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Original article

Seed priming with methyl jasmonate mitigates copper and cadmium toxicity by modifying biochemical attributes and antioxidants in *Cajanus cajan*

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ABSTRACT

Contamination of agricultural soils with heavy metals (HMs) has posed major threat to the environment as well as human health. The aim of this study was to appraise the efficiency of key-antioxidant enzymes in enhancing plants' tolerance to HMs (heavy metals) like copper (Cu) and Cadmium (Cd), under the action of methyl jasmonate (Me-JA) in *Cajanus cajan* L. Seeds of *C. cajan* treated with Me-JA (0, 1 nM) were discretely subjected to noxious concentrations of Cu and Cd (0, 1, 5 mM) and raised for 12 days under controlled conditions in plant growth chamber for biochemical analysis. In contrast to Cd, Cu triggered oxidative stress more significantly (44.54% in 5 mM Cu increase in MDA as compared to control) and prominently thereby affecting plants' physiological and biochemical attributes. By activating the antioxidant machinery, Me-JA pre-treatment reduced HMs-induced oxidative stress, increased proline production, glutathione (41.95% under 5 mM Cu when treated with 1 nM Me-JA treatment) and ascorbic acid content by 160.4 % under aforementioned treatments thus improving the redox status. Thus, in light of this our results put forward a firm basis of the positive role that Me-JA might play in the mitigation of oxidative stress caused due to HMs stress by stimulating antioxidant defense system leading to overall improvement of growth of *C. cajan* seedlings.

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1. Introduction

With industrialization and modernization, various environment polluting factors have exacerbated (Xia et al., 2018) and among these several responsible causes, heavy metals (HMs) display major contribution in environmental degradation posing major threat to plants and other living organisms (Ahmad et al., 2020; Alsahli et al., 2020; Kaya et al., 2020). HMs could not be biodegraded, and exist in the environment indefinitely, and may threa-

ten human health by entering the food chain (Kanwar et al., 2020). Some of these HMs (such as Cd, Pb, and Hg) are not essential, whereas others (such as Fe, Mn, Cu, Mo, and Zn) are essential micronutrients for plant growth but excessive concentrations affect the plant development (Alirzayeva et al., 2017). Concurrent occurrence of cadmium (Cd) and copper (Cu) stresses has been shown to be more detrimental to crop production. However, Cu is a vital micronutrient element in various physiological processes (Kanwar et al., 2020), but an excessive amount of Cu is highly phytotoxic because of its indispensable redox properties (Migocka and Malas, 2018). Cd, on the other hand, is highly phytotoxic and has no established physiological or biochemical roles in plants. Cd and Cu stress conditions do not necessarily have a combined impact on crops, because the results are usually governed on the nature of the interaction between the stress factors (Choudhary et al., 2016). Plants modulate their reactions to both heavy metals to show numerous inimitable responses. For instance, both these heavy metals are known for over-production of ROS (reactive oxy-

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gen species) (Jawad Hassan et al., 2020; El-Beltagi et al., 2020). At toxic levels, the generation and devastation of ROS overcomes the scavenging system and causes oxidative stress outbreaks by inactivation and destruction of proteins, chlorophyll, membrane lipids, and even DNA strand breaks (Ahmad et al., 2019; Kohli et al., 2019; Ahmad et al., 2020). To counterbalance, plants activate a series of detoxification systems to eliminate the detrimental effects of ROS (Hasanuzzaman et al., 2020). Antioxidant detoxification system includes superoxide dismutase (SOD), catalase (CAT), peroxidase (POD). Along with this, non-enzymatic antioxidants include ascorbic acid and glutathione compounds that react with a variety of cellular components and also their key role in Plant defense system and as enzyme cofactors, these antioxidants have an effect on growth and development of plants (Ahmad et al., 2019; Kohli et al., 2019; Ahmad et al., 2020).

Hence, to fully understand the impact of these stress factors on plants, it is very vital to comprehend the essence of these heavy metals. Mittler and his colleagues formed a “stress matrix” to accumulate the effect of various environmental stresses on the growth and productivity of plants (Mittler, 2006; Suzuki et al., 2014). The matrix exhibits that the different stresses could display both positive and negative effects on plants.

Thus, the development of plants with enhanced comprehensive tolerance to both metal stresses involves the use of new-generation phytohormone such as jasmonates that might be involved in the alleviation of heavy metals and improve the physio-biochemical traits that are affected by both metal stresses. Methyl jasmonate (MJ) belongs to a class of cyclopentanone compounds. It is a naturally occurring plant hormone that participates in the response of plants to various environmental stresses through signal transduction pathways (Ahmad et al., 2016; Sirhindi et al., 2020). The level of endogenous methyl jasmonate is increased owing to pathogen infection, wounds, ozone, and stress conditions attributing to heavy metals (Piotrowska et al., 2009). Exogenous or pretreatment application of Me-JA exhibited multi-stress resilience and can decrease Cd damage by diminishing metal uptake (Wei et al., 2021). Evidence shows that the part of jasmonic acid is essential to mitigate the effects of metal stress on diverse plant species (Bali et al., 2019a; Bali et al., 2019b). So far, the research on the effect of Me-JA in reducing the metal stresses in pigeon pea remains to be explored.

Pigeonpea (*Cajanus cajan* (L.) Millsp.) is an economic crop grown in semi-arid tropical regions. In India, it is the second most vital legume food. Pigeon pea has a wide range of adaptability because the maturity time varies greatly. These characteristics make it suitable for cultivation in various environments and cropping systems. Pigeon pea has obtained many benefits at low prices and has become an ideal crop for sustainable agriculture.

Considering all this, our current research envisages to expand our understanding of the mechanism complexed in HMs damage in the *C. cajan* L. and thereby offer a scientific basis for selective breeding which would in turn endorse HMs tolerant species that might help guide pigeon pea production in HMs soil areas.

