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Evaluation of the Toxicity of Copper Oxide Nanoparticles toward Pea Seeds

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ABSTRACT

This work considers the use of copper oxide nanoparticles (CuO NPs) for the pre-sowing treatment of pea seeds as a trace element fertilizer. CuO NPs were chosen since Cu-containing forms are actively involved in the construction of necessary proteins and enzymes, as well as in the processes of growth and development of cells, tissues, and plants. However, many works reported that CuO NPs have a toxic effect on crops as well. Therefore, the purpose of this work was to evaluate the toxicity of CuO NPs towards pea seeds. It was found that the best indicators of changes in the length of roots and sprouts occur in the treatment with 0.1 mg/L CuO NPs. At the same time, the length of roots and sprout of pea seeds at 100 mg/L CuO NPs was the lowest compared to other samples. Preliminarily, the results obtained indicated the potential toxic effect of CuO NPs on pea seeds at concentrations ≥ 1 mg/L. However, anatomical and histological examination showed that the toxic effect starts at 10 mg/L. The results obtained will provide a basis for further study of the multidirectional effect of CuO NPs on other crops at various concentrations to determine the optimal concentrations with growth-stimulating effects for sustainable agriculture.

Keywords: *CuO NPs, Nanoparticles, Ecotoxicology, Sustainability, Agriculture*

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INTRODUCTION

Currently, the problems of studying the positive and negative effects of nanomaterials on biological objects are becoming particularly acute (Klaper *et al*., 2014; Zhang *et al*., 2022). Such studies are becoming extremely relevant, as the range and number of nanoparticles (NPs) entering the environment are expanding (Eweje *et al*., 2019; Serra *et al*., 2019). Therefore, it is necessary to develop methods for assessing the effects of nanoparticles on living organisms, and the development of nanotechnology becomes an integral part of the implementation of the plan for the scientific and innovative development of industry (Ramanathan, 2019; Kumah *et al*., 2023).

Biosafety of nanotechnology, the study of the behavior of nanoparticles in the environment, and living organisms, including plants, is the subject of numerous studies (Sukhanova *et al*., 2018; Abbas *et al*., 2022; Zhang *et al*., 2024). It is worth noting that nowadays nanomaterials are widely used in optics, chemical technologies, medicine, perfumery and cosmetics industry, agriculture, etc. (Shafiq *et al*., 2020; Neme *et al*., 2021; Lan, 2022).

Notably, in experimental studies on the bioassay of NPs, preferences are usually given to plants (Ghosh *et al*., 2019; Orefice *et al*., 2023; Sousa *et al*., 2024). Plants are diverse and accessible objects that are sensitive to external low-intensity factors (Emmanouil *et al*., 2024). It is known that NPs with a size of less than 10 nm are able not only to penetrate a plant cell but also to integrate into the membrane (Das *et al*., 2016; Parkinson *et al*., 2022). It should be noted that plants cultivated *in vitro* are a good model test object for evaluating the effects of NPs that can be introduced into the nutrient medium (Gawas *et al*., 2023; Tansley *et al*., 2024). At the same time, the study of morphogenesis, cytogenetic parameters, and the interaction of NPs with intracellular structures is promising (Singh *et al*., 2020; Cardellini *et al*., 2023).

Interestingly, metal or metal oxide NPs overcoming the membranes of plant cells can affect the cytoplasmic enzyme systems (Gowtham *et al*., 2024; Yu *et al*., 2024). However, studies on the effect of NPs on biological objects and enzymatic systems are extremely ambiguous or contradictory (García-Locascio *et al*., 2024; Gul *et al*., 2024; Rehman *et al*., 2024). At the same time, the dependence of the responses of test objects to the presence of NPs on the level of their biological organization and habitat has not been practically studied, which makes it difficult to analyze the risk of exposure to NPs pollution in natural ecosystems.

This work considers the application of copper oxide nanoparticles (CuO NPs) for the pre-sowing treatment of pea seeds as a trace element fertilizer. CuO NPs were chosen since Cu-containing forms are actively involved in the construction of necessary proteins and enzymes, as well as in the processes of growth and development of cells, tissues, and plants (Printz *et al*., 2016; Rehman *et al*., 2019; Shabbir *et al*., 2020). However, many works reported that CuO NPs have a toxic effect on crops as well (Rajput *et al*., 2018; Naz *et al*., 2020; Yang *et al*., 2020; Xu *et al*., 2023). Therefore, the purpose of this work was to evaluate the toxicity of CuO NPs towards pea seeds.

