Original Research Article

Cadmium priming alleviates salinity induced oxidative stress in pigeon pea

ABSTRACT

Plants are regularly exposed to an adverse environment due to their sessile nature which has negative impacts on plant development and productivity. In this study, the effect of seeds priming with 50 µM $CdCl_2$ in ameliorating the salinity (200mM NaCl) induced oxidative stress in pigeon pea was assessed. Hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) content was reduced in co-stressed (50 µM $CdCl_2$ primed followed by 200 mM NaCl treatment) tissues as compared to salt stressed tissues. The activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and guaicol peroxidase (GPX) were increased in co-stressed tissues as compared to salt stressed tissues. Moreover, the concentrations of ascorbic acid (ASC) and proline were also increased in co-stressed tissues as compared to salt stressed tissues. Thus, a low dose $CdCl_2$ priming provided tolerance to pigeon pea seedlings.by activating the antioxidant machinery.

Keywords: $CdCl_2$ priming, Pigeon pea, Salt stress, Antioxidants, Antioxidative enzymes, Proline

INTRODUCTION

Ecological stress places a significant strain on plant sustainability [1]. Salinity is amongst the most frequent abiotic stresses which trigger the plant's proliferation and developmental operations [2]. As a consequence, there is a massive yearly production loss throughout the world [3]. On a worldwide platform, extensive irrigation has enhanced the salinity of agricultural land to the point that 50% of farmland could be reduced to salinity by 2050 [4]. Salt induced farmland deterioration is indeed a big concern that has a global impact on food yields [5,6].

Plant development is negatively affected by salinity worse than by any certain toxic chemical on the planet [7], salt affects a variety of plant physiological and biochemical systems [8]. The stomatal electric density, plant water linkages, and photosynthetic pigments are all reduced during salt stress. Salinity also decreases the speed of transpiration, photosynthesis, growth, and biofuels generation [9]. The principal cause of reactive oxygen species (ROS) formation in chloroplasts is due to inadequate CO_2 uptake caused by insufficient Na^+ buildup in plants, which leads to hyper-reduction of the electron transport complex (ETC). During stressful situations, hyper-reduction of mitochondrial ETC is another major source of ROS [10]. The

primary ROS that triggers oxidative stress in plants is superoxide radical, hydroxyl radical, singlet oxygen, and hydrogen peroxide [11].

Plant protection towards enhanced ROS through adverse circumstances is linked to the retention of cellular redox equilibrium, which is primarily provided via non-enzymatic and enzymatic antioxidants like catalase, peroxidase, glutathione reductase, glutathione peroxidase, superoxide dismutase, glutathione-S-transferase, monodehydroascorbate reductase, ascorbate peroxidase, and dehydroascorbate reductase [2, 12, 13]. Multiple plant varieties manufacture osmoprotectants including sucrose, glycine betaine, mannitol, trehalose, and proline, which help sustain water relations, stabilize enzymes, protein complexes, and membranes within saline conditions [14-18].

Pigeon pea [*Cajanus cajan* (L.) Millsp.] is the second-largest legume crop in India, behind chickpea, and is primarily planted mostly around the Kharif season. The pigeon pea has a salt sensitivity to its development. In pigeon pea, excessive salt induces physiological drought, and persistent and large treatment is fatal.

Seed priming is a technique for controlling the level of moisture in seeds and the metabolic activities needed for germination. Seed priming improved germination, seedling vigor, and mortality of rice seedlings during normal and severe soil and climatic circumstances [19]. Numerous reports described that seed priming employs organic and inorganic compounds such as choline, chitosan, putrescine, ethanol, paclobutrazol, zinc sulphate, potassium sulphate, copper sulphate, and selenium sulphate to promote crop plant development and tolerances against abiotic stresses [20]. Supplementing selenium (Se) at smaller doses prevents plants from ROS-induced oxidative destruction by triggering antioxidative systems [21]. These are triggering antioxidative and detoxifying processes, and lowering malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) concentrations [21, 22]. Cucumber, rapeseed, canola, and parsley were protected against sodium chloride toxicity by Se priming [23]. Metal-induced defense in plants might be achievable, if a specified concentration of metal has little or no damage to the plant than to the pathogen [24].