2. Material and method

2.1. Plant material and growth conditions

Certified seeds (AL-201, AL-882) of pigeon pea (*Cajanus cajan*) were obtained from the Punjab Agricultural University, Ludhiana, India. The selected seeds with size uniformity and free from infection were used for the further experiments. The seeds of *C. cajan* were initially treated with 0.01% solution of mercury hypochlorite for 5 min and were methodically rinsed beneath the tap water and later with double distilled water (DDW). After the surface steriliza-

tion, the seeds were subjected to pre-sowing soaking treatments in DDW which was used as control and 1 nM concentration of Methyl-Jasmonate for 8 h. After the seed soaking treatments, the seeds were raised in the various concentrations (1 mM, 2 mM, 5 mM) of Cu as CuCl₂ and Cd as CdCl₂ individually in petri-plates (diameter 18 cm) for up to 3 days and later shifted to brown towel papers (14.5x42.5 cm) placed in beakers containing toxic concentrations of Cu and Cd and the seedlings were raised in an artificial plant growth chamber with standard temperature of 25°C, light (16 h Dark/ Light period; intensity of 200 μmol (photon) m⁻²s⁻¹) and the relative humidity 80%. Experimental design contained triplicates for each Cd and Cu treatments treated with Me-JA. After 12 days, seedlings were harvested to evaluate various biochemical responses of treated and untreated seedlings exposed to Cu and Cd stress.

The following combinations were used:

- Control DDW
- 1 nM MeJA
- 1 mM Cd
- 5 mM Cd
- 1 nM MeJA + 1 mM Cd
- 1 nM MeJA + 5 mM Cd

2.2. Hydrogen peroxide (H₂O₂) and malondialdehyde (MDA) estimation

For estimating the H₂O₂ level in *C. cajan* seedlings, method of Velikova et al. (2000) was followed. Crushing of known weight of fresh plant tissue was done in trichloroacetic acid (0.1%) followed by centrifugation for 15 min at 12,000 rpm later dissolving the supernatant in (0.5 mL) potassium phosphate buffer (10 mM) and 1 M potassium iodide (1 mL). The absorbance is noted at 390 nm. The final result of H₂O₂ level was stated in μg g⁻¹ Fresh Weight (FW).

Heath and Packer (1968) method was followed to measure the MDA content. Homogenization of fresh plant material with known weight was done in 20% trichloroacetic acid (TCA) (v/v) (2 mL) and 0.5% thiobarbituric acid (TBA) (v/v) and then heated for 30 min at 95°C followed by immediate cooling in order to halt the reaction in the ice bath. The reading of absorbance was taken spectrophotometrically at 532 nm and 600 nm after 10 min of centrifugation at 10,000 rpm.

2.3. Estimation of proline content

For estimation of proline content, 500 mg of fresh leaf samples were homogenized in 3 mL of aqueous sulfosalicylic acid following (Bates et al., 1973) followed by centrifugation of 12 min at 11,500 × g. Glacial acetic acid and ninhydrin (1 mL each) was added to the one mL of supernatant pursued by 1 hr of boiling at 100 °C and was instantly cooled in order to halt the reaction. Toluene (2 mL) was used to remove the red color and absorbance of chromophore was recorded at 520 nm. The standard curve was prepared for estimation of the proline content.

2.4. Enzyme assay

Fresh plant tissue of known weight was homogenized in 3 mL of 100 mM potassium phosphate buffer (v/v) (pH 7.0) in pestle-mortar (pre-chilled) which was later centrifuged at 4°C and 13,000 rpm for 15 min. And the supernatant was used for protein content determination as well as further enzymatic analyses.

For, protein content estimation, absorbance was recorded at 750 nm and BSA (Bovine Serum Albumin) was employed as standard following Lowry et al. (1951).

SOD (EC 1.15.1.1) enzyme activity was recorded as per Kono (1978) and it is totally based on the rate of reduction of Nitro blue tetrazolium (NBT) dye due to superoxide radicals, generated by auto-oxidised hydroxylamine hydrochloride. Reaction mixture was prepared in 1.3 mL of sodium carbonate buffer (w/v), 100 μ L Triton X-100 (v/v), 500 μ L NBT (w/v) and the reaction initiation takes place after adding hydroxylamine hydrochloride (100 μ L) (w/v) later adding the enzyme extract (70 μ L) after 2 min. Absorbance was noted at 540 nm.

CAT (EC 1.11.1.6) activity recorded after following Aebi (1984) where 3 mL of 100 mM phosphate buffer (v/v) (pH 7.0) and 150 mM of H₂O₂ (v/v) was added to 100 μ L enzyme extract to prepare reaction mixture and the reaction was instigated upon addition of H₂O₂. To estimate CAT activity, absorbance decrease at 240 nm was noted.

Chance and Maehly (1955) was followed to estimate POD (EC 1.11.1.1) activity was estimated following and monitoring of tetra guaiacol formation was done at 436 nm ($\epsilon = 26.6 \text{ mM}^{-1}\text{cm}^{-1}$) and H₂O₂ was the substrate used. The method defines a unit of peroxidase as the enzyme needed for the production of 1 mM of tetra guaiacol/minute.

2.5. Estimation of antioxidant compounds

2.5.1. Glutathione reduced (GSH)

Law et al. (1983) was followed to determine the content of GSH and plant homogenate was prepared in 5 mL of 10% trichloroacetic acid (v/v) followed by 15 min of centrifugation at 15,000 rpm. 100 μ L of 5, 5'-Dithiobis, 2-nitrobenzoic acid (DTNB) 6 mM, glutathione reductase (50 μ L) and 700 μ L of 0.3 mM NADPH was added in 150 μ L of supernatant. The reagents were primed in 125 mM NaH₂PO₄ buffer and 6.3 mM EDTA (pH 7.5) and the standard curve was prepared to analyze the GSH content.