MATERIALS AND METHODS

CuO NPs were obtained by direct deposition in an aqueous medium; copper II acetate was used as a precursor of CuO NPs. The stabilizing agent was hyaluronic acid. Sodium hydroxide was used to precipitate the product. In the first stage, 1.99 g of copper II acetate and 1.99 g of stabilizer were dissolved in 90 mL of distilled water. The solution was heated to 90 °C followed by the addition of 5 mL of 10 M NaOH with continuous stirring for 30 min. The resulting sol was centrifuged at 4000 rpm for 15 min, and the precipitate was dried in a drying chamber at 90 °C (Gvozdenko *et al*., 2022).

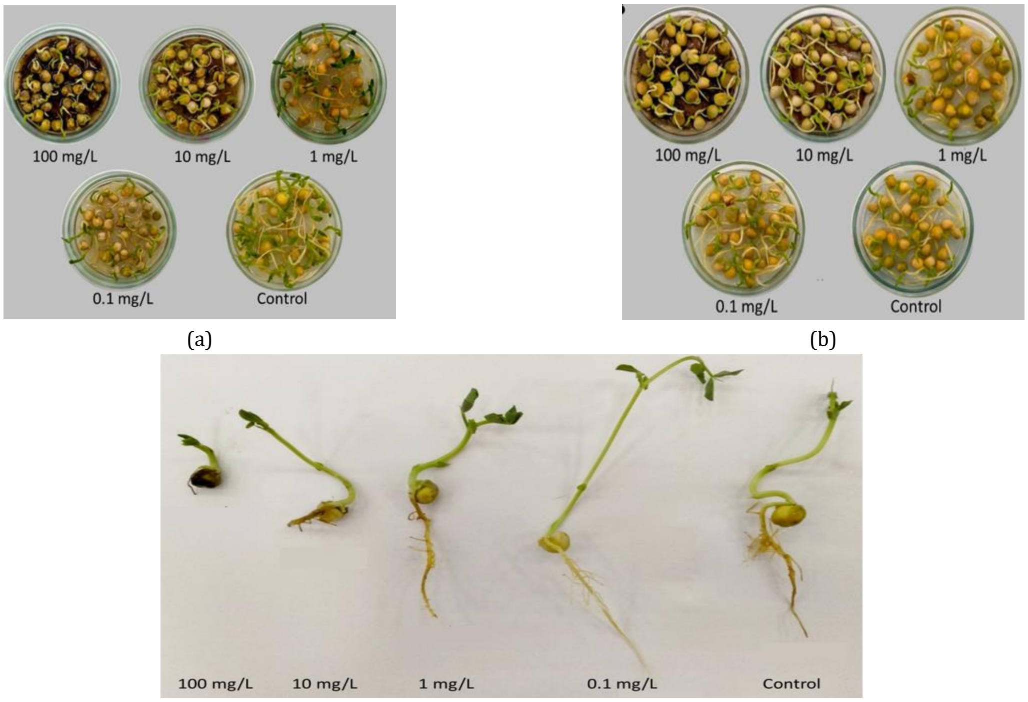
Pea seeds (75 units per group) were placed by 25 units in Petri dishes on filter paper under optimal humidification conditions at a temperature of 20 °C for 7 days. The ratio of the liquid phase and seeds was 4:5. Considering the results of the literature review, the liquid phase was used as follows: distilled water (control group), 0.1 mg/L CuO NPs solution (experimental group 1), 1 mg/L CuO NPs solution (experimental group 2), 10 mg/L CuO NPs solution (experimental group 3) and 100 mg/L CuO NPs solution (experimental group 4). Germination energy, germinability, and linear dimensions of seeding and roots were evaluated according to the ISTA (2006) standard every 3 days during 9 days of germination (Blinov *et al*., 2023; Nagdalian *et al*., 2024).

For the histological study, histological micropreparations of seeding from each group were prepared using the microtome MZP-01 Technom with the microtome cooler OMT-28-02E (KB-Technom, Yekaterinburg, Russia). Histological sections were made at a distance of 5 mm from the seed with the average thickness of the cut of 0.05 mm. Micropreparations were stained with phloroglucinol (Lenreactive, St. Petersburg, Russia) in the presence of hydrochloric acid (Lenreactive, St. Petersburg, Russia) and were processed on a Levenhuk D870T microscope (Levenhuk, Tampa, FL, USA) with a Levenhuk C510 digital camera at magnification 100×. Micrographs were processed in the Levenhuk ToupView 3.7 program (Levenhuk, Tampa, FL, USA) (Nagdalian *et al*., 2023).

