Research Article

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Antioxidant activity as a response to cadmium pollution in three durum wheat genotypes differing in salt-tolerance

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Abstract: Durum wheat is commonly used in various food industry industries and cultivated worldwide. A serious problem with the species cultivation is its capability to accumulate cadmium (Cd) in the grains. The aim of this study is to investigate whether antioxidant activity may be used as a marker of Cd tolerance in durum wheat. The experiment involved three durum wheat genotypes/lines differing in salt tolerance. The plant response to Cd was appraised based on the activity of ascorbate–glutathione (AsA–GSH) cycle enzymes, ascorbate-to-dehydroascorbate ratio, reduced-to-oxidized glutathione ratio (GSH:GSSG), as well as Cd content in the seeds. The highest activity of dehydroascorbate reductase, monodehydroascorbate reductase, and glutathione reductase was noted in control plants of salt-sensitive cultivar “Tamaroi.” In the presence of Cd, activity of these enzymes was considerably reduced. “Tamaroi” plants demonstrated also the highest Cd content in the grain. In conclusion, we identified the cultivar “Tamaroi” as most susceptible to cadmium, and the level of durum wheat sensitivity to the element can be evaluated based on a significant decrease in the activity of AsA–GSH cycle enzymes and GSH:GSSG ratio.

Keywords: antioxidant enzymes, ascorbate–glutathione cycle, heavy metal stress, cadmium, durum wheat

1 Introduction

Durum wheat (Triticum turgidum L. subsp. durum (Desf.) Husn.) is one of the cereals most commonly used in the food industry and cultivated around the world [1]. The species is highly valued for its grain protein content, especially gluten and gluten, high fiber content, low glycemic index, and high levels of vitamins and valuable micronutrients [2,3]. Tolerance to salinity is a crucial factor in durum wheat cultivation [4]. Salinity tolerance mechanisms in plants cause excretion of salt ions, control of ion uptake by roots and their transport to the leaves, and activation of the antioxidant system [5]. Australian researchers identified two loci, Nax1 and Nax2, in Triticum monoccocum, with genes responsible for the excretion of sodium ions from xylem, and consequently limited the accumulation of Na⁺ in the leaves. A cross with a salt-sensitive cultivar “Tamaroi” produced a new line containing these genes and tolerant to high salt concentrations [4]. Durum wheat accumulates large amounts of cadmium (Cd) ions in the grains [6]. The European Union has proposed a limit of Cd concentration in food that should not exceed 0.2 mg kg⁻¹ fresh weight of a product [7]. In humans, even small amounts of Cd can be toxic and cause permanent organ damage [8,9]. Crop plants growing at a higher content of Cd show many physiological disorders, such as inhibition of seed germination and plant growth [10], leaf rolling, chlorosis and necrosis [11], disturbed distribution of nutrients [12], reduction of photosynthesis efficiency and chlorophyll content, and imbalance of water uptake and stomatal closure [13,14]. Zook et al. [15]
and Jalil et al. [16] noticed that durum wheat plants are more effective accumulators of Cd in the grain than *Triticum aestivum* L. They reported significant disparities between species and cultivars in the amount of absorbed Cd content and their tolerance to its poisonous effects. Some cultivars of durum wheat have a genetically determined potential for accumulation of significant amounts of Cd [17]. For example, one of the major genes in *Cdul* locus is responsible for Cd tolerance of durum wheat [18–21]. Hart et al. [22] reported that the Cd level in the grain was regulated not only by genetic propensity but also by physiological factors including Cd absorption from the soil solution, ion transport from root to shoot, Cd sequestration in subcellular compartments, and phloem movement into the grain during fruit development. The analysis of Cd accumulation mechanisms should account for the strong influence of soil properties on the content of bioavailable forms of this element [23]. To become tolerant to Cd toxicity, plants have developed a number of protective mechanisms, including higher activity of enzymatic and nonenzymatic antioxidants [24], adjustment of the influx and efflux of heavy metals [25], and regulation of the levels of heavy metal chelators, phytochelatins, and metallothioneins [26]. Cd does not participate in redox reactions but triggers overproduction of reactive oxygen species (ROS), including H$_2$O$_2$ [27]. Cd increases lipid peroxidation, protein oxidation, and nucleic acid oxidation. Also, Cd inactivates several enzymes by binding with their sulphydryl groups (–SH) and increases free Fe concentration by its replacement with various proteins [28,29]. Some studies reported that exposure to Cd induces specific alterations in the mitochondrial structure and function in animals [30]. Tolerance to toxic Cd content depends on plant capacity to scavenge or detoxify activated oxygen species. The key molecules in these processes are glutathione (GSH) and ascorbate (AsA), i.e., nonenzymatic antioxidants. They are components of the cellular antioxidant defense system, i.e., the ascorbate–glutathione cycle (AsA–GSH cycle) [31,32]. They act as cofactors for numerous enzymes and signaling molecules regulating pivotal cellular processes. Ascorbate reacts with singlet oxygen, hydrogen peroxide, superoxide, and hydroxyl radicals. Glutathione is a tripeptide with a sulphydryl group (L-S-glutamyl-L-cysteinyl-glycine) and is recognized as a key molecule in the detoxification system. The fundamental function of GSH consists of thiol-disulphide interactions, in which reduced glutathione (GSH) is continuously oxidized to a disulphide form (GSSG) [31]. The AsA–GSH pathway comprises four enzymes: ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) [33]. According to Hasanuzzaman et al. [34], all enzymes of the ascorbate–glutathione cycle work closely together, which may additionally improve Cd tolerance. Figure 1 shows a simplified scheme of the ascorbate–glutathione cycle.