Previous studies have demonstrated that low dose of 50µM $CdCl_2$ priming prevent *Fusarium* infection in wheat [25]. The aim of the study was to unravel the role of 50µM $CdCl_2$ priming in alleviation of salinity induced oxidative stress in pigeon pea.

2. MATERIALS AND METHODS

2.1.SEED PRIMING AND NaCl TREATMENT

Healthy and uniform seeds of pigeon pea (*Cajanus cajan* L.) (PRG-176), were procured from the Center for Pulses Research (CPR), Berhampur, Odisha, India. The seeds were surface sterilized with 0.1% $HgCl_2$ solution after being rinsed with distilled water. The seeds were divided evenly; the first half was soaked with distilled water (control) and the second half with 50 µM $CdCl_2$ solution (metal treated). Both the halves were kept in conical flasks and mouths closed with cotton plugs and kept in dark condition at room temperature (RT) for 48 h. The seeds were then transferred into sterilized petri plates with the base covered

with muslin fabric, sealed with parafilm, and covered with dark polythene, and maintained in the dark for 48 h before being incubated under light for 12 h [26].

Once they developed two leaves, these were transferred into a test tube for 10 d for further growth and development.Ten days old seedings of both control and primed seeds were exposed to salinity stress (200 mM NaCl) for 7 d and plants were harvested for futher physio-biochemical analyses.

2.2.CHLOROPHYLL CONTENT

The chlorophyll was extracted from fresh leaves of each group by using 80% acetone, the chlorophyll content was measured in a UV-VIS spectrophotometer following the method proposed by [27].

2.3.RELATIVE WATER CONTENT

The relative water content of plant seedlings from each group was calculated using the method of [28].

RWC (%) = (FW – DW) / (TW – DW) × 100

2.4. ASSAY OF BIOMASS

Fresh weight (FW) was recorded from freshly detached leaves and subjected to oven drying at 70ºC for 24 h to record dry weight (DW). The biomass content of plant seedlings from each group was calculated using the method of [29].

Total biomass = [(FW- DW)/FW] x 100

2.4. ASSAY OF OXIDANTS

2.4.1.ESTIMATION OF HYDROGEN PEROXIDES (H_2O_2)

The H_2O_2 content was assayed using the method described by [30]. 0.1 g of the shoot tissues of every sample were taken and homogenized independently in a sterile mortar and pestle with 2 ml 0.1% (w/v) TCA before centrifugation at 12,000 rpm for 15 min at 4ºC. The intensity was read at 390 nm employing a UV spectrophotometer with 0.5 ml of the supernatant, 0.5 ml of 10 mM phosphate buffer with pH-7.0, and 1 ml of 1M KI in a cuvette. The H_2O_2 content was calculated by an extinction co-efficient 0.28 μM^{-1} cm^{-1} and was quantified as $\mu M\ g^{-1}$ F.W.

2.4.2. ESTIMATION OF MDA

Malondialdehyde (MDA) was assayed using the method describe by [31]. 0.5 ml of 5%TCA was used to homogenize the shoot tissues and centrifuged at 12,000 rpm for 20 min. 2 ml TBA reagent (0.5% TBA in 20% TCA) was added to 0.5 ml supernatants for the MDA estmation. The absorption of homogenates was

evaluated at 532 nm and nonspecific turbidity was corrected by subtracting absorbance at 600 nm, with units of $mM^{-1}cm^{-1}$.

2.4.3. HISTOCHEMICAL DETECTION OF HYDROGEN PEROXIDE

The localization of H_2O_2 was carried out histochemically in leaf samples using the method described by [32]. Healthy leaves of each experimental setup were dipped in DAB solution (1mg ml^{-1}, pH-4.0) in a sterilized glass beaker for 12 h under light at RT. The leaves were then dipped in 95% ethanol and boiled for 20min to decolorize them and after cooling, the presence of H_2O_2 was discovered using a light microscope (brown spots).