The experiments were carried out in threefold biological and fivefold analytical repetition. All parameters obtained were submitted to one-way analysis of variance (ANOVA) and Student's T-test ($p < 0.05$) through the statistical package STATISTICA for Windows (Statsoft, Tulsa, USA). Data on roots and seeding length were statistically processed using Python 3.10 software with the Jupyter Notebook web-based interactive computing platform using the *pandas, numpy, sklearn, matplotlib, and seaborn* libraries (Source). Microsoft Excel 2010 and Origin software were also used for histograms and graphs creation based on the results of the data processing (Nagdalian *et al*., 2024).

RESULTS AND DISCUSSION

Visual observation of *in vitro* germinating pea seeds showed that by the 3rd day of the experiment, there was a pronounced inhibition of seed growth and development when treated with 100 mg/L CuO NPs. Interestingly, seeds treated with 1 and 10 mg/L CuO NPs also turned out to be less developed than the control sample. At the same time, at 0.1 mg/L CuO NPs, an unexpected effect of growth and development stimulating effect was achieved, which can be checked and compared in **Figure 1**.



(a) (b)

(c)

Figure 1. Control and experimental groups of pea seeds on day 3 (a) and day 9 (b, c) of observation.

Notably, the visual effect was supported by data on linear dimensions of roots and sprouts of pea seeds of experimental and control groups. The collected data were mathematically and statistically processed and presented in several options of interdependences **(Table 1, Figure 2)**.

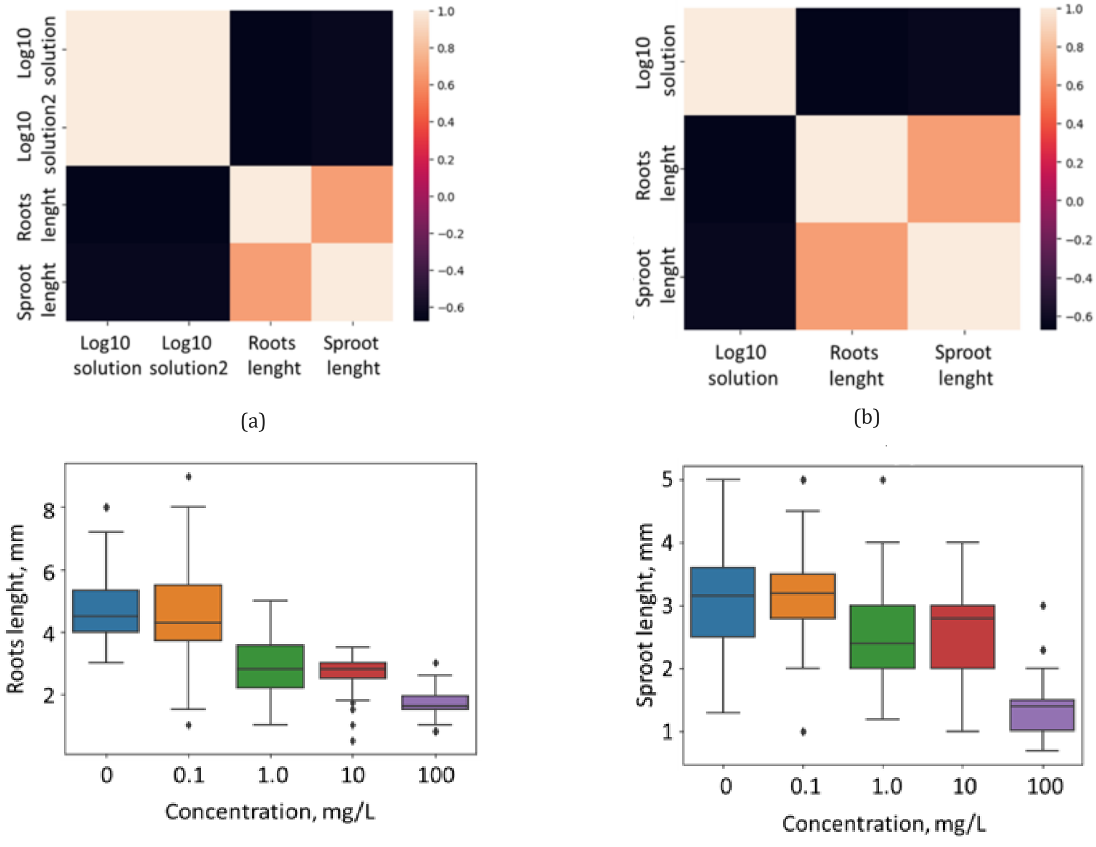
Table 1. ANOVA of dependence of roots and sprout length on the concentration of CuO NPs.