Our hypothesis was that the degree of durum wheat tolerance to salinity caused by NaCl coincides with the tolerance to Cd and that the antioxidant activity of AsA-GSH cycle maybe a marker of this tolerance. We compared the response to soil contamination with Cd applied at 3 and 5 mg kg$^{-1}$ DM (dry matter) in three genotypes of durum wheat: Polish line SMH87 with moderate tolerance to salinity stress, Australian NaCl-sensitive cultivar “Tamaroi,” and NaCl-resistant line BC$_5$Nax2. The defense response of the studied durum wheat genotypes included evaluation of AsA–GSH cycle enzyme activity, ascorbate-to-dehydroascorbate ratio, reduced-to-oxidized glutathione ratio, and Cd content in the seeds.

**Figure 1:** Schematic representation of antioxidant enzymes in the ascorbate–glutathione cycle investigated in this study (according to Szymańska and Strzałka [35]).
2 Materials and methods

2.1 Plant material

This study involved three genotypes of spring durum wheat. SMH87 line was obtained from Dr Jarosław Bojarczuk from Plant Breeding Center in Smolice, Plant Breeding and Acclimatization Institute Group (Poland). In our preliminary study, we identified SMH87 as moderately tolerant to salinity. BC5Nax2 line and cultivar “Tamarol” were obtained from Dr Richard A. James from CSIRO Plant Industry (Australia). The Australian genotypes of durum wheat differed in their salt tolerance: cultivar “Tamarol” was sensitive, while BC5Nax2, containing Nax2 locus with salt tolerance genes, was tolerant to salt stress.

