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Morpho-Physiological and Antioxidative Responses of Wheat Seedlings to Different Forms of Selenium

Ana Vuković Popović [1], Ivna Štolfa Čamagajevac [1],*, Rosemary Vuković [1], Magdalena Matić [2], Dharmendra K. Gupta [3] and Zdenko Lončarić [2]

[1] Department of Biology, University of Osijek, 31000 Osijek, Croatia; avukovic@biologija.unios.hr (A.V.P.); rosemary@biologija.unios.hr (R.V.)

[2] Faculty of Agrobiotechnical Sciences Osijek, University of Osijek, 31000 Osijek, Croatia; maticm@fazos.hr (M.M.); zloncaric@fazos.hr (Z.L.)

[3] Ministry of Environment, Forest and Climate Change, New Delhi 110003, India; gupta.dharmendra@gov.in

* Correspondence: istolfa@biologija.unios.hr

Abstract: Selenium (Se) deficiency in human and animal nutrition is primarily due to low levels of Se in soils. It can be prevented by enriching crops, such as wheat, with Se through agronomic biofortification. Although Se is not essential for plants, it shows a dual effect on their metabolism depending on its concentration. This study aimed to elucidate the impact of five different concentrations (0.4, 4, 20, 40, and 400 mg/kg) of selenate and selenite on the oxidative status and antioxidative response of wheat (*Triticum aestivum* L., cv. Kraljica) shoots and roots. According to morpho-physiological analyses, selenite was found to have a lower toxicity threshold than selenate. The measurement of oxidative stress biomarkers showed that Se did not cause oxidative damage to wheat seedlings due to the activation of detoxification mechanisms at the biochemical level, which depended on the type of tissue, concentration, and form of applied Se. Treatment with 20 mg/kg of selenate can be recommended for wheat seedling biofortification due to a sufficient increase in Se accumulation in shoots without signs of toxicity. These results contribute to a better understanding of wheat seedlings' physiological and biochemical responses to Se and the development of more effective biofortification strategies.

Keywords: shoots; roots; selenate; selenite; oxidative status

1. Introduction

As a result of anthropogenic actions and climate changes, the composition and quality of the soil have been disturbed [1,2]. There are various techniques to improve the condition and health of the soil [3,4], including agronomic biofortification. This technique is an effective strategy for enhancing the concentration and bioavailability of micronutrients in soils and the edible parts of plants through fertilization [5]. Selenium (Se) is one of the most important biologically active micronutrients necessary for the proper functioning of many organisms, including humans and animals [6,7]. Plants are the primary source of dietary Se [8,9]. Since staple crops, like wheat, are consumed by a large portion of the population due to their nutritional value, they represent an obvious target for biofortification strategies that will increase the dietary Se intake in Se-deficient areas [10,11]. Moreover, wheat can concentrate sufficient levels of Se without causing any damage to its growth or yield [12]. Since biofortification increases the concentration of Se in plants and soil, it is necessary to investigate how the increased concentration and different forms of Se affect the plant itself.

Selenate and selenite are mainly used in agronomic biofortification as they are the most readily available forms of Se in soils and waters [13–19]. It is known that plants uptake selenate and selenite by different mechanisms [20–23]. However, due to the similar chemical properties to sulfur (S), both forms are metabolized by the S assimilation pathway to SeCys and SeMet. The nonspecific incorporation of Se instead of S into cysteine and

methionine, and finally the incorporation of SeCys and SeMet in proteins, is one of the main mechanisms of Se toxicity [24,25].

The role of Se in plants depends primarily on its chemical form and concentration but also on the plant species, developmental stage, and plant organ. Although it is not essential for higher plants, Se shows a dual effect on their metabolism. Low concentrations benefit overall growth and development, while at higher concentrations, it becomes toxic [26–28]. Selenium interferes with numerous metabolic pathways, and its effect is visible at the morpho-physiological and biochemical levels [28]. Although Se in lower concentrations has a positive effect on plant growth, Ramos et al. [18] emphasized that selenate and selenite did not affect the shoot and root growth equally and that the influence depended on the form of Se. Some studies showed that increased biomass due to exposure to Se resulted from increased mineral intake and increased photosynthetic efficiency, which includes, among other things, an increase in concentrations of photosynthetic pigments [28]. At higher concentrations, regardless of the chemical form, Se has the opposite effect on most of the parameters mentioned above and can decrease overall plant growth and development [29]. Morpho-physiological toxic symptoms of Se in plants include reduced biomass [28], photosynthetic efficiency [30,31], and germination rate [32]. Toxicity can also manifest through symptoms such as chlorosis, necrosis, various other leaf damages, and drying [30].

Morpho-physiological changes due to the presence of Se are accompanied by changes at the biochemical level [33]. These changes are related to the formation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), and changes in the antioxidative defense system. Excessive accumulation of ROS leads to oxidative damage of biologically important molecules such as DNA, proteins, and lipids [34]. To protect themselves from the negative consequences of ROS accumulation, plants have developed an antioxidant defense system that can be enzymatic or non-enzymatic [35]. Examining the influence of selenate and selenite on the antioxidant capacity of lettuce leaves, Rios et al. [36] determined that selenate is less toxic than selenite as selenite caused a higher accumulation of H_2O_2 and increased lipid peroxidation (LPO); these values were significantly lower after exposure to selenate, followed by higher antioxidative enzyme activities [36]. It is also known that increased antioxidative capacity, in the form of increased activities of antioxidant enzymes, improves Se tolerance in some plant species [37]. Contrary to lower Se concentrations, higher concentrations promote the formation of ROS, whose excessive accumulation causes oxidative damage [38]. An increase in ROS concentration due to exposure to higher concentrations of Se has been recorded in species such as beans [39], cucumber [40], quinoa [41], and rice [30]. The accumulation of ROS promotes LPO, thereby impairing cell integrity, which can lead to cell death [29]. Therefore, the level of LPO is often monitored as a biomarker of oxidative damage [17,26,28,42]. Increased LPO levels may be accompanied by a decrease in antioxidative enzyme activities [26], but also by an increase in the activity of enzymes such as catalase (CAT) [43,44] and guaiacol peroxidase (GPOD) [39], which are important for H_2O_2 detoxification.

Despite numerous studies on the influence of Se on the oxidative and antioxidant status of plants, the mechanism of its action still needs to be further elucidated. It should be emphasized that there is a lack of investigations about the effect of Se on the plant's metabolism without previously exposing the plant to stress. The aim of this study was primarily to investigate the effect of different concentrations (environmentally relevant and sublethal concentrations) and chemical forms of Se (selenate and selenite) on the oxidative status and antioxidative response of wheat seedlings and observe how they will reflect on their morpho-physiological characteristics. We hypothesize that selenate and selenite will increase the concentration of Se in wheat seedlings depending on the applied concentration and the chemical form of Se followed by a tissue-specific response. Additionally, we assume that different forms of Se will activate different antioxidative mechanisms in shoots and roots.

