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Response of *Lepidium sativum* to soil contamination with zinc in molecular and nanoparticle form

Lidia Mielcarz-Skalska*, Beata Smolińska

Institute of General Food Chemistry, Faculty of Biotechnology and Food Science,
Lodz University of Technology, 4/10 Stefanowskiego Str., 90-924 Lodz, Poland

*E-mail address: lidiamielcarz@gmail.com

ABSTRACT

Increasingly frequent use of zinc nanoparticles in everyday life increases the concentration of this element in the environment. At the same time, research into the influence of zinc nanoparticles on living organisms is still lacking. The aim of this experiment was to investigate the effects of zinc oxide in molecular and nanoparticle form on *Lepidium sativum*. Studies have shown that the influence of zinc on growth and physiological features of *Lepidium sativum* depended on the form and the concentration of this metal in the soil. All of the analyzed zinc forms have activated the enzymatic antioxidant system of the plant, which demonstrates its sensitivity to soil contaminants.

Keywords: *Lepidium sativum*, nanoparticles, zinc, zinc-soil contamination, fitotoxicity

1. INTRODUCTION

Zinc is a natural component of the fauna and flora world. In different amounts occurs in rocks, soil, water and air. It is essential for the proper development and health of plants, animals and humans. This element, naturally occurring in the environment, gets into the environment in addition due to human activities. It is widely used in the industry: cosmetics, pharmaceuticals, paints and plastics, metal coatings to protect against corrosion and many others.

Municipal waste water, plant protection products and combustion of fossil fuels also increase their concentration in the environment. In recent years an additional source of zinc are, increasingly used, nanoparticles. Despite their widespread use, the impact of zinc nanoparticles on plants and other organisms is still not fully understood [1,2].

The zinc found in organic compounds is readily absorbed by plants. This applies both to acidic and alkaline soils. The concentration of zinc in living organisms is proportional to its amount in food. In the case of absorption of zinc by plants, the concentration depend on the bioactive fraction, not necessarily the total amount in the soil [1]. Zn is an essential micronutrient but both its deficiency and excess are extremely dangerous. Many researchers deal with the effects of different amounts of zinc on plants. The results are very diverse. Zinc is an essential element for plants, but it is toxic in high concentrations. Studies show that zinc phytotoxicity mainly consists of incomplete and delayed germination and deformations in the root zone [3,4].

Dicotyledonous plants are more susceptible than monocotyledons to excess zinc sensitivity, according to some researchers, due to the displacement of contaminants at an earlier stage of development from the roots to the shoots. Another explanation is the capacity of the root system to and the ratio of ground to ground mass [5]. Studies by Zhang et al. had shown that the more zinc in the soil the smaller the yield was. Already at 50 mg / kg dry weight of soil were observed to fall by 3-18% relative to the control plants, but at a dose of 250 mg / kg dry weight of soil was already a fall of 14-65% in comparison to control plants. Higher concentrations caused even a complete germination inhibition [6]. Zinc accumulation in plant tissues has also been studied. It has been shown that the largest accumulation occurs in the root part of the plant [6].

However, Cakmak at al. showed that zinc deficiency is also harmful to plants. In all studied species (cotton (*Gossypium hirsutum* L.), wheat (*Triticum aestivum* L.), tomato (*Lycopersicon esculentum* L.) and apple (*Malus domestica*)), zinc deficiency caused less growth of the shoot. Leaves of tested plants had symptoms of chlorosis [7]. In case of contact with a toxic substance, there is often a greater reduction of roots than shoots. This is related to the direct contact of soil pollutants with plants. Plants often accumulate impurities in the root zone, limiting their translocation to the aboveground parts, so that the toxicity to that part of the plant is higher and appears to inhibit growth [2].

The presence of high concentrations of heavy metals can cause oxidative stress in living cells. The result is the formation of free radicals that contribute to cell damage [8]. Plants can neutralize the harmful effects of heavy metals. In response to unfavorable environmental conditions, plants activate the antioxidant system: non-enzymatic (consisting of flavonoids, phenolic acids, polyphenols, ascorbic acid, tocopherols and glutathione) and enzymes (composed of several enzymes that play a key role in scavenging free radicals from the cell: POD, SOD, CAT, glutathione transferase).

Our experience was to investigate the response of *Lepidium sativum* to soil loading with different concentrations of zinc oxide, both in molecular and nanoparticle sizes of <50 nm and <100 nm. For this purpose we conducted 14 day *L. sativum* cultures on soils contaminated, in three concentrations: 1, 10 and 100 mg Zn / kg soil.

We then made biometric measurements and plant dye levels and investigated the response of the antioxidant system. In our research we focused on the content of phenolic acids, flavonoids, pyrogallol peroxidase and superoxide dismutase.

2. MATERIALS AND METHODS

2. 1. Plants material

L. sativum was cultivated on soil with bulk zinc and nanoparticles of zinc soil, at three different concentrations: 1 mg / kg soil, 10 mg / kg soil, and 100 mg / kg soil. Growing conditions was: day / night system - 14h / 10h; temperature 22/19 °C; air humidity 50%; soil moisture 35%. Plants were grown for 14 days. After this time, plant material was collected, biometric measurements were made, phenolic acids, flavonoids, plants dyes, SOD and POD were measured.

2. 2. Determination of zinc content in plants

Determination of Zn concentration in the plant was made by atomic absorption spectrometry (AAS) method, after prior microwave mineralization.

