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Gibberellic Acid Alleviates Cadmium-Induced Seed Germination Inhibition through Modulation of Carbohydrate Metabolism and Antioxidant Capacity in Mung Bean Seedlings

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Abstract: Gibberellins (GA) are the decisive players in seed germination whose functionality could be adversely affected by the presence of cadmium (Cd); however, the underlying mechanisms are unclear. Eco-toxicological effects of Cd (0, 25, 50, 75, and 100 µM) on the early stages of ontogenesis in a mung bean variety (ML-2056) were investigated. Seed germination characteristics along with Cd-tolerance index were recorded at the seventh day of germination. Additionally, endogenous gibberellic acid (GA3) level, amylase activity, oxidative stress, and the antioxidant defense system were also investigated in Cd-stressed germinating seedlings. Results revealed that Cd reduced seed germination and interfered with GA synthesis in a concentration-dependent manner. Further, to validate the role of GA in Cd-tolerance, experiments were executed to explore the effect of seed priming with GA3 and its biosynthesis inhibitor paclobutrazol (PBZ) on ML-2056 under Cd stress. Application of GA3 improved the activities of amylase and carbohydrate-metabolizing enzymes, the antioxidant defense system, and sustained lower H_2O_2 and TBARS contents, whereas PBZ caused a significant reduction in growth and decreased endogenous GA3 content in Cd-stressed ML-2056, suggesting a crucial role of GA synthesis in reversing Cd-induced negative effects. Overall, GA synthesis played a crucial role in mitigating Cd toxicity in mung bean, which might be used as a criterion for developing Cd-stress-tolerant genotypes.

Keywords: amylase; antioxidants; paclobutrazol; cadmium; carbohydrate metabolism; gibberellins

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1. Introduction

Heavy metals (HMs) are natural non-biodegradable constituents of the Earth's crust that accumulate in excess and persist indefinitely in the ecosystem due to prevalent anthropogenic activities [1]. Contamination of arable soils with HMs is an ever-growing worldwide problem and a great environmental menace. Amongst various HMs, cadmium (Cd) is a non-essential element, which raises concern owing to its toxicity to both plants and animals even at low concentrations coupled with its propensity for bio-enrichment through the food chain [2]. Agricultural soils are constantly being polluted with Cd due to the increased use of irrigation water containing industrial effluents, synthetic fertilizers, pesticides, sewage sludge, and livestock and poultry manure [3]. The Agency for Toxic Substances and Disease Registry (ATSDR) has listed Cd at the seventh position on its priority list of hazardous substances [4]. At the whole plant level, Cd interacts with many vital processes, resulting in poor growth and low biomass production. The sensitivity of

plants to Cd toxicity is influenced not only by Cd concentration and plant species but also varies with plant developmental stages [5].

Seed germination and subsequent early seedling growth represent important phases of the plant life cycle, which are highly sensitive to HMs including Cd, since defense machinery is not fully developed during early developmental stages [6]. The germination phase is the first line of exchange of materials between plant and environment, which commences with imbibition of water by the dry seed and terminates with the extension of the embryonic axis and appearance of the radicle through seed coat [7,8]. In the course of imbibitions, an inactive quiescent seed is changed into a dynamic metabolizing system. Gibberellins (GA) (synthesized in embryos) stimulate the aleuronic layer to synthesize hydrolytic enzymes (α-amylase) which diffuse into the endosperm and catalyze the degradation of reserve materials for embryo growth [9]. Thus, seed germination is a highly complex transitional stage, which is regulated by both exogenous and endogenous factors. When medium surrounding the seed is contaminated with Cd, an impediment in germination is observed [10], which could be co-related with several disorders in the physiological and biochemical sequence of events linked with germination metabolism. Cd-induced alterations in seed germination would be detrimental for the seedling establishment and healthy growth of plants, ultimately compromising crop yield. Thus, examining the impact of Cd on this stage would be the novel approach to comprehend the toxic mechanisms instigated with the hyper-accumulation of Cd in plants and, hence, needs critical exploration which will merit further investigations.

Exogenous application of plant growth regulators (PGRs) is considered a promising approach under adverse environmental stress conditions, which enhance plant endurance. In the past, several researchers have demonstrated the potential of PGRs in alleviating HM stress in plants, such as GA, salicylic acid, jasmonic acid, auxins, brassinosteroids, etc. [11–14]. Among these, GA (a multi-functional signaling molecule) in particular has been found to be associated with seed germination, water uptake, dormancy breaking, synthesis of hydrolases, and defensive mechanisms inducing plant stress tolerance [12,15]. It has been reported that the endogenous content of GA is influenced by its rate of production and activity of antioxidant systems [16]. GA production has been reported in sorghum (*Sorghum bicolor*) seeds during germination [17]. Furthermore, GA could result in an increased net photosynthetic rate, amylase activity, carbohydrate metabolism, and an activated antioxidant defense system, which could ameliorate Cd-triggered growth inhibition and oxidative disruption [18–20]. Paclobutrazol (PBZ), a chemically synthesized PGR, is a triazole-type inhibitor of GA biosynthesis that hinders plant growth and development [21]. It blocks the action of ent-kaurene oxidase, an enzyme in the GA biosynthetic pathway that facilitates the oxidation of ent-kaurene to ent-kaurenoic acid [22].

Legume species are well known for their ability to fix atmospheric nitrogen, increase soil fertility, and restore disturbed ecosystems leading to increased crop productivity. These characteristics together make legumes extremely interesting crop plants for evaluating the toxic effects of HMs [23]. Among various legume crops grown in India, mung bean (*Vigna radiata*) occupies an important place because of its 25% excellent protein quality and high digestibility due to low flatulence [24]. It is an important source of food for human diet, feed and fodder for animals, and green manure. Mung bean stands third after chickpea (*Cicer arietinum*) and pigeon pea (*Cajanus cajan*) in India among pulses. It occupies a 2.936-million-hectare area and contributes 1.28 million tons in pulse production in the country [24]. In recent studies, sprouts of mung bean after germination have shown significant biological activities including secondary metabolites production [25]. In addition, the initial phases of germination are suggested to enhance the nutritional and therapeutic properties of mung bean because critical biosynthetic enzymes are activated at this time. However, previous reports have highlighted the sensitive behavior of mung bean against Cd stress leading to impeded crop productivity and yield [26,27]. In contrast to numerous plant species belonging to grasses and cereals, recent research has shown that leguminous crops are more vulnerable to Cd. As a result, even at lower metal concentrations, leguminous crops such as

mung bean experience severe biomass output restrictions. The basis for selecting Cd stress in mung bean in our study is that little information is available with regard to Cd tolerance in mung bean (especially at the germination stage), resulting in slow advancement in improvement of this particular plant species.

Since the last decade, phytohormones have been the zealous topic of interest among researchers worldwide; however, the imperative roles of GA3 in Cd response and tolerance, especially at the seed germination stage, have not been thoroughly explored. Keeping the plausible justification in mind, the present study was undertaken to decipher the mechanism of Cd stress tolerance in mung bean under the application of GA3. The experiment was conducted in three sets to (i) determine the response of mung bean variety ML-2056 towards Cd stress in terms of germination, seedling behavior, and various physiological and biochemical attributes, (ii) decipher the mechanism(s) of Cd tolerance induced by application of GA3, and (iii) examine the influence of PBZ, given as a GA biosynthesis inhibitor, on Cd tolerance in mung bean. It is expected that the information acquired through the present investigation would impart insights into the role and mechanism(s) of GA3-mediated Cd tolerance ability at the seed germination and seedling stages, which in turn might assist in crop improvement programs for developing Cd stress tolerance.

2. Materials and Methods

2.1. Plant Material, Germination Conditions and Treatments

Seeds of mung bean (variety ML-2056) were obtained from Punjab Agricultural University, Ludhiana, Punjab, India. Healthy seeds of uniform size and color were surface sterilized in 0.1% sodium hypochlorite solution (*v*/*v*) for 5 min, rinsed 3–4 times and soaked in distilled water for 12 h at 25 ± 2 °C. In the first set of experiments, the seeds were evenly placed in sterilized glass Petri dishes (15 cm diameter) lined with a double layer of sterilized filter paper circles (Whatman No. 1) and moistened with 10 mL of cadmium sulphate ($CdSO_4$) solution with varying concentrations of Cd (0, 25, 50, 75, and 100 µM). In the second set of experiments, seed priming was conducted with GA3 solution (10 µM) for 12 h in 50 mL beakers. Primed seeds were then placed in Petri dishes to germinate under 50 and 75 µM Cd, singly and in combinations. In the third set of experiments, seeds were pre-soaked with PBZ (10 µM; 12 h) and were allowed to germinate in Petri dishes under 50 and 75 µM Cd, alone and in combinations. Control plants were maintained by moistening the filter paper with 10 mL of de-ionized water. Twenty-five seeds were used and placed in each Petri dish (25 × 4 × 17 = 1700; where 4 = no. of replicates; 17 = total number of treatments; therefore, 100 seeds per treatment were maintained) for germination, covered with the lid, and incubated in a seed germinator at 25 ± 2 °C and 70% relative humidity for 7 days. The experiment was conducted with a completely randomized design, each treatment being replicated three times. In order to maintain an adequate moisture level and avoid Cd depletion, the test solutions were renewed every day. The germinated seeds were counted every day, so the minimum radicle length emergence (RLE) at 2 mm was considered as the germination count. Seven days after sowing (DAS), the Petri plates were evaluated for various traits.

2.2. Measurement of Tolerance Index (TI)

Tolerance index was calculated by the equation according to [28].

$$\frac{\text{Mean root length in metal solution}}{\text{Mean root length in control}} \quad (1)$$

Root length was measured at 7 DAS.

2.3. Measurements of Seed Germination Traits

Germination percentage was calculated by employing the formula given by [29].

$$GP = n/N \times 100 \quad (2)$$

where n = number of seeds germinated after seven days; N = total number of seeds in the Petri plates.

