Size-dependent biological effects of copper nanopowders on mustard seedlings

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Abstract. Correlation between the size of copper nanoparticles and effects they produce on white mustard seedlings were studied in the paper. Copper nanopowders with average particle sizes of 45 and 200 nm with concentrations of 0.01–1000 wt ppm of seeds were used to treat white mustard seeds. Nonlinear dose effects were displayed by copper nanopowders obtained by chemical synthesis with the particle size of 35-60 nm. For 45 nm nanoparticles the highest biological activity with regard to germinating energy, 3-day stem and root weight was registered at 5.0 wt ppm concentration, while for root length and weight of the 7-day stems the 1.0 wt ppm concentration had the most pronounced effect. Copper submicron powder with average particle size of 200 nm displayed some effect only after 7th day of the experiment at high concentrations (from 100 to 1000 wt ppm) according to the measurements of length and weight of the seedlings. The obtained results can be used in the development of copper nanoparticles-based preparations for pre-sowing treatment of seeds.

1. Introduction

The effects produced by low doses (LD) of biological active substances have been attracting attention of scientists since the end of the 20th century both in Russia and abroad. Small doses cause biological effects that are comparable or even stronger than those observed under the action of higher doses of the same substance [1-3]. The nature of the biological effects caused by various factors in SD indicates that the cause of this phenomenon is the processes associated with the action of a particular regulatory signal in biological systems [4-6]. All living organisms have a uniformly organized system the function of which is to perceive, read, spread and destroy information continuously received from the environment. The biological effect produced by chemicals and physical-chemical factors in SD should be associated with the transfer of information that is universal for any biological objects, which with a high degree of reliability functions in the system: agent (preparation) - cell and its structures. The system of supramolecular structures of the cell microenvironment satisfies these requirements [7-9].

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We have undertaken a prolonged study of the biological activity of metal nanopowders of trace elements at various concentrations, and the research results show that even at low concentrations metal nanoparticles can have some biological effect. Therefore, a detailed study of the effect of "small doses" of nanomaterials is relevant and is of scientific interest.

If we take into consideration that this effect can manifest itself both at the level of macromolecules and cells [10-13] and at the level of a living organism and even a population and that small and ultra-small doses are those, the effectiveness of which cannot be explained with the help of traditional concepts, then doses of trace elements in nano-form, characterized by quantum-mechanical properties, in the amount of 0.01-10 wt ppm of seeds can be referred to as “low or ultra-low” as compared to salts of trace elements used in doses of 100-500 wt ppm of seeds depending on the crop and soil and climate conditions.

The studies of Polischuk, Nazarova, Churilov D G [14-16] and Churilov G I [17,18] revealed that the energy of nanoparticles (NPs) of biogenic metals (iron, cobalt and copper) of a certain size (25-60 nm) stimulate the processes of self-organization of biological systems and their adaptation to external conditions. The peculiarities of chemical interaction of nanoparticles with a liquid medium are among the determining factors in stimulating the development of plants which makes it possible to use nanometals as micronutrients and growth stimulants having a prolonged effect. It is assumed that nanocrystalline metals have great potential in mineral nutrition and energy impact and due to uncompensated bonds they easily form complex compounds with organic substances. As a result, they activate the synthesis of various enzymes affecting carbohydrate and nitrogen metabolism, amino acid synthesis, the reactions of photosynthesis and cell respiration [19].

One of the characteristic properties of the effect of low dose (LD) is a non-monotonous, “discontinuous” dose-effect relationship. In most cases, the maximum activity is observed at a certain dose range, separated by a so-called “dead zone” or decrease in the index. For metal nanoparticles the oscillatory nature of the dose dependence of the magnitude of the studied parameters is observed, as well as the presence of a "dead zone" (dose interval between two peaks of biological effects, where biological activity does not manifest itself), which is caused, in our opinion, by the wave nature of the propagation of spatial rearrangements of supramolecular structures under the influence of the energetic effect of nanoparticles. In each case the significance of the most important biological processes, including participation in gene expression, has been revealed and proved [20-22].

2. Materials and methods

This study is aimed at revealing the influence of copper nanoparticle size on white mustard seedlings. Copper nanopowders are being actively studied as an alternative form to copper-based micronutrient fertilizers and hormone-based growth stimulants. Ecological safety and stimulating activity of growth and development were revealed for this preparation. In the first set of experiments nanopowders with particle size of 35–60 nm at concentrations of 0.01–100 wt ppm of seeds were studied. In the second set of experiments nanopowders with particle size of 200 nm were used. Copper nanopowders (figure 1) were obtained at NUST “MISiS” by chemical precipitation of metal hydroxides from salt solutions, followed by their low-temperature reduction in hydrogen flow with subsequent passivation [23,24].

