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Antioxidant response of *Lepidium sativum* L. to oxidative stress induced by exposure to chosen heavy metals

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Abstract

Soil contamination with heavy metals is a significant concern, resulting in adverse effects on soil properties and limitations on both its productivity and its environmental capabilities. The aim of this study was to determine the response of *Lepidium sativum* L. to oxidative stress induced by the addition of zinc (Zn), nickel (Ni) and copper (Cu) separately to the soil in which the plants were cultivated. The range of metal ion concentrations was determined in accordance with the soil quality standards set by the Ministry of Environmental Regulation (Dz.U.02.165.1359) on September 9, 2002. The quantity and activity of selected antioxidants belonging to enzymatic and non-enzymatic antioxidant systems were then evaluated.

This study compared the antioxidant responses of the plant to different heavy metals. Based on the obtained results, it can be seen that heavy metal ions induced oxidative stress, which caused an activation of the antioxidant response system through the enhanced production of secondary metabolites such as phenolic compounds, ascorbic acid, and glutathione, as well as an increase in the activity of particular enzymes: catalase (CAT), superoxide dismutase (SOD), guaiacol peroxidase (POD), and glutathione transferase (GSTs). It can thus be concluded that in response to oxidative stress caused by Zn, Ni and Cu, *L. sativum* plants have evolved efficient enzymatic and non-enzymatic defence mechanisms to counteract the excessive production of reactive oxygen species. These responses allow plants to survive in adverse environmental conditions and help us evaluate whether the imposed standards influenced *L. sativum*'s metabolic changes.

1. INTRODUCTION

Lepidium sativum, commonly known as garden cress, is an edible herbaceous plant belonging to the Brassicaceae family. It is characterized by its rapid growth and has been historically employed in both medicinal and culinary contexts. *Lepidium sativum* is classified as an aromatic botanical species that has been associated with beneficial impacts on physiological well-being in humans [Vazifeh et al. 2022]. Its elevated antioxidant activity has made it an active ingredient of interest in pharmaceuticals, alternative medicine, natural therapies and nutraceuticals [V. Kumar et al. 2020]. The plant's various important phytochemical constituents have also been found in the processes of nociception and blood coagulation; garden cress seeds have thus been implicated in the treatment of asthma, pain, and inflammation [Ghante et al. 2011; Gupta, Gupta 2023].

L. sativum's antioxidant properties make it a valuable model food plant for studying the complex reactions of plants that have been exposed to biotic or abiotic stressors.

Various plants can be used as indicators of heavy-metal soil pollution, but the most useful ones for this purpose are those commonly found on a regional or even continental scale, and those with a high capacity for bioaccumulation of trace elements. The Organization for Economic Co-operation and Development (OECD) thus recommends *L. sativum* as an indicator plant for studying environmental stress, particularly when exposed to metal ions during germination under standard conditions [Buso et al. 2020]. Human activity over the past century has caused significant environmental degradation. One of the world's most severe environmental problems is the overabundance of heavy metals in soil from sources such as industrial waste, agricultural fertilizers, and roadways [Georgiadou et al. 2018; Vardhan et al. 2019]. The increased concentration of metals in soil leads to higher metal ion accumulation in plants, though the degree and rate of accumulation of specific metals varies by plant species and growth stage [Georgiadou et al. 2018].

Heavy metals that are present in the environment influence all living organisms. Although some of heavy metals are

essential for cellular metabolism (Cu, Zn, and Ni), others, like Cd and Pb, are not [Drif et al. 2019]. The toxic effect of heavy metals lies mainly in their ability to bind to proteins, which may lead to the inactivation of crucial enzymes (e.g. ATPases, phosphatases, dehydrogenases) involved in the basic cellular metabolic processes [Dubey et al. 2018]. The adverse effects of heavy metals are primarily associated with the increased production of reactive oxygen species (ROS), which damage cells [Drif et al. 2019; Georgiadou et al. 2018].

Plant species that have been chronically subjected to stress conditions like heavy metal exposure have developed mechanisms to counteract the disruption caused by oxidative stress [Georgiadou et al. 2018]. The early response of plants to stress factors is associated with changes in their transcriptional profile, leading to the synthesis of proteins and metabolites that activate mechanisms to counteract adverse conditions. Abiotic stress factors can cause inhibition of defence responses in plants and thus reduce plant growth and biomass. Moreover, stressful conditions may cause overproduction of reactive oxygen species (ROS) [H. Zhang et al. 2020]. ROS are a universal tool used by cells to fight pathogens and limit the development of infections. The concentration of ROS increases when cells are exposed to abiotic stress factors, such as inappropriate temperature, inappropriate moisture content, or soil contamination with heavy metals. Some heavy metals, e.g. Mn, Zn, Cu, Ni, are essential for the proper functioning of living organisms, as they can act as a co-factor in many enzymes. For example, Zn is a component of dehydrogenases; Mn and Cu are components of oxidases; and Ni is a component of ureases. Even metals that are essential for living organisms in lower concentrations, where they may take part in their metabolic functions, are toxic in excessive concentrations. In order to protect their own tissues, plants possess an efficient antioxidant system with both enzymatic and non-enzymatic mechanisms. Enzymes include catalase (CAT); superoxide dismutase (SOD); glutathione peroxidase (GPX); and glutathione transferase (GSTs). Non-enzymatic antioxidants may include polyphenols, flavonoids, glutathione and ascorbate acid [Dumanović et al. 2021].

L. sativum, also referred as garden cress, is a fast-growing herbaceous plant that exhibits diverse processes to environmental stress. In addition to activating antioxidant proteins, garden cress may utilize various different methods to deal with environmental stress. For instance, *L. sativum* has the ability to regulate its levels of phytohormones. These include abscisic acid (ABA), which is crucial for the plant's reaction to abiotic stressors, including drought [Nguyen et al. 2023]. Additional hormones, such as jasmonic acid and salicylic acid, may potentially participate in stress signalling.

Abiotic stress occurring during *L. sativum*'s germination and growth may influence the activity of stress-responsive genes. These genes in turn may participate in signalling networks that facilitate the plant's adaptation to environmental stressors [Ahanger et al. 2017].

Transcription factors and regulatory proteins are essential for coordinating these responses.

Garden cress must also maintain its ion balance in order to deal with the effects of salt stress. Garden cress, like many other plants, has the ability to adapt to drought or salinity stress by modifying its osmotic potential. In this process, the plant stockpiles suitable solutes, such as proline and soluble carbohydrates, which aid in preserving cell turgor and safeguarding against dehydration [dos Santos et al. 2022]. Ongoing research is being carried out on stress-response mechanisms in garden cress, with specificities being subject to the nature and severity of the stress. Obtaining a comprehensive understanding of these systems is crucial to developing efficient strategies for enhancing stress tolerance in crops and other plant species.

The objective of this study was to determine the response of *L. sativum* to oxidative stress caused by the addition of different concentrations of Zn, Cu and Ni to the plant's soil. The selection of metal ion concentrations was consistent with the Polish Ministry of Climate and Environment's Ordinance of September 9, 2002 for soil classified as B (farmlands) (Dz.U.02.165.1359). This study also evaluated whether the imposed standards influenced *L. sativum*'s metabolic processes. The quantities and activity of selected compounds and enzymes that form plant antioxidant systems were tested. Non-enzymatic systems were also monitored, including the total polyphenols, flavonoids, glutathione and ascorbic acid content. Oxidative stress induced by heavy metal ions was expected to stimulate the activity of several enzymes; thus, the activity levels of CAT, SOD, POD and GSTs were also measured and compared with those of the control sample. The observed changes in ascorbic acid and glutathione contents upon increasing the concentration of heavy metal ions were analysed to find correlations between the changes in the activities of the relevant enzymes. The antioxidant responses of *L. sativum* to different heavy metals were also compared.