2.5.2. Ascorbic acid (ASA)

Method of Chinoy et al. (1976) was followed to measure MDA content. A known amount of fresh plant sample was crushed in metaphosphoric citrate buffer at pH 5.4 pursued by the centrifugation for 5 min at 3000 rpm. The supernatant was collected and to estimate ASA content. The reaction mixture was prepared by adding 8 mL of 2, 6- dichlorophenol indophenols dye to 2 mL of plant homogenate and absorbance was recorded at 530 nm. A standard calibration curve was prepared in order to evaluate the content of ASA, later expressed as mg g⁻¹ fresh weight.

2.6. Growth measurement

Twenty five seedlings of each treatment were selected arbitrarily and were measured for root length and shoot length manually with measuring scale and were expressed in centimeters (cm).

2.7. Statistical analysis

The data compiled was evaluated using the analysis of variance (ANOVA) test. The values are represented in the form of mean \pm standard error of three replicates. For multiple comparisons, Tukey's test ($P < 0.05$) was used in Graph Pad Prism Version 8.

3. Results

3.1. Effect of Cu and Cd on oxidative stress markers

3.1.1. H₂O₂ content

The exposure to selected HMs toxicity provoked a dose-responsive elevation in H₂O₂ content and elevation was severe

with increasing concentration of HMs (Cu/Cd) (Fig. 3a). The exogenously applied Me-JA alone significantly induced the H₂O₂ content (18%) in AL-201 while in AL-882 had dramatically decreased (28%) in comparison with their respective control seedlings. In combinations, Me-JA mitigated the toxic effects of both Cu and Cd and reduced H₂O₂ content by 16% in 1 nM + 5 mM Cu (AL-201) and 50% reduced in 1 nM + 5 mM Cd (AL-882) stress seedlings, compared with that of 5 mM Cu and Cd treatment, respectively.

3.1.2. Lipid peroxidation

Membrane damage due to exposure to selected HMs (Cu and Cd) provoked a dose-responsive elevation in MDA content, which is presented in Fig. 1A-B. Treatment with Cu stress showed maximum accumulation of MDA content (44% in AL-201) than Cd stress (41% in AL-882) in contrast with that of untreated control seedlings. The application of Me-JA alone reduced the level of MDA content over their respective untreated control seedlings which was 45% in AL-882 but had no significant effect on AL-201. Furthermore, Me-JA application with Cu and Cd significantly altered the level of MDA content in both *C. cajan* varieties. Maximum alleviation in MDA content was noted under 1 nM Me-JA + 5 mM Cd treatment which was 22% even as 14% was reported in the case of 1 nM Me-JA + 5 mM Cu treatment as compared to only 5 mM treated seedlings respectively.

3.2. Proline content

The effects of both Cu and Cd with and without Me-JA on the concentration of osmoprotective proline content are shown in Fig. 2A. At 5 mM Cu treatment in AL-201, proline content increased sharply (428%) while under Cd stress only 91% elevation in proline content was found in comparison to control seedlings. However, combined appliance of Me-JA with Cu further enhanced proline content which was 53% while Me-JA with Cd showed very deep decline in the proline content that was 86% as compared with only 1 mM Cu and 5 mM Cd treated seedlings. Moreover, Me-JA alone treated seedlings resulted in enhanced proline content by 177% in AL-201 and only 14% in AL-882 over the untreated control seedlings, respectively.

3.3. Effect of Cu and Cd on total protein content

As depicted in Fig. 2B, both Cu and Cd induced stress caused a dramatic reduction in soluble protein content, where the maximum reduction was noted for 5 mM Cd (AL-882) treatment that was 56% and only 22% reduction was reported under Cu stress seedlings (AL-201) in compared to untreated control seedlings. In contrast, the treatment of Me-JA mitigated unpleasant effect of both Cu and Cd stress and further enhanced the total soluble sugar content in *C. cajan* seedlings. About 88% enhancements was found at 1 nM Me-JA + 5 mM Cd in AL-882 seedlings while only 32% was recorded at 1 nM Me-JA + 1 mM Cu in AL-201 seedlings as compared with their respective Cd and Cu (5 mM) alone treatment.

3.4. Effect of Cu and Cd on antioxidant enzymes activity

The *C. cajan* seedlings showed significant change in antioxidant enzyme activities when they are treated with different HMs stresses. The results show that under the same stress conditions, all antioxidant enzyme activity patterns were different from each other.

A remarkable difference noted in SOD activity in both the varieties of *C. cajan* seedlings (Fig. 3A). Results showed a dose-responsive elevation in SOD activity under Cu stress which was 120% in 1 mM and 129% in 5 mM Cu stress seedlings while; SOD activity was decreased under Cd stress seedlings except 5 mM con-

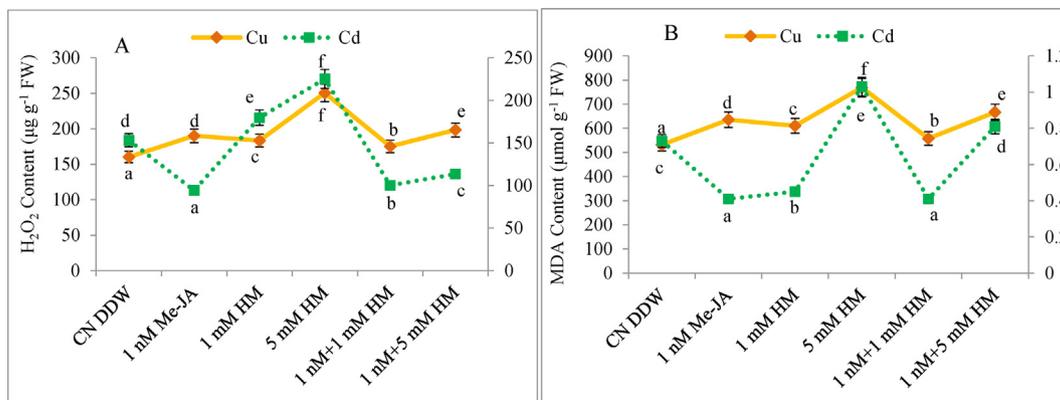


Fig. 1. H₂O₂ (A) and MDA (B) of *C. cajan* under different treatments of Cu and Cd (0, 1 mM, 5 mM) individually or in combination with MeJA (0, 1 nM, MeJA) on oxidative stress markers. Different letters indicate their statistically significant differences among the treatments (P < 0.05) by applying Tukey's correction for multiple comparisons.