2. Materials and Methods

2.1. Plant Material, Growth Condition, and Treatment

In this research, seeds of the Croatian winter wheat variety (*Triticum aestivum* L., cv. Kraljica) originated from the Agricultural Institute Osijek were selected and subjected to different concentrations and forms of Se in the germination stage. The Variety Kraljica was used as the most widespread and high-yielding variety in production in the Republic of Croatia. It belongs to the A2 quality group and shows good resistance to lodging [45,46].

Before germination, wheat seeds were sterilized with 96% ethanol and washed a few times in dH$_2$O. Additional sterilization was done with a sodium dichloroisocynurate solution (Izosan-G, PLIVA, Zagreb, Croatia) containing 0.001% Tween for 8 min. Seeds were rewashed a few times in dH$_2$O and left overnight at 4 °C. The next day, 50 seeds were planted on vermiculite in Petri dishes (Ø 90 mm). Seeds were planted in seven biological replicates for control and seven for each Se treatment (Figure 1). Vermiculite was previously soaked with 20 mL of Hoagland's solution [47] with the addition of Se. Selenium was applied as selenate (Na$_2$SeO$_4$) and selenite (Na$_2$SeO$_3$) to final environmentally relevant and sublethal concentrations of 0.4, 4, 20, 40, and 400 mg/kg. The average Se concentration in soils worldwide is 0.4 mg/kg [48], which is within the range of normal Se levels, while toxicity in soils occurs between 30 and 324 mg/kg [49].
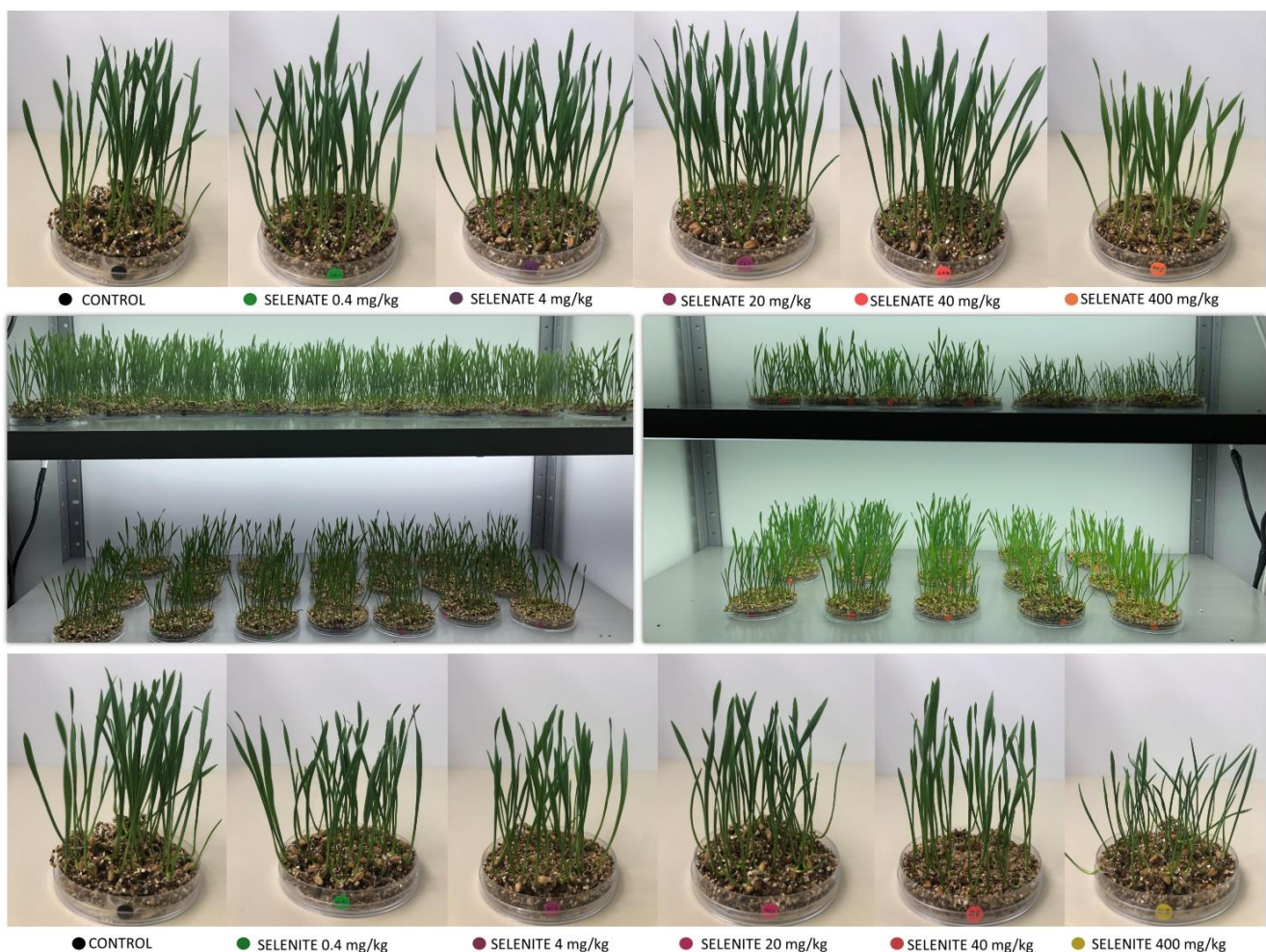

Figure 1. Wheat seedlings on vermiculite seven days after treatment with different concentrations of selenate and selenite. Control plants were grown without selenium.

Control plants were grown on vermiculite without Se, only with the addition of Hoagland's solution. Wheat seedlings were grown under a 16/8 h light/dark photoperiod at 25/20 °C day/night temperature with regular watering. After seven days of growth, seedlings were sampled for morpho-physiological and biochemical analyses. For biochemical and most morpho-physiological analysis, wheat shoot and root tissue were frozen in liquid nitrogen and macerated in 10 mL stainless steel jars containing a grinding ball (Ø 20 mm) for 1 min at 30 Hz using a Tissue-LyserII bead mill (Qiagen, Hilden, Germany). Proteins and metabolites were extracted from the tissue powder aliquots using an appropriate extraction solution.

2.2. Total Se Determination

For the Se concentration estimation, wheat shoots and roots were dried in an oven at 105 °C for 24 h. The dry wheat tissue was ground to a fine powder using a metal laboratory ultracentrifugal mill (Retsch ZM 200, Haan, Germany). To an aliquot of the milled powder, 10 mL of the mixture HNO_3:H_2O_2 (5:1) was added, and the homogenates were then heated in a microwave oven (CEM Mars 6, Charlotte, NC, USA) at 180 °C for 60 min. After cooling, 5 mL of concentrated HCl was added to the reaction mixture to reduce Se^{6+} to Se^{4+}. Se concentration in shoots and roots was determined using optical emission spectrometry with inductively coupled plasma (ICP-OES, model Perkin Elmer Optima 2100 DV, Waltham, MA, USA), where rice flour IRMM-804 was used as a reference material.