2. 3. Determination of the content of vegetable dyes

The extract was prepared by mixing 0.1 g of plant material with 2.5 ml DMSO. The samples were left for about one hour in the dark at room temperature and then incubated at 65 °C (water bath) for 30 minutes. The content of chlorophyll dyes was determined by UV / VIS8453 spectrophotometer at the following wavelength: chlorophyll a - $\lambda = 663$ nm, chlorophyll b - $\lambda = 645$ nm, carotenoids - $\lambda = 470$ nm, anthocyanins - $\lambda = 534$ nm. The content of vegetable dyes was calculated according to the Arnon formula (1949) [9] with modification by Richardson et al. (2002). The amount of individual dyes was given in mg/g fresh weight.

2. 4. Determination of the content of phenolic acids

For the determination of phenolic acids, 1 g fresh weight of the plant was weighed, homogenized with 5 ml of 80% methanol and shaken for 1 h at room temperature. Subsequently, 0.7 ml of extract, 0.1 ml of hydrochloric acid solution, 0.1 ml of Arnov reagent and 0.1 ml of sodium hydroxide were collected and mixed. The reference sample consisted of a sample containing distilled water instead the sample. Absorbance was measured 30 seconds after the addition of the sodium hydroxide solution to the reference sample, at a wavelength of 490 nm. The content of phenolic acids is expressed in mg CAE/g fresh weight.

2. 5. Determination of flavonoids content

For the determination of flavonoids, 1 g fresh weight of the plant was weighed, homogenized with 5 ml of 80% methanol and shaken for 1 h at room temperature. The total flavonoid content was determined using a colorimetric method using $AlCl_3$ aluminum chloride. Then, after 30 minutes incubation at room temperature, the absorbance at zero wavelength (without the presence of extract) was measured at 425 nm. The content of flavonoids was expressed on the basis of the calibration curve as mg of quercetin per 1 g fresh weight. Plants (mg QE / g fresh weight).

2. 6. Determination of superoxide dismutase activity SOD

The test sample contained 50 µl of plant extract, 1 ml of TRIS-EDTA pH 8.2 and 1 ml of 0.2 mM pyrogallol solution. 50 µl of distilled water was added instead of the extract to the control. Absorbance was measured after 10 minutes of addition of pyrogallol at 420 nm.

2. 7. Determination of pyrogallol peroxidase activity POD

The designation was made according to method B. Chance, A.C. Maehly (1955) [10].

3. RESULTS AND DISCUSSION

3. 1. Analysis of biometric parameters

The results of the biometric measurements (Fig. 1) for one of the crop variants (for the contamination of various zinc-particle and nanoparticle forms at a concentration of 10 mg) were presented. Dependencies in this variant correspond to the remaining variants of the experiment. The results show that statistically significant differences exist between the length of the entire plant at exposure to 100 nm zinc nanoparticles and the control sample. Total length reduction of the whole plant was about 15%. By analyzing all the biometric parameters of the crop in this crop variant, it can be seen that the highest length reduction was recorded for the root zone of the plant.

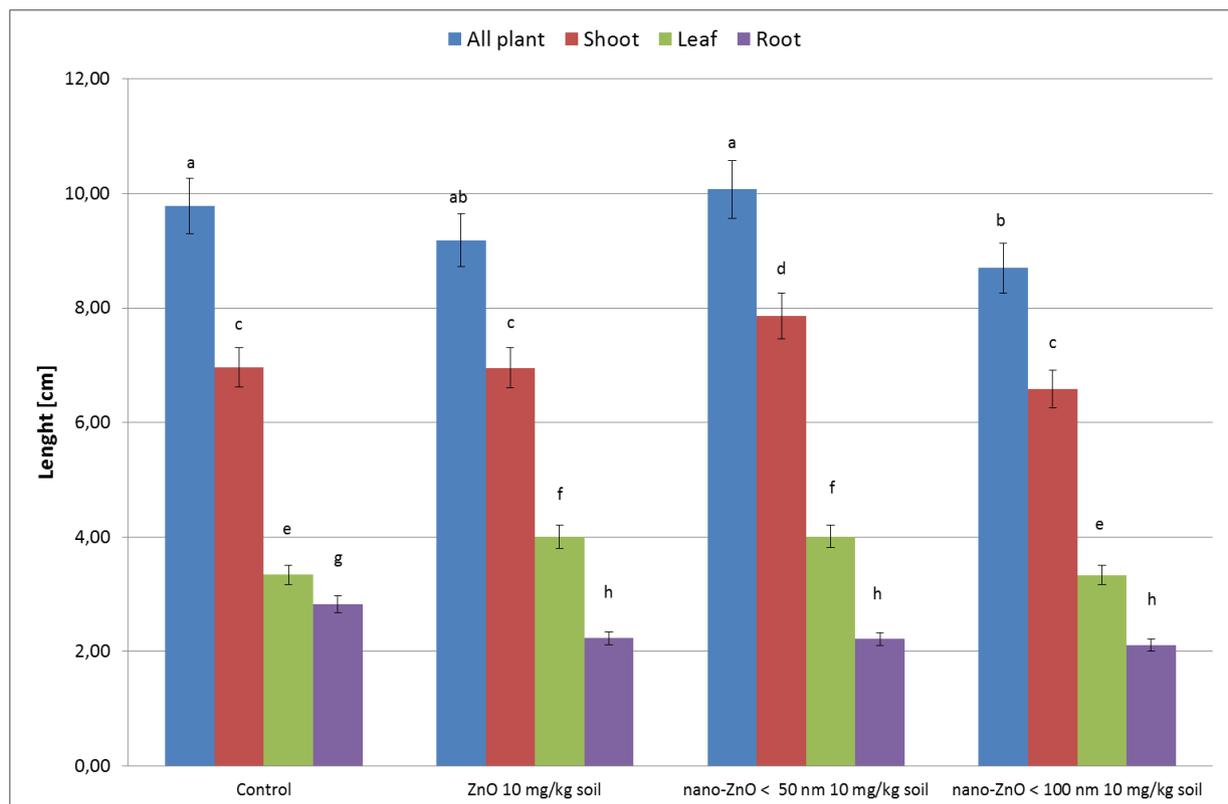


Figure 1. Comparison of the length of individual plant parts for the concentration of 10 mg Zn /kg of soil

