INTERACTIVE EFFECTS OF NaCl AND $CdCl_2$ ON ANTIOXIDANT ENZYME ACTIVITIES AND SOME BIOCHEMICAL COMPOUNDS IN TWO TOMATO GENOTYPES

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SUMMARY

In this study, the combined effects of salt and cadmium stresses on the enzymes of the antioxidative defence system were investigated in two *Lycopersicon esculentum* (*Lycopersicon e.*) genotypes, namely, salt-sensitive Rheinlands Ruhm and salt-tolerant var. *cerasiforme*. Plants were grown under salt (100 mM and 200 mM NaCl), cadmium (100 µM and 200 µM $CdCl_2$), and their combinations for eight weeks in controlled environmental conditions. Chlorophyll a and chlorophyll a/b ratio significantly increased under salt stress in salt-tolerant var. *cerasiforme*, but decreased in salt-sensitive Rheinlands Ruhm. Chlorophyll contents were decreased under cadmium and salt stress combinations in both genotypes. Superoxide dismutase (SOD) activity almost remained unchanged in both cultivars. Ascorbate peroxidase (APX) activity in *Lycopersicon e.* var. *cerasiforme* was found to be higher than in Rheinlands Ruhm, whereas glutathione reductase (GR) activities decreased in Rheinlands Ruhm under all stress treatments. In *cerasiforme* variety, CAT activity significantly increased under single and combined stress treatments, and it seems that APX and CAT may play a more important role than SOD and GR in this salt-tolerant tomato variety at compartments affected from salt and cadmium stresses. Also, cadmium contents were higher in roots of salt-sensitive Rheinlands Ruhm, compared to the tolerant *cerasiforme*. We concluded that the genotype *cerasiforme* has higher tolerance to cadmium stress than that of Rheinlands Ruhm, and may be cross-tolerant to both salt and cadmium stresses.

KEYWORDS: Cadmium; cross tolerance; *Lycopersicon esculentum*; oxidative stress; salt.

INTRODUCTION

Soil contamination with heavy metals has become a world-wide problem, leading to losses in agricultural yield, but also hazardous health effects when they enter the food chain [1]. The main sources of contamination in agricultural soils are fertilizer impurities (Cd^{+2}), and the use of refuge-derived compost and sewage sludge. Disposal of household, municipal and industrial wastes has given rise to even more widespread cadmium soil concentrations [2]. Cadmium is a toxic trace pollutant for plants, animals and humans. It has been revealed that Cd is strongly phytotoxic and causes growth inhibition, and even plant death due to its interaction with photosynthesis, respiration and nitrogen assimilation in plants [3, 4].

Tomatoes are important greenhouse crops in semi-arid regions of the Mediterranean countries. In these regions, soil and ground water salinity are serious problems that affect both tomato yield and quality [5]. Water is also a limited resource that must be conserved, and high salinity may cause ionic imbalance and toxicity in plants [6].

Aerobic oxidative metabolism represented a major evolutionary step for living organisms, allowing a more efficient utilization of the energy stored in chemical bonds. On the other hand, the use of molecular oxygen as the final electron acceptor posed a risk of oxidative damage due to the production of partially reduced intermediates, known as reactive oxygen species (ROS) [7]. ROS are partially reduced forms of atmospheric oxygen (O_2). They typically result from the excitation of O_2 to form singlet oxygen (O_2^1), or from the transfer of one, two or three electrons to O_2, respectively, a superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) or a hydroxyl radical (HO^-). ROS are capable of unrestricted oxidation of various cellular components, and can lead to the oxidative destruction of the cells [8, 9]. The cells are normally protected against these ROS by the operation of intricate antioxidative enzymatic (superoxide dismutase, SOD; EC 1.15.1.1, ascorbate peroxidase, APX; EC 1.11.1.11, glutathione reductase GR; EC 1.6.4.2

and catalase CAT; EC 1.11.6) and non-enzymatic (ascorbate, tocopherol, carotenoids, glutathione and phenolic compounds) systems [10, 11].

