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The effects of Selenium phytotoxicity on two wheat (*Triticum aestivum*) cultivars differing in Se tolerance and the role of antioxidant enzymes in the tolerance mechanism

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Abstract

Wheat seedlings were hydroponically grown in Hoagland solution containing various levels of Se. Tolerance response to Se toxicity was investigated by determining the level of thiobarbituric acid reactive substance (TBARS), proline and chlorophyll content, the growth parameters, and the activity of antioxidant enzymes. The toxic level of Se treatment significantly retarded the seedling growth. A substantial amount of proline accumulation was also observed in response to toxic Se concentration, but it was more pronounced in putative-sensitive cultivars. Chlorophyll content significantly decreased in Se-intoxicated seedlings and increased at the lowest Se dose in both cultivars. Severe and mild chlorosis was observed in putative-sensitive and tolerant cultivars at the highest Se level. Alterations in the activities of glutathione reductase (GR, 1.6.4.2), glutathione S transferase (GST, 2.5.1.18), guaiacol peroxidase (GPX, 1.11.1.7), catalase (CAT, 1.11.1.6), and ascorbate peroxidase (APX, 1.11.1.11) and superoxide dismutase (SOD, EC 1.15.1.1) were determined. TBAR level did not significantly increase in putative tolerant cultivars as an indicator of membrane lipid peroxidation. However, a significant increase was observed in putative-sensitive cultivars in response to higher selenium concentrations. In higher Se treatment groups, CAT and GST activities significantly increased in putative Se tolerant cultivars. However, excluding SOD, the activity of all the studied enzymes was increased considerably in putative-sensitive cultivars in a dose-dependent manner. Higher antioxidant enzyme activities and a substantial amount of proline accumulation did not significantly contribute to overcoming Se phytotoxicity in wheat seedlings grown in media supplemented with toxic selenium levels.

Keywords: Antioxidant enzymes, proline, ROS, Se phytotoxicity, TBARS, wheat

Introduction

Selenium is a naturally occurring trace element, higher in some areas of the world. It is chemically similar to Sulphur, and the essentiality of Se for plants was considered controversial [1,2]. Although plants are deemed to have a low tolerance to higher concentrations of Se, several studies indicate that a trace amount of Se may have a beneficial effect on the metabolism of higher plants [3, 4]. Studying plant Se metabolism is crucial since the primary source of Se for human and animal nutrition depends on a plant-based diet [5]. Hence, some plants, including wheat, can be used for biofortification purposes for human and animal diets [6, 7, 8]. Alternatively, the capabilities of some plant species to absorb and allocate Se could also be utilized to accomplish phytoremediation of high selenium-containing soil. Plants' roots readily uptake selenate and can translocate through sulphate transporters to other organs, including seeds [9]. In Arabidopsis plants, Se becomes toxic at higher concentrations due to malformed selenoproteins and oxidative stress [10]. The level and the available form of Se influence the uptake of selenium and the physiological responses of plants considerably. Although most plant species are Se-sensitive, the accumulated Se may reach extraordinary concentrations when Se-accumulator plants are grown on selenium-rich soil. Those plants may have a mechanism to incorporate Se into selenoproteins that may also disturb the cell metabolism of plants. Some other Se-tolerant plants have selenocysteine methyltransferase activity that prevents the incorporation of selenium into nonspecific proteins by methylating selenocysteine.

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For instance, the amount of hydrogen peroxide and superoxide was significantly lowered in transgenic Arabidopsis plants, overexpressing the methyl transferase of Indian mustard *Brassica oleracea*, regardless of total Se accumulation [11]. Selenium also alleviates heavy metal-induced oxidative stress in low concentrations [12].

Besides antioxidant characteristics, the pro-oxidant characteristic of Se is known for plants by triggering the production of reactive oxygen species (ROS). Numerous studies indicate that the over-production of ROS at higher doses of Se is one of the main reasons for Se-induced phytotoxicity in plants, leading to lipid peroxidation and causing oxidative stress. The extremely high amount of ROS elicits radical chain reactions that might impair chlorophyll membranes and affect cell metabolism. A recent study indicated reactive nitrogen species and phytohormones as new players in plant selenium toxicity [13]. This study aims to investigate the role of antioxidant enzymes (APX, CAT, GR, GST, SOD and GPX) in tolerance response against Se-induced phytotoxicity by using two wheat cultivars differing in tolerance response. Growth parameters, membrane lipid peroxidation, chlorophyll and carotenoid content and proline accumulation in shoot tissues were investigated to characterize the contribution of antioxidant enzymes in ameliorating Se-induced phytotoxicity.

Materials-Methods

2.1. Plant growth

Seeds of wheat cultivars (*Triticum aestivum* L.) were obtained from Ankara Central Agricultural Research Institute (Türkiye). Thirty wheat cultivars were screened for Se sensitivity and tolerance. Based on symptom severity, two cultivars were selected: Selenium tolerant (Gökgöl) and Se sensitive (Ikizce). Seeds were surface-sterilized with 10% sodium hypochlorite solution for 10 minutes, washed and planted on cheesecloth-coated plastic dishes containing Hoagland solution and grown for seven days in a 25 °C growth chamber for 16 hours: eight hours dark light Cycle at a light intensity of 40 mmol $m^{-2}s^{-1}$. The experiment was designed in triplicate, and each Petri dish contained 20 seedlings. Se was applied on day eight and continued for three days in Hoagland solution containing 1, 10 and 20 mg/L sodium selenate. Hoagland solution without Se addition was used in the control group. The solutions were changed every 24 hours. Following the 10 days of growth in Se solution, the leaves were harvested for various experimental analyses.

2.2. Determination of Chlorophyll and Carotenoid Concentration

The method described by Inskeep and Bloom [14] with slight modifications was used. Leaf tissues were ground in liquid nitrogen into a fine powder. Ice-cold acetone at a ratio of 10 mL per g fresh weight was added in a cold room at 4°C and under exceptionally low light intensity. Tubes were vortexed for a few seconds, and the outer surface of the sample tubes was tightly covered with aluminum paper. Then, the tubes were left overnight for extraction of chlorophyll at 4°C. Samples were centrifuged at 2500 rpm at 10 °C for 20 min, transferred to new tubes, and absorbance was determined with a spectrophotometer at 663 nm, 647nm and 470 nm. Pigment content was calculated in µg mL^{-1} using the following equations:

Chl a =12.25*E663-2.79*E647

Chl b=21.50*E647-5.10*E663

Chl a + b =7.15*E663 + 18.71*E647

Carotenoid = (1000*E470-1.82 *Chla-85.02 *Chl b)/198

2.3. Lipid Peroxidation

The method described by Jambunathan [15] was used with slight modifications. Freshly harvested leaf tissue (0.5 gr) was extracted in 10% trichloroacetic acid, and the homogenate was centrifuged at 12000 g at 25 °C for 10 minutes. 1 ml of supernatant was removed, and 20% TCA solution containing 0.5% TBA was added. The mixture was incubated for 30 min at 95°C and re-centrifuged. The supernatant was measured spectrophotometrically at 532 nm. MDA contents were calculated using 155 mM^{-1} cm^{-1} extinction coefficient. Spectrophotometric readings obtained at 600 nm were used to subtract nonspecific turbidity.