Similar results were obtained by Jain et al. [11]. They investigated the effect of excess zinc on maize (*Zea mais* L.), oat (*Avena sativa*), pea (*Pisum sativum*) and sunflower (*Helianthus annuus* L.). Researchers have observed a marked inhibition of sunflower and pea growth at a dose of 33 mg Zn / kg sand. All the plants were also reduced, both the root and the shoot. Other species showed a similar reaction at a concentration of 66 mg Zn / kg sand.

Disante et al. [12] also showed growth inhibition at elevated zinc concentrations in soil. Also, studies by Wang et al. [5] on the effect of zinc nanoparticles on maize growth showed a decrease in yield in the highest concentration of Zn. Similar correlations were also received by Zhang et al. (studies on a tea) [3], as well as Wu and in and Jiang et al, who studied rice [13,14].

3. 2. Tolerance index

The study (Fig. 2) demonstrated the stimulating effect of zinc oxide nanoparticles on <50 nm particle size and 1 mg concentration, probably due to its small size and ease of migration. Inhibition of growth was observed at all concentrations of bulk zinc oxide and zinc oxide nanoparticles on <50 nm particle size at 10 mg and 100 mg and <100 nm particle size concentrations at 1 mg and 10 mg.

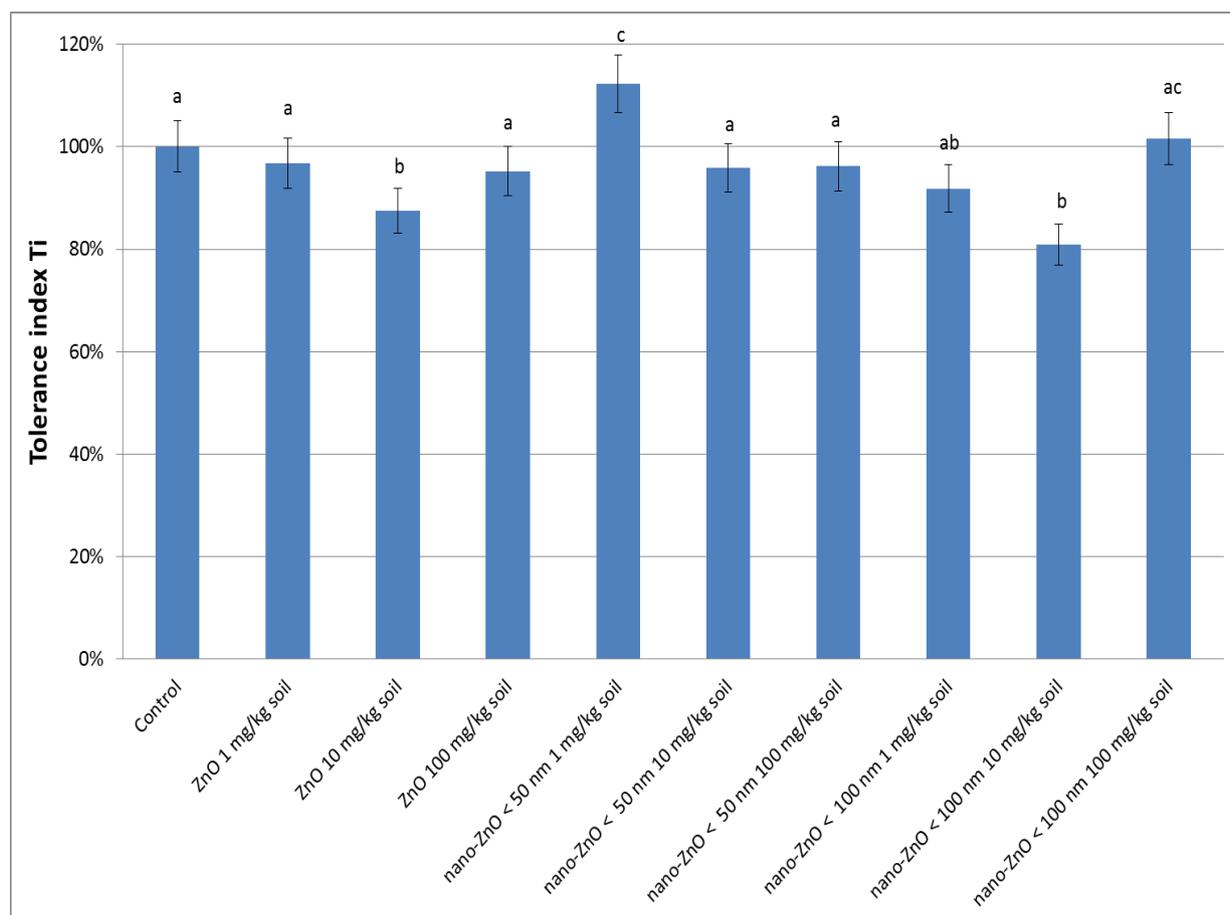


Figure 2. Tolerance index for individual variants of experience

3. 3. Zinc content in *Lepidium sativum*

The results (Fig. 3) show that *Lepidium sativum* accumulates in its shoots low concentrations of Zn. Comparing the Zn concentrations in the plant in various variants of the experiment, it can be seen that the plant collects Zn in its upper parts proportionally to the concentration in the soil. The higher the Zn concentration in the soil, the higher the Zn concentration in the plant, regardless of the form of Zn used for soil contamination. The highest concentration of Zn was recorded in the shoots on a substrate contaminated with Zn nanoparticles of size >50 nm.