Under field conditions, multiple stress factors, such as combined salt and cadmium stresses act simultaneously on the plant. In plant cells, these stress conditions promote the formation of harmful ROS, which may initiate destructive oxidative processes, such as chlorophyll bleaching, lipid peroxidation, protein oxidation, and damage of nucleic acid [9, 13]. Heavy metal [3, 13], salt [9, 14] and drought [15] stresses in different crop plants have been observed. However, no information is available on the relationship between salt tolerance and the level of antioxidant enzymes under Cd stress.

Much of the previously evidenced effects is based on the response of plants under a single stress factor. In the current study, the effects of salt and Cd, singly and combined, on the activity of the antioxidant enzymes, pigment content and the lipid peroxidation of two different salt-tolerant tomato cultivars were investigated, based on two hypotheses (relationship between salt tolerance and Cd stress, and cross tolerance between salt and Cd stress).

MATERIALS AND METHODS

Plant materials and stress applications

In this study, the relatively salt-tolerant *Lycopersicon esculentum* var. *cerasiforme* (LA 1310) and non-tolerant *Lycopersicon esculentum* cv. Rheinlands Rhum (LA 0535) were used. The seeds were obtained from the Tomato Genetic Resource Center, Department of Vegetable Crops, University of California, Davis, USA. Seeds were applied to 3 % sodium hypochlorite for 30 min to sterilize the surface, rinsed in distilled water and treated with 0.1 M H_2SO_4 for 1 h and embedded for 24 h with aerated water. After imbibitions, the seeds were planted into plastic pots containing perlit (3000 g). Plants were grown at 26/22 °C (day/night) temperatures at 65 ± 5 % relative humidity (RH) in a growth chamber with 480 µmol.m^2.s^{-1} light (16/8 h day/night cycle). Pots were watered with 0.5N Hoagland solution [16], containing $CdCl_2$ (M_0 control, M_1 100 µM, M_2 200 µM Cd^{2+} as $CdCl_2$) and NaCl (S_0 control, S_1 100 mM and S_2 200 mM NaCl), and their combinations were applied at the end of eight weeks. Seedlings were grown under cadmium and salt stress for one week, and then harvested. Samples of leaves used for biochemical analyses were flash-frozen in liquid N_2 and stored at -50 ºC.

Cadmium analysis

The heavy metal content was determined by atomic absorption (Hitachi 180-80). The dried root tissues were digested in perchloric acid and nitric acid (1:3), and then dry-ashed overnight at 400 °C. The residues were dissolved in nitric acid (0.1 % v/v).

Extraction and analysis of chlorophylls

The extraction of chlorophylls was carried out according to Porra et al. [17], and leaves were homogenized with acetone (80 %). Chlorophyll a and b were measured at 647 and 664 nm with a Perkin Elmer Lambda EZ 200 spectrophotometer), and then chl a/b ratio and total chlorophyll content were determined.

Lipid peroxidation

Lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA), according to Karabal et al. [18]. Aliquots (0.2 g) of leaf tissues from control and treated plants were cut into small pieces, and homogenized by adding 1 ml of 5 % trichloroacetic acid (TCA) solution. The homogenates were then transferred into fresh tubes and centrifuged at 12,000 g at room temperature for 15 min.

Equal volumes of supernatants and 0.5 % thiobarbituric acid (TBA) in 20% TCA solution (freshly prepared) were added into a new tube, and incubated at 96 °C for 25 min. The tubes were transferred into an ice-bath, and then centrifuged at 10,000 g for 5 min. The absorbance of the supernatants was recorded at 532 nm, and corrected for non-specific turbidity by subtracting the absorbance at 600 nm using 0.5 % TBA in 20% TCA solution as the blank. MDA content was determined using the extinction coefficient of 155 mM^{-1} cm^{-1}.