2.2 Experimental treatments

Seeds were sterilized with 70% ethanol for 1 min and placed in Petri dishes (Ø = 9 cm) on filter paper wetted with distilled water. The seeds germinated in the dark at 4°C for 21 days. Next, they were sown into pots (4 dm³) filled with soil classified as degraded chernozem, formed from loess, the first soil quality class of very good wheat complex. Preliminary analysis of the soil used in the experiment revealed the presence of Cd ions at a concentration of 0.43 mg Cd kg⁻¹ DM of soil. The Cd content in the soil was determined according to Baran et al. [36]. Based on data published by Tóth et al. [37], a dose of 3 mg Cd kg⁻¹ DM was used in the experiment as average soil pollution with Cd ions, while a dose of 5 mg Cd kg⁻¹ DM of soil corresponded to the maximum Cd content determined in Europe. The germinating seeds were sown into the soil contaminated with 3 CdSO₄·8 H₂O at three concentrations: 0 (control), 3, and 5 mg of pure Cd per 1 kg DM of soil. Cd salt was evenly distributed in the entire volume of the pot. After adding the salt at both concentrations, soil salinity did not exceed 0.2 mS cm⁻¹. Its conductivity was measured according to Płazek et al. [38]. Each pot harbored five seedlings. Each treatment (genotype/Cd dose) contained 10 pots. The plants were cultivated to full seed ripening phase in air-conditioned glasshouse at 22 ± 3°C/18 ± 1°C day/night, in daylight (March–May) supplemented with light intensity (AGRO Philips sodium lamps) of 400 μmol m⁻² s⁻² PPFD (photosynthetic photon flux density), up to a 16 h photoperiod. Relative humidity was 65 ± 2%/75 ± 1% day/night. The plants were fertilized with Hoagland medium [39] once a week to ensure proper nutrition. The experiment was performed twice in 2018 and 2019, and the data presented are the means of the results obtained.

2.3 Measurements

2.3.1 Hydrogen peroxide assay

Hydrogen peroxide in flag leaf material was determined using the Amplex Red Hydrogen Peroxide/Peroxidase Assay kit of Invitrogen (Oregon, USA). Leaf material (0.1 g) was homogenized in 0.5 cm³ of 50 mM potassium phosphate buffer (pH 7.5). The homogenates were centrifuged (19,000 g, 10 min, 4°C), and H₂O₂ concentration was measured colorimetrically in the supernatant according to the manufacturer’s protocol. Hydrogen peroxide content was calculated from the standard curve prepared with H₂O₂ solutions. Results are expressed in µM H₂O₂ g⁻¹ fresh weight. The measurements were taken in five replicates for each genotype/treatment with using Synergy 2 Microplate Reader (BioTek, USA).

2.3.2 Antioxidant assays

The concentration of reduced AsA, DHA, reduced (GSH) glutathione, and the activity of APX, DHAR, and GR in flag leaves were measured spectrophotometrically as described by Harrach et al. [40]. The activity of MDHAR was determined according to Hossain et al. [41]. All assays were performed at 25°C using the Ultrospec 2100 pro UV/visible spectrophotometer (Amersham, Umeå, Sweden). The measurements were taken in five replicates for each genotype/treatment.

2.3.2.1 Low-molecular-weight antioxidant assays

For determination of low-molecular-weight antioxidant content, flag leaf material (0.1 mg) was homogenized in 0.5 cm³ of 5% (w/v) metaphosphoric acid at 4°C and centrifuged (19,000 g, 30 min, 4°C). The supernatant was used for the assays. The content of AsA was detected as described by Foyer et al. [42] using ascorbate oxidase. Metaphosphoric acid extracts (125 μL) were neutralized with 25 μL of 1.5 M triethanolamine. The reaction mixture contained 133.3 μL of 150 mM sodium phosphate buffer (pH 7.4), 66.7 μL of H₂O, 2 cm² of 100 mM sodium

medium [39] once a week to ensure proper nutrition. The experiment was performed twice in 2018 and 2019, and the data presented are the means of the results obtained.
phosphate buffer (pH 5.6), and 1 unit of ascorbate oxidase. The extinction was measured immediately at 265 nm after the preparation of solution, and then, there was a decrease in the absorbance. The level of DHA was calculated as a difference between total ascorbate and AsA according to Harrach et al. [40]. Total ascorbate was determined after a reduction of DHA with diethyldithioleol. Neutralized leaf extracts (45 µL) with 54 µL of 150 mM sodium phosphate buffer (pH 7.4) and 27 µL of 10 mM dithiolethirole were incubated for 15 min at room temperature. Total ascorbate levels were measured as mentioned earlier. The standard curve was created by known concentrations of AsA and DHA prepared in 5% metaphosphoric acid. GSH and GSSG were determined by the recycling method, using GR, according to Law et al. [43]. Metaphosphoric acid extracts (100 µL) were neutralized with 36 µL of 1 M triethanolamine. Initially, total glutathione was estimated, and then, to determine GSSG, GSH was derivatized with 2-vinylpyridine to the neutralized samples. The amount of GSH was estimated as a difference between these two assays. To determine total glutathione content, we prepared 1 cm³ of a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.5), 2.5 mM EDTA-Na₂, 1 mM 5,5'-dithio-bis (2-nitrobenzoic acid), 1 unit of GR, 0.2 mM NADPH, and 20 µL of the neutralized sample. Absorbance increases at 412 nm after the addition of GR and NADPH. Before measuring GSSG concentration, the neutralized samples were mixed with 8 µL of 2-vinylpyridine and incubated at 25°C for 1 h. Oxidized glutathione was determined as described earlier, using a 50 µL sample and the reaction mixture of a total volume of 1 cm³. Total glutathione and GSSG content were estimated based on a standard curve generated with stock solutions of GSH and GSSG prepared in 5% metaphosphoric acid.