2.5.ASSAY OF ENZYMATIC ANTIOXIDANTS

2.5.1.ESTIMATION OF SUPEROXIDE DISMUTASE ACTIVITY (SOD, EC 1.15.1.1)

The SOD content from each sample was calculated by using the method proposed by [33]. The activity of SOD to prevent the photochemical degradation of nitro blue tetrazolium (NBT) was used to estimate SOD activity. 0.5 g of tissue from each sample was taken and was homogenized with 2ml of 50mM of phosphate buffer containing 1mM EDTA and 2% PVP and then centrifuged at 13,000 rpm for 20 min at 4ºC and supernatant was collected.50mM phosphate buffer with pH 7, 0.3 ml of 130 mM methionine, 0.3 ml of 750 µM NBT, 0.3 ml of 10 mM EDTA, 0.3 ml of 20 µM riboflavin, 0.25 ml of distilled water, and 50 µl extracted sample was taken in sterilized test tubes and placed under fluorescent lamp for 10 min. After 10min the absorbance was checked at 560 nm. The quantity of enzymes necessary to block the photoreduction activity of the NBT by 50% was denoted by one unit (U) of SOD activity. The enzyme performance was expressed as U g^{-1} f.w. as well as the SOD activity was calculated utilizing provided equation.

% of inhibition= [1- Absorbance of each sample / Absorbance of the control] × 100

2.5.2.ESTIMATION OF ASCORBATE PEROXIDASE ACTIVITY (APX, EC 1.11.1.11)

An APX activity of every sample was determined using the protocol outlined by [34]. In 2 ml of 50 mM phosphate buffer solution containing 1mM EDTA and 2% PVP, 0.5 g of tissue from each sample was homogenized properly and centrifuged at 13,000 rpm for 20 min at 4ºC and the supernatant was collected into a sterilized eppendorf. In a cuvette 1ml of the reaction mixture comprising 600 µl of 50 mM phosphate buffer solution, 100 µl of 1 mM EDTA, 100 µl of 5mM ascorbic acid, 100 µl of H_2O_2, and 100 µl of the sample extract were taken. A UV-VIS spectrophotometer was employed to measure the intensity at 290 nm for 3 min. The ascorbate extinction coefficient of 2.8 nm^{-1} cm^{-1} was used to calculate the enzyme function by monitoring the changes in absorbance at 290 nm for 3 min. 1unit of enzyme action (U g^{-1}f.w.) was established as the amount of enzyme necessary to catalyze the oxidation of 1 mol of ascorbate in 1min.

2.5.3.ESTIMATION OF CATALASE (CAT, EC 1.11.1.6)

The activity of catalase was determined by [35]. 0.5 g tissue was homogenized in 2 ml of 50 mM phosphate buffer solution including 1mM EDTA and 2% PVP. The extracts were centrifuged at 4ºC at 13,000 rpm for 20 min. The supernatant was kept on ice for assay. The reaction mixture containing 50 µl of 30 mM H_2O_2, 2.9 ml of 50 mM of enzyme extract was taken in a cuvette and the change in absorbance was recorded at 240 nm for 3 min. The activity was measured using extinction coefficient 40 mM cm^{-1} and was expressed as U g^{-1} f.w.

2.5.4.ESTIMATION OF GUAICOL PEROXIDASE ACTIVITY (GPX, EC 1.11.1.9)

The GPX activity was assayed by the following method of [36]. 0.5gm of tissue from each sample was taken and homogenized with 50mM PBS buffer solution containing 1 µM EDTA and 2% PVP. At 4ºC, the supernatants were centrifuged for 20 min at 13,000 rpm. The reaction mixture contained 50 mM PBS buffer, 30 mM guaiacol, and 40 mM H_2O_2 along with enzyme extract was carried out for 30min and the absorbance was read at 470 nm. One minute of GPX activity equals the quantity of enzyme catalyzing the oxidation of one mole of guaicol in one minute, and enzyme action was measured in units of U g^{-1} f.w.

2.6.ASSAY OF ANTIOXIDANTS

2.6.1.ESTIMATION OF ASCORBATE CONTENT

The ascorbate contentwas measured by the following method of [37]. 0.1 g stored tissue of shoots were taken and homogenized with 4% TCA in sterilized mortar and pestle. Lysates were placed in a 2 ml eppendorf tube and centrifuged for 10 min at 2,000 rpm at 4ºC. Supernatants were collected and a pinch of charcoal was added to it. The samples were kept for 5min at RT and centrifuged for 10 min at 2,000 rpm at 4ºC to remove charcoal. The aliquotes were used for estimation of ascorbate. 0.5ml charcoal-treated supernatant was transferred to the sterilized test tube. 1.5 ml of 4% TCA, 0.5 ml of 2% dinitrophenyl hydrazine, and 2 drops of 10% thiourea solution was merged with prepared charcoal aggregate and maintained at 37ºC for 3 h. Osazone crystal was formed and dissolved in 85% H_2SO_4 under cold conditions. The absorption was read at 540 nm, and measured in µg g^{-1} f.w.