Germination rate was calculated by the equation according to [30].

$$GR = G1/1 + G2/2 + \ldots . + G_i/G_i \quad (3)$$

where G1 = number of seeds germinated at day 1, G2 = number of seeds germinated at day 2, and so on.

Germination index was expressed as the equation according to [29].

$$GI = \Sigma\ (Gt/Dt) \quad (4)$$

where Gt = number of germinated seeds on day t; Dt = time corresponding to Gt in days.

Coefficient of velocity was measured by the method according to [31].

$$CVG = N_1 + N_2 + \ldots\ \ldots + N_x/100 \times N_1T_1 + \ldots\ \ldots + N_xT_x \quad (5)$$

where N = number of seeds germinated each day; T = number of days from seeding corresponding to germination.

Vigor index was measured at 7 DAS by the equation according to [32].

$$VI = \text{Seedling length} \times \text{Germination percentage} \quad (6)$$

Vitality index was calculated by the equation employed by Li et al. [33].

$$VI = \sum (Gt/Dt) \times S \quad (7)$$

where Gt = number of germinated seeds on day t; Dt = time corresponding to Gt in days;

$$S = \text{seedling length.} \quad (8)$$

Mean daily germination was calculated by the method according to [30].

$$MDG = GP/d \times 100 \quad (9)$$

where GP = total germinated seeds; d = period of germination.

2.4. Determination of Total Proteins and Lignin Content

Total proteins were determined according to the method of [34]. An amount of 100 mg of plant tissue (radicle and plumule) was homogenized in 5 mL phosphate buffer (pH 7.0). The extract was centrifuged at 15,000× *g* for half an hour. The residue was re-extracted with 3 mL phosphate buffer and the supernatants were pooled together. An amount of 0.1 mL of this extract was added to 5 mL of protein reagent and the contents were mixed thoroughly. The OD was taken at 595 nm on a Double Beam UV-190 spectrophotometer (Labnics Equipment, Glasgow, UK) against reagent blank prepared from 0.1 mL of appropriate buffer and 5 mL of protein reagent. The weight of protein was plotted against the corresponding absorbance, resulting in a standard curve used to determine the protein in an unknown sample.

Lignin content was determined following the method described by Gutsch et al. [35]. Powdered deep frozen root tips were mixed with 40 mL of 80% methanol, sonicated for 10 min, and shaken for 4 h at room temperature. The homogenates were subsequently centrifuged at 3700× *g* and the pellet was washed five times with 80% ethanol. The isolated cell wall residues (CWRs) were dried at 45 °C for 24 h and served as material for analyzing lignin. An amount of 5 mg of CWRs was digested with 2.6 mL of 25% acetyl bromide in glacial acetic acid for 2 h at 50 °C. Samples were cooled on ice and transferred to a solution containing 10 mL of 2 M sodium hydroxide (NaOH) and 12 mL glacial acetic acid. Subsequently, 1.75 mL of 0.5 M hydroxyl ammonium chloride was added, the

volume was adjusted to 30 mL with glacial acetic acid, and it was centrifuged at 3000× *g* for 15 min. Lignin content was measured spectrophotometrically (280 nm, extinction coefficient ε = 22.9 g^{-1} cm^{-1}) and was expressed in µmol g^{-1} dry matter.

2.5. Measurement of Endogenous Gibberellic Acid (GA3)

For quantifying GA3, one replicate each of control and treated seed samples was evaluated on the 2nd day of imbibition when the first seedling emergence was visible. GA3 was extracted, purified, and quantified according to the procedure as previously described by [36] and [37] and modified by [38]. Seed samples were grounded in liquid nitrogen, homogenized in 10 mL cold 80% aqueous extraction medium containing 4 parts methanol and 1 part *v*/*v*, and maintained at 4 °C overnight. The leached liquor was centrifuged at 13,000× *g* for 15 min, and the supernatant was collected and evaporated to 1/3 of the initial volume under low pressure. It was then discolored with an equal volume of petroleum ether 3–5 times and the pH was adjusted to 8.0. Afterwards, 0.1 g polyethylene polypyrrolidone (PVPP) was added, and then the solution was shaken for 30 min to remove phenols. The solution was filtered; the pH was readjusted to 3.0 and extracted 3–5 times with the same volume of ethyl acetate. The solution was concentrated to dryness and the residues were dissolved in a phosphate buffer with a pH of 3.5. All the hormones were purified using solid phase extraction (SPE) and the test solution was filtered using a microporous poly tetra fluoroethylene (PTFE) membrane (0.22 mm). For RP-HPLC analysis, the extracts in the vials were injected into an HPLC system equipped with a Waters e2695 separations module (Waters, Milford, MA, USA), Waters 2489 UV detector (Waters, Milford, MA, USA), and Waters symmetry® C18 column (4.6 mm × 250 mm, 5 mm) using acetonitrile, methanol, and 0.6% acetic acid (5:50:45, *v*/*v*/*v*) as the mobile phase [35,36,39]. An amount of 10 mL of the test sample was allowed to flow through column, with elution at a flow rate of 1 mL/min at 30 °C. Standard solution of GA3 (purchased from Sigma-Aldrich, Bangalore, India, as gibberellic acid, 90% HPLC grade) was freshly prepared in ethanol by an appropriate dilution of the stock solution. The retention time of GA3 was determined using its standard compounds. GA3 was identified based on the retention time and absorbance (determined spectrophotometrically of the eluting peaks by comparison with standard compounds.

2.6. Measurement of Total Amylase Activity

The activities of total amylase in seeds germinated in different concentrations of Cd solution were measured by the method of 3,5-dinitrosalicylic acid (DNS) [40]. The DNS reagent was obtained from 1 g of 3,5-dinitrosalcylic acid dissolved in 20 mL 2N NaOH, adding 30 g double tartrate of natrium and potassium and completed with distillate water to obtain 100 mL of solution. Enzyme extract was prepared by first freezing the weighed amount of cotyledons (1 g) in liquid nitrogen to prevent proteolytic activity followed by grinding with 10 mL extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA). Brie was passed through 4 layers of cheesecloth, the filtrate was centrifuged for 20 min at 15,000× *g*, and the supernatant was used as the enzyme. The reaction mixture was prepared by mixing 0.5 mL of seedling extract of different concentrations of Cd present in different test tubes and 1 mL of DNS in each test tube. Afterwards, 10 mL of water was added to each test tube and they were placed in a boiling water bath for 5 min. The reaction mixture was cooled at room temperature and absorbance of each sample was measured at 546 nm and expressed as mg $seed^{-1}$ h^{-1}.

2.7. Measurement of Total Soluble Sugars

Total soluble sugars (TSS) were estimated by the method of [41]. An amount of 500 mg freshly harvested plant material (plumule) was crushed in 5 mL of 95% (*v*/*v*) ethanol. The insoluble fraction of the extract was washed twice with 5 mL of 70% ethanol. All soluble fractions were centrifuged at 3500× *g* for 10 min. The supernatants were pooled together for estimation. TSS were analyzed by reacting 0.1 mL of the alcoholic extract

with 3 mL of freshly prepared anthrone and placing them in a boiling water bath for 10 min. After cooling, the absorbance at 625 nm was determined on a Double Beam UV-190 spectrophotometer (Labnics Equipment, Glasgow, UK). A standard curve was prepared using graded concentrations of glucose.

2.8. Measurement of Reactive Oxygen Species (ROS)

Superoxide anion (O_2^-) was estimated by the procedure adopted by [42]. The seedling samples (radicle and plumule) were homogenized in 3 mL of 65 mM phosphate buffer (pH−7.8) followed by centrifugation at 4500× *g* for 10 min. The reaction mixture contained 0.9 mL of 65 mM phosphate buffer, 0.1 mL of 10 mM hydroxylamine hydrochloride, and 1 mL of supernatant plant extract. After incubation at room temperature (25 °C) for 20 min, 1 mL of 17 mM sulphanilamide and 1 mL of 7 mM *α*-napthyl were added. After the reaction at 25 °C, 1 mL diethyl ether was added and centrifuged at 1500× *g* for 5 min. The absorbance was recorded at 530 nm using a Double Beam UV-190 spectrophotometer (Labnics Equipment, Glasgow, UK). A standard curve with NO_2^- was established to calculate the production rate of O_2^- from the chemical reaction of O_2^- and hydroxylamine and was expressed as μM g^{-1} FW^{-1}.

Hydrogen peroxide (H_2O_2) content was measured in plant tissues as described by [43]. The plant sample (100 mg) was extracted with 5.0 mL of trichloro-acetic acid (TCA), 0.1% (*w*/*v*), in an ice bath and the homogenate was centrifuged at 12,000× *g* for 15 min. To 0.5 mL of the supernatant, 0.5 mL of phosphate buffer (pH 7.0) and 1.0 mL of potassium iodide were added. The absorbance of the mixture was measured at 390 nm. H_2O_2 content was determined using an extinction coefficient of 0.28 μM^{-1} cm^{-1} and expressed as μ M g^{-1} FW.

Lipid peroxidation was determined by estimating the content of TBARS as described by [44]. Fresh leaf tissues (500 mg) were ground in 0.25% 2-thiobarbituric acid (TBA) in 10% TCA using a mortar and pestle. After heating at 95 °C for 30 min, the mixture was rapidly cooled in an ice bath and centrifuged at 10,000× *g* for 10 min. To 1 mL aliquot of the supernatant, 4 mL of 20% TCA containing 0.5% TBA were added. The absorbance of the supernatant was read at 532 nm and corrected for non-specific turbidity by subtracting the absorbance of the same at 600 nm. The content of thiobarbituric acid reactive substances (TBARS) was calculated using the extinction coefficient (155 mM^{-1} cm^{-1}).