### 2.3.2 Antioxidant enzyme activity assays

For the detection of AsA–GSH cycle enzyme activity, flag leaf material (0.1 mg) was homogenized at 4°C in 0.5 cm³ 50 mM Tris–HCl buffer (pH 7.8) containing 1 mM EDTA-Na₂ and 7.5% (w/v) soluble polyvinylpyrolidone. The suspension was centrifuged (12,000 g, 20 min, 4°C), and the supernatant was used to measure the total soluble enzyme activity. The APX activity was determined by following the oxidation of ascorbic acid at 290 nm (extinction coefficient of ascorbic acid was 2.8 mM⁻¹ cm⁻¹) according to Nakano and Asada [44]. The reaction mixture (2.25 cm³) consisted of 2 cm³ of 50 mM Tris–HCl buffer (pH 7.8), 100 µL of 5.7 mM ascorbic acid, 100 µL of 11.25 mM H₂O₂, and 50 µL of the leaf extract. The control reaction was performed using the buffer instead of H₂O₂ solution. Results of the APX activity are expressed in nMAsA mg⁻¹ protein min⁻¹. The DHAR activity was estimated by following the reduction of DHA at 265 nm (extinction coefficient of ascorbic acid was 14 mM⁻¹ cm⁻¹), as described by Klapheck et al. [45]. The assay mixture contained 2 cm³ of 50 mM sodium phosphate buffer (pH 7.5) with 2.5 mM EDTA-Na₂, 100 µL of 22.8 mM GSH, 100 µL of 11.5 mM DHA, and 100 µL of the leaf extract. The control reaction mixtures contained the buffer instead of the supernatant. Results of the DHAR activity are expressed in nM AsA mg⁻¹ protein min⁻¹. The measurement of the MDHAR activity was based on monitoring the consumption of NADH at 340 nm (extinction coefficient of NADH was 6.2 mM⁻¹ cm⁻¹). The reaction mix consisted of 2 cm³ of 50 mM Tris–HCl buffer (pH 7.8), 100 µL of 22.7 mM ascorbic acid, 100 µL of 2.6 mM NADH, 6.6 units of ascorbate oxidase, and 100 µL of the leaf extract. The control reaction mix contained the buffer instead of ascorbate oxidase. Results of the MDHAR activity are expressed in nM NADH mg⁻¹ protein min⁻¹. The GR activity was assayed by the decrease in absorbance at 340 nm due to the oxidation of NADPH (extinction coefficient of NADPH was 6.2 mM⁻¹ cm⁻¹) according to Klapheck et al. [45]. The reaction mixture contained 2 cm³ of 50 mM Tris–HCl buffer (pH 7.8), 100 µL of 2.4 mM NADPH, 300 µL of 4.6 mM GSSG, and 100 µL of the leaf extract. The control reaction was performed with the buffer instead of GSSG solution. Results of the GR activity are expressed in nM NADPH mg⁻¹ protein min⁻¹.