Enzyme assays

Frozen leaves (1 g) were homogenized in 5 ml of 0.1 M potassium phosphate buffer (pH 6.8) containing 100 mg PVP and 0.1 mM EDTA. The homogenate was centrifuged at 16,000 g for 5 min at 4 ºC, and the supernatant was immediately used for the following enzyme assays. Total SOD (superoxide dismutase; EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reaction of nitro blue tetrazolium (NBT), according to the method of Beyer and Fridowich [19]. One unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of the reduction of NBT, monitored at 560 nm. To determine APX (ascorbate peroxidase; EC 1.11.1.11) activity, fresh leaf tissue (1 g) was homogenized in 1.5 ml of extraction medium containing HEPES 200 mM (pH 7.8), EDTA 2 mM, $MgCl_2$ 5 mM, DTT 1 mM, and sodium ascorbate 4 mM. The crude extract was centrifuged at 16,000 g for 5 min at 4 ºC, and the supernatant was used for the measurements according to the method of Bonnet et al. [20]. A decrease in absorbance at 290 nm was observed, when ascorbate was oxidized. APX activity was calculated using an extinction coefficient of 2.8 mM^{-1} cm^{-1} at 290 nm. CAT (catalase; EC 1.11.1.6) activity was assayed by measuring the rate of decomposition of H_2O_2 at 240 nm, as described by Aebi [21],and activity was determined by the reduction of the absorbance in 30 seconds. GR (glutathione reductase; EC 1.6.4.2) activity was measured by following the change of glutathione (GSSG)-dependent oxidation of

NADPH at 340 nm, according to the method of Carlberg and Mannervik [22].

Statistical Evaluation

At least, duplicate measurements of three replicates were used to determine the effects of four independent variables (control, salt, Cd and salt+Cd combination) on antioxidative response, lipid peroxidation and pigment composition. Data were evaluated by analysis of variance (ANOVA) using the Statistica for Windows software package.

RESULTS

The difference between the Cd content in roots of two cultivars was found to be statistically significant ($P<0.001$). The accumulation of Cd was approx. three-fold higher in Rheinlands Ruhm than that in *cerasiforme* (Fig. 1). In *cerasiforme*, the increase of Cd accumulation was related to both increase of salt and Cd level. However, this relation was not observed in Rheinlands Ruhm.

The effect of salt, Cd and salt+Cd treatments on the total chlorophyll content between *cerasiforme* and Rheinlands Ruhm was also found to be statistically significant ($P<0.001$). Chlorophyll a (chl a) contents in *cerasiforme* increased with increasing NaCl concentration in the nutrient solution (Table 1). In contrast, chl a and chl b contents decreased significantly in Cd- and NaCl-treated- Rheinlands Ruhm and Cd-treated *cerasiforme*. Chl a content decreased in salt+Cd combinations, mainly in S_1M_2-treated *cerasiforme*. Besides, chl a and chl b concentrations decreased considerably depending on the Cd increase in Rheinlands Ruhm, whereas chl a/b ratio increased in *cerasiforme* under salt stress. Small but significant increase in this ratio was observed mainly in Cd- and salt+Cd-treated Rheinlands Ruhm, whereas that in *cerasiforme* was decreased.

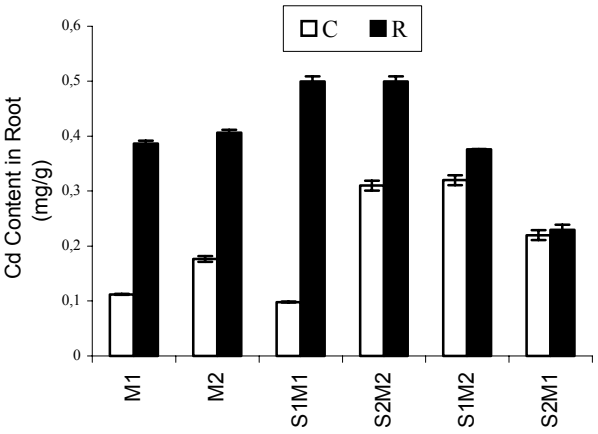

FIGURE 1 - Cd accumulation in *L. esculentum* var. *cerasiforme* and Rheinlands Ruhm, treated with Cd and combined salt+Cd. The data represent the means ±SE of three replicates (M_0 control, M_1 100 µM, M_2 200 µM Cd, and S_0 control, S_1 100 mM, S_2 200 mM NaCl).

