). Mean values are significant at P≤0.05.

Morphological Attributes

Root Length: The overall length of roots reduced with cadmium and the percentage decline was 23.64% (Cd$_{0.2mM}$), 34.15% (Cd$_{0.4m\boxtimes}$), and 41.28% (Cd$_{0.6mM}$) in comparison to control. The loss of root length was comparatively lesser with EBL supplementation *i.e.*, with EBL$_{1.0\mu M}$ upto 12.57% (Cd$_{0.2mM}$), 12.20% (Cd$_{0.4m\boxtimes}$), and 28.71% (Cd$_{0.6mM}$); and with EBL$_{3.0\mu M}$ 12.57% (Cd$_{0.2mM}$), 12.20% (Cd$_{0.4mM}$), and 28.71% (Cd$_{0.6mM}$) to that of control (Figure 2a).

Shoot Length: The overall length of shoots also reduced with cadmium upto 17.48% (Cd$_{0.2mM}$), 44.72% (Cd$_{0.4m\boxtimes}$), and 52.20% (Cd$_{0.6mM}$) to that of control. EBL supplementation has lowered the plant height losses to 10.33% (Cd$_{0.2mM}$), 16.26% (Cd$_{0.4mM}$), and 26.67% (Cd$_{0.6m\boxtimes}$) using EBL$_{1.0\mu M}$; and 23.41% (Cd$_{0.2m\boxtimes}$), +2.44% (Cd$_{0.4mM}$), and 47.40% (Cd$_{0.6mM}$) using EBL$_{3.0\mu M}$ treatments in comparison to control (Figure 2b).

Fresh Weight: In Cd alone treatments (Cd$_{0.2, 0.4, 0.6mM}$), the fresh weight of plants declined up to 22.43%, 41.12%, and 53.27% respectively. The fresh weight loss with EBL$_{1.0\mu M}$ supplementation was comparatively lesser *i.e.*, 1.87%, 13.08%, and 25.23%; and with EBL$_{3.0\mu M}$ was 36.45%, 18.69%, and 18.69% in Cd$_{0.2, 0.4, 0.6mM}$ treatments, respectively (Figure 2c).

Dry Weight: Dry weight of plants also declined with Cd$_{0.2, 0.4, 0.6mM}$ alone treatments upto *i.e.*, 31.19%, 53.61%, and 68.30%, respectively when compared to control. This loss was checked to 2.58% (Cd$_{0.2m\boxtimes}$), 19.07% (Cd$_{0.4m\boxtimes}$), with EBL$_{1.0\mu M}$; and 44.33% (Cd$_{0.2mM}$), 14.43% (Cd$_{0.4mM}$), and 31.70% (Cd$_{0.6mM}$) with EBL$_{3.0\mu M}$ supplementation (Figure 2d) in comparison to control.