### 2.3.3 Determination of Cd content in the grains

The content of Cd was determined as described by Ostrowska et al. [46]. The grains were collected from plants in each treatment and dried separately in an air flow dryer at 65°C for 48 h, weighed, and powdered in a ball mill MM400 (Retsch, Haan, Germany). For determination of hygroscopic water, the ground samples were dried at 105°C and later mineralized in a chamber furnace at 450°C for 12 h. The residue was dissolved in diluted nitric acid (acid:water ratio of 1:2; v/v). The content of the element was determined using the ICP-OES method in PerkinElmer Optima 7300DV apparatus (Norwalk, CT, USA). Reference material NCS DC73348 (China National Analysis Center for Iron & Steel) was applied to each analytical series as described by Fuentes et al. [47]. Analyses of chemical element content were done in five replicates for each Cd treatment/wheat line.
2.3.4 Statistical analysis

Two-way analysis of variance and Duncan’s multiple range test (at $P < 0.05$) were performed using the statistical software of STATISTICA 13.0 (Stat-Soft, Inc., Tulsa, OK, USA). Data were represented as means ± SE (standard error), and linear correlation coefficients (Pearson’s) were putative as statistically significant at $P < 0.05$.

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and discussion

3.1 Visual symptoms of Cd treatment

All cultivated plants showed no symptoms of Cd toxicity such as necrosis or leaf rolling; however, Cd accelerated seed maturation and early plant drying (Figure 2). In some cases, the plants grown in soil contaminated with 3 mg Cd kg$^{-1}$ DM, and even with the higher dose showed greater vigor than control plants. This effect was most evident before flowering. Most often, however, the observed differences between plants resulted from their genotypic diversity.

Our observations showed that plants growing in soil contaminated with 5 mg kg$^{-1}$ DM Cd generally did not differ in appearance from those grown at 3 mg kg$^{-1}$ DM Cd, and even some of them looked more viable. It is difficult to unambiguously explain this phenomenon. It might be due to so-called hormesis effect. Hormesis involves stimulation of various parameters in living organisms by stress factors of low intensity [36,48]. The effect was reported in plants treated with low concentrations of heavy metals [49]. Moral et al. [12] proved lower tomato yield during Cd presence but no differences in mean fresh weight of the fruit. However, these authors stated that Cd negatively affected the plant growth and root and stem length, and also fresh weight decreased with increasing concentrations of Cd. An analysis of Cd accumulation mechanisms should account for a strong influence of soil properties on the content of bioavailable forms of this element [23]. The study by Dai et al. [23] revealed that the introduction of nutrients, namely, nitrogen and sulfur, could be a significant factor determining wheat response to Cd contamination in the soil. It should be noted that by increasing the dose of Cd used as sulfate, the dose of sulfur also increased. Khan et al. [50] stated that coordination between the main N and S assimilation pathways can strengthen plant defense mechanisms and effectively alleviate Cd negative effects. Gill et al. [51] notified that high Cd content in soil affects photosynthesis process and alignment between carbon, nitrogen, and sulfur metabolism.

3.2 Hydrogen peroxide content

Hydrogen peroxide production under Cd stress was specific for each studied genotype (Figure 3). The lowest level of this compound was recorded in control plants of SMH87, and its content in the leaves gradually increases with an increase in Cd dose. “Tamaroi” control plants produced the highest amount of H$_2$O$_2$, and contrary to

![Figure 2: Plants of the studied genotypes, in the heading phase, growing in the soil without Cd (control) (a) and in the soil contaminated with 3 mg Cd kg$^{-1}$ DM (b) and 5 mg Cd kg$^{-1}$ DM (c).](image-url)
plants of this cultivar (Figure 4a–c). In BC$_5$Nax$_2$ line, 3 mg Cd kg$^{-1}$DM enhanced the hydrogen peroxide amount, while higher metal dose reduced its level, which was still higher than that of the control.

