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ORIGINAL ARTICLE

Changes in Physiological Parameters and Some Antioxidant Enzymes Activities of Soybean (*Glycine max* L. Merr.) Leaves Under Cadmium and Salt Stress

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The combined effect of cadmium stress (0,5 mM $Cd(NO_3)_2$) and salt stress (100 mM NaCl) on growth, lipid peroxidation and activities of some antioxidant enzymes were studied in soybean (*Glycine max* L. Merr.) leaves. Shoot lenghts were not changed under all groups. But fresh and dry weight were decreased under salt treatment alone and Cd treatment alone. The decrease was more in the group of combined of Cd and salt treatment. Although APX activity increased under salt treatment alone and combined of Cd and salt treatment, GR activity increased under Cd treatment alone and combined of Cd and salt treatment. Nevertheless DHAR activity increased only in combined of Cd and salt stress. MDA content increased under all groups but it was more in the combined of Cd and salt stress which indicates that salinity is more harmful with cadmium stress in soybean plants. Thus, it was concluded that some of antioxidant enzymes (APX, GR, DHAR) increased their activity under combined of Cd and salt treatment but they were not efficient to protect oxidative damage from soybean plants by alleviating the lipid peroxidation.

Key words: cadmium; salt stress; soybean; antioxidant enzymes; Glycine max L. Merr.

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Key words: cadmium; salt stress; soybean; antioxidant enzymes; Glycine max L. Merr.

Abbreviations: Cd: Cadmium; APX: Ascorbate peroxidase; GR: Glutathione reductase; DHAR: Dehydroascorbate reductase; MDA: Malondialdehyde

Salinity is one of the most important environmental stress in the world which inhibits growth and productivity of crops. It has been estimated that more than 20 % of all cultivated lands around the world contain salt levels high enough to cause salt stress to crop plants (Moud & Maghsoudi, 2008). Neverthless, the problem of salinity becomes more toxic when plants are exposed to Cd ($CdNO_3$) stress by inducing physiological disturbes (Shafi et al., 2009). Also it was known that salt stress can enhance Cd content in soil and promotes the uptake of this metal by formating the Cl^--Cd complex in plants (Weggler et al., 2000). Cadmium (Cd) is a toxic, non- essential metal rapidly taken up by roots and accumulated in various plant tissues which also hamper the crop

growth and productivity worldwide like salinity (Gill et al., 2011). This metal disrupts the uptake of macro and micro elements and carbohydrate *metabolism* (Gussarson et al., 1996; Moya et al., 1993) and this causes to chlorosis and reduction in photosynthetic rate. Thus it is important that to investigate both of these stress factors in plants reveal a threat for agricultural areas for increasing the crop yield and growth.

Previous studies demonstrated that both Cd and NaCl produces oxidative stress by generating free radicals and reactive oxygen species (ROS) as superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (Muhling & Lauchli, 2003; Xu et al., 2010). This molecules react with proteins, lipids and nucleic acids and lead to lipid peroxidation. Increase in the production of ROS are scavenged by antioxidant system which includes superoxide dismutase (SOD EC, 1.15.1.1) peroxidase (POX EC, 1.11.1.7) catalase (CAT EC, 1.11.1.6), ascorbate peroxidase (APX EC, 1.11.1.11), glutathione reductase (GR EC, 1.8.1.7) and dehydroascorbate reductase (DHAR EC, 1.8.5.1) enyzmes in plants. There are many studies that there is a correlation with increasing antoxidant enzyme activities with coping oxidative stress in plants. Siripornadulsil (2002) and Wu et al. (2003) also mentioned the plants can reduce cadmium toxicity through the production of ROS scavengers. Similarly, it is well known that salt stress can also enhance the antioxidant enzyme activities in plants for alleviating the oxidative damage (Azevedo- Neto et al., 2006).