The novelty of this work lies in its comprehensive approach to studying the effects of metal ions on plant metabolism. The specific response under investigation was a change in the concentration of enzymatic and non-enzymatic antioxidants.

2. MATERIALS AND METHODS

2.1. Experimental conditions

2.1.1. Soil

All experiments were conducted in a sandy loam soil with a density of $1.2 \pm 0.1 \text{ g/cm}^3$. pH, organic carbon, total nitrogen, available phosphorus and chosen heavy metal concentration were determined in accordance with methods described by Smolińska [2015].

Acetates of zinc ($\text{Zn}(\text{CH}_3\text{COO})_2$) (Sigma-Aldrich); nickel ($\text{Ni}(\text{CH}_3\text{COO})_2$) (Sigma-Aldrich); and copper ($\text{Cu}(\text{CH}_3\text{COO})_2$) (Sigma-Aldrich), in concentrations given in Table 1, were

used as soil pollutants. A suitable dry weight of the soil was emptied onto a plastic foil and then combined with a solution of salts of heavy metals ($\text{Zn}(\text{CH}_3\text{COO})_2$, $\text{Ni}(\text{CH}_3\text{COO})_2$ and $\text{Cu}(\text{CH}_3\text{COO})_2$) to obtain their respective ion concentrations (given in Table 1). Contaminated soil was left for seven days in order to bond the heavy metals with the matrix of the soil, and then subsequently it was properly hydrated. The selection of metal concentrations was based on the Ordinance of the Polish Minister of the Environment dated 09.09.2002, which establishes standards for soil and earth quality (Dz.U.02.165.1359). The lowest concentration of metals introduced to the soil did not surpass the permissible levels of heavy metal concentration in soil specified in the Ordinance. The subsequent concentrations showed evidence of significant soil contamination with heavy metals.

The experiment was conducted on nine different variants of polluted soil (three different heavy metals as soil pollutants in three different concentrations) and a control soil sample that was untreated by heavy metals. 200 g of soil were put into a plastic pot and supplemented by either Zn, Ni or Cu to obtain the concentrations given in Table 1. Soil samples were homogenized and left for seven days to stabilize. Each variant of the experiment was performed in triplicate.

2.1.2. Greenhouse studies

The greenhouse experiment was conducted in soil prepared as detailed in section 2.1.1. 10 g of *L. sativum* seeds (organic certified seeds PL-EKO-07, Toraf Company) were introduced into each pot. The experiment was conducted at temperatures of 22/19 °C and a photoperiod of 14 hours/10 hours (day/night) for six weeks after sowing. Plants were irrigated using distilled water at a volume that maintained a soil humidity of 35%. After six weeks, all the aboveground plant parts from each variant of cultivation were harvested. The percentage of unexploded seeds in each repetition was below 5%. Collected plants were at the same stage of development.

L. sativum shoots were washed with deionized water to remove soil particles and weighed. All investigations on plant antioxidative systems, both non-enzymatic and enzymatic, were performed on the aboveground parts of fresh plant samples. For analysis of heavy metal concentration, plant shoots were air-dried to their dry weight and introduced to further analysis.

2.2. Concentration of heavy metals in aboveground parts of *L. sativum*

Determination of plant mineral composition was performed using the ICP-MS method (Elan DRC-e, Perkin Elmer, SCIEX, USA) after acid mineralization of the aboveground plant samples. 0.3 ± 0.01 g dry weight of plant shoot sample was mixed with 7 mL of concentrated nitric acid (HNO_3) (Sigma-Aldrich) and mineralized with

the use of microflow mineralizer (Speedwave Two, Berghof, Eningen, Germany). The mineralized samples were then filtered and diluted with distilled water to a volume of 10 mL. The concentrations of Zn, Ni and Co were determined using an inductively coupled plasma excitation mass spectrometer (ICP-MS) and a DRC chamber, which eliminated spectral and matrix interferences. Quantitative analysis was performed with reference to a standard scale made in the laboratory, and rhodium (103Rh) was used as the internal standard. All determinations were provided in three replicates.

2.3. Determination of hydrogen peroxide (H_2O_2) concentration in *L. sativum* shoots

H_2O_2 measurements were performed in accordance with the method described in detail by Smolińska and Bonikowski [2018]. 1 g of plant samples was homogenized with 0.1% trichloroacetic acid (TCA) (Sigma-Aldrich), and then centrifuged for 15 minutes at 4° C at 15,000 rpm. 0.5 ml of the supernatant of each variant and 1 ml of 1 M potassium iodide (KI) (Sigma-Aldrich) were added to 0.5 ml of 10 mM phosphate buffer (Sigma-Aldrich), pH = 7.0, and mixed. The absorbance at 390 nm was measured. The H_2O_2 content was expressed as the concentration of H_2O_2 in mM in 1 g of FM.

2.4. Determination of total antioxidant activity

The total antioxidant activity was determined using the DPPH method [Georgiadou et al. 2016; Su et al. 2007]. 1 g of *L. sativum* shoots was homogenized with 80% methanol (Sigma-Aldrich) and shaken for 1 hour at room temperature. The homogenates were centrifuged at 12,000 rpm for 30 min, and the supernatants were used for further analysis. The test sample contained 3 mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich) solution and 40 µl of the test extract solution. Absorbance at 517 nm was measured 30 minutes after the initiation of the reaction. All measurements were performed in triplicate, and the average absorbance value for each extract was calculated. Total antioxidant activity was expressed as the amount of Trolox equivalents (TEAC) (in µM Trolox (Merck)/g⁻¹ FM (fresh mass)) based on the calibration curve. The results of the assay were expressed as free radical neutralization capacity (% inhibition), calculated according to the following equation:

$$\% \text{ inhibition} = 100 \cdot \frac{(A_0 - A_{av})}{A_0}$$

where A_{av} is average absorbance at 517 nm after 30 minutes and A_0 is the absorbance of the DPPH radical solution.

2.5. Analysis of non-enzymatic antioxidant system

2.5.1. Determination of total polyphenol content

The total phenolic compound (TP) content was determined using the colorimetric Folin-Ciocalteu (F-C) method with modifications [Kai et al. 2007]. The assay was performed by adding 0.1 mL of Folin-Ciocalteu reagent (Merck) to 0.1 mL of the test extracts, which had been obtained from plant shoots using an 80% methanol solution (Sigma-Aldrich) as described in section 2.3. After three minutes of incubation in a dark place at room temperature, 1 mL of 7% Na₂CO₃ (Sigma-Aldrich) was added to the samples and incubated for another 60 minutes. Next, the absorbance at 725 nm was measured against the blank (without extract) sample. All measurements were performed in triplicate and the average absorbance value for the solution was calculated. The polyphenol content was determined from the standard curve, where a methanolic solution of gallic acid was used as the standard. The total polyphenol content was determined as the amount of gallic acid (Sigma-Aldrich) equivalents per gram of fresh weight of the plant (mg GAE/g⁻¹ FW).

2.5.2. Determination of flavonoid content

1 g of fresh aboveground plant tissue was homogenized with 5 mL of 80% methanol (Sigma-Aldrich) and shaken for 1 hour at room temperature. The total flavonoid content was determined by the colorimetric method using aluminium chloride AlCl₃ [Doe et al. 2013]. 1.4 mL of acetic acid/ethanol mixture (1:19) and 100 µL of 5% AlCl₃ (Sigma-Aldrich) were added to 1 mL of the extract. Then, after a 30-minute incubation at room temperature, the absorbance was measured against a blank sample (without the presence of extract) at 425 nm. All measurements were performed in triplicate and the average absorbance value was calculated for each sample. The determination was performed with reference to a standard curve for quercetin (Sigma-Aldrich). The flavonoid content was determined from the standard curve and expressed as mg of quercetin equivalent per 1 g fresh weight of plant (mg QE/g⁻¹ FW).