Cd is not a redox metal, and it cannot catalyze Fenton reaction that affects the ROS production in plant cells. Higher H$_2$O$_2$ level noticed in plants of SMH87 and BC$_5$Nax$_2$ lines cultivated in contaminated soil presumably results from the decreased H$_2$O$_2$ scavenging rate or the increased H$_2$O$_2$ synthesis in enzymatic or non-enzymatic reactions. Hydrogen peroxide production might be associated with cellular integration processes and/or adaptation to environmental conditions [52]. Sarker and Oba [53] detected very low amounts of hydrogen peroxide in drought-sensitive genotype of *Amaranthus* sp. versus more tolerant genotypes. As described further in the article, salt-sensitive “Tamaroi” was recognized as considerably more sensitive to Cd ions than SMH87 and BC$_5$Nax$_2$, so the reduction in hydrogen peroxide under Cd stress may be a marker of plant sensitivity to various environmental stresses. Similar
results were reported by Plažek and Žur [54]. These authors concluded that crop resistance to pathogens depended on low activity of catalase and high amount of hydrogen peroxide.

### 3.3 Enzyme activity

The lowest APX activity was recorded in SMH87 plants, and it was unaffected by increasing Cd concentration (Figure 4a). “Tamaroi” plants showed a decrease (by 21%) in the APX activity under both Cd concentrations. The highest APX activity was detected in the leaves of BC\textsubscript{5}$\text{Na}$\textsubscript{2} line, and it declined rapidly (by about 86%) with the increasing Cd amount in the soil.

The pattern of the GR activity was specific for each studied genotype (Figure 4b). It was the lowest in SMH87 and did not change in the presence of 3 mg Cd kg\textsuperscript{-1} DM but dropped at 5 mg Cd kg\textsuperscript{-1} DM. Very high GR activity in control “Tamaroi” plants gradually decreased in the plants grown in the contaminated soil. Only in BC\textsubscript{5}$\text{Na}$\textsubscript{2} plants, the increasing Cd amount enhanced the GR activity. The MDHAR activity was the highest in “Tamaroi” plants, while in SMH87 and BC\textsubscript{5}$\text{Na}$\textsubscript{2} plants, it was considerably lower (Figure 4c). Cd inhibited the MDHAR activity in “Tamaroi” and BC\textsubscript{5}$\text{Na}$\textsubscript{2} plants, while in SMH87 line, it remained unaffected. Cd contamination did not influence the DHAR activity in SMH87 and BC\textsubscript{5}$\text{Na}$\textsubscript{2} lines, while in “Tamaroi,” we saw a decline in the activity of DHAR only at 5 mg Cd kg\textsuperscript{-1} DM (Figure 4d). Similarly, as for the highest MDHAR activity, the highest activity of DHAR was observed in “Tamaroi” plants.

Figure 5a presents ascorbate-to-dehydroascorbate ratio (AsA:DHA). Predominance of ascorbic acid over its oxidized form was visible mainly in control plants of SMH87 and BC\textsubscript{5}$\text{Na}$\textsubscript{2} lines, while in “Tamaroi” control, DHA level was significantly higher than that of AsA. The AsA:DHA ratio decreased in all plants exposed to Cd, and a particularly strong response was noticed in both Australian genotypes. The ratio depended strongly on APX and DHAR activities (Figure 4a and d).

Similarly as for GR, the ratio of reduced to oxidized glutathione (GSH:GSSG) was specific for each studied genotype (Figure 5b). In SMH87 plants, the ratio increased at both applied Cd levels compared with that of the control. Control plants of cv. “Tamaroi” exhibited the highest GSH:GSSG ratio that rapidly (by 75%) declined in plants grown in the soil containing 3 mg Cd kg\textsuperscript{-1} DM. Higher Cd dose boosted GSH amount and in consequence GSH:GSSG ratio. In the case of BC\textsubscript{5}$\text{Na}$\textsubscript{2}, only 5 mg Cd kg\textsuperscript{-1} DM increased the ratio, which indicated an increase in GSSG accumulation. The quantity of hydrogen peroxide correlated positively with the activity of all studied enzymes and with GSH:GSSG ratio, while the latter correlated only with the quantity of H\textsubscript{2}O\textsubscript{2} amount (Table 1). GR activity correlated positively with APX, DHAR, and MDHAR activities. Sarker and Oba [53] observed a minute increase in ascorbate–glutathione content, ascorbate–glutathione redox, and ascorbate–glutathione cycle enzyme activities, which correlated with dramatic increment in hydrogen peroxide in drought-sensitive genotype of *Amaranthus tricolor*.