2.4. Determination of proline concentration

Leaf tissue (0,5 gr) was ground in liquid nitrogen and homogenized in 5 ml of 3% sulphosalicylic acid [16] and centrifuged at 15000g at room temperature. 2 ml of supernatant were transferred to screw-cap tubes, and 2 ml of glacial acetic acid was added and vortexed. 2 ml of acetic acid-phosphoric acid-ninhydrin solution was added to the mixture and mixed and boiled for one hour. After cooling, 4 ml of toluene was added and vortexed. After settling down, the toluene phase was removed and measured spectrophotometrically at 520 nm. Proline standards were treated like samples to prepare a calibration curve, and the amount of proline was calculated [34].

2.5. Extraction of proteins for enzyme activity analysis

Leaf tissues were harvested and ground in liquid nitrogen. 0.5 g of ground tissue was homogenized in 3 ml of 50 mM of Sodium phosphate buffer (pH=6.8) containing 1mM EDTA and 2% PVP. Bradford assay was used to determine the concentration of protein [17].

2.5.1. Catalase activity

Enzyme activity was assayed in 2 ml of reaction mixture containing 20 mM phosphate buffer (pH=7.5), 15 mM H_2O_2 and 40 µl of sample solution. The reaction was initiated by adding H_2O_2, and the change in the absorbance was followed at 240 nm for three minutes. The extinction coefficient of 40 $mM^{-1}cm^{-1}$ was used to calculate the specific activity of the enzyme [38].

2.5.2. Ascorbate Peroxidase activity

Enzyme activity was assayed in 2 ml of reaction mixture containing 50 mM phosphate buffer (pH=6.6), 1 mM H_2O_2 and 0.25 mM ascorbate. The reaction was initiated by adding 40 µl of the enzyme source, and the change in the absorbance was followed for three minutes at 290 nm. The extinction coefficient of 2.8 $mM^{-1}cm^{-1}$ was used to calculate the specific activity of the enzyme [39].

2.5.3. Glutathione reductase activity

Enzyme activity was assayed in 2 ml of reaction mixture containing 100 mM sodium phosphate buffer (pH=7,5), 1 mM EDTA, 0.1 mM NADPH, and 1 mM GSSG. The reaction was initiated by adding 40 µl of enzyme extract, and the absorbance change was followed at 340 nm for five minutes. The extinction coefficient of 6.2 $mM^{-1}cm^{-1}$ for GSSG was used to calculate the specific activity of the enzyme.

2.5.4. Glutathione S-Transferase activity

Enzyme activity was assayed in 2 ml of reaction mixture containing 100 mM sodium phosphate buffer (pH=6,5), 1 mM EDTA, 1 mM CDNB (1-Chloro 2,4-dinitrobenzene), and 2 mM GSH. The reaction was initiated by adding 40 µl of enzyme extract, and the change in the absorbance was followed at 340 nm for five minutes. The extinction coefficient of 9.6 $mM^{-1}cm^{-1}$ for CDNB was used to calculate the specific activity of the enzyme.

2.5.5. Guaiacol Peroxidase activity

Tissue was ground in liquid nitrogen and homogenized in 3 ml of 50 mM sodium phosphate buffer (pH=6,8) containing 1 mM EDTA and %2 PVP. Homogenate was centrifuged at 15000 g for 20 min at 4°C. The supernatant was transferred to new microfuge tubes, and the amount of protein was determined with Bradford assay [17]. Enzyme activity was measured in 25 mM potassium phosphate buffer (pH=7), 10 mM H_2O_2, and 9 mM guaiacol. To initiate the reaction, 20 µl of enzyme extract was added to complete the 2 ml reaction mixture. The reaction was measured spectrophotometrically at 470 nm for two minutes. The extinction coefficient of 26.6 $mM^{-1}cm^{-1}$ was used to calculate the specific activity.

2.5.6. Superoxide dismutase activity

The reaction mixture was prepared to contain 20 mM sodium phosphate (pH=7.5), 0.1 mM EDTA, 10 mM methionine, 0.1 mM p-Nitro Blue Tetrazolium (NBT), and 0.005 mM riboflavin. First, 20 µl of enzyme samples were added to the glass tubes, and the reaction mixture was added to the tubes with a final volume of 3 ml and vortexed. The tubes were capped and exposed to 300 µmol $m^{-2}s^{-1}$ light for 15 minutes. The absorbance values of the samples were measured at 560 nm. The untreated reaction mixture was used as a blank. The samples were exposed to light stimuli and placed in two tubes that did not contain the enzyme extract. SOD 4470 Umg^{-1} (Sigma) obtained from bovine erythrocytes was used in the preparation of the standards [34].

2.6. Statistical Analysis

Duncan's Multiple Range Test (DMRT) is a post-hoc statistical analysis method used after ANOVA to identify specific mean differences between group (SPSS 17.0) [35].

Results

3.1. Effect of Se treatment on plant growth

Except for the 1 ppm Se treatment, plant height was significantly decreased in response to increasing concentrations of Se. In the Ikizce cultivar, growth reduction was 12% and 20% for 10 and 20 mg/L selenium treatments, respectively (Table 1). In the Gökgöl cultivar, 11 and 17% growth reductions were observed for 10 and 20 mg/L Se treatment groups, respectively. All the mean value differences were statistically significant except for 1 mg/L treatment in the Gökgöl cultivar ($p<0.05$).

Plant height and fresh and dry weights of the wheat seedlings were also affected by Se toxicity. However, significant fresh weight, dry weight, and plant height increases were observed in 1 mg/L selenium treatment in the Ikizce cultivar. A substantial decrease in fresh and dry weight was observed for sensitive cultivars in 10 and 20 mg/L Se treatments (Table 1). While dry weight was not changed significantly in Se tolerant cultivars, fresh weight was significantly reduced at 10 and 20 mg/L Se treatments. Fresh weight decreased by 16% and 23% in 20 mg/L Se treatments in the Ikizce and Gökgöl cultivars.