The difference between *cerasiforme* and Rheinlands Ruhm MDA contents was found to be statistically significant ($P<0.001$). Membrane lipid peroxidation in the leaves of the two tomato cultivars, measured as the content of MDA, is given in Fig. 2. MDA content was almost the same in both cultivars without stress conditions (control). All stress treatments increased MDA content in Rheinlands Ruhm more than in *cerasiforme*.

TABLE 1 - Effect of separate and combined salt and Cd stresses on chlorophyll contents of *L. esculentum*, namely, tolerant genotype *cerasiforme* and sensitive genotype Rheinlands Ruhm (M_0 control, M_1 100 µM, M_2 200 µM Cd and S_0 control, S_1 100 mM, S_2 200 mM NaCl).

L. esculentum var. *cerasiforme*	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Chlorophyll a/b ratio	Total Chlorophyll (mg/g)
S_0M_0	1.45 ± 0.001	0.58 ± 0.06	2.50 ± 0.30	2.03 ± 0.07
S_0M_1	0.41 ± 0.05	0.55 ± 0.02	0.73 ± 0.10	0.96 ± 0.06
S_0M_2	0.57 ± 0.05	0.42 ± 0.04	1.35 ± 0.04	0.99 ± 0.08
S_1M_0	1.56 ± 0.12	0.61 ± 0.08	2.58 ± 0.20	2.16 ± 0.20
S_1M_1	0.36 ± 0.003	0.46 ± 0.04	0.78 ± 0.05	0.82 ± 0.07
S_1M_2	0.18 ± 0.02	0.27 ± 0.01	0.68 ± 0.10	0.45 ± 0.01
S_2M_0	1.64 ± 0.002	0.58 ± 0.01	2.87 ± 0.30	2.21 ± 0.06
S_2M_1	1.03 ± 0.116	0.41 ± 0.09	2.65 ± 0.90	1.44 ± 0.13
S_2M_2	0.96 ± 0.08	0.47 ± 0.06	2.04 ± 0.30	1.43 ± 0.10
L. esculentum cv. Rheinlands Ruhm				
S_0M_0	1.47 ±0.12	0.89 ± 0.06	1.65 ± 0.05	2.37 ± 0.2
S_0M_1	1.00 ±0.04	0.67 ± 0.07	1.51 ± 0.2	1.68 ± 0.04
S_0M_2	1.18 ±0.06	0.63 ± 0.02	1.89 ± 0.08	1.81 ± 0.08
S_1M_0	1.05 ±0.03	0.53 ± 0.06	2.01 ± 0.3	1.57 ± 0.04
S_1M_1	1.60 ±0.13	0.89 ± 0.06	1.79 ± 0.2	2.49 ± 0.2
S_1M_2	1.02 ±0.11	0.62 ± 0.06	1.56 ± 0.2	1.63 ± 0.2
S_2M_0	1.03 ± 0.07	0.63 ± 0.03	1.66 ± 0.2	1.65 ± 0.06
S_2M_1	1.85 ± 0.07	0.80 ± 0.01	2.29 ± 0.08	2.65 ± 0.08
S_2M_2	0.65 ± 0.03	0.57 ± 0.06	1.16 ± 0.2	1.22 ± 0.06