Linear correlation coefficients (Pearson’s) were assumed statistically significant at $P < 0.05$ (ns – not statistically significant).

According to Cuypers et al. [30], Cd can induce oxidative stress by inhibiting antioxidants, but it also

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**Figure 5:** Ascorbate (AsA)-to-dehydroascorbate (DHA) ratio (a) and reduced-to-oxidized glutathione ratio (GSH:GSSG) (b) in the flag leaves of three durum wheat genotypes grown in the soil contaminated with Cd at 0 mg (control), 3 mg kg\textsuperscript{-1} DM and 5 mg kg\textsuperscript{-1} DM. Data are represented as mean ± SE in five replicates. Means with different letters (a, b, c,...) are significantly different at $P < 0.05$ using the Duncan multiple range test.
activates several antioxidant compounds. In the present experiment, the investigated genotypes showed considerable differences in Cd-induced response. The activity of individual enzymes of ascorbate–glutathione cycle and AsA:DHA and GSH:GSSG ratios were specific for each genotype. In the case of control “Tamaroi” plants, the activity of most studied enzymes was many times higher than in the remaining genotypes. Also, these plants responded to the increasing Cd content by a reduction of APX, MDHAR, DHA, and GR activities. This effect was not visible or was considerably weaker in SMH87 and BC5Nax2 lines. Particular attention should be paid to the activity of ascorbate peroxidase that occurs in chloroplasts and cytoplasm. Hydrogen peroxide in chloroplasts is mainly removed by APX. In chloroplast stroma, APX concentration is about 37 µM, which is a high value for an enzyme [55]. In our experiments, the highest APX activity was noted in control plants of BC5Nax2; however, increasing doses of Cd in this line severely decreased the enzyme activity by up to 87%, while in “Tamaroi,” it dropped by only 21%. Large differences in the APX activity in the plants treated with Cd were additionally emphasized by the response of SMH87 plants in which no changes in the activity of this enzyme were observed. In “Tamaroi,” both Cd doses drastically decreased glutathione pool, while a reverse pattern developed in SMH87 and BC5Nax2 lines. The response to Cd pollution in salt-resistant BC5Nax2 seemed more similar to moderately salt-tolerant SMH87 and differed from that of salt-sensitive “Tamaroi.” Considering the results described earlier, it could be concluded that significant inhibition of ascorbate–glutathione cycle activity may be a marker of durum wheat sensitivity to Cd. Our findings also suggest that the most salt-sensitive cultivar is also the most sensitive to Cd contamination. This result was confirmed by the analyses of Cd accumulation in the grains. The Cd content was the highest in the grains of the salt-sensitive cultivar and the lowest in the lines moderately tolerant and durable to salinity. The results of our study did not confirm previous reports [31,32] that tolerance to the toxic effects of Cd ions depends on the increased activity of the AsA–GSH cycle enzymes and nonenzymatic low-molecular antioxidants like ascorbic acid. Conversely, the more Cd tolerant cultivars showed a quantitative advantage of glutathione over oxidized glutathione.

### 3.4 Cd content in the grains

In all studied plants, Cd content in the seeds increased significantly at both applied Cd doses compared with the control (Figure 6). The grain of all plants grown at 5 mg Cd kg⁻¹ DM are concentration less Cd than those produced by plants grown at 3 mg Cd kg⁻¹ DM. Cd accumulation in the grains correlated only with the GSH:GSSG ratio and only for salt-sensitive “Tamaroi” \((r = -0.929; \ P < 0.05)\) (Figure 7), while in the case of SMH87 and BC5Nax2, this correlation was insignificant.