2.5.3. Determination of glutathione (GSH) content

Plant tissues (aboveground parts) were homogenized in a mortar with 0.1M phosphate buffer (Sigma-Aldrich) (pH 7.25). For deproteinization, 50% TCA (Sigma-Aldrich) was added and shaken in a vortex for 30 seconds and centrifuged at 3,000 rpm for 10 minutes. The supernatant was transferred to a tube placed in an ice bath. The test and control samples were prepared using the volume of phosphate buffer (Sigma-Aldrich) needed to reach pH 8.2; 0.006 M DTNB (Sigma-Aldrich); 2.5% TCA (Sigma-

Aldrich); and supernatant. The absorbance at 412 nm was measured against the control sample exactly two minutes after the supernatant was introduced. The glutathione concentration was calculated from the standard curve and expressed as mM/g⁻¹ (FW) [Giustarini et al. 2014].

2.5.4. Determination of ascorbic acid (AsA) content

Ascorbic acid (AsA) concentrations were determined using reverse high-performance liquid chromatography with diode array detector (RP-HPLC-DAD). A Shimadzu Performance UFLC liquid chromatograph in a two-channel system, equipped with an autosampler and an SPD-M20A detector, was used for analysis. 2 g of fresh aboveground plant parts (stems and leaves) were placed in a 25 mL volumetric flask, and 7 mL of redistilled water was added. The samples were then extracted using an ultrasonic cleaner for 15 minutes. After extraction, 1 mL of the DTT (Sigma-Aldrich) reducing solution was added to the samples, and extraction was carried out again using the ultrasonic cleaner for 15 minutes. Then, 5 mL of Carrez solution I and 5 mL of Carrez solution II (Merck) were added to the samples. Redistilled water was added to the flasks to fill them up to the 25 mL mark, and they were incubated at room temperature in the absence of light for two hours. A 1 mL aliquot of the filtrate was injected into HPLC system. The concentration of ascorbic acid was determined in relation to the calibration curve for L-ascorbic acid.

2.6. Analysis of enzymatic antioxidant system

In order to determine the activity of catalase (CAT), superoxide dismutase (SOD) and pyrogallol peroxidase (POD), plant material was prepared as follows. The aboveground parts of plants were ground with a mortar and pestle, and the soluble proteins were extracted with the chosen extraction buffer (50 mM phosphate buffer (pH 7.0) (Sigma-Aldrich) 1 mM EDTA (Sigma-Aldrich); 1 mM PMSF (Sigma-Aldrich); and 1% (w/v) polyvinylpyrrolidone (PVP) (Sigma-Aldrich)). The homogenate was centrifuged at 15,000 g for 20 minutes at 4 °C, and the supernatant was used for the subsequent enzyme assays.

2.6.1. Determination of CAT activity (EC 1.11.1.6)

The supernatant was mixed with 50 mM phosphate buffer (Sigma-Aldrich) and 10 mM H₂O₂ (Sigma-Aldrich), and the absorbance of sample was recorded immediately at 240 nm using a UV/VIS8453 Spectroquant Nova 400 spectrophotometer (Merck KGaA, Darmstadt, Germany), and after three minutes of incubation at room temperature [Sarker, Oba 2018]. The supernatant, mixed with 50 mM

phosphate buffer (Sigma-Aldrich) (pH 7.0), was used as a control sample. Catalase activity was defined as 1 U CAT, which is the amount of enzyme required to degrade 1 μM H_2O_2 per minute at 25 °C ($\epsilon = 39.4 \text{ mM}^{-1}/\text{cm}^{-1}$).

2.6.2. Determination of SOD activity (EC 1.15.1.1)

The superoxide dismutase assay was based on determination of the enzyme ability to inhibit the reduction of NBT-nitro blue tetrazoline [Sarker, Oba 2018]. The reaction mixture contained 2 μM riboflavin (Sigma-Aldrich); 13 mM methionine (Sigma-Aldrich); 75 μM nitroblue tetrazolium chloride (NBT) (Sigma-Aldrich); 0.1 mM EDTA (Sigma-Aldrich); 50 mM phosphate buffer (Sigma-Aldrich) (pH 7.8)); and enzyme extract. The reaction was determined to have started once the 2 μM riboflavin had been added to the tubes with the reaction mixture. The tubes were then placed below a light source consisting of two 15 W fluorescent lamps for 15 min. The reaction was stopped by shutting off the light source and transferring the tubes into a dark room. A control sample, consisting of the whole reaction mixture without any exposure to radiation, was used as a reference. The measurement of absorbance was taken at a wavelength of 560 nm. The unit was 1 U SOD/ g^{-1} FW, defined as the amount of enzyme sufficient for a 50% inhibition of the NBT reduction reaction.

2.6.3. Determination of POD activity (EC 1.11.1.7)

The determination of POD was performed with the use of a modified method of Putter [1974] and Stasolla and Yeung [2008]. The reaction mixture contained 3 mL of 0.1 M phosphate buffer (Sigma-Aldrich) (pH 7.0), 20 mM guaiacol (Sigma-Aldrich) and 0.03 mL of 30% H_2O_2 (Sigma-Aldrich). 0.1 mL of plant extract was added to this mixture, and the absorbance was measured at 436 nm. Guaiacol peroxidase activity was expressed as mol/g FW, using the value of molar absorption coefficient of tetraguaiacol ($\epsilon = 26.6 \text{ mM}^{-1}/\text{cm}^{-1}$).

2.6.4. Determination of GSTs activity (EC 2.5.1.18)

Extracts were prepared by homogenizing 1 g of *L. sativum* aboveground material in 5 mL of acetate buffer (Sigma-Aldrich) (pH 6.25). Samples were centrifuged for 20 minutes at 12,000 rpm at 4 °C. The control sample, which contained 0.1 mL of supernatant, 1.35 mL of acetate buffer pH 6.25, and 0.1 mL of 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma-Aldrich), was prepared. For the test sample, 0.1 mL of GSH glutathione was added after three minutes. Absorbance was measured after five minutes at 340 nm.

The changes in absorbance per minute were calculated using the following formula [Rezaei et al. 2013]:

$$\Delta Abs = \frac{A_{340}(\text{read}_2) - A_{340}(\text{read}_1)}{\text{read}_2(\text{min}) - \text{read}_1(\text{min})}$$

$$GSTs \text{ activity } (\text{Mol} \cdot \text{min}^{-1}) = \frac{\Delta Abs \cdot V \cdot DF}{0.00503 \mu \cdot \text{mol}^{-1} \cdot V_{\text{enzyme}}}$$

where V is total volume of the sample, DF is the dilution factor, and V_{enzyme} is the GSH enzyme volume.

2.7. Statistical analysis

Statistica 12.0 software was used for statistical analysis. Conformity of the variables to the normal distribution was tested using the Shapiro-Wilk test. Since the variables did not meet the assumptions for the use of parametric methods, hypothesis verification was performed using non-parametric methods: the Mann-Whitney U test, the Kruskal-Wallis test (along with Dunn's post-hoc test) and Spearman's rank correlation coefficient. A significance level of $\alpha = 0.05$ was assumed. Results were considered statistically significant when the calculated test probability $p < 0.05$.

3. RESULTS

The results of studies on physicochemical properties of soil studied showed that soil used during the greenhouse experiments was classified as slightly acidic, with pH (H_2O) 6.45 ± 0.01 . The concentration of organic carbon, total nitrogen and available phosphorus ($\mu\text{g}/\text{g}^{-1}$ soil dry weight) were 5.47 ± 0.83 , 0.52 ± 0.10 , and 0.38 ± 0.07 , respectively. The amount of the above-mentioned macroelements was considered sufficient for *L. sativum*'s growth requirements. The analysis of soil samples helped to determine the concentration of Zn, Cu and Ni (expressed as $\mu\text{g}/\text{g}^{-1}$ soil dry weight). In the conducted analysis, the obtained amounts for Zn, Cu and Ni were 0.029 ± 0.008 , 0.023 ± 0.003 , and 0.012 ± 0.002 ($\mu\text{g}/\text{g}^{-1}$ soil dry weight), respectively. The trace concentrations of listed metals consist of the natural soil background.