Index	Sum_sq	dF	F	PR (> F)
		Roots length		
Solution	305.844	1.0	130.451	1.083e-26
Residual	1050.341	448.0	NaN	NaN
		Sprout length		
Solution	136.653	1.0	143.399	7.343e-29
Residual	426.925	448.0	NaN	NaN

One-way ANOVA testing **(Table 1)** shows that there is a significant difference in the length of roots and sprouts depending on the concentration of the solution. Since the concentration of the solution varies exponentially, concentration was turned into a logarithm function (Koch, 1966). Interestingly, during logarithmization, there was a problem associated with the mean concentration for control samples (0 mg/L), which is mathematically absurd. Therefore, along with the decimal logarithm (log10_solution) a new function was introduced (log10_solution2) based on Eq. (1) (Reynolds & Stauffer, 2021):

$$\text{log10\_solution2} = \text{log10\_solution} + 2 \tag{1}$$

Thus, the results of statistical data processing are presented in **Figure 2** and **Table 2**.

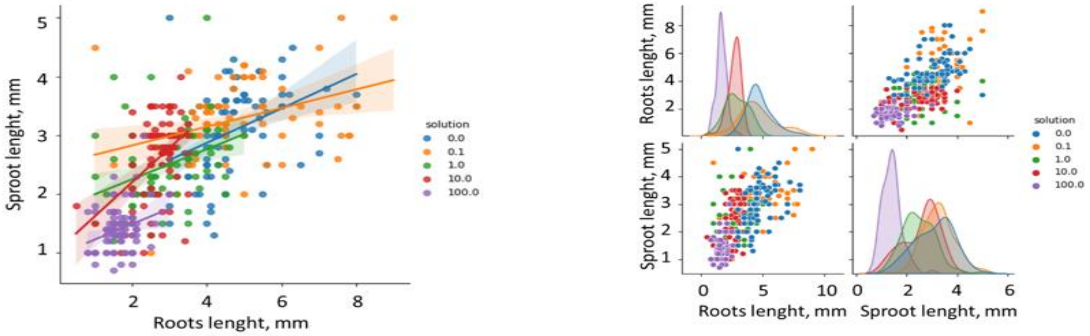


(a)

(b)

(c)

(d)



(e) (f)

Figure 2. Results of mathematical data processing: correlation maps of the length of roots and sprout and logarithm functions of CuNPs concentration (a, b), box plots on the dependence of the length of roots (c) and sprout (d) on CuO NPs concentration, a scatterplot of dependence of sprout length on roots length (e) and the smoothed histograms on the dependence of sprout length on roots length (f). Note: "log10_solution" is a decimal logarithm of concentration and "log10_solution2" is log10_solution + 2 (for instance, 1 instead of -1 or 2 instead of 0, etc.).

Table 2. Statistically data processing.

Index	Number	Solution	Root	Sprout	Log10_solution	Germination_flg	Log10_solution2
Count	450.00	450.00	450.00	450.00	450.00	450.00	450.00
Mean	15.50	22.22	3.05	2.35	0.40	0.92	0.40
Std.	8.66	39.11	1.73	1.12	1.02	0.27	1.02
Min.	1.00	0.00	0.00	0.00	-1.00	0.00	-1.00
25%	8.00	0.10	1.80	1.50	0.00	1.00	0.00
50%	15.50	1.00	3.00	2.50	0.00	1.00	0.00
75%	23.00	10.00	4.10	3.17	1.00	1.00	1.00
Max.	30.00	100.00	9.00	5.00	2.00	1.00	2.00

Where "number" is the number of seeds in the petri dish, "solution" is a concentration of a solution (mg/L), "root" is a root length, "sprout" is a sprout length, "log10_solution" is a decimal logarithm of the concentration, "germination_flg" is many germinated seeds, "log10_solution2" is log10_solution + 2 (for instance, 1 instead of -1 or 2 instead of 0, etc.).