3.2. Effect of Se treatment on chlorophyll content

In response to Se treatments, significant changes in total leaf chlorophyll content were observed in both cultivars ($p<0.05$). The most affected groups were those treated with 10 and 20 mg/L Selenate (Table 2). An approximately two-fold difference between the control and 20 mg/L Se treatments in both cultivars was detected. Chlorophyll contents were significantly increased in 1 mg/L Se treatment in both cultivars.

3.3. Effect of Se Treatments on Carotenoid Content

Although basal carotenoid content was higher in the Se-sensitive Ikizce cultivar (75% high), the decrease was also significant at 10 and 20 mg/L Se treatments. The decrease was almost 40% at 20 mg/L Se treatment and was statistically significant ($p<0.005$). In the Se-tolerant Gökgöl cultivar, Carotenoid content was significantly reduced (21%) in the 20 mg/L Se treatment (Table 2).

3.4. Effect of Se treatment on LPO

A significant increase in TBARS content was observed in 10 and 20 mg/l Se treatments in the Ikizce cultivar. About a 30% increase in TBAR level was determined in 10 mg/L Se treatment ($p<0.05$) (Fig. 1). TBAR level was slightly higher than that of control at 20 mg/L Se concentrations in the Gökgöl cultivar.

Table 1. Effect of Se on plant growth (Fresh and dry weight, Plant height) in Se-tolerant and Se-sensitive wheat cultivars

Wheat Cultivars	Plant Height (cm)	Dry weight (mg)	Fresh weight (mg)
Ikizce			
Control	23,175±0,237d*	0,0308±0,0005ab	0,2388±0,0038c
*1 mg/L**	24,208±0,191e	0,0319±0,0005b	0,2558±0,0037d
10 mg/L	20,255±0,226c	0,0317±0,0006b	0,2118±0,0039b
20 mg/L	18,543±0,222b	0,0294±0,0004a	0,1842±0,0032a
Gökgöl			
Control	20,632±0,168c	0,0372±0,0005c	0,2862±0,0031e
1 mg/L	20,767±0,228c	0,0372±0,0007c	0,2892±0,0048e
10mg/L	18,375±0,153b	0,0388±0,0007c	0,2540±0,0046d
20mg/L	17,625±0,159a	0,0377±0,0006c	0,2383±0,0040c

*Concentrations represent the Se concentrations in the Hoagland solution. Differences in the letters indicate statistical significance at the 5% level in the columns.

3.5. Effect of Se treatment on Proline accumulation

A statistically significant ($p < 0.05$) differences in mean proline content between the control group, 10 mg, and 20 mg/L Se treatment groups were detected. There were about 10- and 25-fold differences between control and 20 mg/L Se treatments in Gökgöl and Ikizce cultivars, respectively (Fig. 2).

3.6. Effect of Se treatment on Antioxidant enzymes

Compared with the control, a significantly higher APX activity was detected ($p < 0.05$) when exposed to 10 and 20 mg/L Se in both cultivars (Fig. 3). In the ikizce cultivar, approximately 3- and 2.5-fold increase in APX activity was observed in the 20 mg/L and 10 mg/L Se treatments groups, respectively. An approximately two-fold increase in APX activity was observed in the Gökgöl cultivar in the 20-ppm Se treatment group. Intrinsic and significantly higher catalase activity was observed in the control group, and all treatment groups in the tolerant cultivar were observed compared to the sensitive cultivars. Although catalase activity was not significantly higher in all treatment groups of the Se-tolerant Gökgöl cultivar compared to the control group, significantly higher CAT activities in 10 and 20 mg/L Se treatments compared with control was observed in the Ikizce cultivar ($p < 0.05$). No significant difference in CAT activity was observed between 10 and 20 mg/L Se treatments in both cultivars (Fig. 3) ($p < 0.05$).

A significant increase in GR activity was detected in 10 and 20 mg/L Se treatments in the Ikizce cultivars (Fig. 3) ($p<0.005$). Approximately 60% increase in the GR activity of the 20 mg/L Se treatment group in Ikizce cultivars was observed. GR activity difference between 10 and 20 mg/L treatment groups was also statistically significant in the Ikizce cultivar ($p<0.005$). GR activity was not significantly increased in the Gökgöl cultivar in all treatment groups compared with the control group ($p<0.005$).

Table 2. Effect of Se on chlorophyll and β-Carotene content in Se-tolerant and Se-sensitive wheat cultivars exposed to Control, 1, 10 and 20 mg/L of Se in Hoagland solutions.

Wheat Cultivars	Chlorophyll a	Chlorophyll b	Total chlorophyll	Carotenoid
Ikizce				
Control	1,2158±0,080^{cd}	0,5692±0,007^{de}	1,8694±0,054^{d}	0,4165±0,018^{cd}
*1 mg/L**	1,4939±0,036^{f}	0,5983±0,015^{e}	2,0923±0,043^{e}	0,4730±0,008^{d}
10 mg/L	0,9177±0,0200^{b}	0,3784±0,012^{b}	1,2961±0,028^{b}	0,3650±0,015^{c}
20 mg/L	0,6222±0,043^{a}	0,2709±0,020^{a}	0,8931±0,062^{a}	0,2811±0,012^{ab}
Gökgöl				
Control	1,3114±0,033^{de}	0,5484±0,017^{d}	1,8654±0,042^{d}	0,3783±0,039^{c}
1 mg/L	1,4479±0,026^{ef}	0,6035±0,005^{e}	2,0513±0,030^{e}	0,3735±0,044^{c}
10 mg/L	1,1008±0,021^{c}	0,4569±0,016^{c}	1,5577±0,035^{c}	0,3545±0,031^{bc}
20 mg/L	0,6987±0,019^{a}	0,2872±0,015^{a}	0,9350±0,065^{a}	0,2620±0,020^{a}

*Concentrations represent the Se concentrations in the Hoagland solution. Differences in the letters indicate statistical significance at the 5% level in the columns.