3.1. The content of Zn, Ni and Cu in aboveground parts of *L. sativum*

The concentrations of Zn, Ni and Cu in *L. sativum* shoots were dependent on the metal concentration in soil. The greater the metal content in the growing medium, the higher its concentration in the plant's aboveground tissues. For the increasing Zn contamination in soil, the increased of metal concentrations in the shoots were 22.3%, 39.6% and then 56.2% relative to the control (Figure 1). The analysis of Ni content in the aboveground parts of *L. sativum* also

showed an increase with the rising Ni concentration in soil. The highest Ni concentration was found in *L. sativum* shoots cultivated in soil treated with 300 $\mu\text{g g}^{-1}$ soil dry weight, which was 73 times higher than that of the control plant sample. In a similar vein, it was observed that all the aboveground parts of plants treated with Cu exhibited elevated levels of the metal in comparison to the control. Notably, the plants treated with the highest concentration of Cu (600 $\mu\text{g/g}^{-1}$ soil dry weight) displayed the greatest accumulation of Cu – over 4 times higher than the control.

3.2. Determination of H₂O₂ in *L. sativum* shoots

L. sativum exposure to Zn, Ni and Cu led to an increase in the H₂O₂ concentration in plant shoots in comparison to the control (Figure 2). The increase of H₂O₂ amount in plant aboveground parts was dependent on both the type of metal used for soil contamination and the concentration of the given metal. As can be seen in Figure 2, the highest concentration of H₂O₂ was found in plant shoots harvested from soil with the highest concentration of heavy metals, regardless of the metal. Comparative analysis of the obtained results showed that plant cultivation in soil polluted by Zn caused a 15.7–22.5% increase in H₂O₂ concentration compared to the control. Increasing the concentration of Cu in soil caused H₂O₂ concentration in plant shoots to increase by about 15.9–46.1% over the control. Increased H₂O₂ concentration (of 19.6–46.1%) was also observed in in *L. sativum*'s aboveground parts after the Ni treatment.

3.3. Determination of free radical DPPH scavenging activity

Free radical DPPH neutralization capacity was correlated with the types of metals used for soil contamination, as well as their concentration. As can be seen in Figure 3, a significant increase in % inhibition of DPPH was observed mostly for *L. sativum* shoots harvested after cultivation in soil treated with Zn and with the highest concentration of Ni. Comparison of the results for the Zn treatment showed that the antioxidant capacity of plant, expressed as free radical DPPH neutralization ability, ranged from 7.6%–21.0% when compared to control. For Zn- and Ni-treated plants, it should be emphasized that % inhibition of DPPH was correlated with the amount of metal accumulated by plant shoots. For Cu-treated plants, the increase in antioxidant activity was insignificant.

3.4. Non-enzymatic antioxidant system of *L. sativum* exposed to Zn, Ni and Cu

Analysis of the non-enzymatic antioxidant system entailed determining the total polyphenols, flavonoids, glutathione (GSH) and ascorbic acid (AsA) concentrations in *L. sativum*

shoots harvested after cultivation in soils polluted by Zn, Ni and Cu, respectively. As can be seen in Figure 4, the total polyphenols content ranged from 12.83–19.98 mg/GAE g⁻¹ FW, depending on the metal used for soil pollution and its concentration. There was a notable enhancement in polyphenol levels in comparison to control sample across all plant variants cultivated in the contaminated soils. For *L. sativum* plants cultivated in Zn-treated soil, the total polyphenol content was not dependent on the concentration of metals used, with polyphenols always increasing around 26.5% in relation to the control (Figure 4).

Polyphenol content varied significantly between plants treated by Ni and those treated by Cu. Applying Ni to soil at different concentrations (50, 100 and 300 $\mu\text{g/g}^{-1}$ soil dry weight) caused polyphenol content to increase by 11.5%, 20.7% and 22.6%, respectively, compared to the control sample.

A similar trend was seen in total polyphenol concentration when soil was treated by increasing the amount of Cu. Cu soil concentrations of 100, 300 and 600 $\mu\text{g/g}^{-1}$ soil dry weight increased polyphenol concentration by 30.0%, 38.3% and 55.7%, respectively. Based on the results, it can be concluded that Cu was the one factor that led to the most dramatic increase in polyphenols in the examined plants' tissues.

Figure 4 shows the flavonoid concentration in *L. sativum* shoots harvested from Zn, Ni and Cu-contaminated soil at different metal concentrations. The results of the study indicated that flavonoid content increased in all variants of provided experiment; however, for Zn-treated soil, the increase was statistically insignificant when compared to the control. The greatest increase in flavonoid concentration was found in *L. sativum* aboveground parts after cultivation in soil polluted by Cu. Treatments consisting of 100, 300 and 600 $\mu\text{g Cu/g}^{-1}$ soil dry weight increased flavonoids by 69.2%, 73.4% and 80.5%, respectively, in relation to the control. A similar tendency was observed for plants cultivated in soil contaminated by Ni, with flavonoid levels similar to those of the Cu-treated plants.

The next examination of the non-enzymatic antioxidant system of *L. sativum* exposed to Zn, Ni and Cu consisted of GSH and AsA determination. As can be seen in Figure 5, GSH concentration in plant shoots was at the highest level in plants harvested after cultivation in soil treated by Cu. In this variant of the experiment, for Cu concentrations of 100, 300 and 600 $\mu\text{g/g}^{-1}$ soil dry weight, the increase of GSH content showed a 75%, 230% and 248%, increase, respectively, in comparison to the control. Plant cultivation on soil polluted by Ni also caused an increase of GSH concentration of 19.6–69.6% compared to the control, but the growth of this parameter was not nearly as remarkable as in plants cultivated in soil polluted by Cu. No significant differences in GSH concentration between the Zn-treated plants and control sample were observed.

Exposure of *L. sativum* to the ions of the tested metals resulted in an increase in AsA content regardless of

the specific metal or its concentration in relation to the control (Figure 5). The greatest increase in AsA concentration was observed for plant samples treated with Zn at a concentration of 500 $\mu\text{g/g}^{-1}$ soil dry weight, which was almost 3 times higher than that of the control. It should be emphasized that the highest concentrations of metals introduced into the soil led to decreased AsA concentration in plant shoots when compared to the medium concentration of metals used in the study, regardless of which metal was used.

3.4. Enzymes activity of *L. sativum* under heavy metal stress

The changes in enzyme activity in *L. sativum* shoots are presented in Figure 6. Increased CAT activity was observed in all variants of the experiments compared to the control, regardless of the metal used for soil treatment. The greatest CAT activity was observed for Cu-polluted soil at a concentration of 100 $\mu\text{g/g}^{-1}$ soil dry weight. In this variant of the experiment, CAT activity was five times higher than in the control sample. It should be also noticed that increasing the concentration of metals in soil beyond this point led to decreased CAT activity in all variants of experiment.

SOD activity was also shown to increase as the concentration of metal ions increased in the soil. An increase of SOD activity – between 26.4 and 30.0% compared to the control – was also observed at various Zn concentrations (Figure 5). For Ni soil contamination, no significant differences in SOD activity were observed as compared to the control sample. When the plants were stressed with Cu, an increase in the activity of this enzyme was found, reaching its peak in plants harvested from soil polluted by 600 $\mu\text{g/g}^{-1}$ soil dry weight. This particular treatment increased SOD activity almost twofold compared to the control.

POD is considered to be the primary indicator of antioxidant stress level in plant cells. The results of this study showed that metal ions of every type tested caused an increase in POD activity. The most evident increase was observed in plant samples cultivated in soil with the highest level concentration of Zn added (1,000 $\mu\text{g/g}^{-1}$ soil dry weight) (Figure 5). The sample with the highest concentration of Cu (600 $\mu\text{g/g}^{-1}$ soil dry weight) showed a 76.6% increase in POD activity in comparison with the plants grown in the control medium.