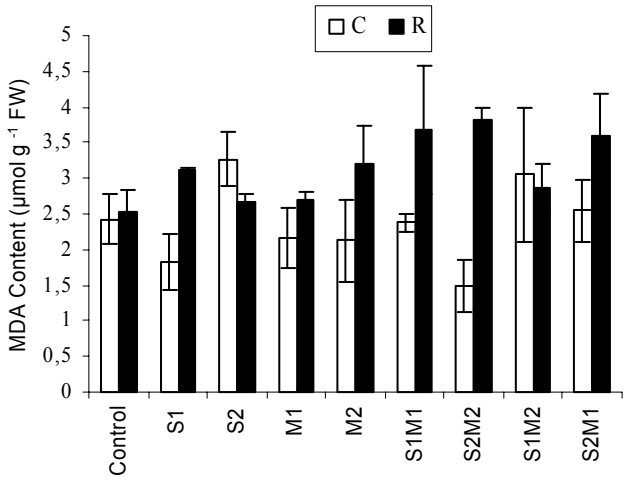

FIGURE 2 - Effect of single and combined salt and Cd stresses on MDA content of *L. esculentum* var. *cerasiforme* and Rheinlands Ruhm. The data represent the means ± SE of three replicates.

The effects of salt ($P<0.05$), Cd ($P<0.05$), and salt+ Cd ($P<0.001$) treatments on SOD activity were again statistically significant between the two cultivars. Their SOD activities remained significantly unaffected by these stress treatments (Fig. 3), but enzyme activity under salt+ Cd combinations was more inhibited in Rheinlands Ruhm variety.

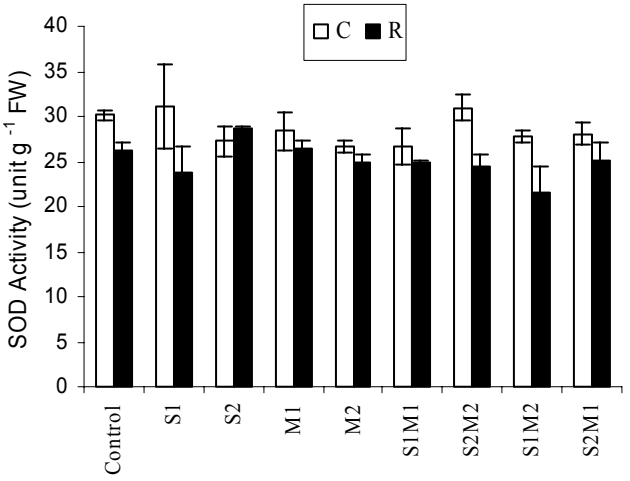

FIGURE 3 - Effect of single and combined salt and Cd stresses on SOD activity of *L. esculentum* var. *cerasiforme* and Rheinlands Ruhm. The data represent the means ±SE of three replicates.

The effects of all treatments on APX activity between the two varieties were also statistically significant ($P<0.001$). APX activity increased significantly in *cerasiforme* and Rheinlands Ruhm under all stress treatments (Fig. 4). A direct correlation was observed between increase of salt levels and that of APX activity in *cerasiforme.* However, APX activity decreased depending on Cd concentration in Rheinlands Ruhm. This enzyme activity was generally lower in Rheinlands Ruhm under salt, Cd and combined salt+Cd stresses.

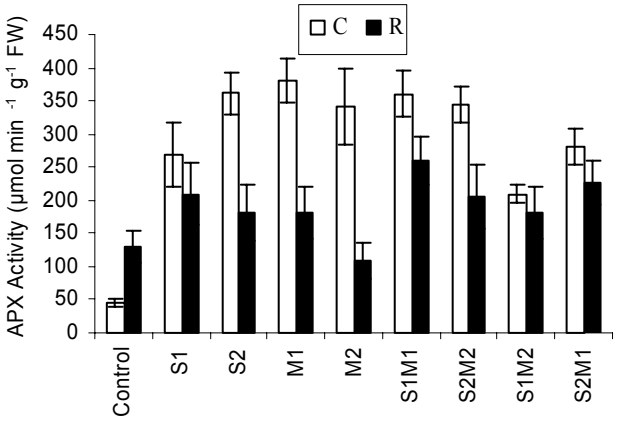

FIGURE 4 - Effect of single and combined salt and Cd stresses on APX activity of L. *esculentum* Cerasiforme and Rheinlands Ruhm. The data represent the means ±SE of three replicates.