2.9. Electrolyte Leakage (EL) in Seedlings

The electrolyte leakage was determined according to the method of [7]. Fresh samples (2.5 mg) of plumule and radicle were cut into thin slices and put into test tubes containing 25 mL of distilled water. Tubes were kept for 24 h at room temperature in the dark. After incubation, electrical conductivity (EC_1) of the solution was recorded using a conductivity meter (BANTE DDS-12W,). The samples were subsequently frozen at −80 °C to completely destroy the tissues and release all electrolytes. Final electrical conductivity (EC_2) was determined after the defrosting of the samples. The EL was expressed as a percentage by the formula:

$$EL\% = (EC_1/EC_2) \times 100. \quad (10)$$

2.10. Antioxidant Enzymatic Assays

Superoxide dismutase (SOD) activity was assayed by monitoring its ability to inhibit the photochemical reduction of nitrobluetetrazolium (NBT) at 560 nm [44]. One unit of SOD activity was taken as that amount of enzyme, which reduced the absorbency reading to 50% in comparison with tubes lacking enzyme. Enzyme activity was expressed as U mg^{-1} protein min^{-1} in all the treated as well as non-treated seedlings (radicle and plumule). Catalase (CAT) activity was assayed by following the decline in absorbance of H_2O_2 at 240 nm according to the method of [45]. One unit of activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μM of H_2O_2 per minute under the assay conditions, and enzyme activity was expressed as μM mg^{-1} protein min^{-1}. Peroxidase

(POX) activity was assayed as the increase in optical density due to the oxidation of guaiacol to tetra-guaiacol [46]. Absorbance due to the formation of tetra-guaiacol was recorded at 470 nm, and enzyme activity was calculated as per the extinction coefficient of its oxidation product tetra-guaiacol € = 26.6 $mM^{-1}cm^{-1}$. Enzyme activity was expressed as U mg^{-1} protein min^{-1}. The procedure of [47] was taken to measure ascorbate peroxidase (APX) activity. The decrease in absorbance of APX at 290 nm was monitored. The assay mixture (1.0 mL) contained phosphate buffer (50 mM, pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 0.1 mM H_2O_2, and enzyme extract. APX activity was calculated using the extinction coefficient 2.8 $mM^{-1}cm^{-1}$. One unit of the enzyme is the amount necessary to decompose 1µM of substrate per min at 25 °C. Glutathione reductase (GR) activity was assayed by the method of [48] of observing the reduced glutathione (GSH)-dependent oxidation of NADPH at 340 nm. An amount of 3.0 mL of the assay mixture contained phosphate buffer (25 mM, pH 7.8), 0.5 mM GSSG, 0.2 mM NADPH, and the enzyme extract. GR activity was calculated using extinction coefficient 6.2 $mM^{-1}cm^{-1}$. One unit of enzyme is the amount necessary to decompose 1 µM of NADPH min^{-1} at 25 °C.

2.11. Fresh Biomass and Dry Biomass of Seedlings

Seven days after sowing, seedlings were harvested and their fresh weight was recorded immediately. For dry weight (DW) determination, radicle and plumule were separated from seeds and oven-dried at 70 °C for 48 h before weighing. The plant weight (fresh and dry mass) was determined by dividing the total weight of samples in each Petri dish by the number of seedlings.

2.12. Radicle and Plumule Length of Seedlings

Radicle length was measured from the root–plumule junction to the tip of the longest root and plumule length was measured from the epicotyls base to the tip of the longest emerging leaf with the help of a simple ruler at 7 DAS.

2.13. Total Chlorophyll Content in Seedlings (Plumule)

Measurement of chlorophyll concentration was made in first fully expanded leaf. An amount of 100 mg of leaf tissue was extracted in 20 mL of 80% acetone. Extracts were measured for chlorophyll (a and b) at 645 and 663 nm by a Double Beam UV-190 spectrophotometer (Labnics Equipment, Glasgow, UK) [49]. The total chlorophyll content of the supernatant was estimated according to [50].

$$Chl_a = 10.63 \times A_{663} - 2.39 \times A_{645}\ Chl_b = 20.11 \times A_{645} - 5.18 \times A_{663} \quad (11)$$

2.14. Seed Water Uptake

Seed water uptake (WU) was evaluated in the early germination stages (the first three days) according to the method of [51].

A total of 25 seeds were placed on a Petri dish with a 5 mL test medium and then incubated at 25 °C in a growth chamber in the dark. At the 3rd day of germination, all germinating seeds were carefully removed, blotted dry, and weighed quickly at different imbibition times (3, 6, and 12 h). The total water uptake (seeds + emerging seedling) was calculated based on the weight changes because of imbibition.

$$WU\ (\%) = \frac{\text{Seed fresh weight} - \text{seed dry weight}}{\text{Seed fresh weight}} \quad (12)$$

2.15. Determination of Starch Content

Starch content was estimated adopting the method of [52]. Dried samples (plumule) were chopped and sieved through a 1 mm sieve. The powdered content (0.1 g) was added to 5 mL of 80% ethanol in a 10 mL centrifuge tube. The mixture was heated at 80 °C for 30 min in a water bath shaker, and then centrifuged at 4000× *g* for 5 min. Then, 80% ethanol was used to extract the pellets and, through evaporation, ethanol was removed. The starch

in the residue was released with 2 mL of distilled water for 15 min in a boiling water bath, and then the mixture was cooled to room temperature. After that, starch was hydrolyzed for 15 min with 9.2 moL L^{-1} $HClO_4$ (2 mL; perchloric acid). After adding distilled water, the samples were then centrifuged at 4000× *g* for 10 min (4 mL). An amount of 4.6 mol L^{-1} $HClO_4$ was employed to isolate the residue (2 mL). In order to increase the supernatants to a volume of 25 mL, they were combined, collected, and mixed with distilled water. The concentration of starch was measured spectrophotometrically at an absorbance of 620 nm using an anthrone reagent and glucose as the standard. Starch content was expressed as mg g^{-1} dry weight (DW).

2.16. Determination of Enzymes Involved in Carbohydrate Metabolism

Sucrose phosphate synthase enzyme (EC 2.4.1.14), sucrose synthase enzyme (EC 2.4.1.13), and soluble acid invertase activity were analyzed following the method of [49]. The activity of ADP-glucose pyrophosphorylase was assayed by the method of [53]. The pyrophosphorolytic activity of ADP-glucose pyrophosphorylase was determined by monitoring the increase in absorbance in the course of conversion of NADP to NADPH at 340 nm.

2.17. Statistical Analyses

Data were analyzed statistically using analysis of variance (ANOVA) by RStudio (version 2022.02.3-492), and the results are presented as a treatment mean ± SE (n = 4). Results obtained from experiments were subjected to one-way analysis of variance (ANOVA). The significance of difference between exposed and control plants were tested by the least significance (LSD) post hoc test. The difference was considered significant at p levels lower than 0.05 ($p \leq 0.05$).

3. Results

3.1. First Set of Experiments

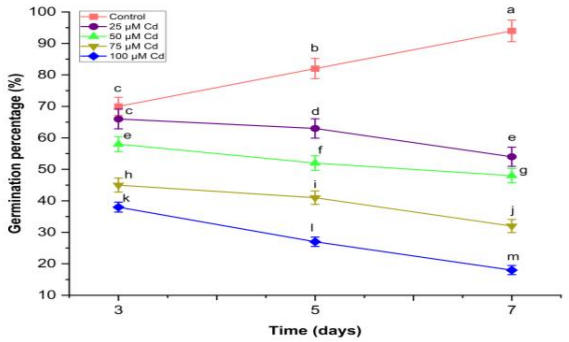
3.1.1. Seed Germination Characteristics under Different Regimes of Cd Stress

The application of different concentrations of Cd (25, 50, 75, and 100 µM) decreased all the seed germination parameters in mung bean in a concentration-dependent manner. Supplementation of the highest Cd treatment, i.e., 100 µM, significantly reduced the germination parameters, viz. germination percentage by 57%, germination rate by 59%, germination index by 59%, coefficient of velocity of germination by 81%, vigor index by 80%, vitality index by 82%, and mean daily germination by 58% in ML-2056 in comparison with their respective controls (Supplementary Table S1).

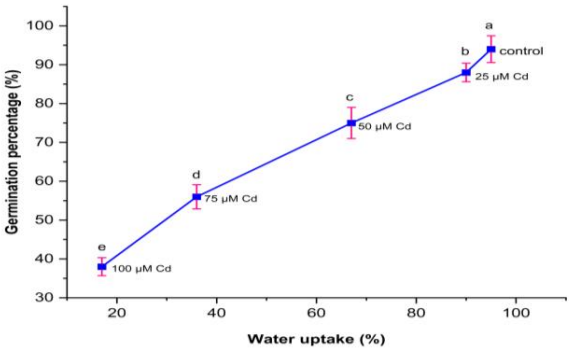
3.1.2. Tolerance Index under Different Regimes of Cd Stress

Tolerance index is a useful indicator for selecting tolerant plant varieties that could be grown in agricultural soils contaminated with high levels of metals. TI was 100% for control seedlings and the index decreased with an increase in Cd doses (Supplementary Table S1). Under 25, 50, 75, and 100 µM Cd, TI was 94, 88, 76, and 52% in ML-2056 (Supplementary Table S1).

In addition, the effect of different concentrations of Cd on germination percentage with time, germination percentage against water uptake, and germination percentage against tolerance index have also been evaluated, and results are depicted in Figure 1a–c. Our results strengthen this point: as Cd stress increased, the germination percentage significantly decreased with time, and this could be due to impaired water uptake, which delimited the growth and development of seeds under different levels of Cd. In addition, the progressive decrease in seed germination has also been found to be correlated with the tolerance index (Figure 1c), which serves as the indicative measure and ascertains that the Cd-induced toxicity increased the sensitivity of the seeds while increasing the level of the Cd stress.