2.3. Morpho-Physiological Analyses

2.3.1. Seed Germination

On the seventh day of germination, the germination percentage was calculated as an indicator of wheat seed viability and potential to emerge. The germination percentage was determined by the number of germinated seeds divided by the total number of planted seeds. The obtained number was multiplied by 100, and germination was expressed as a percentage.

2.3.2. Determination of Shoot and Root Biomass

After determining the germination percentage, the shoots and roots of wheat seedlings were separated to evaluate the morphological characteristics, i.e., biomass. For the biomass estimation, a fresh mass of the shoots and roots was measured immediately after sampling. The fresh weight (FW) of wheat shoots and roots was expressed in grams (g).

2.3.3. Determination of Photosynthetic Pigment Concentration

A fine frozen powder (100 mg) obtained after grinding was homogenized with the cold 80% acetone. Pigments were extracted on ice for 15 min and then centrifuged. The extraction procedure with cold acetone was repeated three more times until the precipitate became colorless. After the reextracted supernatants were collected, their exact volume was measured and diluted to a final volume of 10 mL. This was followed by spectrophotometric measurements of the absorbance at 470 nm, 645 nm, and 662 nm [50]. The concentrations of photosynthetic pigments, chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids were expressed as mg/g of fresh weight.

2.4. Indicators of Oxidative Stress

2.4.1. Determination of Lipid Peroxidation Level

The level of lipid peroxidation (LPO) in wheat shoots and roots was determined according to the method described by Verma and Dubey [51]. This method is based on spectrophotometric measurements of the concentration of reactive substances in thiobarbituric acid (TBARS), mostly malondialdehyde (MDA).

About 200 mg of frozen wheat tissue was extracted with 0.1% (*w*/*v*) trichloroacetic acid (TCA) solution. After a short extraction on ice, homogenates were centrifuged, and the resulting supernatant was mixed with 0.5% (*w*/*v*) thiobarbituric acid (TBA) in a 20% (*w*/*v*) TCA solution. The reaction mixture was incubated in the water bath at 95 °C for 30 min. The intensity of red coloration, which was a result of this reaction, was measured spectrophotometrically at 532 nm and 600 nm. The amount of MDA was calculated using the extinction coefficient (ε = 155 1/mM/cm) and expressed in nmol per g of FW.

2.4.2. Determination of Hydrogen Peroxide

The H_2O_2 content in wheat shoots and roots was measured using the method described by Mukherjee and Choudhuri [52]. Frozen tissue powder (about 100 mg) was extracted with 1 mL of cold absolute acetone. After 15 min of extraction on ice, homogenates were centrifuged, and the supernatant was mixed with titanium oxysulphate and ammonium hydroxide solution to form a titanium-peroxide complex. The resulting white precipitate was dissolved in 2 M H_2SO_4 and centrifuged before measuring the absorbance of the supernatant at 415 nm. The total H_2O_2 content was determined using the standard curve of known H_2O_2 concentrations, and it was expressed as nmol H_2O_2 per g of FW.

2.5. Extraction and Assays of Enzymes

Proteins from the frozen shoot and root powder (approximately 300 mg) were extracted on ice with 1.5 mL of cold potassium phosphate buffer (1/5, *w*/*v*). The homogenates were kept on ice for 15 min and then centrifuged at 20,000× *g* for 15 min at 4 °C for protein extraction. Supernatants were stored at −80 °C and used for spectrophotometric determination of catalase (CAT), guaiacol peroxidase (GPOD), and protein estimation. The enzymes' activities were measured at 25 °C using a LAMBDA 25 UV-Vis spectrophotometer equipped with the UV WinLab v6.0.4 software package (PerkinElmer, Waltham, MA, USA).

CAT (EC 1.11.1.6) activity was determined spectrophotometrically using H_2O_2 as a substrate [53]. The reaction mixture (1.5 mL) consisted of 0.036% H_2O_2 in 50 mM phosphate buffer (pH 7.0) and enzyme extract. The decrease in absorbance was monitored spectrophotometrically at 240 nm for 3 min every 10 s. CAT activity was calculated using the molar extinction coefficient (ε = 0.04 mM/cm) and expressed as U/mg protein.

GPOD (EC 1.11.1.7) activity was estimated by the method described by Siegel and Galston [54] and modified for analysis in a microplate assay. The method is based on the oxidation of guaiacol to tetraguaiacol due to the presence of H_2O_2. The reaction mixture consisted of 18 mM guaiacol solution and 5 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0). The reaction was started by adding the diluted sample, and the increase in absorbance was monitored at 470 nm for 2.5 min every 10 s. GPOD activity was calculated using the molar extinction coefficient (ε = 15.83 mM/cm) and expressed as U/mg protein.

Total protein concentration in wheat protein extracts was determined by the Bradford method [55], modified for microplate assay analysis. The protein extract was incubated in the microtiter plate for 5 min at 25 °C with a Bradford reagent (Sigma-Aldrich, Steinheim, Germany). After a short incubation, the intensity of the resulting blue color was measured at 595 nm. Bovine serum albumin was used as a standard, ranging from 0.1 to 1.4 mg/mL.

2.6. Statistical Analyses

The obtained data from this research were analyzed using Statistica 14.0.0.15 (TIBCO Inc., Palo Alto, CA, USA). The data were presented as the mean of seven replicas ± standard deviation (SD). Considering the normal distribution tested by the Shapiro–Wilks test, differences between treatments were assessed using a one-way analysis of variance (ANOVA), followed by Duncan's test. All tests were performed at a significance level of 5% ($p < 0.05$).

3. Results

3.1. Se Concentration in Shoots and Roots

Selenium concentrations in wheat seedlings changed depending on the applied concentrations and the form of Se (Figure 2A). In wheat shoots, an increase in Se concentration was correlated with applied Se concentrations. Se concentrations in shoots treated with selenate ranged from 1.3 mg/kg to 219.5 mg/kg, which was recorded after exposure to the highest concentration (400 mg/kg). All selenate treatments, except for the lowest applied concentration (0.4 mg/kg), significantly influenced Se accumulation in the shoots compared to the control. Exposure to selenite also increased Se concentrations in shoots compared to the control. However, the range of concentrations was significantly lower than due to exposure to selenite, ranging from 7.7 mg/kg to 53.2 mg/kg.
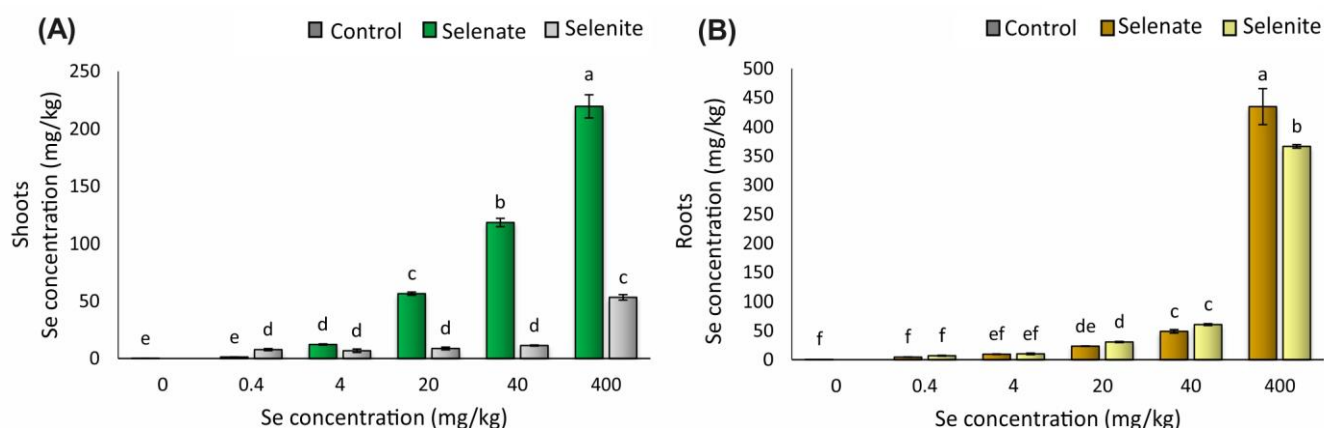