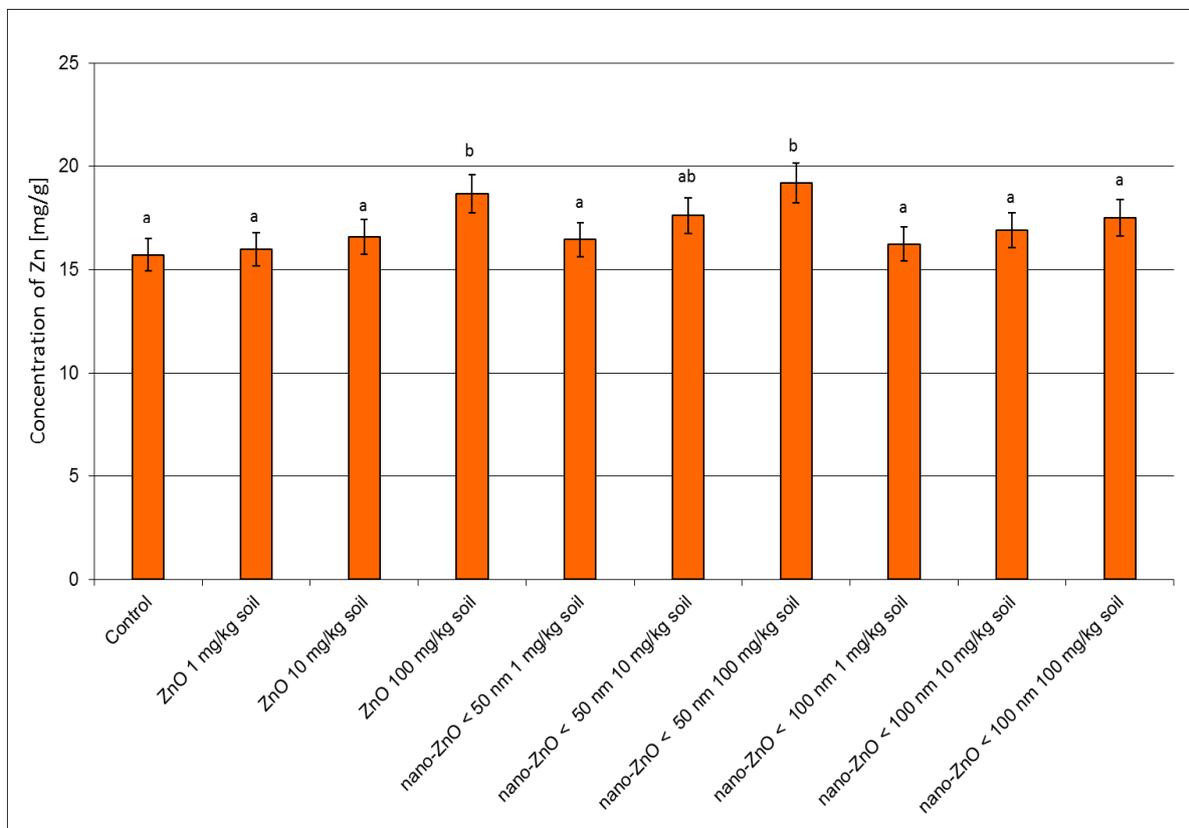


Figure 3. Zinc content in *L. sativum* depending on zinc concentration in soil

Wang et al. [5] also examined the level of zinc taken by plants. The results showed that maize and oats collected higher amounts of zinc in the root than in the shoot (contrary to peas and sunflower). The most sensitive maize collected 38-113 mg of Zn in 5 plants.

For our research only the amount of zinc in shoots of *L. sativum* was checked. However, the inhibitory effect of 15-20 mg Zn on the plant in the case of zinc molecules and nanoparticles of size <100 nm has already been demonstrated. Interestingly, similar concentrations for nanoparticle zinc with a size of <50 nm, in a variant of 1 mg Zn / kg soil, showed a stimulating effect on the growth of *L. sativum*.

3. 4. Non-enzymatic cell metabolites - content of phenolic acids and flavonoids

Flavonoids are natural compounds of the nature of polyphenols, they constitute a very large group of phenolic secondary metabolites. The presence of hydroxyl groups in the flavonoid ring makes them resistant to oxidative stress. The mechanism of action of flavonoids consists in neutralizing free radicals, chelating metal ions and inducing enzymes: superoxide dismutase as well as peroxidases [15].