Soybean is important crop in our world because of it's high protein and oil content. Salinity and cd toxicity inhibits productivity in this plant such as others (wheat, barley and rice). Balestrasse et al. (2004) reported that Cd toxicity caused senescence in soybean nodules by increasing protease activity and ethylene production. Moreover, synergistic effects of Cd and salt stress were determined that inhibition on photosynthesis and growth in soybean (Wei et al., 2007). In aggrement with this results, Ferreira et al. (2002) were reported that some of antioxidant enzymes increased in soybean plants under Cd stress by increasing the lipid peroxidation. In wheat plants, it was observed that cd and salt stress effected more than salt stress alone (Shafi et al., 2009). But there is no report on antioxidant enzyme activities under Cd and salt stress in soybean seedlings. Therefore, in the present study, changes in some physiological parametres and activities of some antioxidant enzymes (APX, GR, DHAR) were determined under both Cd and salt stress in soybean (*Glycine max* L. Merr.) leaves.

MATERIALS AND METHODS

Plant material and experimental design

Seeds of soybean (*Glycine max* L. Merr.) were obtained from the seed company is called May, from Bursa, Turkey. The seeds were sterilized in % 5 hypocloride solution for 15 minutes and then rinsed three times in distilled sterile water. Than they were sown in plastic trays (10 cm x 14cm), containing soil. After germination, seedlings were grown in a growth room, at 25 ^{o}C, (16h day/8h night photoperiod), light intensity of 500 µmol m $^{-2}$s $^{-1}$ and watered with Hoagland solution (Hoagland and Arnon, 1950). 21 days old plants were exposed to 0,5 mM $Cd(NO_3)_2.4H_2O$, 100 mM NaCl, and 0,5 mM $Cd(NO_3)_2.4H_2O$ + 100 mM NaCl in Hoagland solution for 5 days. After that plants were harvested and stored at - 20 ^{o}C for further analysis.

Analyses: Physiological analysis

Determination of fresh and dry weight

6 plants were taken at random divided into

seperate root and leaf fractions. The fresh weights (FW) of shoots were determined. The samples were dried in a forced draft oven at 70 °C for 72 h and then dry weights of shoots (DW) were determined.

Fresh weight of leaves were determined. The leaves were floated on dIH_2O water for 4h under low irradiance and then the turgid tissue was quickly blotted to remove excess water and their turgid weights (TW) were determined. DW was determined after leaves were dried in the oven.

Determination of relative water content (RWC)

After harvest on 5d of all treatment, six leaves were obtained from soybean plants for each species and their FW was determined. The leaves were floated on de-ionised water for 5h under low irradiance and then the turgid tissue was quickly blottedto remove excess water and their turgid weights (TW) were determined. DW was determined after leaves were dried in the oven. Relative water content was calculated by (Smart & Bingham, 1974).

Determination of malondialdehyde content (MDA)

The level of lipid peroxidation in leaf samples was determined in terms of malondialdehyde (MDA) content according to the method of Madhava Rao and Sresty (2000). Content of MDA, which is an end product of lipid peroxidation, was determined using the thiobarbituric acid reaction. MDA concentration was calculated from the absorbance at 532 nm and measurements were corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The concentration of MDA was calculated using an extinction coefficient of 155 $mM^{-1}\,cm^{-1}$.

Estimation of protein content

All operations were performed at 4 °C. For protein and enzyme extractions, 0.5 g of fresh leaf samples were homogenized in 1.5 ml of 50 mM sodium phosphate buffer (pH 7.8) containing 1 mM ethylenediaminetetraacetic acid (EDTA).Na_2 and 2% (w/v) polyvinylpolypyrrolidone (PVPP). Samples were centrifuged at 14,000×g for 30 min, and supernatants were used for the determination of protein content and enzyme activities. Total soluble protein contents of the enzyme extracts were determined according to Bradford (1976) using bovine serum albumin as a standard. All spectrophotometric analyses were conducted on a Shimadzu (UV visible) spectrophotometer.

Ascorbate peroxidase (APX) activity

APX (EC 1.11.1.11) activity was measured according to Nakano and Asada (1981). The assay depends on the decrease in absorbance at 290 nm as ascorbate was oxidized. The reaction mixture contained 50 mM Na-phosphate buffer (pH 7.0), 50 mM ascorbate, 0.1 mM EDTA.Na_2, 1.2 mM H_2O_2 and 0.1 ml of enzyme extract in a final assay volume of 1 ml. The concentration of oxidized ascorbate was calculated by using extinction coefficient of 2.8 $mM^{-1}\,cm^{-1}$. One unit of APX was defined as 1 mmol ml^{-1} ascorbate oxidized min^{-1}.