GST activity significantly increased at 10 and 20 mg/L Se treatment groups of both cultivars, but it was more pronounced at 20 mg/L Se treatment of the Ikizce cultivar ($p<0.005$). There were about 3.5- and 10-fold differences between the control

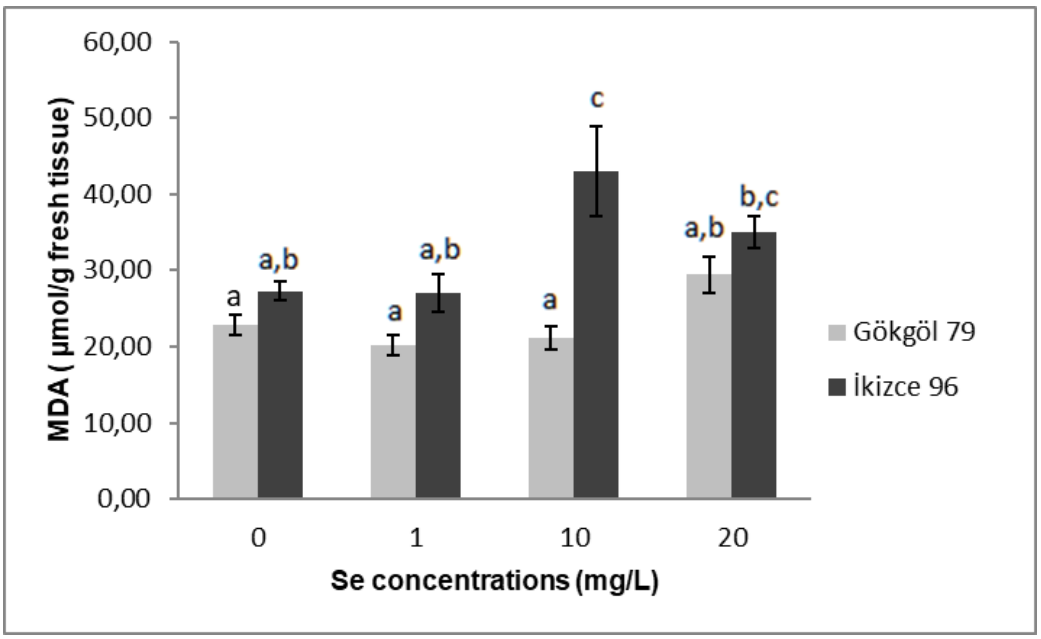

Figure 1. Lipid peroxidation in Se tolerant (Gökgöl) and Se sensitive (Ikizce) wheat cultivars exposed to 0, 1, 10 and 20 mg/L of Se in Hoagland solutions.

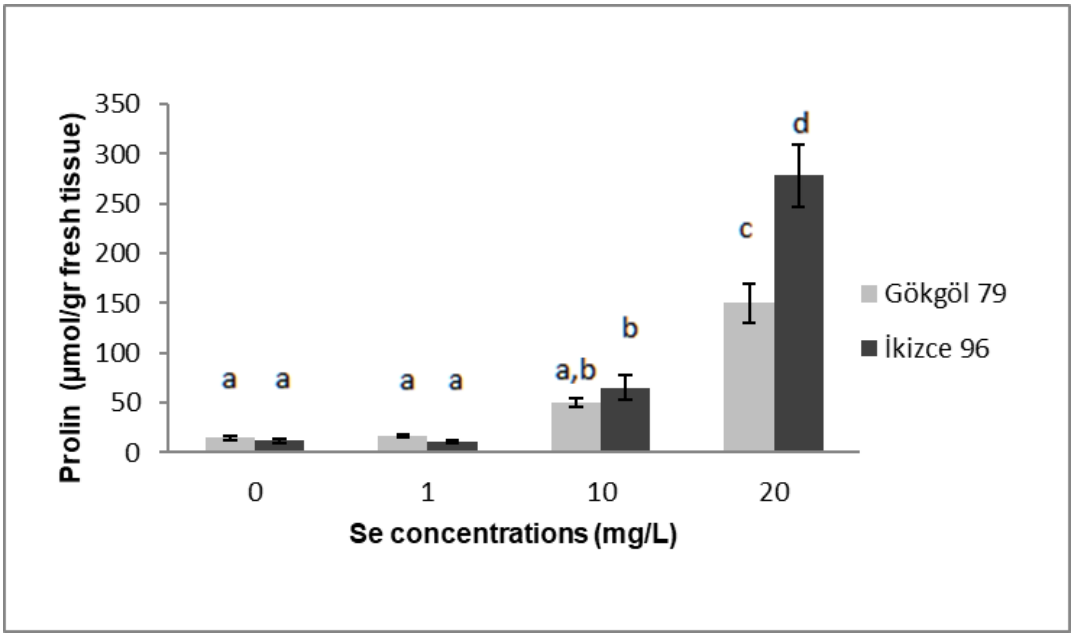

Figure 2. Proline accumulations in Se tolerant (Gökgöl) and Se sensitive (Ikizce) wheat cultivars exposed to 0, 1, 10 and 20 mg/L of Se in Hoagland solutions

and 20 mg/L Se treatment group in Gökgöl and Ikizce cultivars, respectively (Fig. 3). The mean differences between the control groups and all Se treatments were found to be statistically significant ($p<0.05$).

Guaiacol peroxidase activity was increased significantly at 10 and 20 mg/L Selenium treatment groups ($p<0.05$) in both cultivars, but the increase was more conspicuous in the Ikizce cultivar. There were approximately four- and seven-fold increases in Ikizce cultivar at 10 and 20 mg/L Se concentrations, respectively. More than two-fold increase was observed in the Gökgöl cultivar in 20 mg/L Se treatment (Fig 3).

A significant reduction in SOD activity was observed in Se-Sensitive İkizce 96 cultivars in a dose-dependent manner ($p<0.05$). A substantial decrease in activity was also observed in Se-tolerant Gökgöl 79 cultivars.