2.6.2.PROLINE ESTIMATION

The protocol of proline was used to determine the quantity by [38]. 1 ml of 3% sulfosalicylic acid was mixed with 0.1 g leaf tissue and centrifuged for 10 min at 4ºC at 12,000 rpm. The solution mixture was maintained for 1 h at 100ºC in a heated water bath until it became orange, using 500 mM precipitate, glacial acetic acid, and ninhydrin solution. To finish the action, the resulting solution was placed in an icebath. 1ml toluene was added and vigorously stirred for 15-20 sec, after which the color changed to pink. At 520 nm, the solution was read and the µmol g^{-1} f.w. was computed.

2.7.STATISTICAL ANALYSIS

All experiments were performed three times repeatedly and independently with standard error mean (SEM). All the biochemical data were statistically examined and Anova tested for significant (* indicates $p \leq 0.05$ and ** indicates $p \leq 0.01$) difference using GraphPad Prism software.

3. RESULTS AND DISCUSSION

3.1.MORPHOLOGICAL PARAMETERS

Plants have established certain quick methods to counteract salinity induced destruction and acclimatize to a saline environment [13]. Micromolecules like phytohormones and signaling molecules, in complement to their escape strategies, let plants readjust to unfavorable environmental circumstances. $CdCl_2$ pre-treated (50 µM) pigeon pea seedlings showed improved morphological development as compared to control seedlings. High chlorosis and severe wilting symptoms were observed in control seedlings than that of Cd^{2+} pre-treated seedlings when exposed to salt stress. The Cd^{2+} pre-treated pigeon pea seedlings showed a higher amount of total chlorophyll content in the leaf tissues as compared to the control tissues. The elevated chlorophyll content was observed in co-stressed tissues as compared to control tissuesduring salt stress (Fig. 1A).