As it can be seen in Figure 6, an increase in GSTs activity was observed for ions of all the metals tested. Two exceptions occurred in the soil treated with the lowest and medium concentrations of Ni, where there was initially a reduction of GSTs activity compared to the control sample. At high concentrations of Ni, however, an increase in enzyme activity was noted. The most evident increase in GSTs activity was observed in plants grown in soil treated with 600 $\mu\text{g/g}^{-1}$ soil dry weight Cu; GSTs activity here was 60.9% higher than in the control sample.

4. DISCUSSION

Heavy metals can be dangerous for human health. They can be assimilated directly by the human body via dermal contact or inhalation, or through bioaccumulation in the food chain, via ingestion of animal- or plant-derived food sources [Nazir et al. 2011]. The primary mechanism by which plants acquire metal ions is through absorption and assimilation from the soil. Soil has often served as the primary storage medium for waste materials containing high concentrations of heavy metals [Jadia, Fulekar 2008]. Thus, the analysis of metal ion accumulation in plants is crucial to identifying the level to which a specific metal in the soil can accumulate before it becomes toxic toward plants. The evaluation of metal concentration in the aboveground parts of plants is a highly significant parameter, as it serves as an indicator of plants' absorption capacity and reflects their overall uptake capacity for metal ions from the soil solution.

The results of the present study showed that *L. sativum* was able to accumulate Zn, Ni, and Cu. The concentration of heavy metals in plant shoots was dependent on the degree of soil contamination. For higher amounts of Zn, Ni and Cu in soil, the concentration of metals in the plant shoots was also higher. This finding stays in line with the results presented by Sai Kachout et al. [2012], who tested accumulation of Cu, Pb, Ni and Zn by *Atriplex*. The authors concluded that concentration of heavy metals exceeding the levels of 300 mg/kg^{-1} for Zn, 100 mg/kg^{-1} for Cu and 50 mg/kg^{-1} for Ni can be considered to be phytotoxic to plants. It should be emphasized that phytotoxicity of heavy metals can be dependent not only on the metals' concentration, but also on the plant species. This was confirmed by Szczodrowska et al. [2016], who tested the accumulation of Zn, Ni and Cu in *Mentha peperita* L., *Ocimum basilicum* L. and *L. sativum*. Despite significant differences in greenhouse conditions compared to the present study, the results of Zn, Ni and Cu concentration in *L. sativum* shoots showed similar trends. The plants examined in that study bioaccumulated Ni more abundant than Zn and Cu.

Heavy metal phytotoxicity is a result of many factors, including complex reactions that occur in the rhizosphere between plants, soil and microbes. Heavy metals present in the soil can act as toxic substances for living organisms due to their chemical properties [Schutzendubel 2002]. The plant responses to Zn, Ni and Cu presented in the study showed uptake of these metals from soil by plant roots, which were then transported into the aboveground parts of *L. sativum*, where they were stored.

All of tested metals are biologically significant for living organisms. For example, Zn is involved in carbohydrate, auxin and protein metabolism. It also is a cofactor in enzymatic reactions [Ghori et al. 2019]. Zn is a redox-inert metal [Cuajungco et al. 2021]. Ni is classified as a transition element that takes part in the structure of enzymes like SOD. It also plays a role in biochemical processes like

metabolism of hydrogen and maintenance of the cellular redox state, as well as stress tolerance and defence efficiency [Ghori et al. 2019].

Cu is an element that takes part in carbon assimilation and ATP synthesis in plants. It has been established as an important constituent of plastocyanins and cytochrome oxidases [Ghori et al. 2019]. Cu is a redox-active metal that can undergo autoxidation, resulting in oxygen free-radical formation and subsequently in hydrogen peroxide and hydroxyl radical production via Fenton-type reactions [Schutzendubel 2002]. The redox potential of elements, as well as their ability to bind to oxygen, nitrogen or sulphur atoms, influences their phytotoxicity. The binding affinity of metals to the aforementioned atoms can lead to inactivation of enzymes due to, for example, their binding to cysteine residues [Schutzendubel 2002].

The phytotoxicity of heavy metals manifests in a variety of physiological and metabolic changes in plants, including decreased seed germination, plant growth reduction and/or photosynthesis disorders [Schutzendubel 2002]. The exposure of plants to heavy metals may also cause overproduction of the reactive oxygen species (ROS), which leads to cellular damage in plants. Counteracting changes in plant cells that occur under ROS requires activation of the plant antioxidant system, including both non-enzymatic and enzymatic antioxidants.

H_2O_2 is usually produced by superoxide dismutase (SOD) dismutation of oxygen free radicals during electron transport in different compartments of the plant cell and is involved in plant growth, metabolism and stress tolerance [Mariyam et al. 2023]. Its increasing amount in plant tissue indicates oxidative stress.

In the present study, H_2O_2 was tested to determine if plant exposure to Zn, Ni and Cu increases the formation of one of the ROS in *L. sativum* shoots. Based on the results, it can be concluded that all of tested metals increased the concentration of H_2O_2 in aboveground parts of plant, regardless of their concentrations. These findings are in line with the results presented by Mahdavian [2022], who tested the amount of H_2O_2 in two populations of *Peganum harmala* L. seedlings exposed to Zn. The author stated that increased concentration of H_2O_2 in seedlings under Zn treatment indicated oxidative stress. Ginnakoula et al. [2021] showed increased H_2O_2 concentration in leaves of *Citrus aurantium* L. plants treated with increasing amounts of Cu. The growth of H_2O_2 in sweet potato has also been observed with Ni treatment at increasing concentrations [S. Kumar et al. 2022]. H_2O_2 at low concentrations acts as a powerful signalling molecule involved in triggering reactions to various stress conditions [Smolińska, Bonikowski 2018]. At higher concentrations, it causes oxidative burst in organic molecules, which may result in cell death [Mariyam et al. 2023].

The free radical-scavenging activity of *L. sativum* shoots has been investigated in various studies. DPPH parameters show the capability of plants to prevent against oxidative damage caused by heavy metals. Based on the results, it can be stated that Zn (regardless of concentration) and

Ni, in the highest concentrations, increased the DPPH activity in plant aboveground parts when compared to the control. Mandal and Khdal [2023] found the highest DPPH activity in *Holarrhena pubescens* and *Wrightia tinctoria* that were not exposed to heavy metals (their controls). Schutzendubel [2002] found that increasing the concentration of heavy metals in industrial soils led to a moderate decrease of DPPH activity in tested plants, and concluded that decreasing DPPH activity was negatively correlated with heavy metal concentration in industrial soils due to decreased concentrations of phenolic and flavonoid compounds.

In the present study, the total polyphenols and flavonoids increased in *L. sativum* shoots harvested from soil contaminated by increasing concentrations of heavy metals. Polyphenols and flavonoid compounds are abundant for plants. One of their roles consists of controlling the oxidative stress induced by free radicals [Schutzendubel 2002]. The phenolics possess antioxidant properties to chelate metals, mostly via their hydroxyl group. Moreover, these compounds inhibit lipid peroxidation and maintain the integrity of the plant membranes [Ghori et al. 2019]. The increasing concentration of polyphenolic compounds observed in the study (Figure 3) confirm that *L. sativum* plants were able to stimulate their non-enzymatic antioxidant system to neutralize free radicals presented in their cells. The obtained results are consistent with previously published study of Márquez-García et al. [2012], who found an increase in the concentration of flavonoids, phenolic compounds, and total antioxidant potential in the tissues of the endemic species *Erica andevalensis* growing on post-mining soils heavily contaminated with a different heavy metal (Cd).