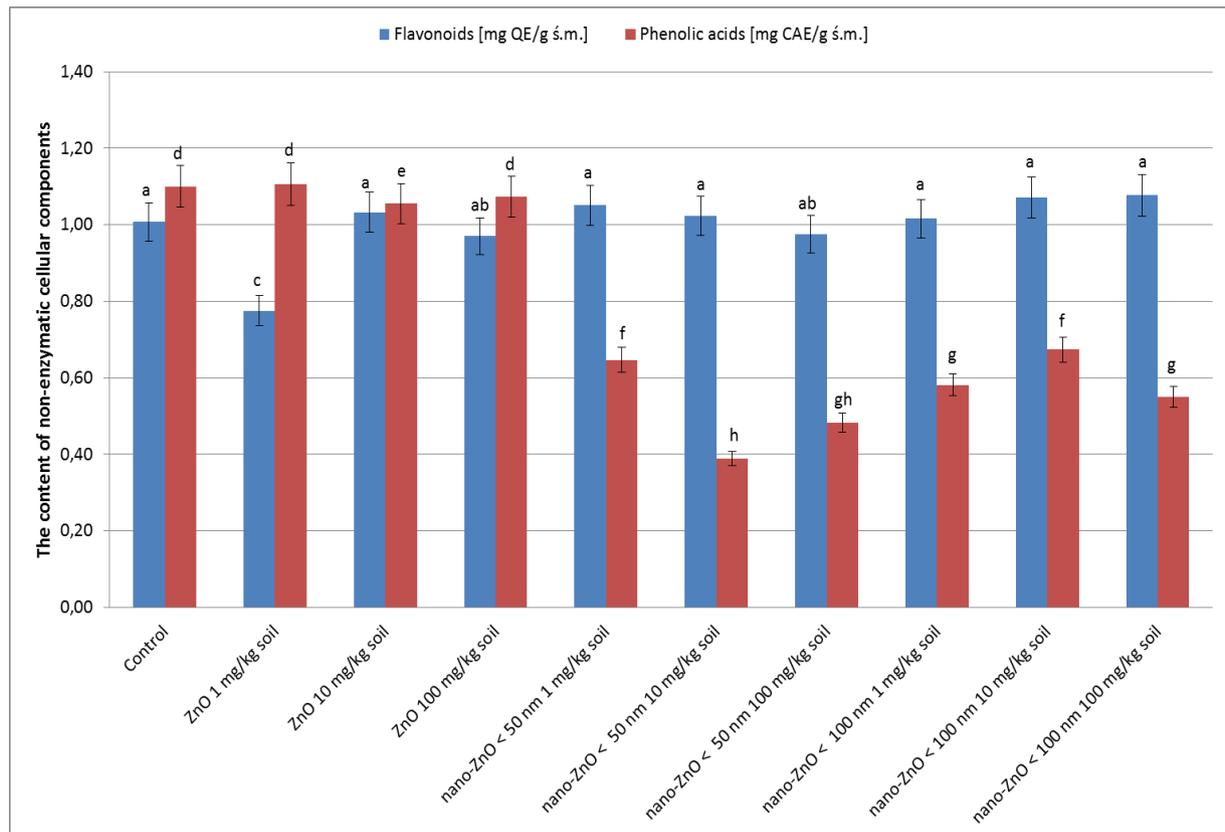


Figure 4. The content of non-enzymatic cellular components in *L. sativum* shoots

The concentrations of flavonoids (Fig. 4) in the shoots of *L. sativum* cultivated in different variants of contamination remain at a similar level, regardless of the variant of the experiment. Only Zn in the molecular form, at a concentration of 1 mg, resulted in a statistically significant reduction in the concentration of flavonoids. Analysis of the concentration of phenolic acids (Fig. 4) in the shoots of plants cultivated on soils contaminated Zn in various forms and concentrations showed a decrease in their content, particularly noticeable for plants cultivated on zinc nanoparticles. This may be due to limited activation of the antioxidant system.

Zhang et al. in received the opposite results when examining the effect of excess zinc on tea. They observed an increase in flavonoid content in tea leaves [3]. However, their study was conducted under hydroponic conditions, which may lead to differences in zinc extraction from the medium. It is easier to transport ions from water than from soil (better accessibility).

3. 5. Plants pigments - chlorophyll a and b, carotenoids and anthocyanins

Carotenoids are non-enzyme antioxidants that can be synthesized by plants under unfavorable environmental conditions. Their role is to remove and deactivate free radicals. Carotenoids are compounds that include xanthophylls and carotenes. Anthocyanins are natural plant dyes that give a variety of colors to flowers and fruits. Their color depends on the pH of the environment and chelation by metal ions. In terms of chemical structure, anthocyanins are glycosides. They are mainly found in flowers, fruits and seeds, but also in plant leaves. Chlorophyll is an organic compound found in green plants (green pigment) that converts light energy into chemical energy that is used in photosynthesis. Chlorophylls easily exchange magnesium ions to divalent metal ions such as iron (gray color), copper (green color) or zinc (also green) [15].