Thus, according to **Figure 2** and **Table 2**, it is possible to determine the effect of CuO NPs on the length of roots and sprout of germinated pea seeds. Notably, the best indicators of changes in the length of roots and sprouts were observed in samples of the first experimental group treated with 0.1 mg/L CuO NPs. At the same time, the length of roots and sprout of pea seeds from the 4th experimental group (100 mg/L CuO NPs) was the lowest among other samples. Samples of the 2nd (0.1 mg/L CuO NPs) and the 3rd (1 mg/L CuO NPs) had an average length of roots and sprout. Preliminarily, the results obtained revealed the potential toxic effect of CuO NPs on pea seeds at concentrations ≥ 1 mg/L. However, to declare the toxicity of CuO NPs, it should be confirmed by other research methods.

In parallel, other integral biological indicators reflecting the effect of CuO NPs on pea seeds are germination energy and germinability (Luo *et al.*, 2024). The results of the calculation of germination energy and germinability of pea seeds of experimental and control groups are shown in **Table 3**.

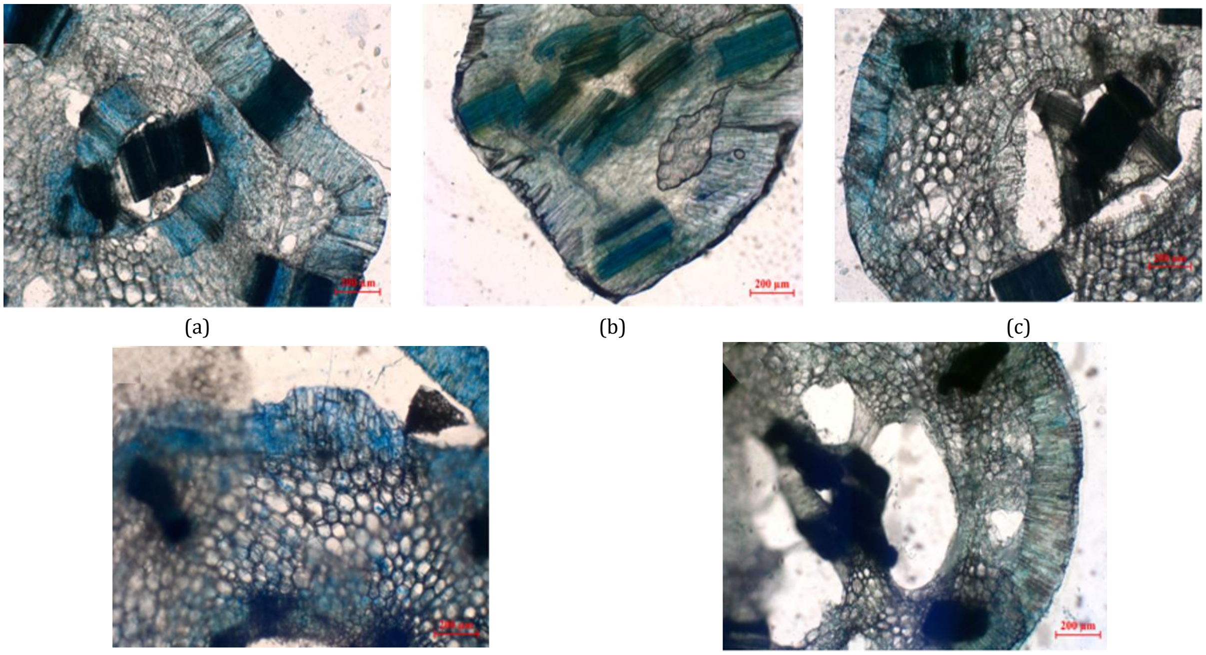
Table 3. Germination energy and germinability of pea seeds of experimental and control groups.