The CAT activity difference between the two cultivars proved to be statistically significant ($P<0.001$). CAT activity in *cerasiforme* significantly increased in all stress treatments, mainly salt+Cd combinations (Fig. 5). The highest CAT activity was found in *cerasiforme* under S_2M_2 combination. In contrast, CAT activity decreased in Rheinlands Ruhm under stress conditions. An approx. five-fold reduction was observed in S_2M_1 combination of Rheinlands Ruhm, compared with its control.

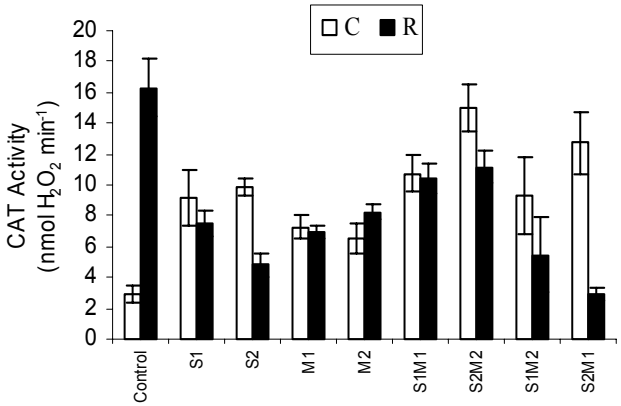

FIGURE 5 - Effect of single and combined salt and Cd stresses on CAT activity of *L. esculentum* var. *cerasiforme* and Rheinlands Ruhm. The data represent the means ± SE of three replicates.

All stress treatments significantly inhibited GR activity of both cultivars ($P<0.001$). The increase of $CdCl_2$ concentration caused a significant decrease of this enzyme activity in both cultivars. However, GR activity in Rheinlands Ruhm decreased more than that in *cerasiforme,* especially under salt stress (Fig. 6).

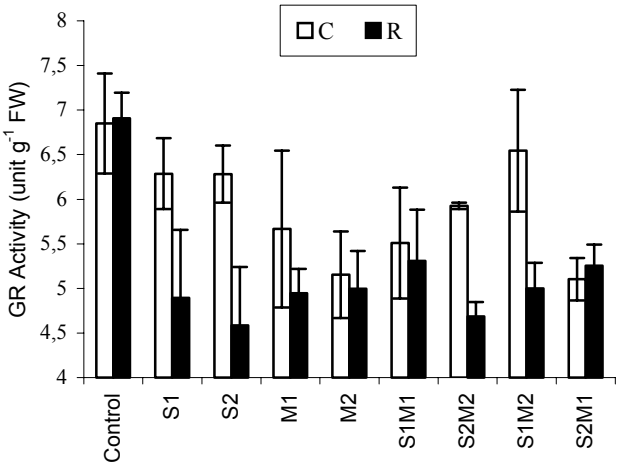

FIGURE 6 - Effect of single and combined salt and Cd stresses on GR activity of *L. esculentum* var. *cerasiforme* and Rheinlands Ruhm. The data represent the means ± SE of three replicates.

DISCUSSION AND CONCLUSION

Chlorophyll a and total chl in *cerasiforme* increased under salt stress, while both pigments remarkably decreased in Rheinlands Ruhm, possibly due to their degradation or inhibition of biosynthesis [23, 24]. Chl a and chl b contents in Cd and salt+Cd combinations decreased, mainly in *cerasiforme*. This may be due to Cd toxicity. Decrease of chl a/b ratio in *cerasiforme* might show better protection of PSII under Cd and salt+Cd combinations. However, low chl b levels in *cerasiforme* and Rheinlands Ruhm cultivars might be a result of PSII sensitivity to these stress conditions [25, 26].