(**a**)



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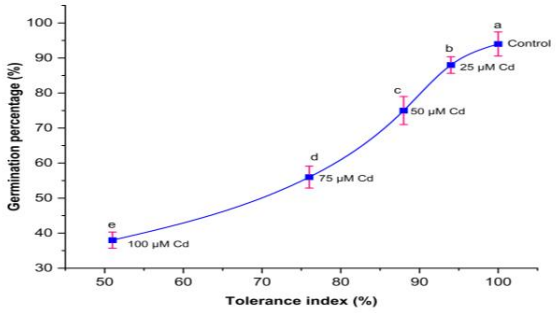

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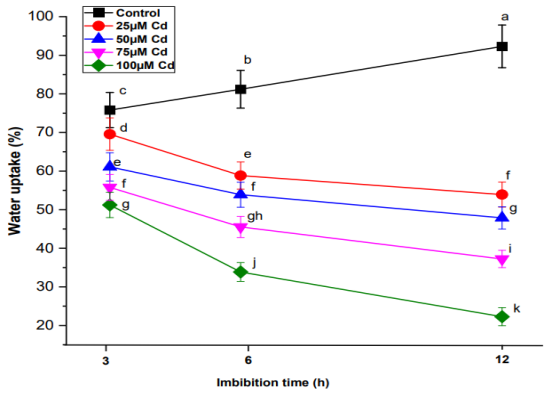


(**d**)

Figure 1. Effect of different concentrations of cadmium (Cd) on (**a**) germination percentage with time, (**b**) germination percentage against water uptake, (**c**) germination percentage against tolerance index, and (**d**) water uptake with time in mung bean seedlings. Values are mean ± S.E. of four replicates. The values containing same letter are non-significant and the values containing different letters are significantly different at ($p \leq 0.05$).

Water is essential for the emergence of seedlings; hence, water contaminated with HMs inhibits seedling appearance by hindering water uptake. In the presence of Cd, the permeability of the seed coat is affected, resulting in a disturbance in the cell–water relationship. Examination of results revealed that the total water uptake (seed and emerging seedling) efficiency significantly decreased with time in proportion to the Cd levels (Figure 1d). Out of the various Cd levels (25, 50, 75, and 100 µM), 100 µM (12 h) proved to be more toxic and decreased the values for water uptake by 75% compared with respective controls (Figure 1d).

3.1.3. Seedling Growth Traits under Different Regimes of Cd Stress

The effects of Cd on radicle and plumule length varied with the concentrations used (Supplementary Table S1). Radicle and plumule length decreased by 5% and 10% in ML-2056 as compared with controls under 25 µM Cd. At the highest Cd level (100 µM), relative to controls, decline in radicle and plumule length was 60% and 65% in seedlings. Reduction in seedling length in turn decreased their fresh and dry biomass, indicating symptoms of phytotoxicity (Supplementary Table S1). At 25 µM Cd, fresh and dry weights of seedlings decreased significantly by 12% and 21%, respectively, as compared with controls. The highest supply of Cd resulted in a sharp decrease in fresh and dry biomass of seedlings and, hence, under 100 µM Cd, 70 and 83% decreases, respectively, were observed in comparison with the controls.

3.1.4. Total Chlorophyll Content under Different Regimes of Cd Stress

Chlorophyll is a pigment molecule found in plants which takes part in photosynthesis. The pigment molecule was significantly affected in the plumule of seedlings under Cd stress leading to a disturbance in the photosynthetic machinery. Total chlorophyll content in plumule declined in response to different Cd levels in mung bean. Under 25 µM Cd, the total chlorophyll content was reduced by 7% in comparison with their respective controls. However, under 100 µM Cd, a drastic reduction in chlorophyll content was observed in mung bean seedlings, which was 60% compared with their respective controls (Table 1).

Table 1. Effect of different concentrations of cadmium (Cd) on total protein content (mg g^{-1} FW), total chlorophyll content (µg mg^{-1} FW), amylase activity (mg $seed^{-1}$ h^{-1}), total soluble sugars (mg g^{-1} FW), endogenous gibberellic acid content (GA3; µg g^{-1} FW), and lignin content (µM g^{-1} DW) in mung bean seedlings.

Treatments	Total Protein Content Radicle	Total Protein Content Plumule	Total Chlorophyll Content Plumule	Amylase Activity Plumule	Total Soluble Sugars Plumule	Endogenous Gibberellic Acid Content	Lignin Content Radical
Control	0.220 ± 0.02 a	0.330 ± 0.017 a	391 ± 16.71 a	1.04 ± 0.05 a	0.820 ± 0.015 aa	8.70 ± 1.08 a	0.141 ± 0.022 e
25 µM Cd	0.189 ± 0.026 a	0.290 ± 0.023 a	363 ± 15.89 a	0.86 ± 0.06 b	0.713 ± 0.023 b	7.22 ± 0.85 b	0.162 ± 0.012 d
50 µM Cd	0.132 ± 0.019 b	0.231 ± 0.037 b	320 ± 11.91 b	0.64 ± 0.10 c	0.565 ± 0.025 c	5.65 ± 0.72c	0.233 ± 0.031 c
75 µM Cd	0.081 ± 0.036 c	0.161 ± 0.044 c	246 ± 12.73 c	0.37 ± 0.04 d	0.352 ± 0.023 d	4.26 ± 0.31d	0.282 ± 0.046 b
100 µM Cd	0.041 ± 0.031 d	0.060 ± 0.024 d	156 ± 13.57 d	0.10 ± 0.01 e	0.164 ± 0.017 e	1.91 ± 0.97 e	0.365 ± 0.033 a

The values containing same letter are non-significant and the values containing different letter are significantly different at ($p \leq 0.05$).

3.1.5. Total Protein and Lignin Contents under Different Regimes of Cd Stress

Protein content in plants is a critical indicator of alterations in metabolism and responds to diverse stresses, including HMs. Cd treatments led to a considerable loss of the protein pool and protein content was found to be in the order plumule > radicle. In response to 25 µM Cd, the total protein contents of the radicle and plumule decreased by 14% and 12%, respectively, as compared with their controls. However, the application of 100 µM Cd reduced the number of proteinaceous molecules in the radicle and plumule to the highest degree (Table 1). Thus, proteins were sensitive targets of Cd stress, as was evidenced by considerable reduction in their contents in both radicles and plumules.

Lignin is a component of the cell wall, which protects it against any injury. Lignin content was determined in the radicle of mung bean seedlings exposed to different Cd concentrations. Results revealed that lignin content increased with an increase in Cd doses, and the increment was found to be greater at higher Cd concentrations than lower treatments. Under 100 µM Cd, lignin content was enhanced by 159% with respect to their controls (Table 1).

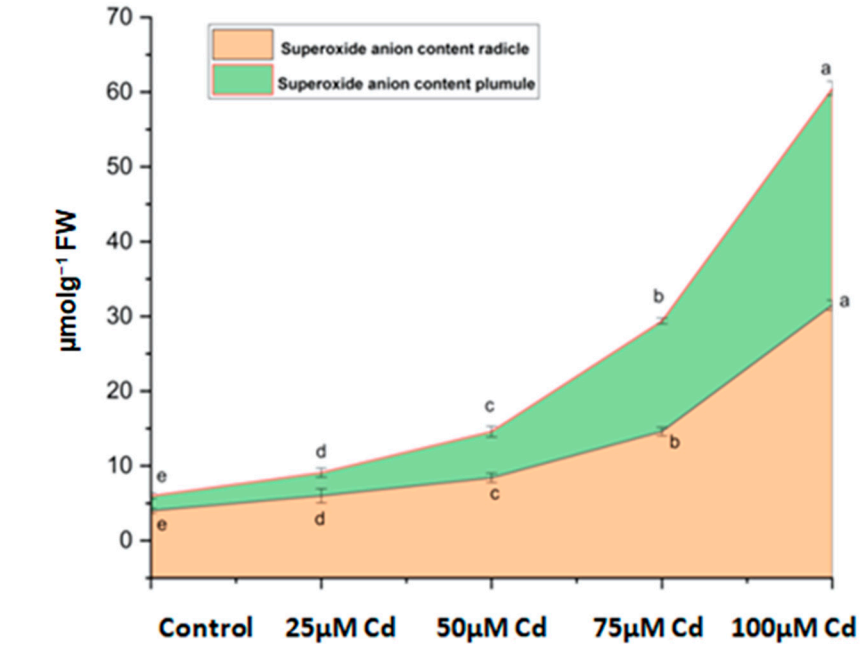
3.1.6. Endogenous Gibberellin Content, Total Amylase Activity, and Total Soluble Sugar under Different Regimes of Cd Stress

Gibberellins (GA) are the key players in seed germination as they assist in the synthesis of hydrolytic enzymes such as amylases. Starch is one of the most abundant storage materials in seeds which are degraded into simple sugars with the help of amylases. The simple sugars are then mobilized towards the embryo to provide nourishment to the germinating seedling. The functionality of GA3 as well as amylases was hampered in the presence of Cd, which in turn decreased the accumulation of soluble sugars. With respect to controls, supplementation of 25 and 100 µM Cd reduced the endogenous GA3 levels by 17% and 78%, respectively (Table 1). Similarly, total amylase activity decreased by 15% and 90% under 25 and 100 µM Cd, respectively, as compared with controls. The highest declension in total soluble sugars was witnessed under 100 µM Cd, which was about 80% compared with respective controls.