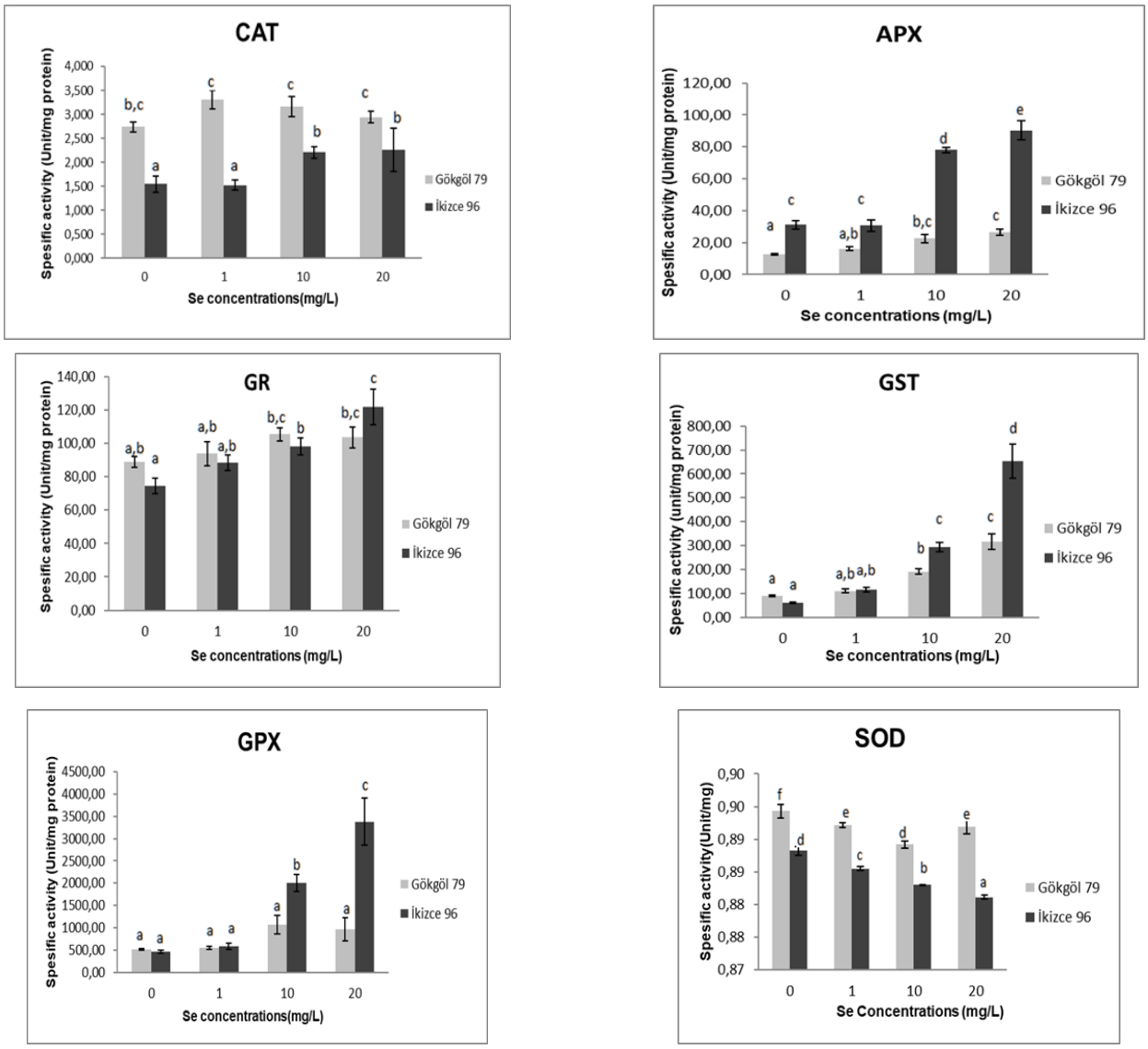

Figure 3. Antioxidant enzyme activities in Se tolerant (Gökgöl) and Se sensitive (Ikizce) wheat cultivars exposed to 1, 10 and 20 mg/L of Se in Hoagland solutions. GST, Glutathione S-transferase, CAT, Catalase; APX, Ascorbate peroxidase; GPX, Guaiacol peroxidase; GR, Glutathione reductase.

Discussion

Wheat is considered among non-accumulators but one of the Se-tolerant plants; however, the critical level of Se-toxicity depends upon the accumulated Se forms. Wheat cultivars may differentially accumulate Se, and threshold toxicity might differ for various wheat cultivars. The critical level of Se for toxicity was found to be different for diverse plant species, and the critical tissue concentration of Se for wheat was found to be 325 mg/kg DW in a study reported by Lyons et al. [18].

A noticeable plant growth retardation was demonstrated where Se treatments were above 10 mg/L in hydroponic cultures during this study (Table 1). Retarded seedling growth in wheat seedlings due to extreme concentration of bioavailable Se could be explained by the interference of Se with plant metabolism, such as nutrient uptake, synthesis of malformed proteins, chlorophyll biosynthesis, plant growth regulators and oxidative stress. In agreement with this study, Molnárová and Fargasová [19] also reported a considerable decrease in the total fresh and dry mass of wheat and barley plants when plants were exposed to higher concentrations of Se as selenite. Notwithstanding, a lower Se dose (1 mg/L) was beneficial and promoted plant growth, as indicated by a significant increase in plant height and fresh and dry weight in this study. In agreement with our results, Boldrin et al. [3] also reported an enhancement in wheat shoot biomass when treated with a lower dosage of Se. The dual effect of Se was also reported by Guerrero et al. [4], indicating the growth stimulant effect at lower doses.

While severe chlorosis was observed mainly on the edges of the leaf in sensitive Ikizce cultivars, the level of chlorosis was lower in the putative tolerant Gökgöl cultivar. Significantly reduced (approximately 50%) chlorophyll accumulation was observed when Se level was 20 mg/L. The Se level in hydroponic culture proportionally inhibited chlorophyll biosynthesis in wheat leaf tissue. The reduction of chlorophyll concentration

in leaves caused by a higher concentration of selenate might be attributed to the interference of Se with chlorophyll metabolisms. The effect of selenium toxicity on chlorophyll biosynthesis in mung bean seedlings was studied by Padmaja et al. [20]. The amount of chlorophyll in dark-grown seedlings was suppressed, and porphobilinogen synthase activity was repressed by Selenium toxicity. As a result, total chlorophyll content in light-grown seedlings was decreased.

Higher concentrations of Se also reduced the level of carotenoids during the current study, and the difference was statistically significant in the 10 and 20 mg/L application doses of both cultivars (Table 2). As natural isoprenoid antioxidants, carotenoids can also scavenge various forms of ROS and protect the photosynthesis machinery by scavenging ROS-like compounds [21].

At higher Se concentrations (20 mg/L) (Fig. 2), the level of TBARS in leaves was significantly increased. The concentration of TBAR was 30% higher at 20 mg/L than that of control in both cultivars. TBAR level was not changed to 20 mg/L Se in the tolerant cultivar, but significantly higher TBAR was observed in the sensitive cultivar at 10 mg/L Se treatment. The Se dosage is the predominant factor affecting the membrane LPO. Although Se has an antioxidative function in trace amounts, this effect of Se appeared to be reduced or reversed at higher concentrations. In addition to several other damages, the pro-oxidant nature of Se contributing to Se toxicity was also reported [22]. Following this study, TBAR level was also increased two- to three-fold when polished and finished wheat were treated with 100 μM selenite in a survey conducted by Labanowska et al. [23].