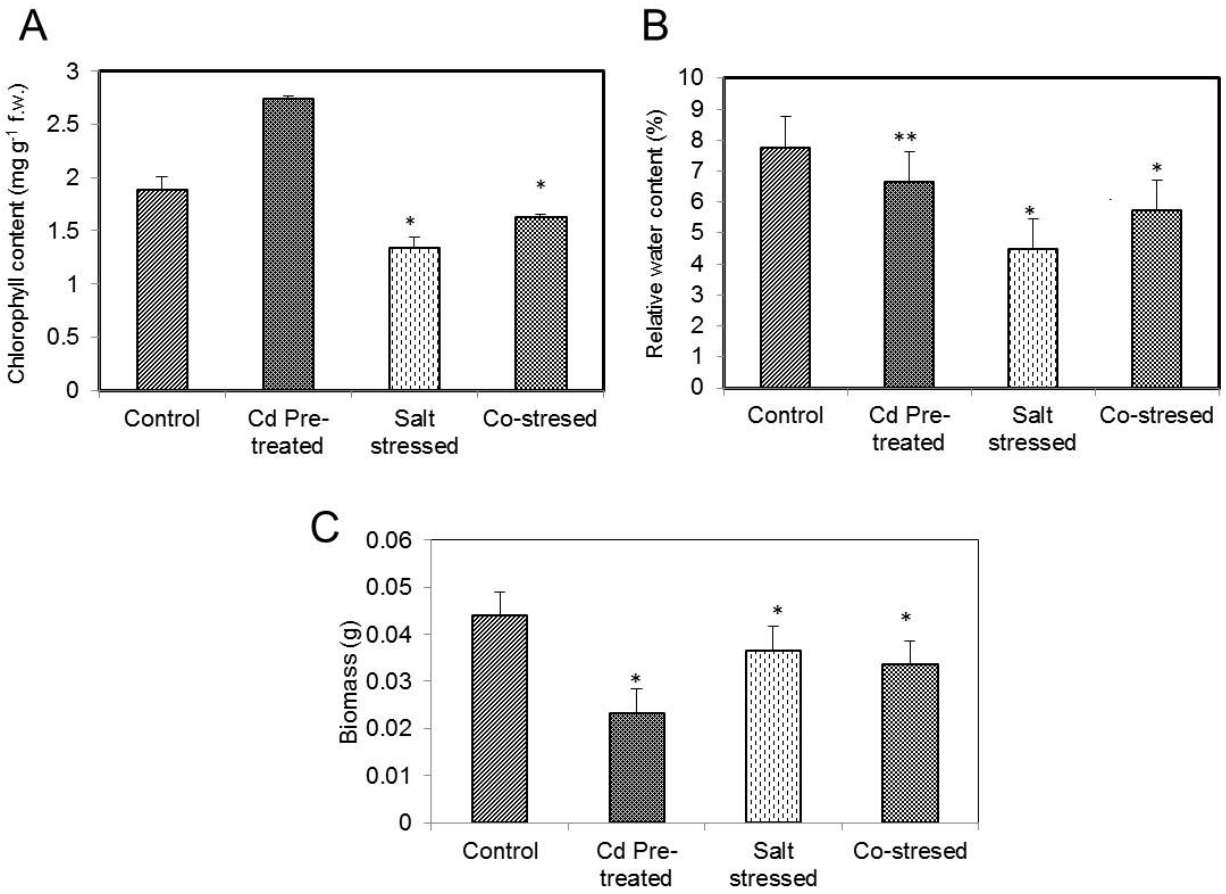

Figure 1 (A-C): Morphological analysis (A) Chlorophyll content, (B) Relative water content (%), and (C) Biomass (g) of shoot tissues of pigeon pea seedlings.

Salinity is amongst the most serious ecological stresses for plants, causing a reduction in growth rate as well as a variety of metabolic disturbances. Even a modest amount of salt can disrupt normal physiological and biochemical pathways, causing the cell to slow down its growth and progression [8, 39]. Plant proliferation is regulated by photosynthetic pigments and photosynthesis rate [13]. The photosynthetic pigments like total chlorophyll content was reduced dramatically in control seedlings when exposed to salt stress similarly reported in rice [40] and *Pisum sativum* [41]. The aggregation of Na^+ and Cl^- ions enhanced with larger salt concentrations, that hampered chlorophyll production by interfering with the Fe^{3+}-containing chlorophyll synthesizing enzymes [42], enhancing the levels of the chlorophyll-degrading enzyme chlorophyllase and reactive oxygen species (ROS) [22]. In co-stressed pigeon pea seedlings, priming with Cd^{2+} (50µM $CdCl_2$) enhanced total chlorophyll content. It was previously reported that Cd^{2+} priming increased total chlorophyll content in *Trigonella foenum-graecum* [43], *Festuca arundinacea* [44], and rice [45].

The relative water content (RWC) in $CdCl_2$ pre-treated pigeon pea seedlings was higher than the control seedlings. The co-stress pigeon pea seedlings showed a significantly high RWC value as compared to the salt-stressed seedlings (Fig. 1B).

It occurred as a result of osmotic stress, which causes the root to become hard and unable to absorb water [2, 8]. This result was analogous to exogenous Ca^{2+} priming favorably influencing stomatal performance by maintaining guard cells turgid [46], guaranteeing CO_2 supply, or regulating stomatal conductance [47]. Turgor pressure is essential for cell expansion [48], however, salinity diminishes turgor pressure, resulting in shorter shoot and root lengths and inhibited growth.

The enhanced biomass was observed in control seedlings as compared to the seedlings under salinity stress. When compared to co-stressed seedlings, salt stressed seedlings had lower biomass (Fig. 1C). [49] reported reduced shoot and root development, and physiological abnormalities (photosynthesis pigment degradation and electron flow impairment) leads to loss of biomass deposition. In our investigation, we found that the biomass was decreases when seedlings were subjected to salt stress.

As salinity promotes abscisic acid (ABA) mediated stomatal closure, which slows CO_2 assimilation and disrupts the Calvin cycle's normal electron flow for carbon breakdown, fresh and dry matter synthesis was diminished [50, 51].