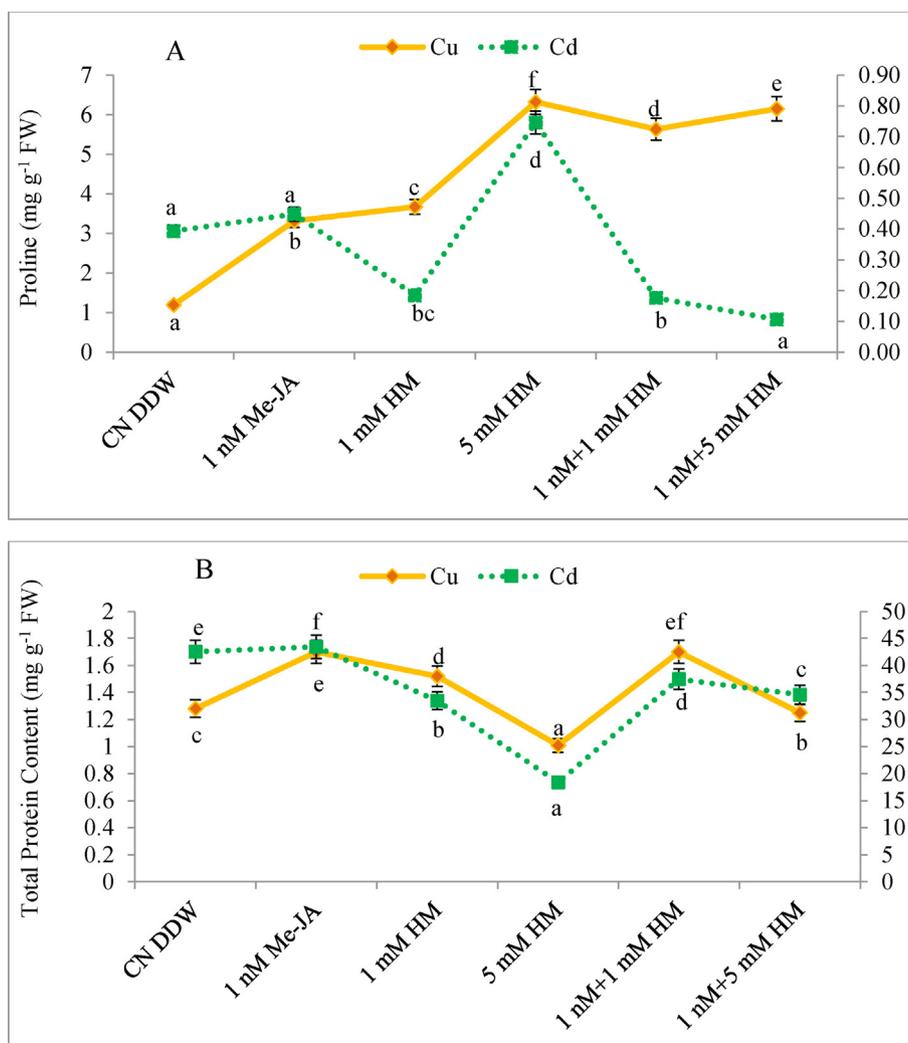


Fig. 2. Effect of different treatments of Cu and Cd (0, 1 mM, 5 mM) individually or in combination with MeJA (0, 1 nM, MeJA) on proline content (A) and total soluble protein content (B) in *C. cajan*. Different letters indicate their statistically significant differences among the treatments (P < 0.05) by applying Tukey's correction for multiple comparisons.

centration which showed an 11% up-regulation in comparison with their respective control seedlings. Exogenously applied Me-JA with Cu and Cd treatments improved the SOD activity in both *C. cajan* varieties. The highest induction of Me-JA application was observed at 1 nM Me-JA + 5 mM Cd treatment where the increase of SOD

activity was 332% in AL-882 and in AL-201 was only 62% (1 nM Me-JA + 5 mM Cu) as compared with their respective control seedlings.

It is obvious from Fig. 3B, that the CAT activity increased with increasing Cu concentration that was 110% in 5 mM while in terms