Glutathione reductase (GR) activity

GR (EC 1.6.4.2) activity was measured according to Foyer and Halliwell (1976). The assay medium contained 25 mM Na - phosphate buffer (pH 7.8), 0.5 mM GSSG, 0.12 mM NADPH.Na_4 and 0.1 ml enzyme extract in a final assay volume of 1 ml. NADPH oxidation was followed at 340 nm. Activity was calculated using the extinction coefficient of NADPH (6.2 $mM^{-1}\,cm^{-1}$). One unit of GR was defined as 1mmol ml^{-1} GSSG reduced min^{-1}. The specific enzyme activity for all enzymes was expressed as in unit mg^{-1} protein.

Dehydroascorbate reductase (DHAR) activity

For the DHAR (EC 1.8.5.1) assay, a reaction mixture containing phosphate buffer (pH 7.0) 0.7 ml, reduced glutathione (GSH) 20 mmol/L 0.1 ml in the phosphate buffer (pH 7.0), 2 mmol/l DHA 0.1 mL, and crude enzyme 0.1 ml was used. DHA was freshly prepared and kept on ice until it was added to the reaction mixture in the cuvette to prevent its fast oxidation at room temperature. The reduction of DHA to ASA was monitored by the increase in absorbance at 290 nm, taking 2.8 $(mmol/l)^{-1}$ cm^{-1} as the absorbance coefficient (Krivosheeva et al., 1996).

Statistical analysis

Each pot was treated as one replicate and all the treatments were repeated three times. The data were analyzed statistically with non- parametric test Mann-Whitney U. Each data point was the mean of six replicates (n=6) and comparisons with p values <0.05 were considered significantly different.

RESULTS

In general cadmium alone, salt alone, and combined of Cd and salt treatment did not change the lenght of (*Glycine max* L. Merr.) seedlings as compared to control group. But there were some differences in the leaves of dry and fresh weight and relative water content under three different stress treatment. Fresh weight of leaves were decreased by 20,46 % and 12,18 % under combined of Cd and salt treatment and cd treatment alone as compared to control group respectively. Similarly dry weight of leaves were decreased under same groups, but it was more remarkable in combined of Cd and salt treatment by 35,2 % and 15,50 % in the group of Cd treatment alone. On the other hand, fresh weight and dry weight of leaves were reduced by 10,79 % and 13,63 % in the group of salt treatment alone (100 mM NaCl) according to control group respectively.

Relative water content was decreased under combined effect of Cd and salt and Cd treatment alone as compared to control group. Also it was decreased under salt treatment alone (100 mM NaCl) by 13,31 %. The combination of Cd and salt treatment led to significiant reduction in 23, 58 % when compared with Cd treatment alone (15,86 %).

APX activity was enhanced in (*Glycine max* L. Merr.) leaves under combined of Cd and salt and salt treatment alone as compared to control group. But it was more pronounced in combined of Cd and salt treatment by 47,61 %. This increase was also significiant by 28,57 % under salt treatment alone (100 mM NaCl). Otherwise cd treatment alone did not change the APX activity in the leaves of this plant. In contrast to APX enzyme activity, GR activity was not changed in (*Glycine max* L. Merr.) leaves under salt treatment alone (100 mM NaCl). Otherwise it was significiantly enhanced in combined effect of Cd and salt stress by 20 % like APX activity. In addition to that, cd treatment alone increased the GR activity by 13,33 % according to control group.

Similar to GR, DHAR activity was not changed in the leaves of this plant under salt treatment alone. Beside this, differently from GR; it was also unchanged in the group of Cd treatment alone. Otherwise DHAR activity was increased only in combined of Cd and salt treatment as compared to control group like APX and GR activities, but this increase (18,18 %), was lower when compared with APX and GR activity. MDA content was increased in the leaves of this plant under all groups as compared to control group. This increase was remarkable in cd stress alone by 43,39 %.

Otherwise, this increase was 17,45 % by salt treatment alone. In the group of combined of Cd and salt treatment MDA content was enhanced by 54,61 % as compared to control group.