3.2.OXIDANTS

3.2.1. H_2O_2 CONTENT

The salt-stressed seedlings had a significantly larger H_2O_2 content than the control, Cd^{2+} pre-treated, and co-stressed tissues. In contrast to salt-stressed tissues, the H_2O_2 content in co-stressed tissues was much lower (Fig. 2).

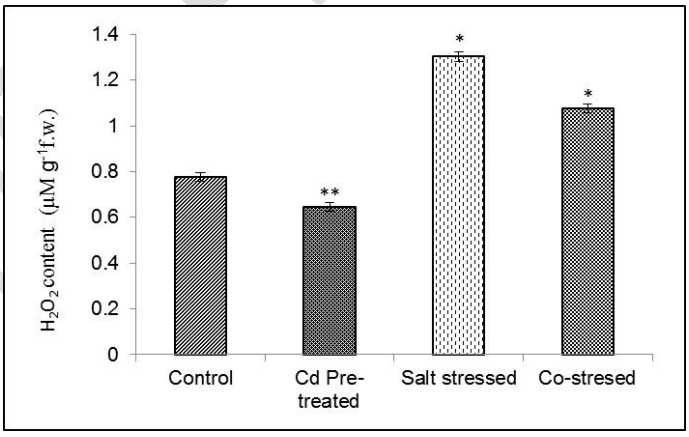

Figure 2: H_2O_2 content of shoot tissues of pigeon pea seedlings.

Salt induced the development of ROS such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), and hydroxyl radicals (OH^-) in plants [52]. ROS production could potentially be caused by an abundance of Na^+ in the cells and a lack of Ca^{2+} and K^+ [53]. As a result, the H_2O_2 content in salt-stressed tissues was significantly higher than in co-stressed, Cd pre-treated, and control tissues in the current investigation, which was consistent with

previous results. It had also been reported that H_2O_2 would be the most stable ROS and its excessive generation and aggregation is poisonous and harmful to plant cells during abiotic stress [54] and severely disrupt normal metabolism by peroxidation of membrane lipid [55].

3.2.2.MDA CONTENT

The MDA content in the salt-stressed tissues was remarkably higher than the co-stressed tissues. However, the MDA content was observed minimum in control and Cd^{2+} (50µM) pre-treated control tissues (Fig. 3).

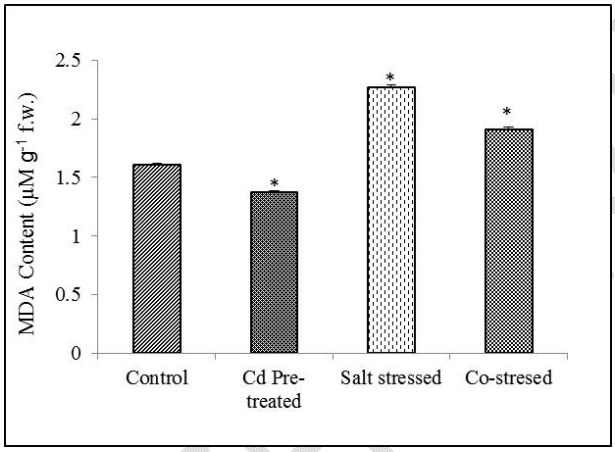

Figure 3: MDA content of shoot tissues of pigeon pea seedlings.

Moreover, increased ROS production in plant tissues causeses cell damage and poses a serious threat to macromolecules like DNA, proteins, and lipids, leading to lipid peroxidation, protein oxidation, and DNA damage [56]. Moreover, when comparing to control tissues exposed to salt, a limiting degree of lipid peroxidation was detected in co-stressed tissues, which leads to less amount of synthesis of malondialdehyde (MDA) as a byproduct of lipid peroxidation.

3.2.3. HISTOCHEMICAL ANALYSIS OF HYDROGEN PEROXIDE (H_2O_2)

Salt stress causes a high amount of ROS that induces tissue damage in the plant. The localized H_2O_2 in the pigeon pea leaves was observed by staining the leaves through oxidation of DAB. Salt stressed pigeon pea leaves showed higher numbers of dark brown spots which were indicative of accumulated H_2O_2 as compared to the co-stressed leaves [57] (Fig. 4).

Figure 4: Histochemical localization of Hydrogen peroxide content (A) Control, (B)Cd pre-treated, (C) Salt stressed, and (D) Co-stressed leaves of pigeon pea seedlings.