Proline accumulation was significantly increased in leaves of Se-treated wheat seedlings in a concentration-dependent manner (Fig. 3). In higher plants, several abiotic stress conditions, including salinity, drought, and heavy metal toxicity, induce proline accumulation [24]. Several mechanisms of proline stress protection exist, such as osmolyte function, chemical chaperone, metal chelator, and ROS scavenger [36]. However, how higher trace element concentrations trigger proline accumulation in higher plants is not clarified. ROS accumulation is considered one of the reasons for triggering proline biosynthesis. Proline has the chelating ability; binding with metal ions can protect heavy metal-stressed plants. Parallel increases in proline and TBARS contents during heavy metal stress suggest a relationship between free radical generation and proline accumulation. Transgenic algae expressing the P5CS gene produced freer proline than the wild type, and the free proline levels were negatively correlated with MDA levels in heavy metal-treated algae [25]. This result indicates the role of free proline as an antioxidant in Cd-stressed cells. However, despite a significant increase in proline accumulation, the membrane damage revealed by LPO was not reduced in wheat seedlings exposed to higher selenate concentrations. Although proline accumulation was significantly higher in Se sensitive cultivar, membrane lipid peroxidation revealed by TBAR content was also higher than that of selenium tolerant cultivar. These findings indicate that proline accumulation alone cannot protect wheat cells against oxidation-based selenium toxicity. However, exogenous proline application decreased the phytotoxicity of selenium by reducing the oxidative stress in bean seedlings in a study reported by Aggarwal et al. [26].

ROS are eliminated or reduced by plants with various mechanisms, including natural antioxidant substances such as reduced glutathione (GSH) and antioxidant enzymes including SOD, POD, CAT, GST and APX which play vital roles in eliminating the ROS in the cells by increasing the activity or quantity under stress conditions [27]. Including trace elements, several abiotic stresses cause damage to plant cells either directly or indirectly by stimulating the elevation of ROS. The result of the present study indicates that Se toxicity causes membrane lipid peroxidation and alters the activities of SOD, CAT, APX, GR, GST and GPX. The activities of antioxidant enzymes were found to be different in Se-tolerant and sensitive cultivars. Especially in APX, GPX and GST, the activity was significantly higher in Se sensitive cultivar when compared with the tolerant cultivar. Although a significant increase in GPX, GR, GST, APX, and CAT activities was observed, the LPO level was also increased, and chlorophyll concentration was decreased dose-dependent (Fig. 1 and Table 2). These results suggest that the induction of antioxidant enzymes is insufficient to protect chloroplasts and cell membranes from Se-induced phytotoxicity. There is supposed to be some other defence mechanism that provides tolerance against Se toxicity. Similar to this finding, Cavalcanti et al. [28] reported that the increase in the activities of SOD, CAT, and POX enzymes did not protect against oxidative damage caused by salt stress in cowpea leaves. Similar findings were also reported by Freeman et al. [29] when they studied *selenium hyperaccumulator* Se-tolerant *Stanleya pinnata* and secondary accumulator Se-sensitive *Stanleya albescens*. They concluded that *S. albescens* might accumulate more toxic forms of Se in its leaves than *S. pinnata*. Expression of genes involved in sulfur assimilation, antioxidant activities, defense, and response to salicylic acid, jasmonic acid, or ethylene in *S. pinnata* was also higher. Considering antioxidant mechanisms provide general tolerance to oxidative stress, the central tolerance to Se is accomplished possibly through various detoxification mechanisms such as volatilization, storage, sequestration, oxidation, transamination, selenation, and transportation. As reported by Freeman et al. [29], higher expression of genes involved in sulfur assimilation may also be one of the primary mechanisms for Se tolerance. In addition, different degradation pathways for synthesized malformed or deformed selenoproteins may also increase selenium tolerance. Zhou et al. [30] described the molecular mechanism of Se tolerance in a recent study in a selenium hyperaccumulator *Cardamine hupingshanensis* seedlings by comparative transcriptomics analysis. Their study identified differentially expressed 25 genes in four pathways responding significantly to selenite in *C. hupingshanensis* seedlings. Among the pathways, storage function, oxidation, transamination, and selenation were the most influenced. Moreover, a different degradation pathway for

malformed selenoproteins also influenced selenium tolerance at different selenite concentrations. A similar approach might be beneficial to determine the nature of Se tolerance by using our Se-sensitive and tolerant wheat cultivars.

The activity of APX was correspondingly increased in wheat cells when exposed to Se toxicity caused by a higher concentration of selenate in hydroponic culture (Fig. 3). These increases were more pronounced in Se-sensitive cultivars, which had more membrane damage, intensive chlorosis and decreased in fresh and dry weight. Like other antioxidant enzymes, several stress conditions, including heavy metals, induce APX activity in eliminating detrimental levels of H_2O_2 from plant cells. Sharma et al. [31] reported that ascorbic acid reduces selenium toxicity in rice (*Oryza sativa* L.) by increasing the antioxidative and metal tolerance. Despite three-fold higher APX activity in Se-sensitive İkizce cultivar, membrane LPO and leaf chlorosis still indicate its failure to protect wheat cells from Se toxicity.

CAT is a universal oxidoreductase that degrades H_2O_2 into water and molecular oxygen. Depending on the dosage, the activity of CAT was significantly increased upon exposure to Se. It s more pronounced in Se-tolerant cultivars (Fig. 4). In agreement with this finding, the application of selenium as a seed treatment (5 mg/L) and foliar spray (100 mg/L) significantly increased antioxidant enzymes such as catalase and peroxidase in soybean in a study reported by Djanaguiraman et al. [32].
GR, GST and GPX activities were significantly increased in wheat seedlings exposed to higher levels of selenium in the current study (Fig. 4). Increases in the activity of those enzymes were more pronounced in Se-sensitive cultivars. Selenate may interfere with the regeneration of GSH. Decreased levels of glutathione were observed in *Stanleya albescens* plants treated with selenite, accompanied by increased superoxide and hydrogen peroxide levels compared with the more tolerant Se-hyper-accumulator [29]. GSH and its derivatives are considered crucial components of detoxification and antioxidative systems in plant cells and participate in various metabolic processes. The level of GSH increases significantly when plants are exposed to a wide range of abiotic stress conditions. Since glutathione is considered an effective antioxidant, neutralizing the effect of ROS on plant cells, it is expected to modify the response of antioxidant enzymes to heavy metal stress [37]. The glutathione level was decreased two-fold after 6 h in Arabidopsis plants treated with 50 mM selenite [33]. Thus, interference of Se toxicity with GSH synthesis in plants may jeopardize plant defense against hydroxyl radicals and oxidative stress. Although GSH is found predominantly in its reduced form in most plant tissues, the oxidized, disulphide form of glutathione (GSSG) can also be detected. Glutathione reductase is a responsible enzyme for the regeneration of GSH from GSSG, which maintains the high GSH/GSSG ratio in plant cells, which might elucidate the increase in the activity of GR under oxidative stress conditions.