Figure 2. Selenium (Se) concentration in wheat shoots (**A**) and roots (**B**) after treatments with five different concentrations of selenate and selenite (0.4, 4, 20, 40, and 400 mg/kg). Control plants were grown without selenium (Se) (0 mg/kg). Results are presented as means ± standard deviation. Differences between treatments were assessed by a one-way analysis of variance (ANOVA), followed by Duncan's test. Different letters indicate significant differences between treatments ($p < 0.05$).

In the roots, the Se concentration also increased as the applied concentrations of selenate and selenite were higher (Figure 2B). In most treatments, it was evident that the roots accumulated more Se after exposure to selenite than to selenate, except with the highest applied concentration. The noted range in roots treated with selenate was from 4.4 mg/kg to 434.4 mg/kg, and in roots treated with selenite, from 6.8 mg/kg to 366.3 mg/kg.

3.2. Morpho-Physiological Traits

3.2.1. Grain Germination

The presence of Se affected the wheat germination rate, mainly when it was applied as selenite (Figure 3A). Moreover, in most treatments, selenite caused a significant reduction in germination compared to both control and selenate treatments. On the other hand, selenate in most treatments did not affect germination compared to the control. The exception was the highest concentration of selenate (400 mg/kg), which reduced germination by 12%. Although both forms of Se applied in the highest concentrations reduced germination compared to the control, the germination rate was 14% lower in the selenite treatment than in the selenate treatment.
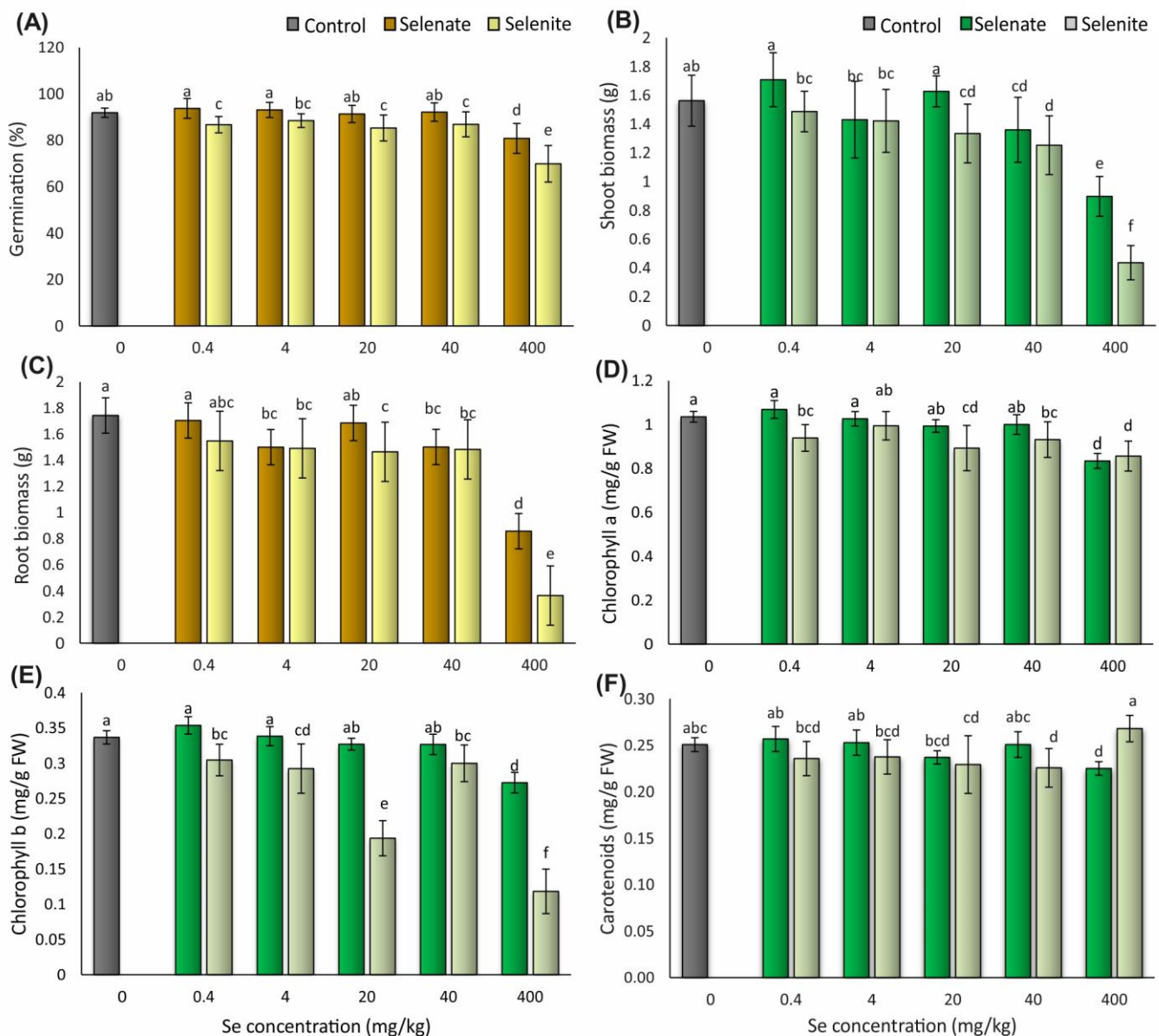
Figure 3. Morpho-physiological traits: germination rate (**A**); shoot and root biomass (**B,C**); chlorophyll a (**D**); chlorophyll b (**E**); and carotenoids (**F**) in wheat after treatments with five different concentrations of selenate and selenite (0.4, 4, 20, 40, and 400 mg/kg). Control plants were grown without selenium (Se) (0 mg/kg). Results are presented as means ± standard deviation. Differences between treatments were assessed by a one-way analysis of variance (ANOVA), followed by Duncan's test. Different letters indicate significant differences between treatments ($p < 0.05$).