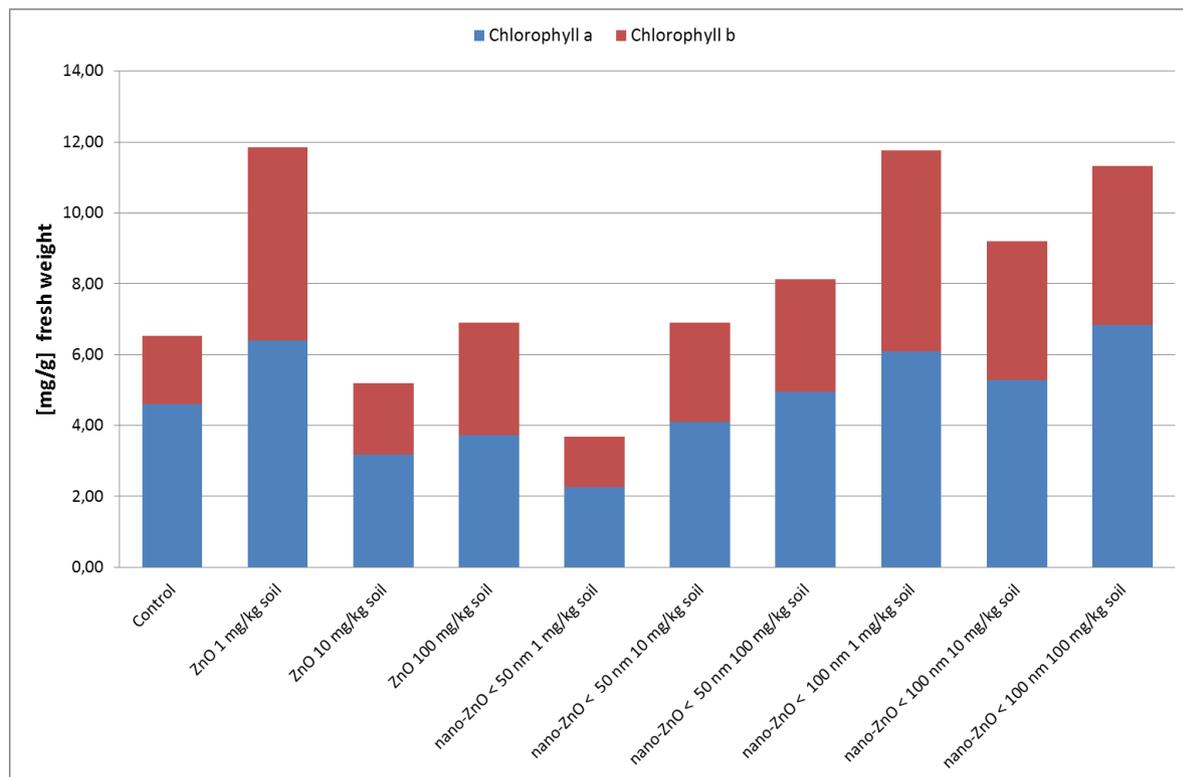


Figure 5. The content of chlorophyll a and chlorophyll b in plants

The obtained results (Fig. 5) showed an increase in chlorophyll a and chlorophyll b in all variants except chlorophyll a for ZnO at <50 nm nanoparticles size in concentration of 1 mg and 10 mg, <100 nm nanoparticles size in concentration of 1 mg. Spectrophotometric analyzes of carotenoid and anthocyanin concentrations in plant cells were performed during exposure to various zinc compounds at various concentrations. The analyzes indicated that the carotenoids and anthocyanin content increased during exposure to Zn ions, regardless of their form and concentration, compared to the control sample. This proves the activation of the plants antioxidant system. The difference in the concentration of these compounds was shown in the graph (Fig. 6). The highest concentration of carotenoids was observed in a plant sample grown

on soil contaminated with ZnO nanoparticles of size >100 nm, at a concentration of 100 mg. Comparing the concentration of carotenoids in plants when cultivated on soil contaminated by any zinc form - zinc concentration in the middle (10 mg Zn / kg soil) caused a decrease in carotenoids concentration, but no correlation was observed for the remaining concentrations.

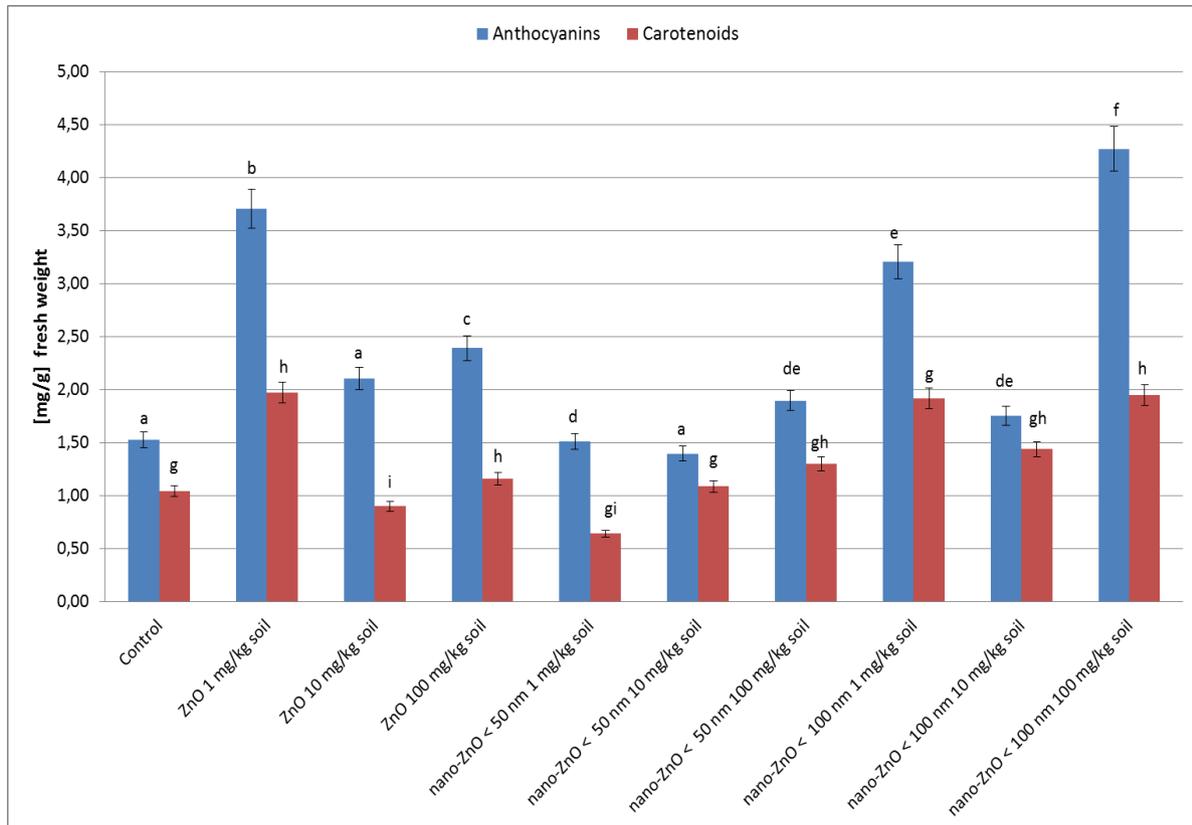


Figure 6. Concentration of carotenoids and anthocyanins for various variants of experiment

Wang et al. studies showed a decrease in chlorophyll a, chlorophyll b and carotenoids irrespective of the dose used (400-32000 mg nano-Zn / kg soil) [3]. Also, Tiecher et al. in studies conducted by them on maize showed a drop of chlorophyll a chlorophyll b at all tested concentrations (30, 90, 180 or 270 mg Zn / kg soil) relative to the control plants [16]. In contrast, the increase in chlorophyll and carotenoid content was observed by Radha et al., in studies conducted on sugarcane (*Saccharum spp.*) with excess zinc content soil [11].