Guaiacol peroxidase is one of the H_2O_2-eliminating enzymes during normal metabolism and stress conditions. GPX favors aromatic compounds such as guaiacol and pyragallol as electron donors. GPX is the critical enzyme in removing H_2O_2 since it is active in the intracellular area (cytosol, vacuole), cell wall and extra-cellular regions. The activity of guaiacol peroxidase was enormously increased in Se-treated wheat seedlings, indicating an increase of H_2O_2 in the cells due to oxidative stress caused by the interference of Se with the metabolism of cells.

There was a significant increase in MDA as an indicator of membrane lipid peroxidation in sensitive cultivars in response to higher doses of Se treatments. In higher Se doses, catalase enzyme activity was significantly higher in the Se-tolerant cultivar. Higher catalase activity might have a critical role in reducing membrane lipid peroxidation in that cultivar since the Se-sensitive cultivar with severe toxicity symptoms and higher membrane lipid peroxidation exhibited higher specific activity in other investigated antioxidant enzymes. Higher proline accumulation in Se-sensitive cultivars also did not provide tolerance against Se toxicity, considering that higher proline accumulation is not a specific mechanism protecting against Se or heavy metal toxicity.

Se toxicity is caused by interfering with several metabolic pathways, including chlorophyll metabolism or chloroplast integrity, protein misfolding, and oxidative stress. Higher activities of antioxidant enzymes may only be part of the tolerance mechanism against oxidative damage caused by Se since the Se-sensitive cultivar with severe toxicity symptoms exhibited higher specific activity in all the antioxidant enzymes except SOD and catalase.

A lower dose of Selenium had a beneficial effect as indicated by higher total chlorophyll content, carotenoid content and fresh and dry weight in 1mg/l treated groups both in Se-sensitive and -tolerant cultivars, proving the dual effect of Se as pro-oxidant in lower and oxidant effect in higher doses. These cultivars with differential responses to Se toxicity could be utilized in further comparative studies, such as functional genomics studies, to elucidate the possible mechanism of Se tolerance in wheat.

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Visualization.

Compliance with Ethical Standards

This article contains no studies that have been performed with human participants.

Declaration of Competing Interest

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

References

1. Pilon-Smits EAH, Quinn CF. Selenium metabolism in plants. In: Hell R, Mendel R, editors Cell biology of metal and nutrients, Berlin: Springer 2010; pp 225-241. https://doi:10.1007/978-3-642-10613-2_10
2. Hasanuzzaman M, Bhuyan MHMB, Raza A, Hawrylak-Nowak B, Matraszek-Gawron R, Nahar K, Fujita M. Selenium Toxicity in Plants and Environment: Biogeochemistry and Remediation Possibilities. Plants (Basel) 2020; 9(12):1711. https://doi:10.3390/plants9121711
3. Boldrin PF, de Figueiredo MA, Yang Y, Luo H, Giri S, Hart JJ, Faquin V, Guilherme LR, Thannhauser TW, Li L. Selenium promotes sulfur accumulation and plant growth in wheat (Triticum aestivum). Physiol Plant 2016; 158(1):80-91. https://doi:10.1111/ppl.12465
4. Guerrero B, Lugany M, Palacios O, Valient M. Dual effects of different selenium species on wheat. Plant Physiol Biochem 2014; 83:300-307. https://doi:10.1016/j.plaphy.2014.08.009
5. Hu W, Zhao C, Hu H, Yin S. Food Sources of Selenium and Its Relationship with Chronic Diseases. Nutrients 2021; 13(5): 1739. https://doi:10.3390/nu13051739
6. Galic L, Vinkovic T, Ravnjak B, Loncaric Z. Agronomic biofortification of significant cereal crops with selenium-a review. Agronomy-Basel 2021; 1:1015. https://doi:10.3390/agronomy11051015
7. Liu Y, Huang S, Jiang Z, Wang Y, Zhang Z. Selenium Biofortification Modulates Plant Growth, Microelement and Heavy Metal Concentrations, Selenium Uptake, and Accumulation in Black-Grained Wheat. Front. Plant Sci 2021; 12:748523. https://doi:10.3389/fpls.2021.748523
8. Ramkissoon C, Degryse F, da Silva RC, Baird R, Young SD, Bailey EH, Mclaughlin MJ. Improving the efficacy of selenium fertilizers for wheat biofortification. Scientific Reports 2019; 9:19520. https://doi.org/10.1038/s41598-019-55914-0
9. Sors TG, Ellis DR, Salt DE. Selenium uptake, translocation, assimilation and metabolic fate in plants. Photosynth Res 2005; 86(3):373-89. https://doi:10.1007/s11120-005-5222-9
10. Van Hoewyk D. A tale of two toxicities: malformed selenoproteins and oxidative stress both contribute to selenium stress in plants. Ann Bot 2013; 112(6):965-972. https://doi:10.1093/aob/mct163
11. Zhou X, Yuan Y, Yang Y, Rutzke M, Thannhauser TW, Kochian LV, Li L. Involvement of a broccoli COQ5 methyltransferase in the production of volatile selenium compounds. Plant Physiol 2009; 151(2):528-540. https://doi:10.1104/pp.109.142521
12. Balakhnina TI, Nadezhkina ES. Effect of selenium on growth and antioxidant capacity of Triticum aestivum L. during development of lead-induced oxidative stress. Russ J Plant Physiol 2017; 64:215-223. https://doi.org/10.1134/S1021443717010022
13. Kolbert Z, Lehotai N, Molnár Á, Feigl G. "The roots" of selenium toxicity: A new concept. Plant Signaling & Behavior 2016; 11:10, e1241935. https://doi:10.1080/15592324.2016.1241935
14. Inskeep WP, Bloom PR. Extinction coefficients of chlorophyll a and B in n,n-dimethylformamide and 80% acetone. Plant Physiol 1985; 77(2):483-485. https://doi:10.1104/pp.77.2.483
15. Jambunathan N. Determination and Detection of Reactive Oxygen Species (ROS), Lipid Peroxidation, and Electrolyte Leakage in Plants, in R. Sunkar (ed.) Plant Stress Tolerance Methods in Molecular Biology 639 © Springer Science+Business Media LLC, 2010; pp 291. https://doi:10.1007/978-1-60761-702-0_18
16. Ábrahám E, Hourton-Cabassa C, Erdei L, Szabados L. Methods for Determination of Proline in Plants. In: R. Sunkar (ed.) Plant Stress Tolerance Methods in Molecular Biology 639 © Springer Science+Business Media LLC, 2010; pp 317. https://doi:10.1007/978-1-60761-702-0_20
17. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248-254. https://doi.org/10.1016/0003-2697(76)90527-3
18. Lyons GH, Stangoulis JCR, Graham RD. Tolerance of wheat (Triticum aestivum L.) to high soil and solution selenium levels. Plant Soil 2005; 270:179–188. https://doi.org/10.1007/s11104-004-1390-1
19. Molnárová M, Fargasová A. Se (IV) phytotoxicity for monocotyledonae cereals (Hordeum vulgare L. Triticum aestivum L.) and dicotyledonae crops (Sinapis alba L. Brassica napus L.). J Hazard Mater 2009; 172:854-861. https://doi.org/10.1016/j.jhazmat.2009.07.096
20. Padmaja K, Prasad DKK, Prasad ARK. Effect of selenium on chlorophyll biosynthesis in mung bean seedling. Phytochem 1989; 28:3321-3324. https://doi.org/10.1016/0031-9422(89)80339-5
21. Grace SG, Logan BA. Energy dissipation and radical scavenging by the plant phenyl propanoid pathway. Phil Trans R Soc B 2000; 355(1402):1499-1510. https://doi:10.1098/rstb.2000.0710