3.2.2. Shoot and Root Biomass

Both applied forms of Se affected shoot and root biomass. Compared to the control, the two highest concentrations of selenate, 40 and 400 mg/kg, decreased shoot biomass by 13% and 43%, respectively (Figure 3B). Selenite applied in three concentrations, 20, 40, and 400 mg/kg, also decreased shoot biomass by 15%, 20%, and 72%, respectively.

The roots responded similarly to Se presence, where 4, 40, and 400 mg/kg of selenate and 4, 20, 40, and 400 mg/kg of selenite also reduced biomass (Figure 3C). Comparing selenate and selenite treatments, shoot biomass was lower after exposure to selenite compared to the same treatments with selenate. Selenite also caused a greater reduction in biomass in roots compared to selenate, after exposure to concentrations of 20 and 400 mg/kg.

3.2.3. Concentrations of Photosynthetic Pigments

Most selenate treatments (0.4, 4, 20, and 40 mg/kg) did not influence the concentration of Chl a and Chl b in wheat seedlings compared to the control, while the highest concentration (400 mg/kg) reduced Chl a by 17% and Chl b by 19% (Figure 3D,E). Unlike selenate, four concentrations of selenite (0.4, 20, 40, and 400 mg/kg) reduced Chl a concentration by 9%, 14%, 10%, and 17%, respectively. Furthermore, all selenite treatments (0.4, 4, 20, 40, and 400 mg/kg) reduced Chl b concentration by 10%, 13%, 43%, 11%, and 65%, respectively. Comparing the influence of the two forms of Se on the chlorophyll content, higher values were recorded in most selenate treatments compared with selenite, especially in Chl b concentration.

Selenate applied in concentrations of 0.4, 4, 20, and 40 mg/kg did not affect carotenoids, while the highest treatment reduced the concentration by 10% compared to the control (Figure 3F). A decrease in the concentration of carotenoids was also recorded due to exposure to selenite in a concentration of 40 mg/kg, where carotenoids were 10% lower compared to the control. Other selenite treatments did not significantly affect carotenoid concentrations.

3.3. Indicators of Oxidative Stress

3.3.1. Lipid Peroxidation Levels in Wheat Shoots and Roots

The oxidative status of wheat seedlings was evaluated by the determination of the LPO level, which was monitored by measuring the content of TBARS. All applied concentrations of both forms of Se significantly decreased TBARS content in the shoots compared to the control (Figure 4A). While selenate reduced LPO levels by 13%, 16%, 23%, 15%, and 16%, respectively, selenite reduced them by 25%, 26%, 36%, 20%, and 10% with increasing applied concentrations.
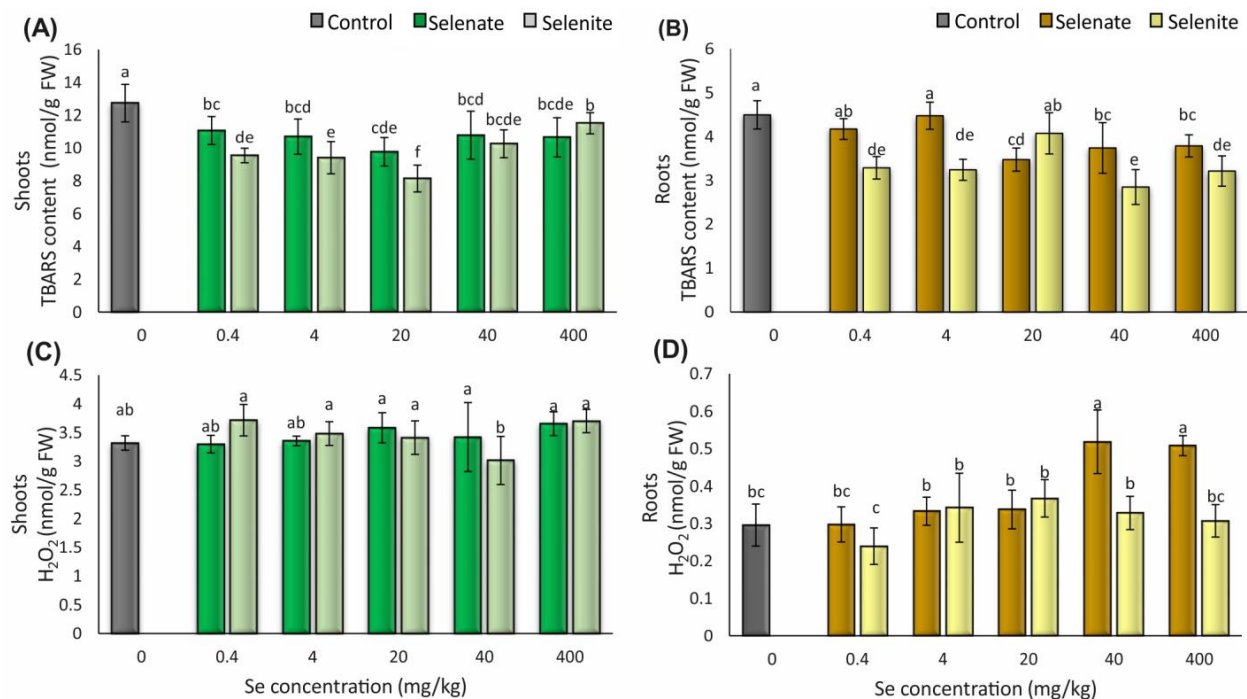

Figure 4. The content of thiobarbituric reactive substances (TBARS) (**A**,**B**); and hydrogen peroxide (H_2O_2) (**C**,**D**) in wheat shoots and roots after treatments with five different concentrations of selenate and selenite (0.4, 4, 20, 40 and 400 mg/kg). Control plants were grown without selenium (Se) (0 mg/kg). Results are presented as means ± standard deviation. Differences between treatments were assessed by a one-way analysis of variance (ANOVA), followed by Duncan's test. Different letters indicate significant differences between treatments ($p < 0.05$).

TBARS content in wheat roots decreased after exposure to the three highest concentrations of selenate (20, 40, and 400 mg/kg) by 23%, 17%, and 16%, respectively, as compared to the control (Figure 4B). Selenite also reduced the amount of TBARS, in four treatments (0.4, 4, 40, and 400 mg/kg) by 27%, 28%, 37%, and 29%, respectively.

3.3.2. The Concentration of H_2O_2 in Wheat Shoots and Roots

In addition to the LPO level, another indicator of oxidative stress measured in wheat seedlings was the concentration of H_2O_2. In wheat shoots, Se did not cause a significant change in the H_2O_2 concentration (Figure 4C).

Similar to the shoots, in roots, Se did not significantly affect the H_2O_2 concentration in most treatments (Figure 4D). Only treatments with 40 and 400 mg/kg of selenate caused an increase in the H_2O_2 concentration by 43% and 42%, respectively, compared to the control.