3.3. ASSAY OF ANTIOXIDANT ENZYMES

The SOD activity was detected higher in the co-stressed tissuesas compared to salt-stressed tissues. Similar results found in rice under water deficit condition [58]. Moreover, the co-stressed tissues showed high SOD activity as compared to the control and Cd^{2+} pre-treated tissues (Fig. 5A).

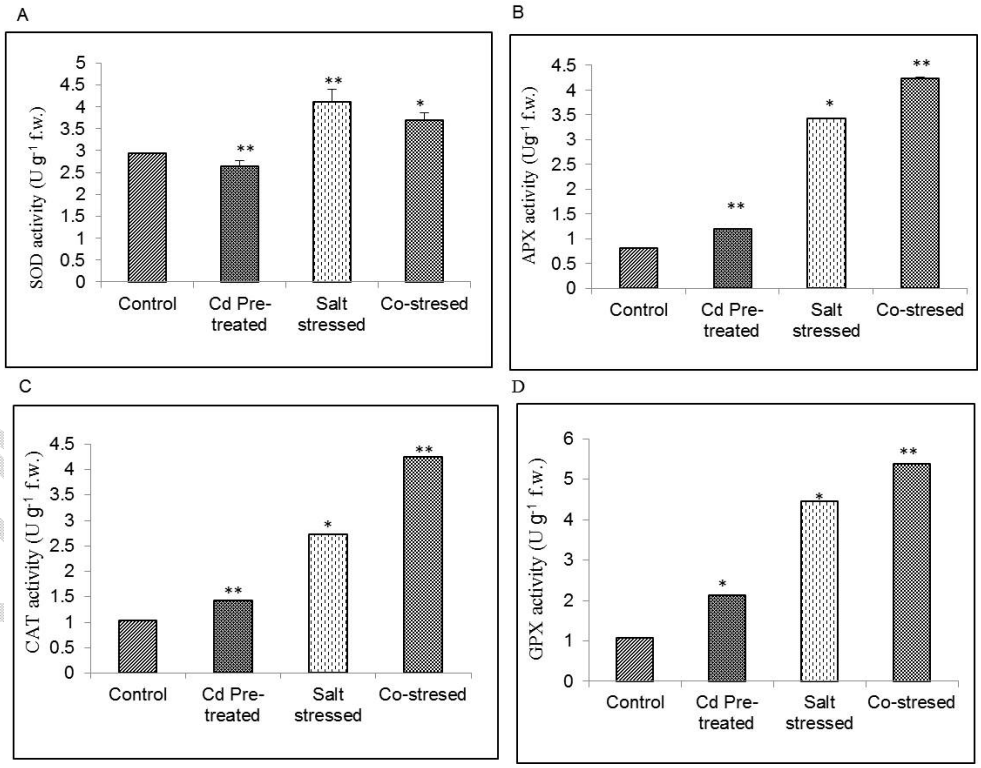

Figure 5 (A-**D):**. Antioxidant activity in shoot tissues of Pigeon pea seedlings. (A) SOD activity, (B) APX activity, (C) CAT activity, and (D) GPX activity .

Salinity stress has a big impact on SOD behavior [2]. Under high metal stress, rice varities showed an increase in SOD activity in the leaves [59]. It could be related to an elevation in superoxide production, which results in enhanced SOD gene expression [60]. The very first enzyme in the detoxifying process, SOD converts superoxide (O_2) radicals to hydrogen peroxide (H_2O_2) [61]. The co-stressed tissues also showed high SOD activity but the Cd^{2+} might be ameliorated the H_2O_2 production and it was previously confirmed by [62] on soyabean nodules.

When plants are exposed to salt stress, ROS formation arises [13]. Throughout this condition, antioxidant enzymes are intended to involve a vital part in the plant's protection against ROS [63]. APX activity was lower in the salt stressed tissues than co-stressed tissues (Fig. 5B) which was previously confirmed by [64] on *Glycine max*.

The ascorbate-glutathione cycle is a crucial hydrogen peroxide detoxifying pathway in plant cells, and APX enzymes play a crucial role in transforming H_2O_2 to H_2O employing ascorbate as a primary electron donor [65].