Moreover, the phenolic compounds that are overproduced under heavy metal exposition may be a substrate for some peroxidases. Although the main polyphenol peroxidases include tyrosinase, catechol oxidase and laccase [S. Zhang 2023], flavonoids act as a possible regulator of lipoxygenase, the enzyme that converts polyunsaturated fatty acids into corresponding peroxidases and hydroperoxidases [Kesawat et al. 2023]. It can be observed in the present study's results that POD activity in *L. sativum* shoots increased with increasing concentration of each of the metals – Zn, Ni and Cu – in relation to the control (Figure 5). This finding is in line with the study presented by Barcelos et al. [2018], who demonstrated that increasing the amount of Ni (10–40 g/ha⁻¹) in the growing medium led to an increase in the POD activity in soybean plants in comparison to the control [Barcelos et al. 2018]. However, the same authors also showed that POD activity decreased after soybean exposition to Ni concentration that exceeded 60 g/ha⁻¹. A similar trend of increasing peroxidase activity was shown by Li et al. [2006]; in a study where microalga *Pavlova viridis* were exposed to increasing concentrations of Cu [Barcelos et al. 2018]; and by León-Morales et al. [2012] in a study of 3- and 5-day-old crops of *Beta vulgaris*. Another non-enzymatic antioxidant that plays a crucial role in plant defence against ROS under heavy metal stress

is glutathione (GSH). GSH is an active compound that is oxidized during the degradation of, and thus controls the levels of, hydrogen peroxide in plant cells. Ghori et al. [2019] found that the ratio of ROS between the oxidized forms of GSH to be an indicator of redox balance and ROS generation in the cell. GSH is involved in the detoxification of heavy metals by conjugating with them and transporting them to cells' vacuoles, where they are detoxified [Ghori et al. 2019]. The concentration of GSH in *L. sativum* plants was found to be elevated when grown in soil that was contaminated with increasing concentrations of Zn, Ni and Cu. The most significant GSH increase was observed in plant shoots treated with the highest concentration of Cu. The present findings suggest that the presence of heavy metal ions leads to the initiation of oxidative stress, thereby facilitating the production of non-enzymatic antioxidants like GSH.

Previous studies found that increased GSH biosynthesis enhanced Ni tolerance in *Thlaspi* [Freeman et al. 2005]. GSH is correlated with the activity of glutathione S-transferase enzymes (GSTs) that are centrally positioned in its network of redox control and cellular detoxification machinery [Nianiou-Obeidat et al. 2017]. Although GSTs enzymes are not core components in antioxidant systems in plants, some studies reported that GSTs proteins were also involved in protection against oxidative stress, serving as bifunctional enzymes – glutathione S-transferase/ glutathione peroxidase. Increasing production of GSTs in plants can enhance tolerance to stressors [Barcelos et al. 2018]. The present study found that activity of GSTs enzymes increased in *L. sativum* shoots exposed to increasing concentration of Zn and Cu when compared to the control (Figure 5). However, the Ni treatment saw only insignificant differences in GSTs activity over the control. Li et al. [2018] showed that Cu treatment decreased GSTs activity in *Oryza sativa* plants compared to the control [Barcelos et al. 2018]. On the other hand, Al-Zahrani et al. [2022] demonstrated that Zn supplementation could cause an increase in GSTs activity in soybean plants induced by Zn supplementation [Barcelos et al. 2018]. Therefore, the present study concludes that *L. sativum* response to Zn, Ni and Cu differs depending on the element used for soil contamination.

Elevated concentrations of GSH and GSTs cannot always be correlated with enhanced tolerance of heavy metals in plants [Ghori et al. 2019]. There is not sufficient evidence to support GSH's role in resistance to heavy metal induced stress [Yadav 2010]. Other antioxidants and repair mechanisms may also participate in the tolerance process. It is known that GSH is vital in the ascorbic acid-GSH cycle, scavenging diverse free radicals and maintaining cellular redox balance [Wu et al. 2020]. However, ascorbic acid (AsA) also plays a significant role in defence mechanisms that occur in plant cells under heavy-metal stress. AsA is crucial for synthesis of phytohormones and effectively counteracts the harmful effect of ROS due to its stability and ability to donate electrons, which are used for the neutralization of hydroxyl, superoxide and tocopherol

radicals [Wu et al. 2020]. Approximately 90% of AsA is localized in the cytosol, and thus AsA is the first line of defence against ROS [Kesawat et al. 2023]. The present study showed that AsA concentration in *L. sativum* increased in all variants of experiments when compared to the control (Figure 4), suggesting that *L. sativum* activated its defence system against ROS produced under Zn, Ni and Cu treatment. Similar findings have been reported by Wu et al. [2020], who tested the response of *Phragmites australis* to Cu stress [Wu et al. 2020]. In this study, a positive correlation between concentration of AsA in *P. australis* and Cu concentration was observed [Wu et al. 2020]. Based on the results of the present study, it can be stated that increased levels of AsA in *L. sativum* shoots exposed to Zn, Ni and Cu separately can signify the plant's tolerance to heavy metal stress conditions.

The conducted research consisted of determining the activity of enzymes that constitute the enzymatic part of antioxidant machinery of plants. Besides of the enzymes mentioned above, the SOD and CAT activity were investigated in *L. sativum* shoots exposed to Zn, Ni and Cu. SOD is a plant's first line enzymatic defence against ROS, performing the dismutation of oxygen radical into hydrogen peroxide [Wu et al. 2020]. Hydrogen peroxide is not only a toxic product of oxidative stress, but is also known to be an important signalling molecule involved in a variety of mechanisms designed to neutralize the effects of abiotic stress. Therefore, oxidative stress levels may be assessed directly by monitoring H_2O_2 concentration, and indirectly by SOD activity.

SOD activity in *L. sativum* shoots increased with increasing concentration of Zn and Cu, regardless of the concentration of those metals in the soil (Figure 5). Plants cultivated in Ni-contaminated soil, however, resulted in an insignificant increase in SOD activity when compared to the control.

Comparative analysis of obtained results with results presented by Li et al. [2006] showed similar trends in SOD activity after exposition to Cu and Zn, separately [Barcelos et al. 2018]. Barcelos et al. [2018] showed that increasing concentrations of Cu in the growing medium led to increased SOD activity in *Pavlova viridis*, while exposing microalgae to increasing concentrations of Zn induced a moderate increase of SOD compared to the control. Another study [Azooz et al. 2012] reported an increase in SOD activity in wheat *Triticum aestivum* L. that was exposed to Cu, which may indicate the participation of Cu in the mechanisms responsible for the defence against ROS.

Dismutation of oxide radicals to hydrogen peroxide is the first step of oxygen radical detoxification. Another stage is the transformation of hydrogen peroxide into water and oxygen. This process is conducted by CAT. The ability of CAT to effectively limit hydrogen peroxide concentration in cells underlines its importance to the physiological plant processes that are activated as defence mechanisms against ROS [Wu et al. 2020]. This study found that CAT activity was higher in *L. sativum* shoots after exposition to Zn, Ni and Cu compared to the control (Figure 5). It should be also emphasized that increasing the concentration of

metals in the soil led to decreased CAT activity in plant's aboveground parts, regardless of which metal was used. These results also confirm the findings of Sida-Arreola et al. [2017], who showed that exposure of *Phaseolus vulgaris* L. to increasing Zn concentrations increased CAT activity compared to the control [Wu et al. 2020]. Barcelos et al. [2018] found that although treating soybean plants with Ni led to increased CAT activity in relation to control, increasing the concentration of Ni in the soil led to a decrease in CAT activity. It should be emphasized that CAT is not the only enzyme that catalyses the dismutation of hydrogen peroxide to water and oxygen. Peroxidases can play the same role, the increasing activity of which is presented in Figure 5.