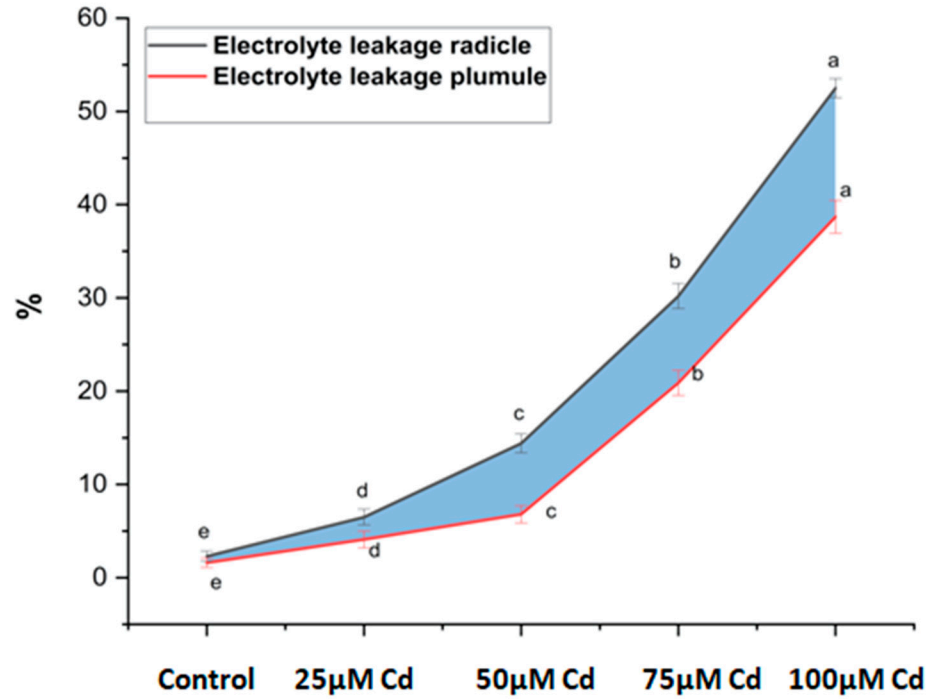
3.1.7. Oxidative Stress Markers under Different Regimes of Cd Stress

The cellular redox state, which is connected to oxidative damage, is a vital determinant of metal phytotoxicity. One of the chief consequences of HM stress is the burst of reactive oxygen species (ROS) leading to oxidative stress, and superoxide anion ($O_2^{\bullet -}$) is one of the ROS formed in plants during stressful conditions. In general, $O_2^{\bullet -}$ production was found to be proportional with increasing Cd concentrations in the germinating medium. $O_2^{\bullet -}$ levels were higher in the radicle than plumule, which suggested that Cd caused oxidative stress mainly at radicle level (Figure 2a). Maximum oxidative burst was observed under 100 µM Cd, in which seedlings exhibited 7- and 13-fold higher $O_2^{\bullet -}$ levels in the radicle and plumule, respectively, relative to control plants.

Membrane permeability is the major function of a cell, as it permits the cell to decide which type of particles can leave and enter the cell. Metal-induced ROS formation results in disruption of membrane integrity and stability, leading to the leakage of cellular contents, rapid desiccation, and even cell death. Alteration in the functioning of cellular membranes due to stress is well expressed in terms of increased permeability, which can be readily measured by the loss of electrolytes. Influence of Cd on the extent of membrane damage in mung bean seedlings was evaluated indirectly with electrical conductivity (EC) measurements of solute leakage from the cells/tissues of the radicle and plumule (Figure 2b). $O_2^{\bullet -}$ contents had a direct bearing on the leakage of ions. Radicle tissues had a higher accumulation of $O_2^{\bullet -}$ and hence exhibited a greater loss of the membrane's selective permeability than plumules. Cd-induced degradation of cell membranes increased proportionally with an increase in metal level in the external medium, which led to leakage of electrolytes. Under 25, 50, 75, and 100 µM Cd, EL was found to be 6, 14, 30, and 62%, respectively, in the radicle and 4, 7, 21, and 39%, respectively, in the plumule of mung bean seedlings as compared with controls. Thus, the results evidently showed that the imposition of Cd caused deleterious effects on the structural integrity and functioning of cellular membranes of mung bean seedlings.



(**a**)



(**b**)

Figure 2. Effect of different concentrations of cadmium (Cd) on (**a**) superoxide anion content and (**b**) electrolyte leakage on radicle and plumule in mung bean seedlings. Values are mean ± S.E. of four replicates. The values containing same letter are non-significant and the values containing different letter are significantly different at ($p \leq 0.05$).

3.1.8. Antioxidant Defense under Different Regimes of Cd Stress

Plants are well stocked with an enzymatic antioxidant defense system (SOD, POX, and CAT) to scavenge different types of ROS, thereby preventing potential cell injury and tissue dysfunction. Activities of antioxidant enzymes generally increase under stressful

conditions, which correlate well with enhanced plant metal tolerance. In light of this, SOD, CAT, and POX activities were estimated in radicles and plumules of mung bean seedlings raised under different Cd levels (Figure 3). In general, activities of SOD, CAT, and POX were higher than controls in seedlings exposed to Cd stress and increased with an increase in metal concentration in the medium. Though the activity of CAT was found to be higher as compared with POX, the percent increase in activity was higher for POX than for CAT. When 25 µM Cd was introduced in the germinating medium, results revealed that the activities of SOD, POX, and CAT increased by 34, 79, and 15% in the radicle and 38, 82, and 16% in the plumule, respectively, in seedlings. The maximum expression of ROS-detoxifying enzymes was witnessed in seedlings raised under 100 µM Cd, indicating that this metal treatment resulted in the maximum production of ROS. As a result, radicles and plumules displayed a 6- and 7 -fold increase in SOD activity, 10- and 12-fold increments in POX activity, and 4- and 5-fold enhancement in CAT activity compared with controls. Thus, mung bean seedlings seemed to be able to respond to Cd stress through stimulation of SOD, POX, and CAT activities.

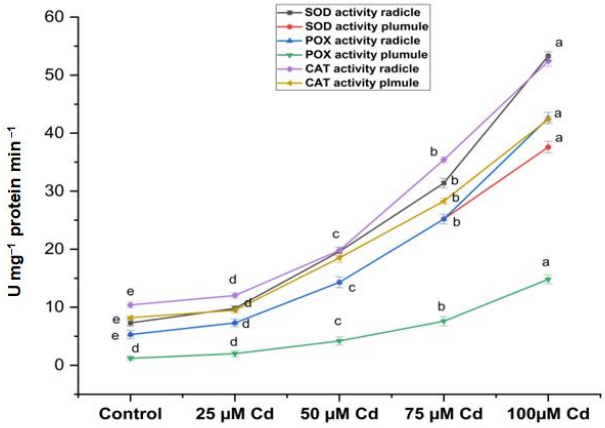


Figure 3. Effect of different concentrations of cadmium (Cd) on SOD activity, POX activity, and CAT activity in radicle and plumule of mung bean seedlings. Values are mean ± S.E. of four replicates. The values containing same letter are non-significant and the values containing different letter are significantly different at ($p \leq 0.05$).

From the results of Experimental Set I, it can be concluded that out of the four levels (25, 50, 75, and 100 µM) of Cd, minimum and maximum effects were generated in response to 25 and 100 µM. However, seedlings were found to be moderately stressed at both 50 and 75 µM concentrations, and showed comparatively less sensitivity towards the dose-dependent behavior of Cd in mung bean seedlings. Therefore, these two concentrations (50 and 75 µM) of Cd were selected for subsequent experiments to study the thematic area.

3.2. Second set of Experiments

3.2.1. Exogenous GA3 Reduces Oxidative Stress via Upregulation of Antioxidant Enzymes under Cd Stress

Supplementation of both 50 µM and 75 µM Cd led to enhanced levels of H_2O_2 and TBARS in the tested variety of mung bean (ML-2056). However, the application of higher concentration of Cd (75 µM) enhanced H_2O_2 and TBARS contents to a higher extent than a lower level of Cd (50 µM), which were 76% and 73%, respectively, when compared with their control plants. The combined supplementation of 10 µM GA3 + 50 µM Cd reduced the H_2O_2 content by about 54% and TBARS levels by about 58% in comparison with their respective 50 µM Cd-treated plants. The application of the same concentration of GA3 to 75 µM Cd-treated plants reduced the H_2O_2 content by about 33% and TBARS content by about 31% when compared with their respective 75 µM Cd-treated plants (Figure 4).

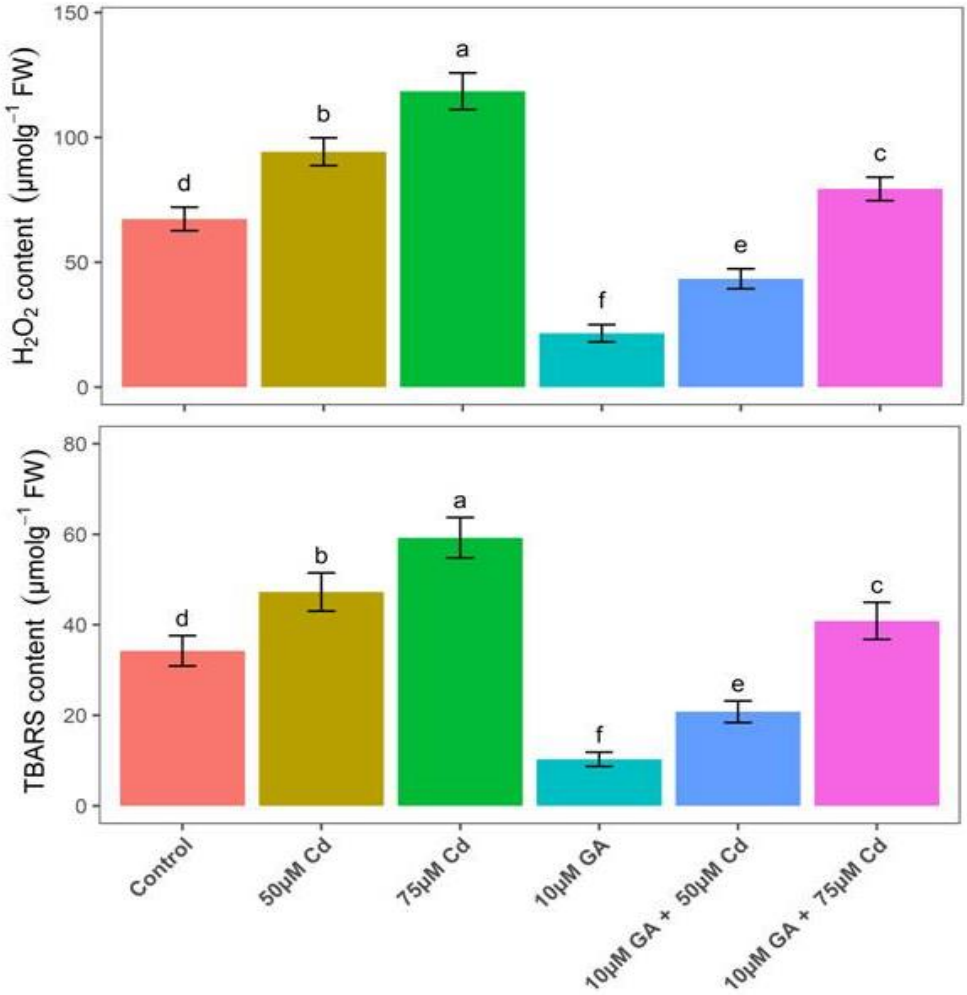


Figure 4. Effect of different concentrations of cadmium (Cd) and 10 µM gibberellic acid (GA3), singly and in combination, on H_2O_2 content and TBARS content in mung bean. Values are mean ± S.E. of four replicates. The values containing same letter are non-significant and the values containing different letter are significantly different at ($p \leq 0.05$).