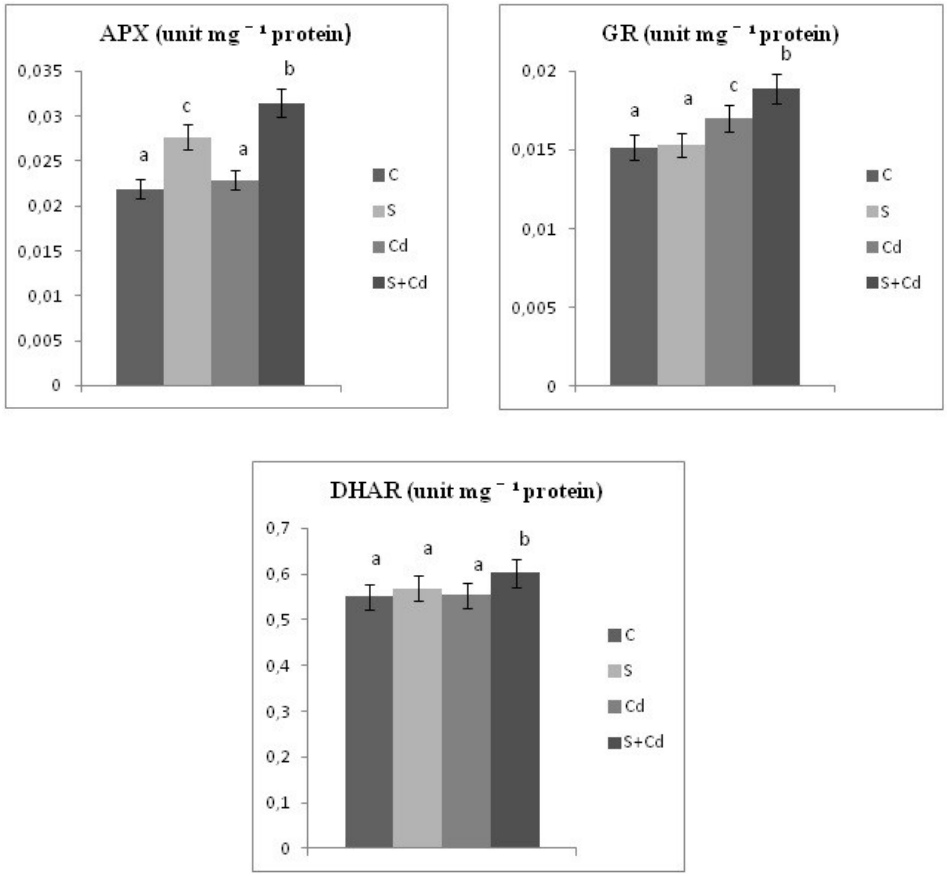

Figure 1. Changes in a) APX b) GR c) DHAR activities in leaves of (*Glycine max* L. Merr.) under stress treatment for 5 days (C, S, Cd, S+Cd). C: control; S: 100 mM NaCl, Cd: 0,5 m M $Cd(NO_3)_2$, S+Cd: (100 mM NaCl + $Cd(NO_3)_2$. The different letters are significantly different ($p < 0.05$) values.

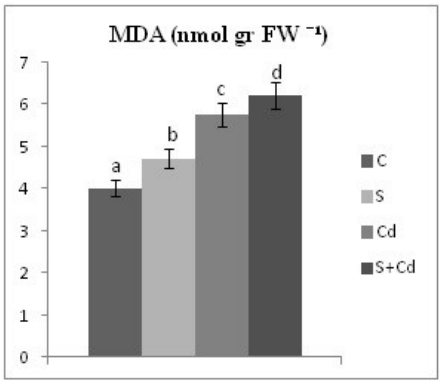

Figure 2. Changes in MDA content (nmol gr FW^{-1}) in leaves of (*Glycine max* L. Merr.) under stress treatment (C, S, Cd, S+Cd). C: control; S: 100 mM NaCl, Cd: 0,5 mM $Cd(NO_3)_2$, S+Cd: (100 mM NaCl + $Cd(NO_3)_2$. The different letters are significantly different ($p < 0.05$) values.

Table 1. Changes in relative water content (%), fresh and dry weight (gr), shoot lenght (cm) in leaves of (*Glycine max* L. Merr.) under stress treatment for 5 days (C, S, Cd, S+Cd). C: control; S: 100 mM NaCl, Cd: 0,5 mM $Cd(NO_3)_2$ S+Cd: (100 mM NaCl + $Cd(NO_3)_2$. The different letters are significantly different ($p < 0.05$) values.