3. 6. Activity of pyrogallol peroxidase (POD)

Pyrogallol peroxidase is considered a key enzyme involved in the removal of reactive oxygen species as it participates in the distribution of H₂O₂ within plant cells. The increase in POD activity is related to lipid peroxidation in the cell, which is caused by an increase in H₂O₂ concentration [15].

Analysis of the results (Fig. 7) showed an increase of POD activity in the shoots of plants cultivated on Zn contaminated soil in particle form, regardless of concentration, and

Zn in the form of nanoparticles size <100 nm, at 10 and 100 mg / kg. These results indicate the activation of the enzymatic antioxidant system, which means that the plant undertakes treatments aimed at neutralizing the toxic activity of Zn in these forms and concentrations. The effect of ZnO nanoparticles size <50 nm on POD activity has been interesting. 1 mg ZnO with a nanoparticle size <50 nm in the soil resulted in a significant increase in POD activity, while a further increase in the concentration of nanoparticles in the soil contributed to the reduction of the enzyme activity relative to the control. This may be due to partial inhibition of POD activity due to toxic effects of ZnO of <50 nm in high concentrations.

A similar correlation was observed by Radha et al., in studies conducted on sugar cane grown on soil with elevated zinc content [11].

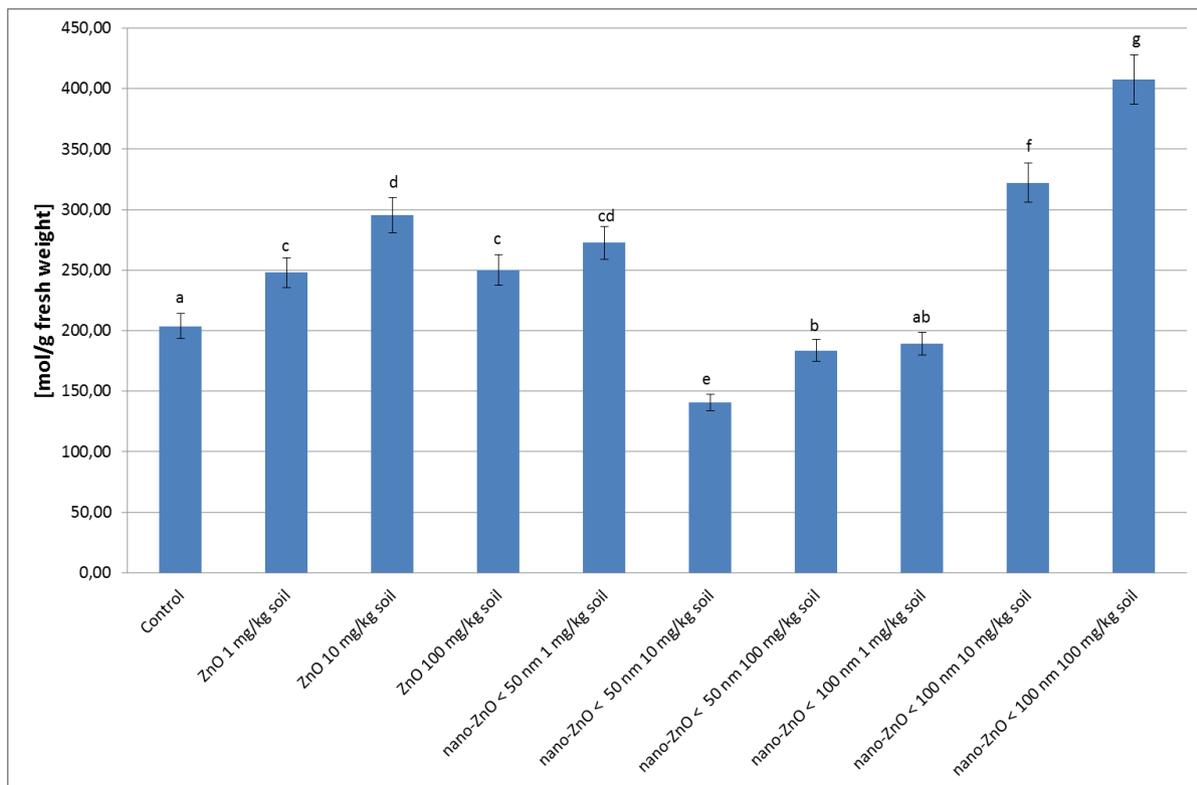


Figure 7. Pyrogallol peroxidase activity according to the variant of the experiment

3. 7. Activity of superoxide dismutase (SOD)

Superoxide dismutase is an enzyme that is the first line of cell defense against the O₂ radical, by converting this radical to molecular oxygen and hydrogen peroxide. The presence of SOD in chloroplasts, mitochondria and peroxisomes has been demonstrated, ie., in cell organelles involved in RFT production [17].