3.4. Antioxidative Enzyme Activities

3.4.1. Catalase Activity in Wheat Shoots and Roots

The specific CAT activity in the wheat shoots and roots is shown in Figure 5. In shoots, CAT activity was unchanged at most of the applied selenate concentrations, except for the highest (400 mg/kg), which increased its activity by 18%, compared to the control (Figure 5A). On the other hand, selenite applied in the three largest concentrations (20, 40, and 400 mg/kg) increased the activity compared to the control by 18%, 10%, and 20%, respectively.
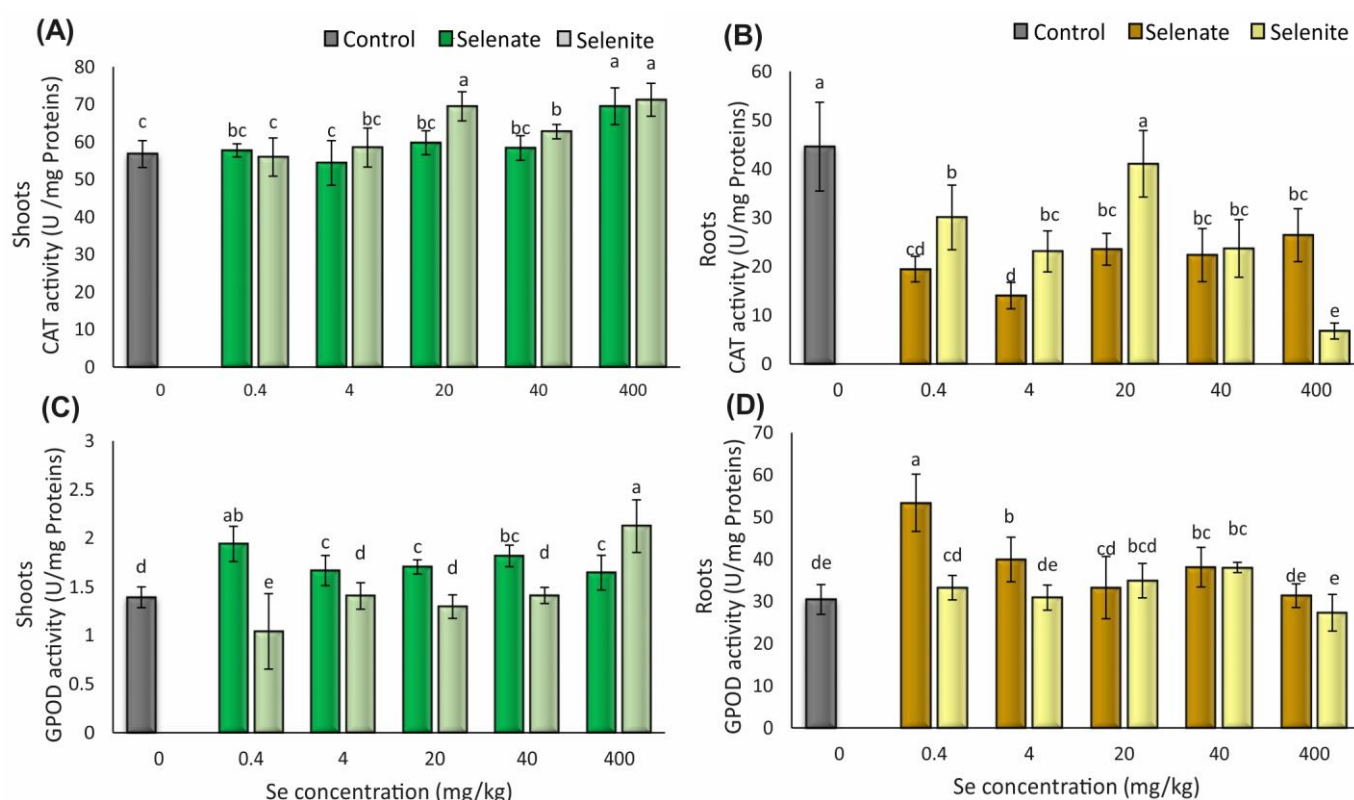

Figure 5. The activity of catalase (CAT) (**A**,**B**); and guaiacol peroxidase (GPOD) (**C**,**D**) in wheat shoots and roots after treatments with five different concentrations of selenate and selenite (0.4, 4, 20, 40, and 400 mg/kg). Control plants were grown without selenium (Se) (0 mg/kg). Results are presented as means ± standard deviation. Differences between treatments were assessed by a one-way analysis of variance (ANOVA), followed by Duncan's test. Different letters indicate significant differences between treatments ($p < 0.05$).

The CAT activity in roots greatly differed from shoots. All treatments, except 20 mg/kg of selenite, caused a statistically significant decrease in specific CAT activity compared to the control (Figure 5B). Thus, selenate decreased the activity by 56%, 69%, 47%, 50%, and 41% as the applied concentrations increased. After exposure to four concentrations of selenite (0.4, 4, 40, and 400 mg/kg), the activity decreased by 33%, 48%, 47%, and 85%.

3.4.2. Guaiacol Peroxidase Activity in Wheat Shoots and Roots

The specific activity of GPOD in wheat shoots increased due to all selenate treatments compared to the control (Figure 5C). The increase in the concentration of selenate (0.4, 4, 20, 40, and 400 mg/kg) was followed by increased GPOD activities by 28%, 16%, 18%, 23%, and 15%, respectively. Compared to the control, the lowest concentration of selenite (0.4 mg/kg) reduced GPOD activity by 25%, 4, 20, and 40 mg/kg of selenite had no effect; and the highest applied concentration increased the activity by 34%.

Selenate treatment also increased GPOD activity in roots, but only in three applied concentrations, 0.4, 4, and 40 mg/kg, by 43%, 24%, and 20%, respectively (Figure 5D). Other concentrations (20 and 400 mg/kg), like most selenite treatments (0.4, 4, 20, and 400 mg/kg), did not affect GPOD activity compared to the control. Regarding selenite, only 40 mg/kg of selenite affected the GPOD activity in the roots, and it was 20% higher than in the control.

4. Discussion

4.1. Se Effect on Its Accumulation in Wheat

Se uptake, distribution, and effect in wheat depend on the growth stage [43] and the form and concentration of applied Se. In this research, the increase in applied concentrations of selenate and selenite caused a linear increase in Se concentration in shoots and roots (Figure 2A,B), which correlates with the results of previous studies [12,18,56,57]. Although there was a linear increase in Se concentration in all treatments, when treated with selenite, 2-fold to 10-fold lower Se concentrations were found in the shoots compared to Se concentrations after selenate treatments. After uptake via sulfate transporters in the roots [58], selenate is translocated through the xylem to the chloroplast, where it begins its reduction to selenite. In contrast, selenite is taken up by passive diffusion [59], via phosphate [22] or silicon transporters [23], after which it is reduced and converted to organic Se forms, which causes less mobility to the shoots [58]. Li et al. [58] detected selenite and organic forms such as MeSeCys in the root extracts and xylem sap from selenite-treated plants. Differences in Se accumulation were determined by Kaur and Sharma [60] in wheat leaves, whereby after exposure to selenate, up to 60-fold higher concentrations of Se were obtained compared with selenite, and they concluded that selenate is a more mobile form. It is important to emphasize that, in our experiment, selenate caused a more significant increase in Se concentration in the shoots than in the roots, except after the application of the highest concentration, where selenate was less translocated into the shoots. Namely, in the treatments with lower selenate concentrations up to 72% of the total uptake Se was translocated to the shoots. In contrast, at the highest concentration of selenate, only 35% was translocated. It can be due to a lack or dysfunction of sulfate transporters. This is supported by the research of Boldrin et al. [61], who found a decrease in *Sultr1* gene expression in several wheat varieties after treatment with 13 μM of sodium selenate.