5. CONCLUSIONS

The results of the study showed that all of tested heavy metals can cause oxidative stress in the aboveground parts of *L. Sativum* plants. Increasing metal concentration in soil led to its increasing accumulation in plant shoots, which was reflected in defence responses in the plant. All of the tested concentrations of Zn, Ni and Cu increased the level of H_2O_2 in plant shoots, which confirmed the presence of oxidative stress. The obtained results showed that *L. sativum* possesses a capacity to overcome unfavourable environmental conditions by increasing its production of both enzymatic and non-enzymatic antioxidants. Although an increase in antioxidant activity was observed in all variants of this study's experiments, comparative analysis showed that plant treatment with Cu caused the highest production of polyphenols, flavonoids and glutathione, as well as increased CAT, SOD, POD and

GSTs activity. Therefore, it seems that Cu, when used in increasing concentrations, stimulates the antioxidant system in *L. sativum* more intensely than Zn and Ni.

This study finds that concentrations of Zn, Ni and Cu that did not exceed the suggested limits set forth by the Polish Minister of the Environment's Ordinance from September 9, 2002 for soil and earth quality standards (Dz.U.02.165.1359) nonetheless did cause metabolic changes in *L. sativum*, increasing the antioxidant activity in the plant. However, despite the high stimulation of *L. sativum*'s antioxidant system by the presence of metals in the soil, the plant's growth and development remained unaffected, confirming the possibility of cultivating edible plants in these soil conditions.

AUTHOR CONTRIBUTIONS

Conceptualization: Smolińska B., Leszczyńska J.; methodology: Smolińska B., Leszczyńska J., Szczodrowska A.; software: Szczodrowska A.; validation: Smolińska B., Leszczyńska J.; formal analysis: Szczodrowska A.; investigation: Szczodrowska A.; writing— Smolińska B., Szczodrowska A., Leszczyńska J., Włodarczyk K.; writing—review and editing: Smolińska B., Leszczyńska J., Włodarczyk K.; supervision: Leszczyńska J., Smolińska B.; project administration: Leszczyńska J., Smolińska B. All authors have read and agreed to the published version of the manuscript.

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REFERENCES AND LEGAL ACTS

- AHANGER M. A., AKRAM N. A., ASHRAF M., ALYEMENI M. N., WIJAYA L., AHMAD P. 2017. Plant responses to environmental stresses – From gene to biotechnology. *AoB PLANTS* 9, 4: plx025. <https://doi.org/10.1093/aobpla/plx025>
- AZOOZ M., ABOU-ELHAMD M., AL-FREDAN M. 2012. Biphasic effect of copper on growth, proline, lipid peroxidation and antioxidant enzyme activities of wheat (*Triticum aestivum* cv. Hasaawi) at early growing stage. *Australian Journal of Crop Science* 6, 4: 688–694.
- BARCELOS J. P. Q., REIS H. P. G., GODOY C. V., GRATÃO P. L., FURLANI JUNIOR E., PUTTI F. F., CAMPOS M., REIS A. R. 2018. Impact of foliar nickel application on urease activity, antioxidant metabolism and control of powdery mildew (*Microspheera diffusa*) in soybean plants. *Plant Pathology* 67, 7: 1502–1513. <https://doi.org/10.1111/ppa.12871>
- BUSO P., MANFREDINI S., REZA AHMADI-ASHTIANI H., SCIABICA S., BUZZI R., VERTUANI S., BALDISSEROTTO A. 2020. Iranian medicinal plants: from ethnomedicine to actual studies. *Medicina* 56, 3: 97.
- CUAJUNGCO M., RAMIREZ M., TOLMASKY M. 2021. Zinc: Multidimensional Effects on living organisms. *Biomedicines* 9, 2: 208. <https://doi.org/10.3390/biomedicines9020208>
- DOEE., AWUA A., ACHORIBOE., SANDRA A. 2013. Extractable phenolic and flavonoid content from selected natural and industry processed spices. *American Journal of Applied Chemistry* 1, 4: 53.
- DOS SANTOS T. B., RIBAS A. F., DE SOUZA S. G. H., BUDZINSKI I. G. F., DOMINGUES D. S. 2022. Physiological responses to drought, salinity, and heat stress in plants: A review. *Stresses* 2, 1: 113–135. <https://doi.org/10.3390/stresses2010009>
- DRIF F., ABDENNOUR C., CIĞERCI İ., ALI M. M., OUARDA M., MESSARAH M. 2019. Preliminary assessment of stress and genotoxicity biomarkers in bivalve molluscs from the Gulf of Annaba, Algeria. *Bulletin of Environmental*