The salt-stressed tissues had lower CAT activity than the co-stressed tissues (Fig. 5C). Rice [66] and *Vigna radiata* had similar results [67]. To eliminate H_2O_2 from plants, CAT converts H_2O_2 to oxygen and water. Hence, the higher CAT activity contributed to better salt tolerance [11].

The co-stressed tissues showed high GPX activity as compared to salt-stressed tissues (Fig. 5D), as also reported by [68] in the roots and leaves of *Oryza sativa*. Our findings showed that increased levels of GPX in seedlings successfully absorb H_2O_2, which is a key modulator of cellular damage under environmental stress.

3.4. ASSAY OF ANTIOXIDANTS

ASC is an antioxidant and a critical substrate for the detoxifying of ROS [69]. Through non-enzymatic and enzymatic detoxification pathways, ASC is one of the most effective antioxidants generated from plants, and it performs a vital role in the lowering of excessive ROS [70]. Whenever plants are stressed by drought, salinity, or even other environmental challenges, the non-enzymatic antioxidants ascorbate and proline accumulate to high levels, according to several repots [71]. Ascorbate concentration was found to be lower in salt-stressed tissues when contrasted to co-stressed in this study (Fig. 6A).

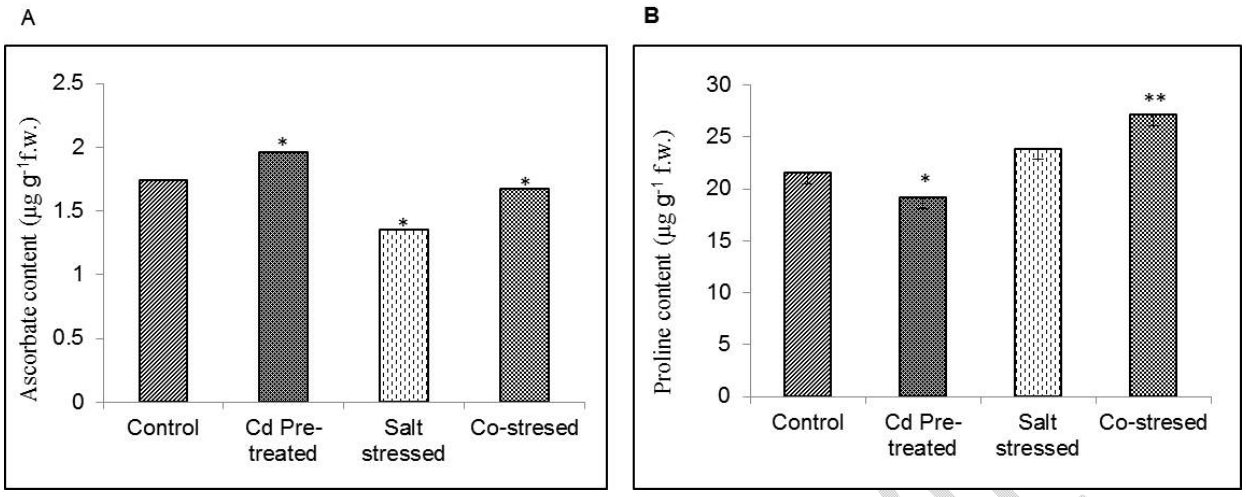

Figure 6 (A-B): Antioxidant contents in the shoot tissues of Pigeon pea seedlings. (A) Ascorbate content, and (B) Proline content.

A low dose of $CdCl_2$ could detoxify the ROS content in co-stressed tissues by accumulating ascorbate content, which might detoxify the ROS to stabilize the redox status. Certain metabolic processes also metabolize MDA and H_2O_2, resulting in the regeneration of reduced ASC in the ASC–GSH cycle [72].

The formation of osmolytes is a normal approach to stress in an attempt to minimize physiological disruption [73]. The increased proline content was observed in co-stressed tissues as compared to salt-stressed tissues (Fig. 6B). The proline content was maximum in co-stressed tissues which indicates that antioxidants might be acting as ROS detoxifiers at the cellular level and protects the integrity of membranes, stability of enzymes or proteins, and provides tolerance to diverse stimuli [41, 74].

4. CONCLUSION

It was concluded that a low dose $CdCl_2$ (50µM) pretrement conferred tolerance to salinity induced oxidative stress in pigeon pea seedlings through the activation of antioxidants defense machinery.

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