The concentration of CuO NPs	Germination energy (%)	Germinability (%)
0 mg/L (Control)	93.30	93.40
0.1 mg/L	95.20	95.20
1 mg/L	90.76	90.76
10 mg/L	90.56	89.56
100 mg/L	87.10	87.10

Table 3 shows that seeds from the 1st experimental group (0.1 mg/L CuO NPs) had the highest germination energy. The percentage of pea germination from the 4th experimental group 4 (100 mg/L CuO NPs) was the lowest among other samples. Samples from the 2nd (0.1 mg/L CuO NPs) and the 3rd (1 mg/L CuO NPs) had average values of germination energy. Thus, it is possible to conclude that CuO NPs have a stimulating effect on pea seed germination at a concentration of 0.1 mg/L. At the same time, it was found that CuO NPs have an inhibition effect on pea seed germination at a concentration of 100 mg/L. Interestingly, the results obtained are consistent with data reported by Kadri *et al.* (2022) and Ochoa *et al.* (2017). It is important to note, that obtaining friendly full-fledged seedlings is an important prerequisite for the formation of high seed yields (Riikonen & Luoranen, 2018). According to **Table 3**, the average germination rate for pea samples was 92.1%. It was found that the best indicator of germinability was observed in pea seeds treated with 0.1 mg/LCuO NPs (95.20%). At the same time, pea seeds treated with 100 mg/L CuO NPs and 10 mg/L

CuO NPs had germinability of less than 90%, which is not suitable for field sowing (Bellaloui *et al.*, 2017).

Results of histological examination of cross-sections of pea sprouts from experimental and control groups are presented in **Figure 3**.



(a) (b) (c)

(d) (e)

Figure 3. a) Micrographs of histological cross-sections of sprouts of pea seeds of the control group, b) the 1st experimental group, c) the 2nd experimental group, d) the 3rd experimental group, e) and the 4th experimental group.

For conciseness, the results of the analysis of histological examination were structured and tabulated **(Table 4)**.

Table 4. Results of anatomical and histological examination.

Concentration of CuO NPs	Epidermis	Mesophyll	Stomatal apparatus
0 mg/L (Control)	Cells are tightly closed and evenly thickened, their walls are not strongly convoluted; the cell membranes are clear-cut, with noticeable pores.	Homogeneous, clearly defined.	Stomata are small and numerous.
0.1 mg/L	Cells are tightly closed, and evenly thickened, the cell walls are not strongly convoluted; the cell membranes are clear-cut, with noticeable pores.	Homogeneous, clearly defined.	Stomata are small and numerous.
1 mg/L	Cells are tightly closed and evenly thickened, their walls are not strongly convoluted; the cell membranes are clear-cut, with noticeable pores.	Homogeneous, clearly defined.	Stomata are small and numerous.
10 mg/L	Cells are tightly closed, have an uneven thickening, and the cell membranes are even-shaped.	Homogeneous, clearly defined.	Stomata are small but not numerous.
100 mg/L	Cells are tightly closed, have an uneven thickening, and the cell membranes are even-shaped.	Crumbly, heterogeneous.	Stomata are small but not numerous.

According to the results of **Table 4**, it can be concluded that treatment of pea seeds with CuO NPs at concentrations of 0.1-1 mg/L leads to improvement in their anatomical and histological parameters (mesophyll is homogeneous, epidermal cells are dense). However, it was found that at concentrations of 10-100 mg/L, anatomical and histological parameters of samples deteriorated with a noticeable decrease in elasticity of the mesophyll and epidermis. Thus, the results of histological examinations confirmed the toxicity of CuO NPs towards pea seeds at concentrations more than 10 mg/L. The results obtained are in line with previous studies on this topic (Mukherjee *et al.*, 2016; Rajput *et al.*, 2018; Essa *et al.*, 2021) and will become a basis for further study of the multidirectional impact of CuO NPs on other crops at various concentrations for determination of optimal concentrations with growth stimulating effect for sustainable agriculture.

CONCLUSION

Metal or metal oxide nanoparticles overcoming the membranes of plant cells can affect the cytoplasmic enzyme systems. However, studies on the effect of NPs on biological objects and enzymatic systems are extremely ambiguous or contradictory. Initially, the results obtained revealed the potential toxic effect of CuO NPs on pea seeds at concentrations ≥ 1 mg/L. In parallel, the anatomical and histological parameters of seeds treated with 10-100 mg/L deteriorated with a noticeable decrease in elasticity of the mesophyll and epidermis. Thus, the results obtained revealed the toxicity of CuO NPs towards pea seeds at concentrations of more than 10 mg/L. This knowledge will become a basis for further study of the multidirectional impact of CuO NPs on other crops at various concentrations for the determination of optimal concentrations with growth-stimulating effects for sustainable agriculture.

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ETHICS STATEMENT: None.

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