MDA is the final product of membrane lipids` peroxidation, and accumulates when plants are subjected to oxidative stress. Therefore, MDA concentration is commonly considered to be a general indicator of lipid peroxidation as well as the stress level [27]. The enhanced MDA content in Rheinlands Ruhm indicated that combined stress factors induced membrane damage.

SOD activities remained significantly unaffected by stress treatments, because the potential oxidative damage could be avoided by the activity of other antioxidant enzymes. Various researchers have reported an increase in SOD activity, when investigating tolerant tomato [28] and beet [9] cultivars under salt stress. In contrast, salinity [29] and Cd stress [4] in various plants decreased or did not significantly change SOD activity [3, 13]. In this study, the nearly unchanged SOD activity may be an *in vivo* effect of Na^+ ions, according to Dionisio-Sese and Tobita [29].

APX activity in the relatively salt-tolerant *cerasiforme* tomato variety increased more than in Rheinlands Ruhm under all stress treatments. A direct correlation was observed between the salt concentrations, in agreement to Bor et al. [9] and Mittova et al. [28]. APX is well-known to be important in detoxification of H_2O_2 [30], because it easily permeates membranes, thus dedrading leaking H_2O_2 in the mitochondrial and peroxysomal membranes. This study also suggests that H_2O_2 in the leaves of *cerasiforme* is more efficiently eliminated by APX.

In contrast to the results obtained with APX, all stress treatments decreased GR activity in both cultivars. Salt, Cd and salt+Cd combinations-induced GR activities were lower in Rheinlands Ruhm than in *cerasiforme*, mainly under salt stress. In *cerasiforme*, GR activity remained unaffected under S_1M_2 stress combination, not aggreeing. with the results of Bor et al. [9], Shalata et al. [14] and Leon et al. [31], who found high GR activity in more tolerant tomato and beet varieties under salinity, and pepper under Cd stress, respectively. Similarly, Bailly et al. [32] reported higher activities of GR in wild drought-tolerant *Phaseolus* species compared to drought-sensitive ones. However, in pea plants no significant changes in GR activity were observed at 40 and 50 µM Cd [24]. The level of GR may vary with tolerance degree, genotype, and concentration of Cd or salt.

Highest CAT activities in *cerasiforme* were observed under S_2M_1 and S_2M_2 combinations. CAT is the most effective antioxidant enzyme in preventing cellular damage by H_2O_2 [9, 24]. CAT activity increased significantly in all stress-treated *cerasiforme* cultivars, but decreased remarkably in Rheinlands Ruhm under these conditions. Our findings support those of Bor et al. [9] and Shalata et al. [14], who also found high CAT activity in tolerant beet and tomato under salt stress, respectively. Similarly, Wu et al. [4] and Vitoria et al. [33] observed Cd-induced activity of CAT in barley and radish, respectively.

In *cerasiforme*, it seems that APX and CAT may play a more important role in H_2O_2 detoxification than SOD and GR. However, Bor et al. [9] and Benavides et al. [34] reported that APX was more effective than CAT in disposition of H_2O_2 cytotoxicity. Our results are in accordance with [14, 28], showing that *cerasiforme* known as stress-tolerant genotype cultivar exhibited higher antioxidant enzyme activities than Rheinlands Ruhm under separate or combined salt+Cd stress. Salt-tolerant and non-tolerant cultivars exhibited different behaviors with regard to APX or CAT activities, and pigment compositions. However, they show similar tendencies concerning SOD and GR activities.

Our results suggest that there are relationships between Cd uptake level and salt stress. The salt-tolerant *cerasiforme* took up less Cd ions, and, thus, especially Rheinlands Ruhm lipid peroxidation may be enhanced by combined stress conditions.

Therefore, we concluded that salt-tolerant genotype *cerasiforme* had higher tolerance to cadmium stress than salt-sensitive genotype Rheinlands Ruhm, and a cross tolerance between salt and cadmium stress may exist.

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