Groups	Relative water content (%)	Fresh weight (gr)	Dry weight (gr)	Shoot lenght (cm)
(C)	54,58 ±0,56 [a]	1,76± 0,07[a]	0,19 ± 0,03[a]	50,1± 0,52[a]
(S)	47,23± 0,88[b]	1,55 ± 0,05 [b]	0,17±0,05[b]	50,47± 0,68[a]
(Cd)	45,92± 0,34[c]	1,57± 0,03[b]	0,16 ± 0,07[b]	50,40 ± 0,69[a]
(Cd+ S)	41,71 ± 0,57[d]	1,40 ± 0,04[c]	0,12± 0,08[c]	50,46 ± 0,70 [a]

DISCUSSION

In the present study, lenght of soybean (*Glycine max* L. Merr.) shoots were not changed under all stress treatments (Table 1). In contrast to our results, Shafi et al (2009) reported that Cd and NaCl treatment decreased in shoot lenght of wheat species. Also in the previous studies, it was shown that a inhibition of shoot lenght of soybean plants was in hydroponic treatments under Cd (Ferreira et al., 2002) and salt stress (Kang et al., 2007). In our study, it can be suggested that Cd (0,5 mM) and salt treatment (100 mM NaCl) for 5 days were short time to inhibit shoot lenght in soil experiment when compared to other studies. These results are in aggrement with Drazic et al. (2004) reported that growth of plants under stress conditions were related with concentration, kind of treatment, duration and plant species. So our results being different from the others were probably due to growth conditions, duration and concentrations.

In the present study, fresh and dry weight of leaves in soybean plant, were decreased under all groups but it was remarkable in the group of both Cd and salt treatment. In parallel with our results, Shafi et al. (2011) mentioned that Cd treatment inhibited dry weight of wheat plants. Moreover Razıuddin et al. (2011) observed excess Cd accumulation in soil can reduce the growth of plants by disturbing the photosynthesis and chlorophyll synthesis. So it can be suggested that both of this stress factors affected the soybean plants more negatively by disturbing the physiological and biochemical parameters. In aggrement with our results, Smykalova and Zamecnikova (2003) observed that NaCl + Cd treatment inhibited the fresh and dry weights of barley plants. Otherwise it's well known that salinity also inhibits fresh and dry weights of plants. As (Farhoudi and Tafti, 2011) mentioned, fresh weight of soybean seedlings significiantly decreased with the increase of salinity levels. Similarly, there was a reduction of this parameters in soybean leaves under salt treatment alone in our experiments (Table 1).

Inhibition of growth can be related with decreasing water content in plants under stress conditions (Mittler, 2002). In the present study, relative water content was decreased under all groups. (Table 1). This is consistent with the findings in *Brassica sp* (Razıuddin et al., 2011). Salt treatment alone reduced the relative water content of soybean leaves but it was higher in the group of Cd treatment alone in our experiment. These findings are in aggrement with previous report in *Atriplex* plants (Levefre et al., 2009). It can be suggested that, decrease in relative water content is related with osmotic stress. Moreover it has been

reported that in *Arabidopsis* plants, proline content was increased under combined of Cd and salt stress (Xu et al., 2010). According to this results, it's clear that Cd toxicity can also tigger the osmotic stress like salinity in soybean plants.

Malondialdehyde (MDA) as the decomposition product of polyunsaturated fatty acids of biomembranes, showed greater accumulation under stress condition (Gossett et al., 1996). In the present study, MDA content was increased in the leaves of soybean plant under all groups as compared to control group (Figure 2). In additional this, combined Cd and salt treatment had a higher than either salt or Cd treatment alone. In aggrement with our results, Shafi et al. (2010) reported that MDA content was increased under salt and Cd stress in wheat plants. Our results are suggested that the enhanced in MDA content under Cd and salt treatment can be related with increasing reactive oxygen species. In literature, there are many reports changing MDA content under salinity in plants (Hernandez and Alamansa, 2002; Yu and Liu, 2003). However as Hegedus et al. (2001) mentioned Cd toxicity also increased MDA content of barley plants. Also it's well known that salt stress may occur oxidative damage, but from this results, it is more toxic when plants are exposed to Cd treatment.