Cd amount increased significantly in the seeds of all the studied genotypes exposed to the metal; however, the highest Cd ion content in the grain was demonstrated by plants growing at lower Cd concentration in the soil (3 mg kg⁻¹ DM). This observation is difficult to explain. We can assume that higher Cd concentration (5 mg kg⁻¹ DM) was toxic enough to block the processes involved in the transport of assimilates and ions to the

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**Table 1:** Correlation between the activity of enzymes involved in the ascorbate–glutathione cycle, GR, reduced-to-oxidized glutathione ratio (GSH:GSSG), and hydrogen peroxide (H₂O₂) determined in all studied durum wheat plants grown under Cd pollution

| Variable | GR    | GSH:GSSG | H₂O₂ |
|----------|-------|----------|-------|
| APX      | 0.711 | ns       | 0.463 |
| DHR      | 0.877 | ns       | 0.593 |
| MDHAR    | 0.607 | ns       | 0.381 |
| GR       | —     | ns       | 0.685 |
| GSH:GSSG | ns    | —        | 0.385 |
| H₂O₂     | 0.686 | 0.385    | —     |

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**Figure 6:** Cd content (µg g⁻¹ DM) in the grains of three durum wheat genotypes grown in the soil contaminated with Cd at 0 mg (control), 3 and 5 mg kg⁻¹ DM. Data are represented as mean ± SE in five replicates. Means with different letters (a, b, c,…) are significantly different at \(P < 0.05\) using the Duncan multiple range test.
seeds. Lu et al. [56] observed a similar phenomenon in *Fagopyrum tataricum*. They stated that in plants exogenously treated with sulfur, increased Cd uptake in root vacuoles and its decreased translocation to the leaves can result from enhanced Cd binding by cell walls, chelation and vacuolar sequestration with nonprotein thiols, and inhibited transport of Cd from roots to shoots. Hart et al. [22] demonstrated that Cd uptake rates in the roots and xylem translocation to the shoots of durum wheat were not accountable for higher Cd accumulation in mature grains. Cd content in the grains correlated negatively with the GSH:GSSG ratio, which indicates that Cd accumulation is accompanied by intense oxidative stress, as evidenced by greater amount of oxidized form of glutathione. Cultivar “Tamaroi” is highly sensitive to both NaCl salinity and Cd pollution. This might indicate that tolerance to these stresses has a common physiological background. However, SMH87 line, which is similar to “Tamaroi,” does not possess Nax2 locus, demonstrated Cd tolerance at the level of BC5Nax2 line. Thus, it can be assumed that the NaCl-resistant genes in this locus do not contribute to Cd tolerance.

4 Conclusion

Durum wheat genotypes differed considerably in the activity of ASA–GSH cycle enzymes in the presence of Cd. Salt-sensitive cv. “Tamaroi” demonstrated the strongest Cd-induced decline in their activity and in the GSH:GSSG ratio. This cultivar accumulated also the highest amounts of Cd in the seeds compared with other genotypes under the study. We concluded that salt-sensitive “Tamaroi” was more susceptible, while moderately salt-tolerant SMH87 and salt-resistant BC5Nax2 were more tolerant to Cd contamination. The study showed that the inhibited activity of AsA–GSH cycle enzymes and a significant decrease in the GSH:GSSG ratio induced by Cd can be recognized as markers of durum wheat sensitivity to this metal. In the future, we plan to explore other mechanisms that block the accumulation of Cd in the seeds. First, we will study durum plants for the expression of genes located at Cdu1 locus in the presence of Cd.

**Abbreviations**

- APX ascorbate peroxidase
- AsA ascorbate
- DHA dehydroascorbate
- DHAR dehydroascorbate reductase
- DM dry matter
- GSH glutathione
- GSSG oxidized glutathione
- GR glutathione reductase
- MDHAR monodehydroascorbate reductase
- NADH nicotinamide adenine dinucleotide, reduced form
- NADPH nicotinamide adenine dinucleotide phosphate, reduced form

**Figure 7:** Correlation between Cd accumulation in the grains (mg kg⁻¹ DM) and GSH:GSSG ratio in cv. “Tamaroi.” Linear correlation coefficients (Pearson’s) were assumed statistically significant at $P < 0.05$. 

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Y = 2.0172 - 0.071x \\
R^2 = 0.8643 \\
r = -0.929; P < 0.05
\]
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Conflict of interest: The authors declare no conflict of interest.

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