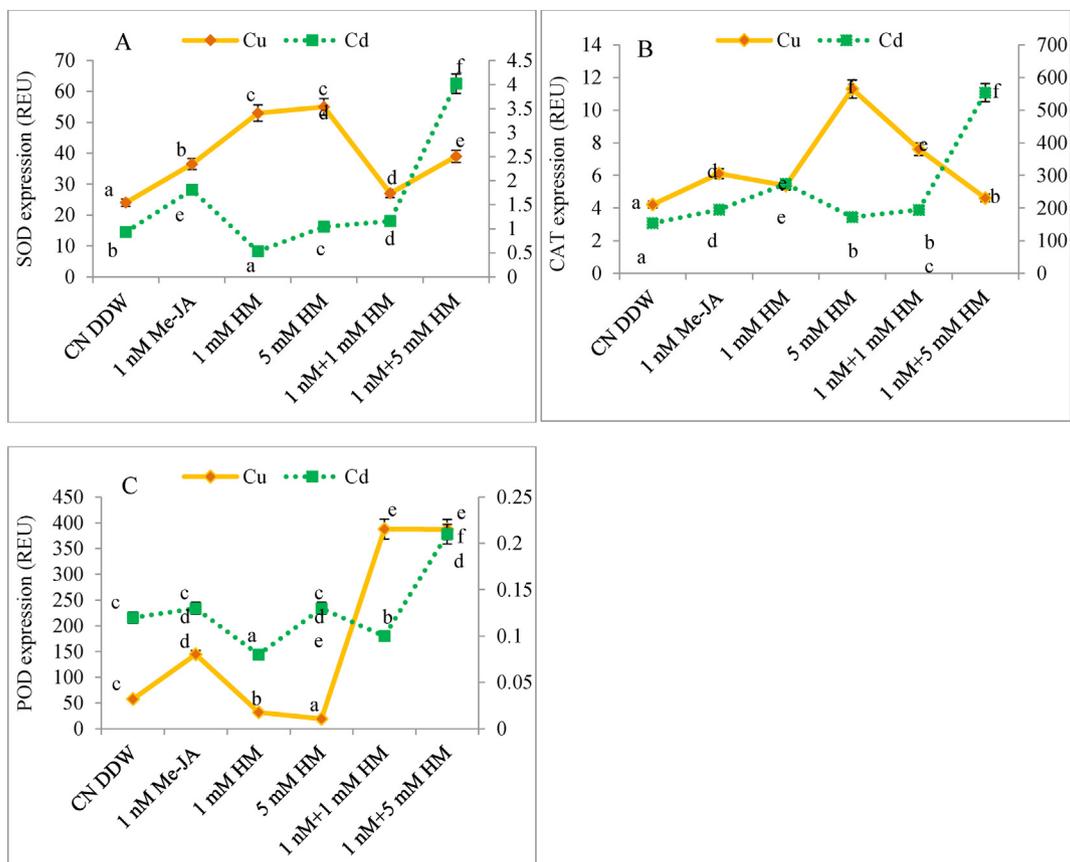


Fig. 3. SOD (A), CAT (B) and POD (C) of *C. cajan* under different treatments of Cu and Cd (0, 1 mM, 5 mM) individually or in combination with MeJA (0, 1 nM, MeJA) on antioxidant enzymes activity. Different letters indicate their statistically significant differences among the treatments ($P < 0.05$) by applying Tukey's correction for multiple comparisons.

of Cd stress, the CAT activity first increased to its lower concentration and then gradually decreased about 59% at 1 mM in comparison with their lower (1 mM Cu) and higher (5 mM Cd) concentration, respectively. Exogenously applied Me-JA alone enhanced the CAT activity in both *C. cajan* varieties. Maximum up-regulation in CAT activity was noted in AL-201 which was 44% and 27% in AL-882 as compared to their control seedlings.

As shown in Fig. 3C, the POD activity were considerably diminished under both Cu and Cd stress. The high concentration of Cu (5 mM) affected POD activity, reducing it by 67% while, for the lower concentration of Cd (1 mM Cd), there was a 31% reduction as compared with their respective control seedlings. However, under Me-JA alone treatment, AL-201 showed significantly higher activity of POD compared with AL-882 where its activity was considerably reduced as compared to AL-201.

3.5. Effect of Cu and Cd on antioxidant compounds

3.5.1. Glutathione reduced (GSH)

The readability of data given in Fig. 4A showed that the GSH content was notably increased by both Cu and Cd stresses. The elevation in GSH content was greater under Cu stress (39%) in AL-201 than those under Cd stress (29%) in AL-882 as compared to their respective control seedlings. Moreover, exogenously applied Me-JA to unstressed seedlings resulted in enhanced GSH content by almost similar in both AL-201 (33%) and AL-882 (37%) as compared to that of untreated control seedlings. In contrast, Me-JA application with Cu and Cd stressed seedlings improved the GSH content. The maximum induction was observed at 1 nM + 1 mM Cd stress in AL-882 where the increase in GSH content was 40% whereas at

1 nM + 1 mM Cu stress in AL-201 only 8% GSH content was induced in comparison with their 1 mM Cu/Cd stressed seedlings.

3.5.2. Ascorbic acid (AsA)

The effects of Cu and Cd with or without Me-JA on the vitamin C content are shown in Fig. 4B. The content of vitamin C was significantly reduced under Cu alone treatment (5 mM) in AL-201, which was a 23% decline, Whereas in Cd stressed seedlings, no significant increase in the vitamin C content was noted in AL-882 as compared with their respective control seedlings. Exogenous Me-JA alone had on considerable effect, but under Cu and Cd stress it significantly alleviated the Cu and Cd toxicity and further amplified the Vitamin C content. The maximum vitamin C content was observed in AL-201, which was 160% (1 nM Me-JA + 5 mM Cu) while in AL-882 was only 7% (1 nM Me-JA + 5 mM Cd) as compared with that of the control seedlings, respectively.

3.6. Effect of Cu and Cd on physiological variables

As depicted in Table 1. HMs stressed reduced seedling length of *C. cajan* L. varieties. Seedlings fed only with Cd showed reduced seedling height by 59%, while under Cu stress showed a 46% reduction in seedling height at 5MM concentration, compared to their respective control seedlings. However, Exogenous Me-JA pretreatment relieved the toxic effect of HMs and improved the seedling height by 20% at 1 nM Me-JA + 1 mM Cu and 54% at 1 nM Me-JA + 1 mM Cd stress relative to the 1 mM Cu and Cd treatments, respectively.