APX plays vital role in plant defence against oxidative stress by scavenging H_2O_2 in chloroplast, ctyosol, mitochondria and peroxisome of plant cells (Asada, 2006). Different reports have shown increased activity of APX in response to stress treatments (Hernandez et al., 1995). In the present study, APX activity was enhanced by 28,57 % in the leaves of soybean under salt stress alone as compared to control group (Figure 1a). In paralel to our results, Yu and Liu (2003) reported that APX activity was enhanced in the leaves of salt tolerant cultivar of soybean plants under salt stress. Otherwise Cd treatment alone did not change the APX activity in the leaves of this plant. Similarly, Ferreira et al. (2002) observed CAT activity did not change under Cd stress in soybean plants. CAT enzyme is also plays role by detoxifying the H_2O_2. The combined of Cd and salt stress had a higher APX activity as compared to control group than either salt or Cd treatment alone (47,61 %). It's clear that salinity induces the APX activity in the leaves of soybean plants with lower MDA content compared to combined of Cd and salt treatment, Cd treatment alone may not be efficient to increase this enzyme activity. The increase of MDA content in the group of Cd treatment alone can be related with unchanged APX activity.

GR is a potential enzyme of the ASC–GSH cycle which plays an essential role in the plant's defence against ROS by maintaining the GSH level (Madhavo Rao et al., 2000). In our experiment, GR activity did not change under salt treatment alone in the leaves of soybean plants (Figure 1b). In agrement with our results, Meloni et al. (2003) mentioned that GR activity did not change under salt stress in cotton plants. In te present study, although APX activity was higher under salt treatment alone, unchanged GR activity can be related with increase MDA content as compared to control group under salt treatment alone. However, combined effect of Cd and salt treatment increased the GR activity by 20 %. Ferreira et al. (2011) observed GR activity in soybean plants did not change under Cd treatment. In contrast to this result, we found that GR activity increased with Cd treatment alone but it was lower than combined Cd and salt stress. Similar with our results, GR activity increased in *Phaseolus vulgaris* plant under Cd treatment (Chaoui et al. 1997).

Because of the unchanged activity of APX in the group of Cd treatment alone, APX and GR enzymes can not complete the ascorbate-glutathione cycle.

In ascorbate-glutathione cycle, monodehydroascorbate is regenerated by NADPH in a reaction catalyzed by MDAR and DHAR catalysis the reduction of DHA to AsA by oxidizing GSH. The oxidative form of glutathione (GSSG) is reduced by GR (Noctor & Foyer, 1998). In addition, GR regulates GSH/GSSG ratio and supplies GSH for DHAR, which convert H_2O_2 to H_2O and reduce oxidized ascorbate, respectively. Although GR also increases $NADP^+$/NADPH ratio. DHAR is thought to play an important role in the oxidative stress tolerance of plants by regenerating ascorbate from dehydroascorbate (Foyer & Mullineaux, 1998).

In our results, DHAR activity was not changed under salt treatment and Cd treatment alone. It was only increased in the group of Cd and salt treatment by (18,8 %) (Figure 1c). In literature there are some reports that DHAR activity increased under salt stress (Mittova et al., 2003; Amor et al., 2006). Nevertheless, Gallego et al. (1999) reported that DHAR activity decreased in the leaves of sunflower plant under Cd treatment. However, it increased in the root of soybean plants under Cd stress (Balestrasse et al. 2001). In our experiment, under salt treatment alone, both GR and DHAR activity were not changed. So it can be said that APX activity was not efficient alone for decreasing MDA content. Otherwise, Cd treatment alone increased only GR activity, not the APX and DHAR. Increase in MDA content in this group can be related with this results. Neverthless, the combined of Cd and salt stress treatment had higher APX, GR and DHAR activity as compared to control group. The MDA content was increased according to control group although this enzyme activity was increased significiantly.

CONCLUSIONS

In the present study, cd and salt treatment alone decreased the physiological parameters (dry and fresh weight, relative water content) but the combined effect of cd and salt treatment was more negative in the leaves of soybean plants. Nevertheles, it's clear that some of antioxidant enzymes (APX, GR, DHAR) increased their activity under combined of cd and salt treatment but they were not efficient to protect oxidative damage from soybean plants by alleviating the lipid peroxidation. Further biochemical and molecular studies are required to clarify the effects of cd and salt stress in the leaves of soybean (*Glycine max* L. Merr.) plants.

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