22. Hartikainen H, Xue T, Piironen V. Selenium as an anti-oxidant and pro-oxidant in ryegrass. Plant Soil 2000; 225:193–200. https://doi.org/10.1023/A:1026512921026
23. Łabanowska M, Filek M, Kościelniak J, Kurdziel M, Kuliś E, Hartikainen H. The effects of short-term selenium stress on Polish and Finnish wheat seedlings-EPR, enzymatic and fluorescence studies. J Plant Physiol 2012; 169(3):275-284. https://doi.org/10.1016/j.jplph.2011.10.012
24. Szabados L, Savouré A. Proline: a multifunctional amino acid. Trends Plant Sci 2010; 15(2):89-97. https://doi:10.1016/j.tplants.2009.11.009
25. Siripornadulsil S, Traina S, Verma DP, Sayre RT. Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae. Plant Cell 2002; 14(11):2837-2847. https://doi:10.1105/tpc.004853
26. Aggarwal M, Sharma S, Kaur N, Pathania D, Bhandhari K, Kaushal N, Kaur R, Singh K, Srivastava A, Nayyar H. Exogenous proline application reduces phytotoxic effects of selenium by minimizing oxidative stress and improves growth in bean (Phaseolus vulgaris L.) seedlings. Biol Trace Elem Res 2011; 140(3):354-367. https://doi:10.1007/s12011-010-8699-9
27. Mittler R. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 2002; 7:405-410. https://doi.org/10.1016/S1360-1385(02)02312-9
28. Cavalcanti FR, Oliveira JTA, Martins-Miranda AS, Viégas RA, Silveir JAG. Superoxide dismutase, catalase and peroxidase activities do not confer protection against oxidative damage in salt-stressed cowpea leaves. New Phytol 2004; 163:563-571. https://doi.org/10.1111/j.1469-8137.2004.01139.x
29. Freeman JL, Tamaoki M, Stushnoff C, Quinn C, Cappa J, Devonshire J, Fakra S, Marcus M, McGrath S, Hoewyk DV, Pilon-Smits EAH. Molecular mechanisms of selenium tolerance and hyperaccumulation in Stanleya pinnata. Plant Physiol 2010; 153(4):1630-1652. https://doi:10.1104/pp.110.156570
30. Zhou Y, Tang Q, Wu M, Mou D, Liu H, Wang S, Zhang C, Ding L, Luo J. Comparative transcriptomics provides novel insights into the mechanisms of selenium tolerance in the hyperaccumulator plant Cardamine hupingshanensis. Sci Rep 2018; 8(1):2789. https://doi:10.1038/s41598-018-21268-2
31. Sharma S, Kaur N, Kaur S, Nayyar H. Ascorbic Acid Reduces the Phytotoxic Effects of Selenium on Rice (Oryza Sativa L.) by Up-Regulation of Antioxidative and Metal-Tolerance Mechanisms. J Plant Physiol Pathol 2014; 2:3. https://doi:10.4172/2329-955X.1000128
32. Djanaguiraman M, Devi DD, Arun K, Shanker J, Sheeba A, Bangarusamy U. Impact of selenium spray on monocarpic senescence of soybean (Glycine Max L.). J Food Agric Environ 2004; 2(2):44-47. https://doi.org/10.1234/4.2004.162
33. Hugouvieux V, Dutilleul C, Jourdain A, Reynaud F, Lopez V, Bourguignon J. Arabidopsis putative selenium-binding protein1 expression is tightly linked to cellular sulfur demand and can reduce sensitivity to stresses requiring glutathione for tolerance. Plant Physiol 2009; 151(2):768-81. https://doi:10.1104/pp.109.144808
34. Akbulut M, Çakır S. The effects of Se phytotoxicity on the antioxidant systems of leaf tissues in barley (Hordeum vulgare L.) seedlings. Plant Physiol Biochem 2010; 48(2-3):160-166. https://doi.org/10.1016/j.plaphy.2009.11.001
35. SPSS Inc. Released 2008. SPSS Statistics for Windows, Version 17.0. Chicago: SPSS Inc.
36. Samuel D, Kumar TK, Ganesh G, Jayaraman G, Yang PW, Chang MM, Trivedi VD, Wang SL, Hwang KC, Chang DK, Yu C. Proline inhibits aggregation during protein refolding. Protein Sci 2000; 9:344-352. https://doi:10.1110/ps.9.2.344
37. Halušková L, Valentovičová K, Huttová J, Mistrík I, Tamás L. Effect of abiotic stresses on glutathione peroxidase and glutathione S-transferase activity in barley root tips. Plant Physiol Biochem 2009; 47(11-12):1069-1074. https://doi:10.1016/j.plaphy.2009.08.003
38. Yilmaz S, Temizgül R, Yürürdurmaz C, Kaplan M. Oxidant and antioxidant enzyme response of redbine sweet sorghum under NaCl salinity stress. Bioagro 2020; 32(1): 31-38. https://revistas.uclave.org/index.php/bioagro/article/view/2684
39. Yilmaz SH, Kaplan M, Temizgul R, Yilmaz S. Antioxidant enzyme response of sorghum plant upon exposure to Aluminum, Chromium and Lead heavy metals. Turk J Biochem 2017; 42(4): 503-512. https://doi.org/10.1515/tjb-2016-0112