Seedlings biomass in terms of Fresh and Dry weight (FW and DW) showed dramatic reduction under Cu and Cd stress. 1 nM

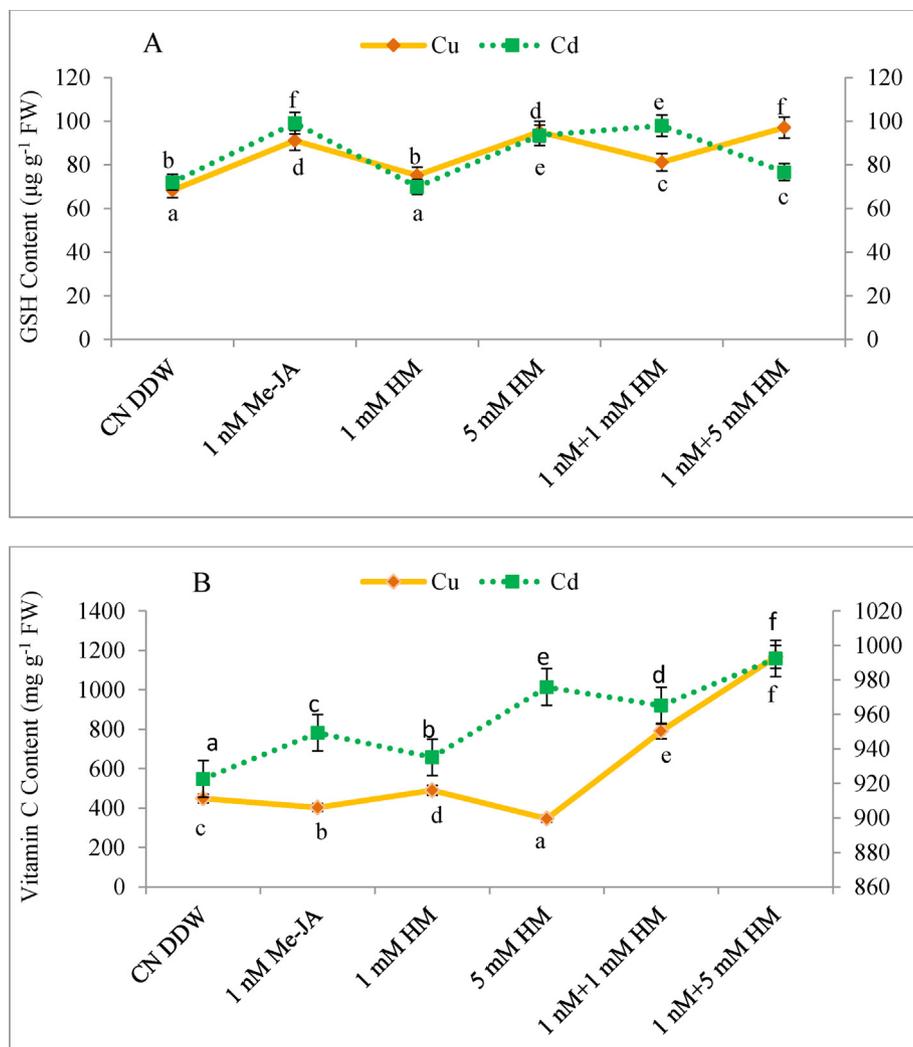


Fig. 4. GSH (A) and Vitamin C (B) of *C. cajan* under different treatments of Cu and Cd (0, 1 mM, 5 mM) individually or in combination with MeJA (0, 1 nM, MeJA) on antioxidant compounds. Different letters indicate their statistically significant differences among the treatments ($P < 0.05$) by applying Tukey's correction for multiple comparisons.

Table 1

Effect of different treatments of Cu and Cd (0, 1 mM, 5 mM) individually or in combination with MeJA (0, 1 nM, MeJA) on seedling length (cm) in *C. cajan*. Different letters indicate their statistically significant differences among the treatments ($P < 0.05$) by applying Tukey's correction for multiple comparisons.

Treatment	Seedling length (cm)	
	Cu	Cd
Control	14.73 ± 1.00 ^d	19.83 ± 0.02 ^f
1 nM Me-JA	15.13 ± 0.70 ^e	16.99 ± 0.51 ^c
1 mM HM	12.58 ± 0.45 ^c	9.67 ± 0.05 ^b
5 mM HM	8.06 ± 0.45 ^a	8.27 ± 0.05 ^a
1 nM Me-JA + 1 mM HM	15.13 ± 1.05 ^{ef}	14.83 ± 0.01 ^d
1 nM Me-JA + 5 mM HM	9.76 ± 1.15 ^b	11.97 ± 0.45 ^c

Me-JA treatment under Cu stress showed alleviatory effect on FW by 10% (1 nM Me-JA + 5 mM Cu) whereas under Cd showed diminution effect by 21% (1 nM Me-JA + 5 mM Cd) as compared to their respective control seedlings. Seedling DW under Cu stress alone was decreased by 20% at 5 mM Cu, whereas in Cd stress, maximum reduction in DW was noted at 1 mM concentration which was 23% as compared to their control seedlings respectively. Supplementation of Me-JA to Cu and Cd fed seedlings resulted in improved water content (WC), which demonstrated the positive effect of Me-JA on Cu and Cd stress (Table 2).

4. Discussion

Plants are important bio-indicators that provide information on the combined and immersive impact of environmental stressors, such as elevated metal concentrations. The negative effects on them act as a warning signal to protect crop productivity and get a good handle on stress reactions. Me-JA plays a regulatory role in many stresses including metal stress. Therefore, in our study, we reported the Cu and Cd responsive alteration in *C. cajan* L. varieties and their regulation after Me-JA exposure signify important research questions.

H₂O₂ is a very harmful ROS, and it increases greatly with the increase of Cu concentrations in AL-201 as compared to Cd concentrations in AL-882 and the results of the current study are in accordance with Mwamba et al. (2016) in *Brassica napus*. It is believed that this is related to the different chemical properties among the two metals. Cu is one of the major effective catalysts of free radical configuration due to its various redox properties, since it is vulnerable in straight to produce ROS by Fenton like reactions or the Haber–Weiss cycle. On the contrary, Cd is a non-redox active metal and can generate ROS indirectly by interacting with the antioxidant molecules or by disrupting the electron transport chain (Andresen and Küpper, 2013).