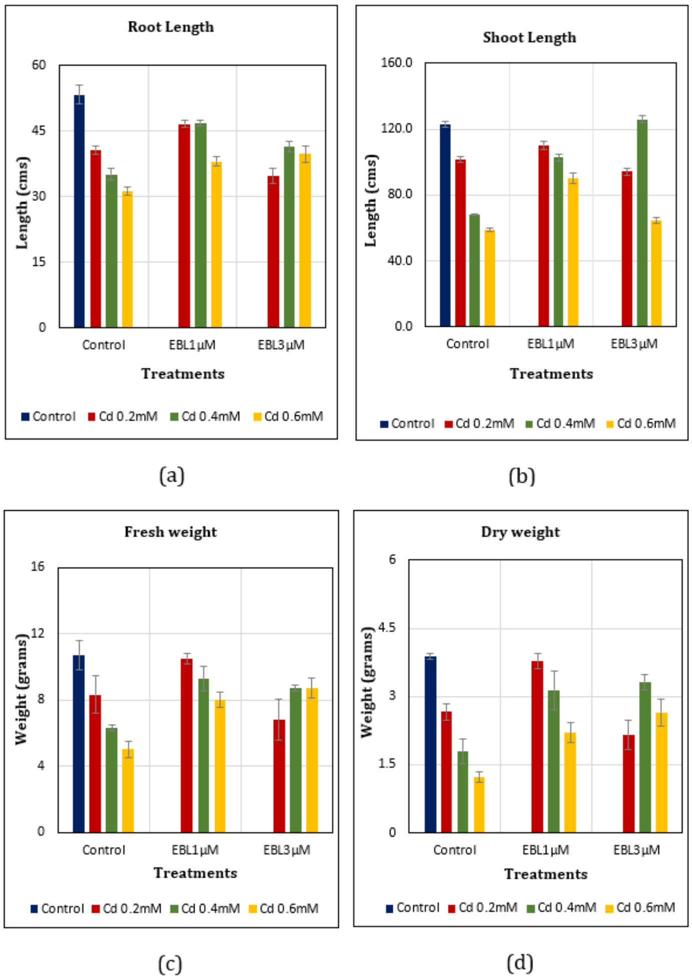

Figure 2 (a-d): Effect of Cd alone and in combination with 24-EBL on (a) Root length ($LSD_{0.05}$=4.02). (b) Shoot length ($LSD_{0.05}$=5.63) (c) Fresh weight ($LSD_{0.05}$=2.10) (d) Dry weight ($LSD_{0.05}$=0.72) in soybean plants. Each value represents the mean ±SE of three replicates (n=3). Mean values are significant at p-value ≤0.05.

Biochemical Attributes

MDA Content: MDA content of leaves as an indicator of oxidative stress increased with $Cd_{0.2,\ 0.4,\ 0.6mM}$ treatments up to 47.71%, 93.49%, and 152.05%, respectively when compared to control. With EBL supplementation, the capacity to reduce MDA content was more in Cd-treated plants lowering it up to 0.48%, 11.20% and 84.70% with $EBL_{1.0\mu M}$; and up to 80.84%, 23.86%, and 34.46% $EBL_{3.0\mu M}$ treatments (Figure 3a).

H_2O_2 Content: Hydrogen peroxide, another indicator of oxidative stress also increased with $Cd_{0.2,\ 0.4,\ 0.6mM}$ treatments showing a rise of 54.84%, 104.84%, and 169.35%, respectively in comparison to control. In the combination treatments, this accumulation was lowered to 12.90%, 25.81%, and 117.74% with $EBL_{1.0\mu M}$ and 58.06%, 37.10% and 114.52% $EBL_{3.0\mu M}$, respectively. It was noticed that EBL interaction was effective with low concentrations of Cd (0.2mM) in reducing elevated levels of H_2O_2 content (Figure 3b).

Ascorbic Acid: Ascorbic acid content of the leaves reduced with Cd treatments lowering it upto 30.65%, 50.00%, and 66.13% in $Cd_{0.2,\ 0.4,0.6mM}$ treatments, respectively, in comparison to control. The decline in EBL application was moderate *i.e.*, 4.84%, 20.16%, and 50.81% using $EBL_{1.0\mu M}$, and 30.65%, 34.68% and 31.45% using $EBL_{3.0\mu M}$, respectively (Figure 3c).

Proline Content: Proline, an osmo-protectant got accumulated in Cd-stressed leaves with a rise of 22.53%, 45.77%, and 81.21% in $Cd_{0.2,\ 0.4,\ 0.6mM}$ treatments, respectively in comparison to control. EBL application further led to a rise upto 42.18%, 78.05%, 102.87% in case of $EBL_{1.0\mu M}$ and 25.68%, 91.10%, and 122.96% in $EBL_{3.0\mu M}$, respectively (Figure 3d).