4.2. Se Effect on Wheat Morpho-Physiological Characteristics

Selenium can influence numerous plant morpho-physiological characteristics, and its response depends primarily on its concentration. Although it is recorded that lower Se concentrations can have a positive effect on germination and the physiological quality of seeds [27,62,63], it has also been reported that the application of lower concentrations has no significant impact on germination [64,65], as in our study after exposure to lower concentrations of selenate (0.4, 4, 20, and 40 mg/kg) (Figure 3A). On the contrary, lower concentrations of selenite (0.4, 20, and 40 mg/kg) inhibited seed germination, leading to the

conclusion that germination mainly depends on the chemical form of applied Se and that lower concentrations of selenite are more toxic than selenate. The highest concentrations of selenate and selenite caused the greatest inhibition of seed germination compared to the control, emphasizing that the lowest germination was recorded at the highest concentration of selenite. Accordingly, numerous previous studies have established that germination is most often inhibited by increased concentrations of Se [27,65–67]. Lapaz et al. [65] investigated the influence of eleven different Se concentrations ranging from 0.1 to 800 mg/L on germination and other morphological characteristics of the *Vigna ungiculata* species. They found that only the highest concentration of Se inhibited germination, while the others did not affect it. El Mehdawi et al. [66] noted that *Arabidopsis thaliana* germination rate decreased at concentrations of Se that are higher than 5 mg/kg DW, while 50% of inhibition was recorded at 10 mg/kg DW, i.e., 125 μM of Na_2SeO_4. The inhibitory effect of Se on wheat seeds may be related to enzyme inhibition, which hydrolyzes metabolites necessary for the development of the plant embryo, as concluded in research by Sreekala and Lalitha [68]. They determined that 0.5 ppm of Na_2SO_3 increased β-galactosidase and β-glucosidase activities in *Trigonella foenumgraecum* species, but concentrations above 1 ppm drastically reduced their activities, which also reduced germination.

In addition to wheat germination rate, treatments with different concentrations of Se affect growth and seedling biomass. Seed germination and biomass are related because lower germination can reduce the total biomass, as shown by the results of this research. Although numerous studies have established a positive influence of lower concentrations of Se on wheat growth and biomass [69–72], this research showed that lower concentrations did not significantly affect the shoot and root biomass. On the contrary, higher concentrations of both forms of Se significantly reduced biomass, where the greatest decline was recorded in treatments with 400 mg/kg of Se (Figure 3B,C). The influence of higher concentrations of Se on biomass reduction has been recorded in numerous plant species, including *Lactuca sativa* L. [18,73], *Sinapis alba* L. [74], *Oryza sativa* [30], *T. aestivum* L. [75,76], *Zea mays* L. [77] and *Brassica napus* L. [28]. Cartes et al. [78] investigated the influence of selenate and selenite (0.1, 0.25, 0.50, 0.75, 1, 1.5, 2, 4, 6, 8, and 10 mg/kg) on the dry biomass of 55-day-old *Lolium perenne* shoots. They defined that only selenate causes a decrease in biomass when the concentration of Se in shoots exceeds 150 mg/kg. Lapaz et al. [65] determined a reduction in the fresh biomass of shoots and roots of *V. ungiculata* after exposure to 40 mg/kg of selenate or more, which correlates with the results of this study. It is important to emphasize that selenite at lower concentrations compared to selenate decreased both shoot and root biomass, indicating a lower toxicity threshold for selenite in wheat seedlings. As the toxicity threshold can be defined as the lowest concentration of Se that causes a significant reduction in the biomass compared to the control, in the shoots and roots of wheat seedlings, the threshold toxicity for selenate was 40 mg/kg. In comparison, for selenite seedlings, the threshold toxicity in the shoots was 20 mg/kg, and in the roots was 4 mg/kg. These results indicate a more toxic effect of selenite on wheat biomass when compared to selenate, where this effect is more pronounced in the roots. The results of this research are correlated with the results of research conducted by Hawrylak-Nowak et al. [29], who noted a reduction in the biomass of shoots and roots of *Cucumis sativus* L. at 80 μM of selenate and 20 μM of selenite, but also with the results of other studies that concluded that selenite is a more toxic form than selenate [36,73,79]. Tian et al. [80] emphasized that higher Se toxicity was associated with low S levels, during which the proportion of Se in proteins increases compared to S. From previous studies [80–85], it is evident that there was less S uptake in selenite treatments, which can cause and explain the higher selenite toxicity in wheat seedlings. In addition to the mentioned mechanism, Se toxicity can be associated with a reduced concentration of photosynthetic pigments and increased oxidative damage, such as increased LPO [30,86].

Although previous studies highlighted the positive influence of lower Se concentrations on the concentration of chlorophyll and carotenoids [87,88], in this study, lower Se concentrations did not significantly affect the content of photosynthetic pigments (Chl a

and carotenoids) in wheat seedlings. Haghighi et al. [89] also concluded that Se did not affect chlorophyll concentration in *Cucumis sativus* L. after exposure to 2, 4, and 6 mg/L of selenite. Moreover, in our study, chlorophyll concentration significantly decreased due to exposure to the highest concentration of selenate, while selenite caused a decrease at a concentration of 0.4 mg/kg. It should be emphasized that selenite had a much more negative effect on Chl b than on Chl a. Together with the germination and biomass reduction, these results confirm the higher toxicity of selenite compared to selenate. High Se concentrations can impair the uptake and content of micro and macronutrients, which can be reflected in photosynthesis [28,29]. This especially applies to nutrients such as Fe and Mg, which are essential components of chlorophyll and Fe-S proteins or participate in their synthesis [90]. Additionally, Se can interfere with enzymes that contain a sulfhydryl group in the active site, such as porphobilinogen synthase, which is involved in chlorophyll biosynthesis [91].

Reducing the chlorophyll concentration in wheat seedlings can cause a decrease in the light energy absorption that will be converted into chemical energy, which can result in reduced production of starch and reduced biomass [42]. Ulhassan et al. [28] also found that in several cultivars of *B. napus* L. treated with 100 μM selenite, higher concentrations of Se minimize the amount of total soluble sugars, which affects biomass reduction.