Table 2

Effect of different treatments of Cu and Cd (0, 1 mM, 5 mM) individually or in combination with MeJA (0, 1 nM, MeJA) on Fresh weight, Dry weight and water content in *C. cajan*. Different letters indicates their statistically significant differences among the treatments ($P < 0.05$) by applying Tukey's correction for multiple comparisons.

Treatment	Fresh weight(mg)		Dry weight (mg)		Water content (%)	
	Cu	Cd	Cu	Cd	Cu	Cd
Control	1707 ± 0.085 ^d	3903.67 ± 1.15 ^f	204 ± 0.102 ^{bc}	567.67 ± 0.58 ^f	88.03 ± 4.40 ^c	85.45 ± 0.02 ^c
1 nM Me-JA	2340 ± 0.12 ^f	2135.33 ± 1.52 ^c	207 ± 0.10 ^{bcd}	305.00 ± 1.00 ^a	91.16 ± 4.55 ^e	85.72 ± 0.03 ^{cd}
1 mM HM	1520 ± 0.076 ^b	2084.00 ± 1.00 ^b	203 ± 0.101 ^b	440.00 ± 0.58 ^d	86.67 ± 4.33 ^b	78.33 ± 0.02 ^b
5 mM HM	1200 ± 0.06 ^a	1437.33 ± 0.58 ^a	164 ± 0.08 ^a	550.33 ± 0.57 ^e	86.33 ± 4.32 ^a	61.71 ± 0.03 ^a
1 nM Me-JA + 1 mM HM	1631 ± 0.08 ^c	3287.33 ± 1.53 ^e	217 ± 0.10 ^c	431.00 ± 1.00 ^c	86.68 ± 4.30 ^{bc}	86.90 ± 0.01 ^{de}
1 nM Me-JA + 5 mM HM	1890 ± 0.09 ^e	3103.33 ± 0.58 ^d	222 ± 0.01 ^d	395.33 ± 0.58 ^b	88.29 ± 4.41 ^d	86.85 ± 0.02 ^d

On the other hand, the effects of Cu and Cd also measured in terms of MDA formation (a by-product of the lipid peroxidation of plasma membrane) was established to be amplified in the present study, because, it is here documented that both Cd and Cu trigger the oxidative stress that destroy the cell membranes and accordingly increase MDA content. Moreover, this finding is consistent with the results of [El-Amier et al. \(2019\)](#) and [An et al. \(2019\)](#) which accounted increase in the MDA content in *Pisum sativum* under Cd stress and cotton under Cu stress respectively. In present study, MDA was significantly increased under Cu stress which indicates oxidative stress in AL-201 seedlings. The main mechanism behind the ROS formation is an outcome of the interaction of HMs with lipid-rich membrane which consequently leads to a conformational change in the membrane by activating the NADPH-oxidase located there, thereby generating ROS. These ROS destroy the double bonds present in the phospholipid bilayer of the plasma membrane, thereby increasing lipid peroxidation ([Yalcinkaya et al., 2019](#)). In the present study, Me-JA pre-treatment reduces the production of H₂O₂ content, which overtly affects the lipid membranes. The present findings are reliable with the results of [Sirhindi et al. \(2015\)](#) who noted that exogenous application of JA reduced the over-production of MDA content in soybean Ni fed seedlings and similar results were obtained by [Bali et al. \(2018\)](#) in tomato under Pb stress. These results suggest that HMs may stimulate the octadecenoic acid pathway that leads to activation of JA-biosynthesis, which act as a signaling molecule implicated in the regulation of antioxidants as well as in the growth-related processes and impart tolerance to plants by reducing the production of ROS ([Santner and Estelle, 2009](#)).

To further investigate the osmotic amendment potential of the *C. cajan* seedlings, we estimated the proline content of the seedlings. Our observations exposed that both Cu and Cd significantly enhanced proline accumulation during all the treatments, whilst AL-201 seedlings tended to build up the maximum proline contents at each Cu concentration. Same results were obtained by [Noreen et al. \(2018\)](#), and [Abdel Latef et al. \(2020\)](#) who showed that plants grown under Cu stress increase proline content. Proline contributes as an osmoprotectant for the detoxification of ROS and protects the membrane ([Ghosh et al., 2021](#)). Increase in the proline accumulation acts as an indicator of the stress condition in *C. cajan* seedlings and this observation confirmed a dynamic link between enhanced proline and MDA content. Although, in our study the combined application of Cu with Me-JA showed less proline accumulation as compared to the individual treatment of Cu but more accumulated as compared to Cd with Me-JA application. Our results are in rationality with earlier findings of [Yan et al. \(2015\)](#) in *Solanum nigrum* under Cu and Cd stress who concluded that JA might stimulate enzymes that trigger the accretion of mRNAs encoding proline-rich proteins and protect the cells from oxidative bursts by scavenging the ROS ([Creelman and Mullet, 1991](#)).

As illustrated in [Fig. 2B](#), the total soluble protein content demonstrates noteworthy toxic effects of Cu and Cd treatments on both varieties of *C. cajan* seedlings, which is an important

parameter to determine the phytotoxicity of HMs. The maximum reduction in protein content was record in AL-201 under Cu stress. These results projected that both Cu and Cd are lethal and extensively reduce the protein content of *C. cajan*, which may be due to augmented protease activity and other catabolic enzymes. The present result is supported by [Banu Doğanlar \(2013\)](#) who accounted that, decreased level of protein content could be explained by the fact that toxic harms formed by the Cu and Cd might amend the cytochrome oxidase activity and different respiratory pathways. Moreover, supplementation of Me-JA improved soluble protein content under multiple stress conditions.