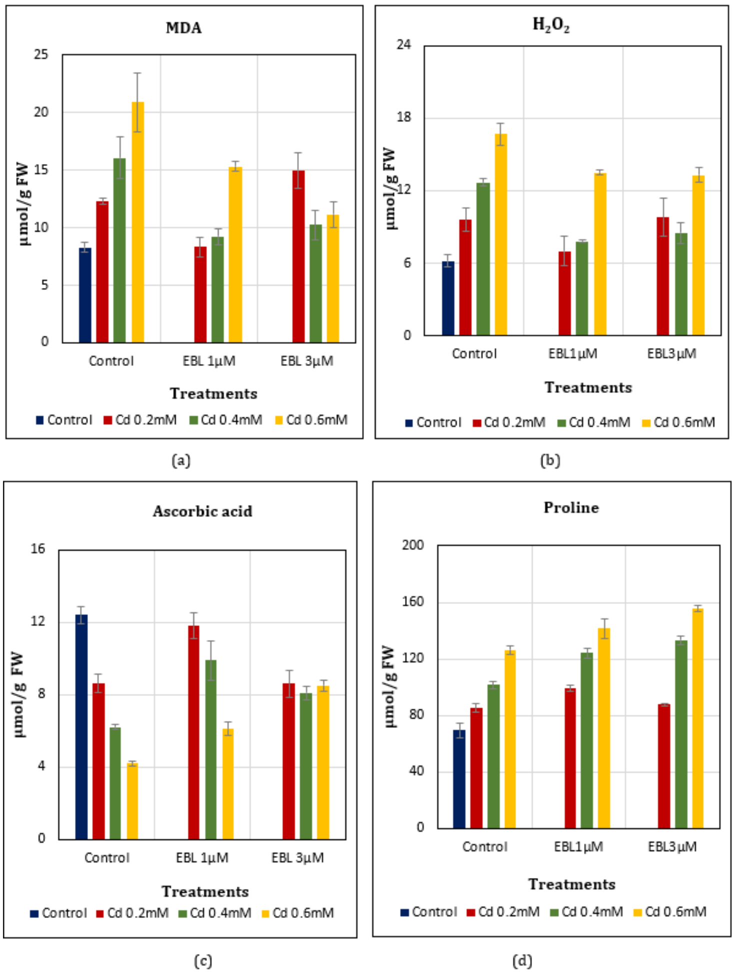

Figure 3 (a-d): Effect of Cd alone and in combination with 24-EBL on (a) MDA content ($LSD_{0.05}$=3.84) (b) H_2O_2 content ($LSD_{0.05}$=2.49) (c) Ascorbic acid content ($LSD_{0.05}$=1.65) (d) Proline content ($LSD_{0.05}$=10.8) in soybean plants. Each value represents the mean ±SE of three replicates (n=3). Mean differences are significant at p-value ≤ 0.05.

Discussion

Cadmium effects on the crop plants include the inhibited plant growth, seed germination and disrupted water balance [33]. Our findings clearly indicate that Cd-induced stress diminished growth parameters like root-shoot length and fresh-dry biomass accumulations, lowered photosynthetic pigments and RLWC accompanying the increased leakage of ions. Earlier reports have also indicated that elevated Cd levels impede cell and overall plant growth of crops like alfalfa, wheat, and spinach [34]. Such inhibitions by cadmium were attributed to the interference with cell division and cell elongation rate mainly through irreversible inhibition of proton pump [35]. The present study has indicated that supplementing EBL to Cd-stressed soybean minimized the loss of growth attributes. Consistent with our findings, many reports have suggested the exogenous application of EBL enhanced the growth attributes of Cd stressed crops such as tomato, radish, cucumber and chickpea [36,37]. EBL is involved in cell elongation, regulation of genes responsible for XTHs (Xyloglucan Endotransglucosylase/Hydrolase) activity that influence cell wall modification and expansion, cellulose synthesis, and sucrose synthesis [38].

Numerous studies have highlighted the primary site of action of Cd as photosynthetic pigments like chlorophyll and carotenoid [39], up regulating chlorophyllase activity and degradation of chlorophyll [40]. Cadmium also inhibits the production of δ-amino-laevulinic acid and proto-chlorophyllide reductase, essential for the biosynthesis of chlorophyll [41]. Our study has confirmed the promotory role of EBL in synthesis of carotenoids in Cd stressed soybean leaves. The exogenous application of 24-EBL promotes accumulation of carotenoids and chlorophyll by up regulating their genes in T_1 generation [42]. The reduced RLWC of cadmium stressed leaves also improved with EBL application in our case. This decreased RLWC was attributed to reduced water absorption under stress conditions [43]. It was reported earlier that EBL supplementation restores RLWC of Cd-stressed cucumber seedlings [44].

Cadmium induced ROS production cause peroxidation of the critical cellular components such as lipids, proteins, and hampers the metabolic processes [45]. Elevated levels of H_2O_2 followed by MDA disrupt the membrane fluidity causing the electrolyte leakage, inhibited enzyme activity and protein channelling [46]. In our study, various stress indicators were elevated like electrolyte leakage, MDA content, H_2O_2 content, proline content and accompanied by reduced ascorbic acid contents. MDA, the by-product of polyunsaturated fatty acid decomposition in cell membrane, is a recognised marker of the oxidative stress [47]. Brassinosteroids can modify the structure and stability of membranes under stress [48]. Therefore, EBL application promotes the membrane stability accompanying reduced lipid peroxidation [49]. Earlier reports have also indicated that application of EBL reduced the MDA, H_2O_2 and ion leakage under heavy metal stress [50,51]. EBL application allows proline accumulation that protects the cells from oxidative damage by maintaining nutrient uptake through water transport [52,53]. The EBL supplementation was able to restore ascorbic acid content in our case that gets oxidized to dehydroascorbic acid with ROS [54]. Both proline and ascorbic acid levels were able to increase the stress tolerance against Cd. Hence, a significant improvement of these stress indicators with 24-EBL supplementation improving the Cd stress tolerance is reported in soybean.

Conclusion

Cd above a particular threshold in the soil disrupts the normal physiological and metabolic functions of plants. The present study has indicated that 24-EBL application as a foliar spray to Cd-stressed soybean plants ameliorates the toxic effects by improving growth profile, physiological and biochemical attributes. 24-EBL application has improved the level of stress indicators against Cd induced oxidative stress proving the efficacy of the treatment.

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Conflict of Interests

There is no conflict of interests.

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