Analysis of SOD activity (Fig. 8) showed an increase in enzyme activity in plants cultivated on Zn-contaminated soil. Exposure of the plant to zinc nanoparticles resulted in a decrease in SOD activity in ground parts of the plant, regardless of their size and concentration.

A similar effect was obtained by Blasco et al., who conducted a hydroponic study of the effect of zinc on cabbage (*Brassica rapa*). They showed that concentrations of 0.05 and 500 μM Zn resulted in an increase in SOD activity [18]. A similar relationship was observed by Radha et al., In studies conducted on sugar cane (*Saccharum spp.*) on soil with elevated zinc content [11].

In the case of zinc nanoparticle studies, the opposite effect was obtained by Wang et al. Their maize studies showed a slight increase in SOD activity at a concentration of 32,000 mg nano-Zn / kg of soil and no significant effect at lower concentrations [5].

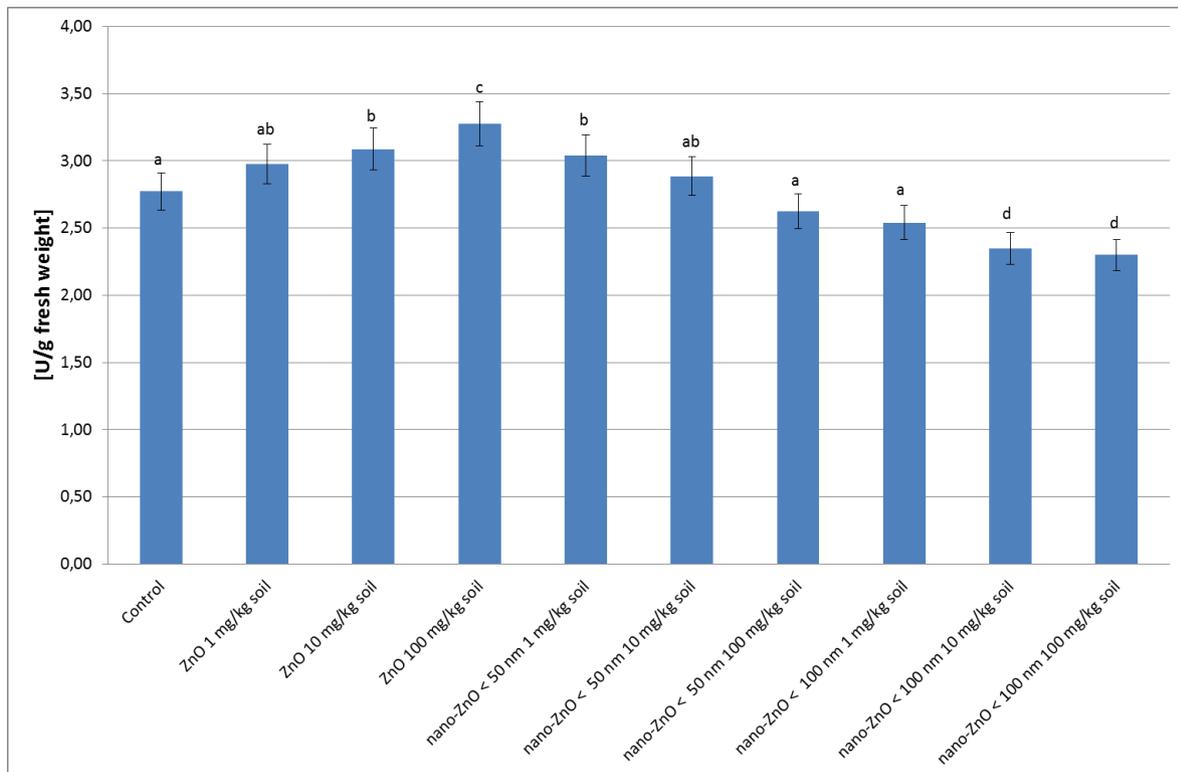


Figure 8. Activity of superoxide dismutase in *L. sativum* cultivated on soil of different concentration and zinc form

4. CONCLUSIONS

In the case of zinc molecules and nanoparticles with a size of <100 nm, a negative effect on the root of *L. sativum* at 100 mg Zn / kg soil can be observed. In the conducted experiment, reduction of the root part of the plant, both biomass and length, takes place more often than the reduction of the shoot of the plant. No negative effect was observed with nanoparticle zinc <50 nm size. Greater inhibition of growth on soil with Zn in concentration 10 mg Zn/kg soil and nanoparticle size of <100 nm and 10 mg concentration than correspondingly higher concentrations may result from oxidative shock at lower concentrations and activation of antioxidant systems at higher concentrations. In all analyzed variants, increased accumulation of Zn was observed in the upper parts of the plant with an increase in its

concentration in the soil. In the case of molecular zinc no activation of the non-enzymatic antioxidant system was observed, whereas the enzymatic system was activated. Zinc nanoparticles of size <50 nm caused partial degradation of chlorophyll, and an increase in SOD and POD activity. Nanoparticle of size <100 nm reduced the concentration of phenolic acids in plant shoots. In this variant, a significant increase in SOD activity was also observed, indicating activation of the enzymatic antioxidant system of plants.

Biometric measurements and tolerance indexes were made for whole plants, but further analyzes (enzymatic and non-enzymatic oxidative systems) were only carried out on the above-ground parts of plants. Reduction of the length and biomass of the root system, suggest toxic effects of zinc nanoparticles on this part of the plant. In order to confirm these hypotheses it is necessary to carry out further research on the remaining metabolites. It is necessary to undertake further research aimed at detailed analysis of the root part of the plant (Zn accumulation and physiological response to Zn soil contamination).

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