Plants have an armory of the enzymatic network that scavenge ROS and counter the harmful effects. While, the activity of enzymatic antioxidant differs as it depends on different plant varieties and exposure concentration of HMs that affects plants growth ([Sharma and Dietz, 2009](#)). The activation of enzymatic activities plays a vital role in the detoxification and thus represents the altered redox status of cell under stressful conditions. Among all, SOD is supposed to serve as a frontline enzymatic antioxidant defense, that at a very high pace catalyses the dismutating of superoxide radicals into hydrogen peroxide and there after subsequently convert them into H₂O by the activity of CAT and POD ([Ahmad et al., 2010](#)). In the current study, exposure of AL-201 seedlings to the selected Cu stress resulted in significant increase in the enzymatic activity, which was progressively increased in a dose-dependent manner, except for POD activity ([Fig. 3C](#)), while under Cd-stressed AL-882 seedlings, all enzymatic activities reduced initially and then increased with increasing concentration. The current study reported that higher accumulation of H₂O₂ surpassed ROS-scavenging potential in HMs stressed *C. cajan* seedlings. The present results are reliable with those of [Pandey et al. \(2009\)](#) who reported boosted enzymatic activity in spinach under HMs stress that might be caused via overproduction of the free radicals. However, exogenous application of Me-JA modulates the antioxidant enzyme activities in both Cu and Cd stressed seedlings compared to their untreated control seedlings. Furthermore, an addition of Me-JA induced improved growth and redox status possibly by boosting enzymatic activity. Also, exogenous application of Me-JA acts to maintain the antioxidant defense of both the varieties of *C. cajan* seedlings in adaptation to Cu and Cd stress. Our observations are comparable to the previous reports of [Chen et al. \(2014\)](#) in *K. obovata* and [Piotrowska et al. \(2009\)](#) in *W. arrhiza* L. who reported that Me-JA reduced the HMs toxicity by declining the oxidative stress. Therefore, it is anticipated that, Me-JA application further improve the activity of antioxidant enzymes, which helps to enhance its efficiency in scavenging ROS.

Ascorbic acid and glutathione (GSH) have been found to play a significant role in shielding the plants from stress. ASA is considered to be the main redox factor in plants. Here the decrease in vitamin C content was reported in Cu stress AL-201 seedlings, whereas in Cd stressed AL-882 seedlings no significant effect on vitamin C content was noted. Our findings are in accordance with the results of [Ahn et al. \(2012\)](#) in *Brassica rapa*. In the current study,

we found that Me-JA regulates the metabolism of ascorbic acid, as observed with the significant increase in vitamin C content under Cu stress seedlings (Fig. 4B). Previously, Wolucka et al. (2005) reported in tobacco plants and *Arabidopsis* that Me-JA improves the stress tolerance of plants by regulating synthesis and also recycling of ascorbic acid. Elevation in vitamin C by Me-JA might be due to the stimulation of Me-JA responsive genes which are encoding the vitamin C biosynthesis.

In the same way, glutathione is one more major compound in the antioxidant system. GR reduces GSSG to GSH and further reinforce the detoxification process (Ahmad et al., 2010; Ahmad et al., 2019). Kaya et al. (2020) reported that toxicity of heavy metals increased the GSH content in pepper and this increase in GSH redox balance acts as an antioxidant defense which persuades the cellular signaling pathway under stress conditions (Foyer and Noctor, 2011). However, in our study, a similar trend for amplified GSH content was observed in Cu-treated AL-201 seedlings (Fig. 4B). Dar et al. (2015) made an observation that Me-JA might be held responsible for regulating genes involved in GSH biosynthesis and metabolism under heavy metal stress conditions. The results of the present study are in coherence with the aforementioned results as it was observed that Me-JA upregulated GSH activity under Cu and Cd stress conditions. On the basis of Shan and Liang (2010) previous results, it was speculated that Me-JA acts as a signaling molecule to induce the expression of detoxification enzymes and related genes under stress conditions. The application of Me-JA regulates the metabolic pathways of both ASA and GSH at the transcriptional level and plays a foremost role in the tolerance of Cu and Cd-induced stress conditions.

The studied seedling growth of both the varieties of *C. cajan* L. was negatively influenced by elevating the concentration of Cu and Cd. The impact was conflicted when HMs were applied with co-application of Me-JA. Evidences have shown that exogenously applied Me-JA activates seedling growth by regulating various defense systems (Bali et al., 2019a; Bali et al., 2019b; Sirhindi et al., 2020). The present result is in concurrence with Bali et al. (2018) who found that, exogenously applied Me-JA was reported to counter the HMs stress and restore plant growth.

5. Conclusion

In conclusion, Cu induced oxidative stress proved to be more toxic on *C. cajan* seedlings by affecting their physiological and biochemical properties as compared with Cd toxicity. In contrast, Me-JA pretreatment might be a competent approach for successful tolerance of *C. cajan* seedlings under Cu and Cd stress. Me-JA maintained a redox imbalance by regulating antioxidant defense machinery. Thus, our results revealed synergistic or antagonistic diversity in the redox regulation of Me-JA pretreated seedlings under Cu and Cd stress. Nevertheless, superfluous facts regarding the metal-specific participation of oxylipin biosynthesis in the cellular responses are essential that will be a sustainable approach to augment crop yield and form exciting topics for further research.

Ethics approval

Not applicable.

Consent to participate

All authors consent to participate in the manuscript publication.

Consent for publication

All authors approved the manuscript to be published.

Availability of data and material

The data supporting the conclusions of this article are included within the article. Any queries regarding these data may be directed to the corresponding author.

Author contributions

Geetika Sirhindi designed the research and Shruti Kaushik conducted experiments. Poonam Sharma helped in Methodology and Project administration. Gurvarinder Kaur and Anil Kumar Singh helped in writing the manuscript. Fahad A. Al-Misned, Hesham M Shafik analyzed the data.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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