In response to 50 µM and 75 µM Cd, the activity of SOD increased by about 43% and 78%, APX by about 46% and 52%, and GR by about 49% and 54%, respectively, when compared with control plants of mung bean variety ML-2056. The single supplementation of 10 µM GA3 under unstressed conditions reduced the SOD activity by about 36% and further increased the APX and GR activity by about 120% and 130%, respectively, in comparison with their respective control plants. The combined applications of 10 µM GA3 + 50 µM Cd and 10 µM GA3 + 75 µM Cd reduced the SOD activity by about 48% and 33% and enhanced the APX activity by about 86% and 92% and GR by about 90% and 95%, respectively, when compared with their respective 50 µM and 70 µM Cd-treated plants (Figure 5).

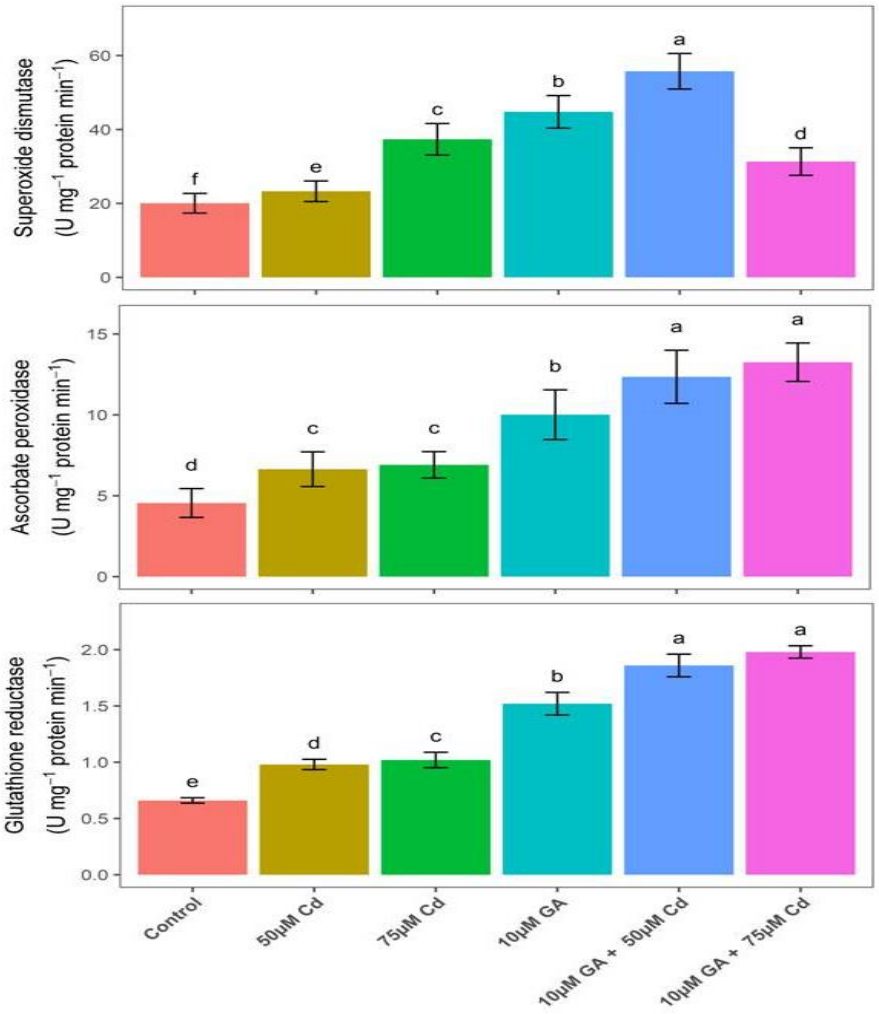


Figure 5. Effect of different concentrations of cadmium (Cd) and 10 μM gibberellic acid (GA3), singly and in combination, on SOD, APX, and GR activity in mung bean. Values are mean ± S.E. of four replicates. The values containing same letter are non-significant and the values containing different letter are significantly different at ($p \leq 0.05$).

3.2.2. Exogenous GA3 Increases Plant Dry Matter (PDM), Chlorophyll, Endogenous GA3 Content, and Amylase Activity under Cd Stress

Plants of mung bean variety ML-2056 exposed to both 50 μM and 75 μM Cd experienced reduction in PDM, chlorophyll, GA3 content, and amylase activity compared with their respective control plants (Figure 6). However, the 75 μM Cd stress exhibited higher detrimental effects and, hence, the PDM decreased by 83%, chlorophyll content by 37%, endogenous GA3 levels by 51%, and amylase activity by 64% compared with controls. The amount of 10 μM GA3 was more effective in alleviating the negative effects of Cd on the above mentioned respective parameters under 50 μM Cd than 75 μM Cd (Supplementary Table S2). As a result, supplementation of 10 μM GA3 to 50 μM Cd-stressed plants increased the PDM, chlorophyll, endogenous GA3 content, and amylase activity by about 58%, 43%, 52%, and 54%, respectively, in comparison with 50 μM Cd-stressed plants (Figure 6).

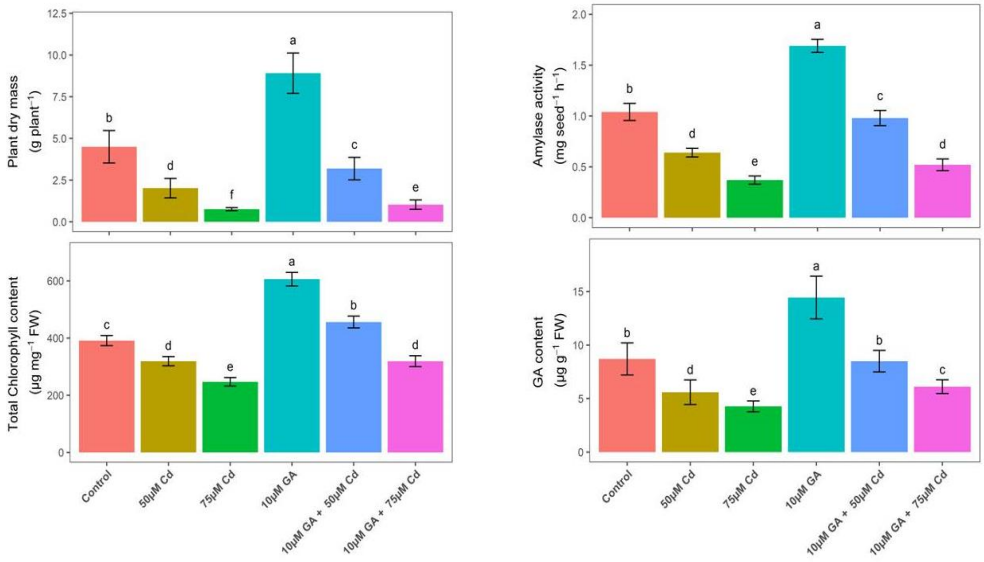


Figure 6. Effect of different concentrations of cadmium (Cd) and 10 µM gibberellic acid (GA3), singly and in combinations, on plant dry mass, amylase activity, total chlorophyll content, and GA3 content in mung bean seedlings. Values are mean ± S.E. of four replicates. The values containing same letter are non-significant and the values containing different letter are significantly different at ($p \leq 0.05$).

3.2.3. Exogenous GA3 Increases Starch Content and Activities of Carbohydrate-Metabolizing Enzymes under Cd Stress

Both 50 and 75 µM Cd reduced the contents of total soluble sugars, starch, along with the activity of ADP-glucose pyrophosphorylase in ML-2056 (Figure 7). However, higher concentrations of Cd interfered with the carbohydrate metabolism to a higher extent than lower concentrations. The combined applications of 10 µM GA3 + 50 µM Cd and 10 µM GA3 + 75 µM Cd enhanced the total soluble sugars by about 54% and 47%, starch content by about 66% and 49%, and activity of ADP-glucose pyrophosphorylase by about 69% and 47%, respectively, when compared with 50 µM and 75 µM Cd-treated plants (Figure 7). The concentrations of 50 µM and 75 µM Cd increased the activities of carbohydrate-metabolizing enzymes, viz. sucrose phosphate synthase by 129% and 125%, whereas activities of sucrose synthase decreased by about 30% and 52% and soluble acid invertase by about 35% and 65%, respectively, when compared with their respective control plants (Figure 7). Under unstressed conditions, the single supplementation of 10 µM GA3 increased the activities of sucrose phosphate synthase, sucrose synthase, and soluble acid invertase by about 139%, 44%, and 48%, respectively, in comparison with their respective control plants. However, the combined applications of 10 µM GA3 + 50 µM Cd and 10 µM GA3 + 75 µM Cd increased the activities of sucrose phosphate synthase by about 165% and 160%, sucrose synthase by about 66% and 53%, and soluble acid invertase by about 59% and 48%, respectively, when compared with their 50 µM and 70 µM Cd-treated plants (Figure 7).