- Contamination and Toxicology 102: 555–559. <https://doi.org/10.1007/s00128-019-02583-4>
- DUBEY S., SHRI M., GUPTA A., RANI V., CHAKRABARTY D. 2018. Toxicity and detoxification of heavy metals during plant growth and metabolism. *Environmental Chemistry Letters* 16, 4: 1169–1192. <https://doi.org/10.1007/s10311-018-0741-8>
- DUMANOVIĆ J., NEPOVIMOVA E., NATIĆ M., KUČA K., JAČEVIĆ V. 2021. The significance of reactive oxygen species and antioxidant defense system in plants: A concise overview. *Frontiers in Plant Science* 11. <https://doi.org/10.3389/fpls.2020.552969>
- FREEMAN J., PERSANS M. W., NIEMAN K., ALBRECHT C., PEER W., PICKERING I., SALT D. E. 2005. Increased glutathione biosynthesis plays a role in nickel tolerance in *Thlaspi* nickel hyperaccumulators. *The Plant Cell* 16, 8: 2176–2191. <https://doi.org/10.1105/tpc.104.023036>
- GEORGIADOU E. C., GOULAS V., NTOUROUT., MANGANARIS G. A., KALAITZIS P., FOTOPOULOS V. 2016. Regulation of on-tree vitamin E biosynthesis in olive fruit during successive growing years: the impact of fruit development and environmental cues. *Frontiers in Plant Science* 7. <https://doi.org/10.3389/fpls.2016.01656>
- GEORGIADOU E. C., KOWALSKA E., PATLA K., KULBAT K., SMOLIŃSKA B., LESZCZYŃSKA J., FOTOPOULOS V. 2018. Influence of heavy metals (Ni, Cu, and Zn) on nitro-oxidative stress responses, proteome regulation and allergen production in basil (*Ocimum basilicum* L.) plants. *Frontiers in Plant Science* 9. <https://doi.org/10.3389/fpls.2018.00862>
- GHANTE M. H., BADOLE S. L., BODHANKAR S. L. 2011. Health benefits of garden cress (*Lepidium sativum* Linn.) seed extracts. In *Nuts and Seeds in Health and Disease Prevention* (pp. 521–525). Elsevier.
- GHORI N.-U.-H., GHORI T., HAYAT M., IMADI S. R., GUL A., ALTAY V., OZTURK M. 2019. Heavy metal stress and responses in plants. *International Journal of Environmental Science and Technology* 16: 1807–1828.
- GIANNAKOULA A., THERIOS I., CHATZISSAVVIDIS C. 2021. Effect of lead and copper on photosynthetic apparatus in citrus (*Citrus aurantium* L.) plants. The role of antioxidants in oxidative damage as a response to heavy metal stress. *Plants* 10, 1: 155. <https://doi.org/10.3390/plants10010155>
- GIUSTARINI D., FANTI P., MATTEUCCI E., ROSSI R. 2014. Micro-method for the determination of glutathione in human blood. *Journal of Chromatography B* 964: 191–194. <https://doi.org/10.1016/j.jchromb.2014.02.018>
- GUPTA S., GUPTA R. 2023. Research update on the therapeutic potential of garden cress (*Lepidium sativum* Linn.) with threatened status. *Current Drug Research Reviews* 16, 3: 369–380. <https://doi.org/10.2174/0125899775273877231023102011>
- JADIA C., FULEKAR M. 2008. Phytoremediation: The application of vermicompost to remove zinc, cadmium, copper, nickel and lead by sunflower plant. *Environmental Engineering and Management Journal* 7, 5: 547–558. <https://doi.org/10.30638/eemj.2008.078>
- KAI M., VANSELOW K., SEBASTIAN L., RALF H., RUSER A., ULF-PETER H. 2007. Determination of DPPH radical oxidation caused by methanolic extracts of some microalgal species by linear regression analysis of spectrophotometric measurements. *Sensors* 7, 10: 2080–2095. <https://doi.org/10.3390/s7102080>
- KESAWAT M. S., SATHEESH N., KHERAWAT B. S., KUMAR A., KIM H.-U., CHUNG S.-M., KUMAR M. 2023. Regulation of reactive oxygen species during salt stress in plants and their crosstalk with other signaling molecules – Current perspectives and future directions. *Plants* 12, 4: 864. <https://doi.org/10.3390/plants12040864>
- KUMAR S., WANG M., LIU Y., FAHAD S., QAYYUM A., JADOON S. A., CHEN Y., ZHU G. 2022. Nickel toxicity alters growth patterns and induces oxidative stress response in sweetpotato. *Frontiers in Plant Science* 13. <https://doi.org/10.3389/fpls.2022.1054924>
- KUMAR V., TOMAR V., RANADE S. A., YADAV H. K., SRIVASTAVA M. 2020. Phytochemical, antioxidant investigations and fatty acid composition of *Lepidium sativum* seeds. *Journal of Environmental Biology* 41, 1: 59–65.
- LEÓN-MORALES J., RODRÍGUEZ-MONROY M., JIMÉNEZ G. 2012. Betacyanin accumulation and guaiacol peroxidase activity in *Beta vulgaris* L. leaves following copper stress. *Acta Societatis Botanicorum Poloniae* 81, 3: 2083–2480. <https://doi.org/10.5586/asbp.2012.019>
- MAHDAVIAN K. 2022. The role of organic acids and thiol compounds in detoxification and tolerance of Zn stress in two populations of harmel. *Russian Journal of Plant Physiology* 69, 3: 58. <https://doi.org/10.1134/S1021443722030098>
- MARIYAM S., BHARDWAJ R., KHAN N. A., SAHI S. V., SETH C. S. 2023. Review on nitric oxide at the forefront of rapid systemic signaling in mitigation of salinity stress in plants: Crosstalk with calcium and hydrogen peroxide. *Plant Science* 336: 111835. <https://doi.org/10.1016/j.plantsci.2023.111835>
- MÁRQUEZ-GARCÍA B., RECAMALES Á., CORDOBA F. 2012. Effects of cadmium on phenolic composition and antioxidant activities of *Erica andevalensis*. *Journal of Botany* 2012,1: 936950. <https://doi.org/10.1155/2012/936950>
- NAZIR A., MALIK R., AJAIB M., KHAN N., SIDDIQUI M. 2011. Hyperaccumulators of heavy metals of industrial areas of Islamabad and Rawalpindi. *Pakistan Journal of Botany* 43, 4: 1925–1933.
- NGUYEN C. H., YAN D., NAMBARA E. 2023. Persistence of abscisic acid analogs in plants: Chemical control of plant growth and physiology. *Genes* 14, 5: 1078. <https://doi.org/10.3390/genes14051078>
- NIANIOU-OBEIDAT I., MADEIS P., KISSOUDIS C., VOULGARIS G., CHRONOPOULOU E., TSAFTARIS A., LABROU N. E. 2017. Plant glutathione transferase-mediated stress tolerance: Functions and biotechnological applications. *Plant Cell Reports* 36, 6: 791–805. <https://doi.org/10.1007/s00299-017-2139-7>
- PUTTER J. 1974. *Methods of Enzymatic Analysis*. 2nd English ed. H. U. Bergmeyer, ed. London, Academic Press.

- REZAEI M. K., SHOBBAR Z.-S., SHAHBAZI M., ABEDINI R., ZARE S. 2013. Glutathione S-transferase (GST) family in barley: Identification of members, enzyme activity, and gene expression pattern. *Journal of Plant Physiology* 170, 14: 1277–1284. <https://doi.org/10.1016/j.jplph.2013.04.005>
- SAI KACHOUT S., MANSOURA A., RANIA M., LECLERC J., REJEB M., OUERGH Z. 2012. Accumulation of Cu, Pb, Ni and Zn in the halophyte plant *Atriplex* grown on polluted soil. *Journal of the Science of Food and Agriculture* 92, 2: 336–342. <https://doi.org/10.1002/jsfa.4581>
- SARKER U., OBA S. 2018. Catalase, superoxide dismutase and ascorbate-glutathione cycle enzymes confer drought tolerance of *Amaranthus tricolor*. *Scientific Reports* 8, 1: 16496. <https://doi.org/10.1038/s41598-018-34944-0>
- SCHUTZENDUBEL A. 2002. Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. *Journal of Experimental Botany* 53, 372: 1351–1365. <https://doi.org/10.1093/jexbot/53.372.1351>
- SMOLIŃSKA B. 2015. Green waste compost as an amendment during induced phytoextraction of mercury-contaminated soil. *Environmental Science and Pollution Research* 22: 3528–3537. <https://doi.org/10.1007/s11356-014-3601-5>
- SMOLIŃSKA B., BONIKOWSKI R. 2018. Activation of non-enzymatic antioxidants by *Lepidium sativum* L. exposed to Hg during assisted phytoextraction. *CLEAN – Soil, Air, Water* 46, 2: 1700667. <https://doi.org/https://doi.org/10.1002/clen.201700667>
- STASOLLA C., YEUNG E. C. 2007. Cellular ascorbic acid regulates the activity of major peroxidases in the apical poles of germinating white spruce (*Picea glauca*) somatic embryos. *Plant Physiology and Biochemistry* 45, 3: 188–198. <https://doi.org/https://doi.org/10.1016/j.plaphy.2007.02.007>
- SU L., YIN J.-J., CHARLES D., ZHOU K., MOORE J., YU L. 2007. Total phenolic contents, chelating capacities, and radical-scavenging properties of black peppercorn, nutmeg, rosehip, cinnamon and oregano leaf. *Food Chemistry* 100, 3: 990–997. <https://doi.org/https://doi.org/10.1016/j.foodchem.2005.10.058>
- SZCZODROWSKA A., KULBAT K., SMOLIŃSKA B., LESZCZYŃSKA J. 2016. Accumulation of metal ions in selected plants from *Brassicaceae* and *Lamiaceae* families. *Biotechnology and Food Science* 80, 1: 29–42.
- VARDHAN K. H., KUMAR P. S., PANDA R. C. 2019. A review on heavy metal pollution, toxicity and remedial measures: Current trends and future perspectives. *Journal of Molecular Liquids* 290: 111197. <https://doi.org/10.1016/j.molliq.2019.111197>
- VAZIFEH S., KANANPOUR P., KHALILPOUR M., EISALOU S. V., HAMBLIN M. R. 2022. Anti-inflammatory and immunomodulatory properties of *Lepidium sativum*. *BioMed Research International* 2022: 3645038. <https://doi.org/10.1155/2022/3645038>
- WU J., HU J., ZHAO L., MA F. 2020. Responses of *Phragmites australis* to copper stress: A combined analysis of plant morphology, physiology and proteomics. *Plant Biology* 23, 2: 351–362. <https://doi.org/10.1111/plb.13175>
- YADAV S. 2010. Heavy metals toxicity in plants: An overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. *South African Journal of Botany* 76, 2: 167–179. <https://doi.org/10.1016/j.sajb.2009.10.007>
- ZHANG H., ZHAO Y., ZHU J.-K. 2020. Thriving under stress: How plants balance growth and the stress response. *Developmental Cell* 55, 5: 529–543. <https://doi.org/10.1016/j.devcel.2020.10.012>
- ZHANG S. 2023. Recent advances of polyphenol oxidases in plants. *Molecules* 28, 5: 2158. <https://doi.org/10.3390/molecules28052158>