4.3. Se Effect on Wheat Oxidative Status and Antioxidative Response

The effect of Se on plants, which is visible at the morpho-physiological or biochemical level, is mediated by redox state changes, the formation of ROS, and the activation of antioxidative mechanisms. Our results showed that Se application had no impact on cellular H_2O_2 content in shoots, while root H_2O_2 content was affected only by the two highest concentrations of selenate (Figure 4C,D). The TBARS results show that the increase in H_2O_2 did not cause negative consequences in the wheat roots, contributing to its role in cell signaling. The LPO level is one of the most important biomarkers of oxidative stress that can determine the degree of oxidative damage in cells. It is known that lower Se concentrations can reduce LPO levels, while higher concentrations have a negative effect and increase peroxidation [18,38]. In addition, Se can protect various plant species from abiotic stress-mediated oxidative damage, as seen through a reduction in LPO followed by enhanced regulation of detoxification defense systems [92–94]. Both applied forms of Se reduced LPO levels in all treatments in wheat shoots (Figure 4A). The reduction was also recorded in the roots after exposure to selenite and the two highest concentrations of selenate (Figure 4B). Se ions are assumed to protect membranes and play a key role in reducing LPO levels. Filek et al. [95] investigated wheat plastid membrane properties and concluded that Se ions can induce changes in fatty acid composition by increasing its unsaturation. They connected those changes with decreased LPO levels and concluded that Se ions can protect cell membranes from oxidative damage. Many studies involving lipid monolayer research confirm that Se ions can bind to specific membrane domains and thus affect their properties [96–98]. In addition, an active antioxidative defense system contributes to maintaining low LPO levels.

Hydrogen peroxide can be directly or indirectly converted to O_2 and H_2O by several enzymes, such as CAT and GPOD, and thus prevent its accumulation and negative consequences. The increased activities of these enzymes in the shoots and roots kept the concentration of H_2O_2 unchanged in most treatments. CAT is an enzyme with a low affinity for H_2O_2 and is active at very high concentrations of H_2O_2 [99], while lower concentrations of H_2O_2 are removed by enzymes such as GPOD [100]. Unchanged CAT activity in wheat shoots due to exposure to the four lowest concentrations of selenate and lower concentrations of selenite may be related to the lower concentrations of H_2O_2 produced preferentially removed by other enzymes. As in this research, Lara et al. [12] also observed an unchanged amount of H_2O_2 and CAT activity in wheat after treatments with 0, 12, 21, 38, 68, and 120 g/ha of Na_2SeO_4. On the other hand, the increased activity of CAT in the shoots after exposure to higher concentrations of Se is a critical H_2O_2 detoxification mechanism. This is supported by the research of Kaur and Sharma [60], which determined

the presence of several new CAT isoenzymes in the wheat leaf after exposure to higher doses of selenate and selenite. Silva et al. [101] noticed that both selenate and selenite increased CAT activity in leaves of *Vigna unguiculata* (L.) Walp., but not equally in all treatments. While selenate increased CAT activities only at higher applied concentrations (20, 40, and 60 g/ha), selenite increased CAT activities in all treatments (2.5, 5, 10, 20, 40, and 60 g/ha) [101]. Similarly, in this research, selenite in wheat shoots increased CAT activity in most treatments, while the increase due to exposure to selenate was recorded only at the highest concentration (Figure 5A). In addition, the results of this study showed that CAT activity is also tissue specific. Contrary to the CAT activity in shoots, where it was increased or unchanged, Se inhibited its activity in roots (Figure 5B). Chioti and Zervoudakis [102] reported differences in CAT sensitivity between shoots and roots of different plant species. Concerning sensitivity to the inhibitor, they concluded that CAT is monofunctional in the shoots of the investigated species, while in the roots, it is a bifunctional enzyme. Monofunctional CAT has a common action that converts H_2O_2 into H_2O and O_2 in two steps, while bifunctional, in addition to the usual CAT activity, also exhibits peroxidase activity with an electron donor present [103]. The different response of CAT between shoots and roots is also discussed in the research conducted by Gayatridevi et al. [104]. After exposure to salicylic acid and *Fusarium* sp., they observed a different number of CAT isoforms in shoots and roots in different genotypes of *Cicer arientum* L. In addition, they determined a significant difference between shoots and roots in sensitivity to salicylic acid, as well as the activities of individual isoforms were completely inhibited by individual treatment.

In addition to CAT, an important role in H_2O_2 detoxification is also played by the enzyme GPOD, which is activated at much lower concentrations of H_2O_2 compared to CAT [105]. Numerous previous studies have established that CAT and GPOD react similarly to Se, and an increase in the activity of one enzyme is often accompanied by an increase in the activity of the other, regardless of the form of applied Se [41,62,87,106,107]. However, in wheat seedlings, we found different results. While selenate increased GPOD activity in the shoots and partly in the roots, in most treatments, selenite did not change it (Figure 5C,D). Different ways of selenate and selenite uptake, translocation, and assimilation in plants may be the cause of ROS production in different cellular compartments. So, different detoxification mechanisms are activated depending on the location of ROS accumulation. In wheat, GPOD was found in the cytosol, cell wall, and vacuole [108]. Given the localization of GPOD in cells, active transport of selenate could result in the formation of H_2O_2 in the cell wall but also in the vacuole, where selenate can be accumulated [109]. At the same selenate treatments, CAT activities were unchanged or significantly reduced, and the opposite response of CAT and GPOD activity was also recorded in the research of Saidi et al. [110]. They determined that pretreatments with 5 and 10 μM of selenate reduced GPOD activity and increased CAT activity in *Helianthus anuus* leaves exposed to Cd. Furthermore, Jóźwiak and Politycka [40] noticed an increase in GPOD activity and a decrease in CAT activity in the roots of *Cucumis sativus* L. after treatment with 5 and 10 μM of selenite. As the concentration of H_2O_2 in cucumber roots did not change, they concluded that GPOD was responsible for maintaining its low concentrations. Therefore, the GPOD activity would be one of the key H_2O_2 detoxification mechanisms in wheat after exposure to selenate in shoots and to lower selenate concentrations also in the roots.

5. Conclusions

Wheat responses to different chemical forms of Se were monitored at the morpho-physiological and biochemical levels. Morpho-physiological analyses such as seed germination, shoot and root biomass, and chlorophyll and carotenoid concentrations revealed that selenite has a lower toxicity threshold than selenate. Measurement of oxidative stress indicators, LPO and H_2O_2, showed that Se did not cause oxidative stress in wheat seedlings. Thus, the removal of H_2O_2 from the shoots and roots was performed by different mechanisms depending on the chemical form and concentration of the applied Se. H_2O_2 originated from selenate treatment that primarily removes GPOD both in shoots and roots. Shoot

H_2O_2 originated from selenite treatment that primarily removes CAT, which is evident from the increased activities in most treatments.

This research contributes to a better understanding of wheat seedlings' biochemical and morpho-physiological responses to Se. It also contributes to the development of new insights into the mechanisms of toxicity depending on concentration, chemical form, and type of plant organ. Treatment with 20 mg/kg of selenate can be recommended for wheat seedling biofortification due to a sufficient increase in Se accumulation in shoots without signs of toxicity. The insight into the biochemical mechanisms of Se tolerance obtained by this research contributes to the development of more effective biofortification strategies.

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