3.3. Third Set of Experiments

Paclobutrazol Decreases PDM and Endogenous GA3 Content under Cd Stress

Supplementation of 50 µM and 75 µM Cd reduced the PDM by about 56% and 82% and intrinsic GA3 levels by about 38% and 53%, respectively, when compared with their respective control plants (Figure 8). However, further results concerning the application of 10 µM PBZ obtained in our study confirm the GA3-mediated alleviation of detrimental effects of Cd stress on mung bean plants. PBZ acted as an inhibitor of GA and did not exhibit a positive response in ML-2056 against Cd stress; instead, it exerted negative effects.

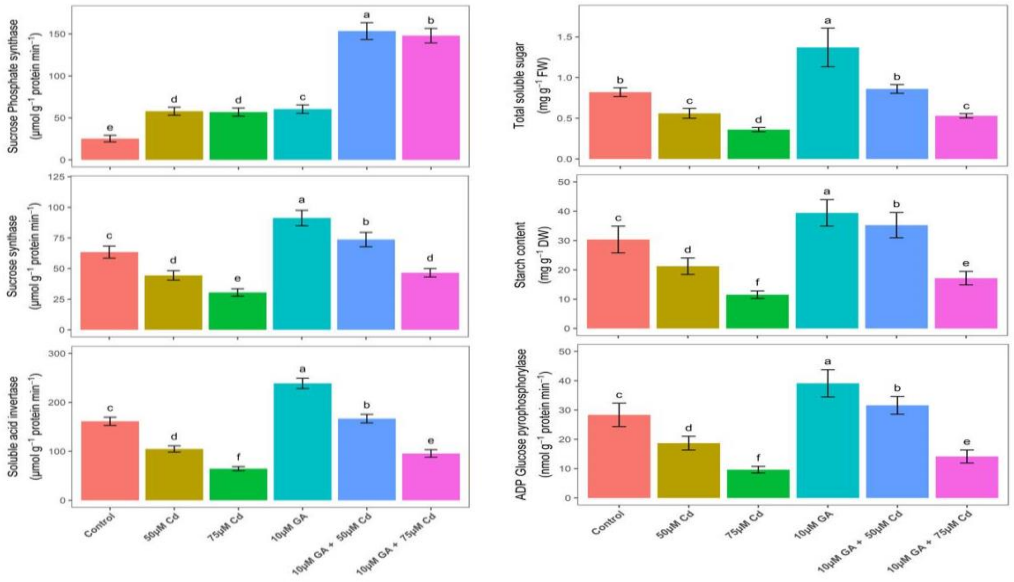


Figure 7. Effect of different concentrations of cadmium (Cd) and 10 µM gibberellic acid (GA3), singly and in combinations, on sucrose phosphate synthase, total soluble sugars, sucrose synthase, starch content, soluble acid invertase, and ADP-glucose pyrophosphorylase in mung bean. Values are mean ± S.E. of four replicates. The values containing same letter are non-significant and the values containing different letter are significantly different at ($p \leq 0.05$).

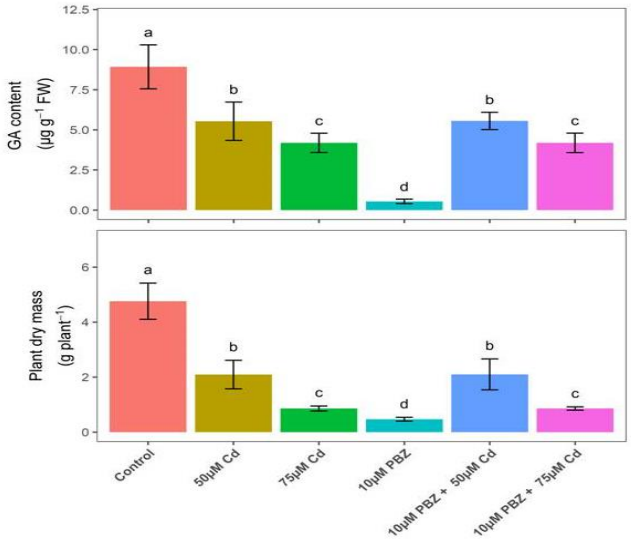


Figure 8. Effect of different concentrations of cadmium (Cd) and 10 µM paclobutrazol (PBZ), singly and in combinations, on endogenous GA3 content and plant dry mass in mung bean. Values are mean ± S.E. of four replicates. The values containing same letter are non-significant and the values containing different letter are significantly different at ($p \leq 0.05$).

4. Discussion

Cd toxicity is a serious menace for sustainable crop production around the globe. The present study elucidates the physio-biochemical facet of Cd stress, primarily in terms of gibberellins, in mung bean at the germination stage. Moreover, to date, studies dealing with exogenously sourced GA3 and PBZ-mediated stress tolerance through GA biosynthesis induction and/or inhibition in plants are scanty. To the best of our knowledge, this is the first study reporting exogenously applied GA3-mediated enhancement in Cd tolerance in mung bean, especially at the seed germination and seedling establishment stage, which has

been confirmed through the experiments pertaining to seed priming by PBZ, acting as a GA biosynthesis inhibitor.

According to the results, all the studied traits were affected by Cd, and there was a significant difference between control and stressed seeds. Our results revealed that ML-2056 had a high capacity of metal TI under Cd toxicity, which could be due to its inborn ability to endure the stress. Seed germination characteristics (germination percentage (GP), germination rate (GR), and germination index (GI) reduced as Cd concentrations increased in the growing media, which could be ascribed to the presence of toxic Cd in the germination medium. Energy production is essential for seed germination and its obstruction influences the synthesis of proteins, RNA, and DNA as energy is involved in these processes [32]. Therefore, the decline observed in GP of mung bean might be the result of poor seed viability due to decreased energy generation by the growing embryo in response to Cd. Our findings are in agreement with those reported earlier [6,54], in which Cd stress decreased the GP, GI, GR, and vigor index in different crops. Another possible reason for reduction in seed germination could be Cd-induced reduction in water absorption and transport [55]. Evidence indicates that Cd passes through the cytosol via calcium channels located in the plasma lemma, thereby altering cell–water relations [56]. According to [57], metals influence seed germination by two methods: (i) firstly due to the toxicity and (ii) secondly by affecting water uptake during the imbibition process. Few studies on pea [55] and sorghum [58] have linked inhibition of germinating seeds to decline in osmotic potential of the germination medium, especially in the presence of HMs such as Cd and Cu, causing difficulty in the absorption of water by grain. In the present study, we also witnessed a decline in water uptake by seeds of mung bean, authenticating the Cd-induced osmotic effect on germinating seeds resulting in limitation of maximum water uptake. Studies reported in *Vicia sativa* (common vetch) and *Vicia faba* (faba bean) [59,60] have correlated the metal-induced decrease in seed germination with several disturbances in the chain of events of germination metabolism. Due to reduction in GP, GR, and GI, other parameters such as vitality and vigor indices along with mean daily germination (MDG) were adversely affected in response to Cd in mung bean. In the present study, seedling size (length of the radicle and plumule) decreased under Cd stress in a concentration-dependent manner. This might be due to inhibition in cell division (reduction in meristematic cells), suppression in cell expansion, and enlargement and modifications in many physiological processes of the developing seedlings, consequently reducing the growth and biomass accumulation [61]. Because of reduction in the radicle and plumule length, the biomass (fresh and dry weights) of seedlings under study decreased significantly with an increase in Cd concentration. Such results have been inferred in different plants under Cd stress [7,62]. Ghani [63] also reported negative effects of Cd on the plumule and root length in different varieties of mung bean. The breakdown of stored food materials in seeds by Cd could also be one of the reasons for the retarded or inhibited seedling growth [64]. Furthermore, impaired carbohydrate synthesis due to the inhibitory effect of Cd on carbohydrate metabolism has also been shown to inhibit shoot and root growth [65]. Our study further revealed that Cd had a detrimental effect on total chlorophyll content in accordance with the Cd doses, which could be due to the inhibition of the chlorophyll biosynthesis pathway, breakdown of pigments or their precursors [66], or the replacement of magnesium (Mg^{2+}) with Cd^{2+} in chlorophyll frameworks [67–69]. Protein content of germinating seedlings (radicle and plumule) also decreased significantly under various Cd concentrations. Oxidative stress is one of the reasons for the disturbance in the protein metabolic pathway and damage to the protein structure. In our study, high levels of Cd affected protein contents of both the radicle and plumule by triggering the generation of O_2^- [70]. Deleterious effects of Cd on seedling cellular membranes were also reflected in terms of increased ionic leakage, clearly evidencing the impairment of membrane integrity associated with the increased generation of O_2^-. As a consequence of the loss of solutes, various macromolecules, such as glucose, soluble sugars, amino acids, etc., are leaked out, causing osmotic imbalance [7], ultimately reducing germination and seedling establishment, which were also observed in our study.

Our results also confirmed increased lignin content in mung bean radicles exposed to Cd^{2+}, which could be due to increments in the phenylpropanoid pathway [71]. In our experiments, activities of antioxidant enzymes (SOD, POX, and CAT) were elevated under Cd stress in mung bean. As POX is engaged in the cross-linking of hydroxyproline-rich glycoproteins with phenolic acids, enhanced POX activity increases the ability of plants to cope with Cd-induced oxidative stress with a rigid cell wall by synthesizing complex compounds, such as lignin, which functions as a secondary protection mechanism against oxidative damage. Enhanced lignin accumulation has been reported previously in soybean, lupine, and mung bean supplemented with Cd^{2+}/Pb^{2+} [71,72].

Seed germination is controlled by several extrinsic as well as intrinsic factors and occurs when conditions are favorable. Amongst such factors, PGRs such as gibberellins (GA) play an important role in seed germination. The present study revealed that accretion of Cd in the cotyledons resulted in decreased GA3 levels, which in turn decreased the total amylase functionality, consequently reducing the rate of starch hydrolysis and depleting energy and substrates required for seed germination and seedling growth [73,74]. Germination and post-germination of seeds are characterized by the mobilization of reserves from storage tissues and the transfer of solubilized derivatives to the growing embryonic axis. Differential decline observed in germination and radicle as well as plumule growth by Cd could be attributable partly to a lack of products of seed reserve mobilization [5,60]. Inhibition in the amylase activity in cotyledons of Cd stressed mung bean seeds further provoked the decrease in total soluble sugars content, thereby decreasing optimal growth and development of embryonic axis. Decreased soluble sugar contents detected in the mung bean embryo tissues could also be due to an obstruction in sugar mobilization by Cd from cotyledons to the embryo [75]. This suggests that Cd toxicity impaired not only the breakdown of starch by inhibiting amylase activity but also the translocation of soluble sugars, consequently decreasing the availability of nutrients for extension of the embryonic axis. Inhibition in amylase activity might have resulted from a direct effect of Cd ions on the enzyme by displacing the Ca^{2+} ions that are essential for amylase activities [76] in germinating rice seeds under Cd stress. Additionally, GA3 has been reported to enhance sulfate assimilation, which promotes GSH/phytochelatin production, which is important for plant defense mechanisms [77]. Consequently, the Cd-induced decrease in endogenous GA3 levels observed in our study might have interfered with the defense machinery of mung bean seedlings, resulting in low growth and biomass under Cd stress.

In the present study, seed priming with GA3 in stress-free and Cd-treated seedlings accelerated the amylase activity (Figure 6). GA accelerates the synthesis and translation of mRNAs required for amylase activity, which in turn is responsible for the breakdown of seed reserves [78]. Starch is the most copious storage substance in all seeds, and evidence suggests that, in germinating seeds, starch is broken down chiefly via the amylase pathway [79]. Further, seed priming with GA3 was effective in supporting the growth and alleviated the adverse impacts of Cd by enhancing the total chlorophyll content, endogenous GA3 content, carbohydrate metabolism, and the antioxidant defense system in variety ML-2056, clearly validating the role of GA3 in inducing Cd tolerance. Growth enhancements can also be linked to an increase in antioxidant machinery following GA treatment. The study of [20] in mung bean divulged that the implementation of GA ameliorated Cd stress and eased the deleterious effects on plant growth and biomass by lowering oxidative stress markers and increasing the nutritional status of plants. It has been reported that application of GA to Cd-stressed plants improves total chlorophyll content, which could be attributed to the activation of chlorophyll biosynthetic genes [12]. Moreover, exogenously sourced GA3 has been shown to boost the endogenous GA3 content in plants and confer resilience to Cd stress by stimulating the antioxidant system, thereby detoxifying ROS which maintained the cellular osmotic adjustment and shielded the photosynthetic machinery from the damaging impact of Cd. Carbohydrates/sugars (particularly sucrose) are the most significant product of photosynthesis and their abundance serves as the foundation for plant growth and development [80]. Under Cd stress circumstances, total soluble sugar

content, ADP-glucose pyrophosphorylase, and carbohydrate-metabolizing enzymes (viz. sucrose synthase and soluble acid invertase) in leaves generally decrease, as the oxidative pentose phosphate pathway related with NADPH production (ROS-detoxifying metabolic pathway) becomes disrupted. The decreases in carbohydrate metabolism under Cd stress have been related to the reduced carbon assimilation as well as the limited allocation of concurrent photosynthates in both source and sink organs [19]. Moreover, decreased plant dry mass (PDM) is also associated with a reduction in carbohydrate metabolism under Cd stress [20].

Li et al. [81] reported a significant increase in the activity of sucrose phosphate synthase (SPS) under Cd stress and, in line with this; we also found a higher activity of sucrose phosphate synthase under Cd in our tested variety of mung bean. The increased activity of SPS in the leaves (plumule) could result in the accumulation of hexoses, which might have conferred stress tolerance to ML-2056. It is well documented that the carbohydrate metabolism of plants under Cd stress can be revived by exogenously sourced GA3, implying that GA facilitates the activation of several specific genes involved in a broad range of cellular processes including growth, development, and stress acclimatization [82]. It is proposed that carbohydrate accumulation following the exogenous application of GA could be due to the increased photosynthetic efficacy as well as changes in endogenous hormones (especially GA) [83]. Furthermore, the influence of GA on carbohydrate metabolism to ameliorate stress could be related to the activation of most of the major enzymes of the Calvin cycle [84] and sucrose-synthesizing enzymes, i.e., SPS and sucrose synthase (SS) associated with source–sink partitioning, i.e., extracellular invertase [85]. Because of the cumulative effect of these enzymes, enhanced growth and tolerance were witnessed in mung bean seedlings. The results of this study further reported that the variety ML-2056 treated with Cd had higher levels of H_2O_2 and TBARS content, whereas exogenously applied GA3 reduced the production of ROS, thereby mitigating Cd-induced oxidative damage. Likewise, [86] revealed that exogenously sourced GA was efficacious in lowering H_2O_2 and TBARS accumulation in *Trigonella foenum-graceum* (fenugreek). Moreover, production of H_2O_2 is strongly linked to superoxide generation, O_2^- [87]. High levels of H_2O_2 and TBARS could reduce the photosynthetic efficiency of PSII and PSI, thus influencing plant growth and economy [88]. Recently, [89] also observed a decline in H_2O_2 and TBARS content in the vicinity of GA in *Solanum lycopersicum* (tomato).

In the present research, it was found that increasing levels of Cd accelerated the enzymatic defense systems (CAT, APX, and GR), which was further up-regulated by exogenous application of GA and the influential boost in the enzymatic activities was witnessed in seedlings exposed to co-application of GA3 and Cd. It has been demonstrated that GA ameliorates the negative impact of a variety of abiotic stresses by boosting the antioxidant enzyme activity, which might strengthen the plant growth [77,90], and which was also observed in our study. It has been speculated that the positive influence of GA with respect to various types of stresses could be due to the increased expression of antioxidant genes [11,12]. Seed priming treatment of PBZ substantially reduced the plant dry mass of seedlings, which was attributed to its inhibitory action on the synthesis of sterols, prerequisite compounds for GA biosynthesis [91], which in turn is implicated in cell division [21]. GA inhibition by PBZ in *Raphanus sativus* (radish) has been proved through LC-MS analysis [92]. Our results are consistent with the findings of [93], which indicated that PBZ is a plant growth retardant that suppresses olive tree growth length. In our study, PBZ treatment resulted in decreased endogenous GA levels in ML-2056. It has been reported that PBZ inhibits KO, a cytochrome P450 monooxygenase class enzyme of the GA biosynthesis pathway [94], thus reducing the level of endogenous GA in plants. Additionally, [95] also reported the down-regulation of KO and other GA biosynthetic enzymes under high-temperature stress on PBZ application. All in all, our findings demonstrated that exogenous GA is an apt mediator for conferring Cd resistance in mung bean seedlings, which was further confirmed through PBZ-mediated reduction in growth and endogenous GA3 levels.

5. Conclusions

The current study highlighted the substantial response of mung bean to Cd stress on short-term exposure during the germination stage. Overall, Cd treatments adversely affected the seed germination and embryo growth of mung bean in a concentration-dependent manner. Our results suggested that the inhibition of seed germination after exposure to Cd was partly the consequence of impairment in seed water uptake efficiency, but primarily due to interference in the reserve mobilization process from cotyledons to the growing embryonic axis, which was reflected as decline in GA3, total amylase activity and soluble sugar content. Cd-mediated O_2^- generation resulted in solute leakage, ultimately impeding seed germination. However, exogenously applied GA3 (through seed priming) was found to be effective in sustaining the morpho-physiological and biochemical characteristics, including amylase activity, total chlorophyll content, endogenous GA3 content, carbohydrate metabolism, and ultimately growth under Cd stress and stress-free conditions, by influencing the activities of ROS-detoxifying enzymes, mainly SOD, APX, and GR. The enhanced activity of antioxidant enzymes mediated by exogenous GA3 maintained the capability of mung bean seedlings to withstand excess Cd. The enhanced adaptability of seedlings to Cd stress induced by GA3 could also be attributed to their ability to alleviate Cd-induced oxidative stress, as indicated by lower ROS (H_2O_2 and TBARS contents). Moreover, exogenously applied GA3 was found to be involved in the regulation of photosynthesis and growth in mung bean under Cd stress, authenticating the role of GA3 in boosting Cd tolerance. This study also revealed that PBZ triggered changes in GA3 levels, resulting in a reduction in plant growth attributes, further confirming the positive impact of GA3 in stimulating stress resilience. Further investigations are required to obtain deep insights into the interference mechanisms of Cd with vital germination-related factors at the proteomic, metabolic, and molecular levels. Additionally, future studies should be directed towards exploring GA3-induced stress-responsive genes responsible for bestowing Cd tolerance to plants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su15043790/s1. Table S1. Effect of different concentrations of cadmium (Cd) on tolerance index (%), germination percentage (%), germination index, coefficient of velocity of germination, vigor index, vitality index, mean daily germination (%), fresh biomass per seedling (gm), dry biomass per seedling (gm), and radicle and plumule length (cm) in mung bean seedlings. Table S2. Comparative effect of different concentrations of cadmium (Cd) singly or in combination with gibberellic acid (GA3, 10 μM; μg g^{-1} FW) and paclobutrazol (PBZ, 10 μM) on endogenous GA3 content in mung bean seedlings at 7 DAS.

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