Nanoparticle suspensions were prepared in distilled water. Sample quantities (from 1 mg to 10 g in increments of 10 times) were weighed using a ViBRA HT analytical balance (Shinko Denshi, Japan, accuracy ± 0.0001 g), poured into a preliminary prepared container with 1 l of water and stirred with a glass rod for 20 seconds. Then the suspension was dispersed using ultrasound for 5 minutes (power - 300 W, frequency - 23.740 kHz) according to the developed sample preparation method. An IKA MagicLab MK (IKA Werke, Germany) laboratory machine was used as a mechanical disperser. The speed of the homogenizer when manufacturing suspensions of NPs using mechanical effects was 20,000 rpm, pressure 2.5 bar and processing time 5 and 10 minutes. Distilled water for control experiments was treated in the same way without adding nanoparticles (NPs). Then the resulting suspensions were used for treating mustard seeds.
Studies were conducted at the Nano Center for the AIC at Ryazan State Agrotechnological University Named after P.A. Kostychev. Seeds were placed in Petri dishes, 50 seeds in each in an 8-fold replication. Then the Petri dishes with seeds were placed in a thermostat where they germinated at a constant temperature (t = 23 °C). Germinating energy and viability were determined. The length of sprouts and roots of each plant was measured with a ruler. The mass of seedlings was measured with a digital analytical balance Ohaus. The control seeds were soaked in distilled water, and the test seeds - in a solution of copper nanopowder (NP Cu) of a certain concentration (table 1).

Statistical data manipulation was carried out using the electronic software package Statistica 6.

| #  | Variant     | Concentration, wt ppm of seeds |
|----|-------------|--------------------------------|
| 1  | Control     | Seeds soaked in distilled water for 30 minutes |
| 2  | NP Cu, 35-60 nm | 0.01; 0.05; 0.1; 0.5; 1.0; 5.0; 10.0; 100.0; 500.0; 1000.0 |
| 3  | NP Cu, 35-60 nm | 0.1; 0.25; 0.5; 1.0; 1.5; 2.0; 2.5; 3.0; 3.5; 4.0; 4.5; 5.0; 5.5; 6.0; 6.5; 7.0; 7.5; 8.0; 8.5; 9.0; 9.5; 10.0 |
|    | 2nd set of experiments |                                  |
| 1  | Control     | Seeds soaked in distilled water for 30 minutes |
| 2  | NP Cu, 200 nm | 0.01; 0.05; 0.1; 0.5; 1.0; 5.0; 10.0; 100.0; 500.0; 1000.0 |

In order to reveal the dose-effect relationship we have identified the parameters suitable for clear demonstration of stimulating and depressing effect or the lack of any effect. These parameters are germinating energy, laboratory viability, length and weight of 7-day-old seedlings of mustard seeds in the laboratory.

3. Results and discussion
In the first set of experiments the effect of copper nanopowder suspension with a particle size of 35-60 nm on the growth and development of white mustard in the laboratory was studied. The results are presented in table 2 and in graphs (figures 2-4).
Figure 2. Germinating energy (a) and viability (b) of white mustard seeds treated with NP Cu 35-60 nm.

In the range of nanoparticles concentrations of 0.01-1000 wt ppm of seeds for copper NPs (Cu), a periodic increase in germination (peaks were noted at 0.5 and 5.0 wt ppm) and germinating energy (figure 2) was observed with maximum concentrations at 0.1 and 5.0 wt ppm. Starting from 100.0 wt ppm, both parameters dropped significantly, since high concentrations inhibit the growth and development of mustard seeds.

The dependence between the NPs dose and length of the white mustard stem and root also has several peaks and falls (figure 3): the first jump in the stem length was observed at 0.01-0.05 wt ppm (+67 %), the second one was at 1.0-5.0 wt ppm (+92-96 %), while the maximum value of the parameter was recorded at 10.0 wt ppm (+128 %). The root length measurements showed peaks at 0.01 wt ppm (+23.9 %), 0.1 wt ppm (+52.2 %) and 1.0 wt ppm (+81.5 %). Starting from 10.0 wt ppm there was a sharp decrease in the root length and then with an increase in the concentration of nanoparticles in the nutrient medium, deviation from the control in the stem and root lengths became negligible.

Figure 3. Length of white mustard stem (a) and roots (b) treated with NP Cu 35-60 nm.

A similar dependence is observed for the weight of stems, both on the third and seventh days after germination (figure 4). The weight of 3-day stems was maximum at 5.0 wt ppm (+62.4 %). The maximum weight of 7-day stems was observed at 1.0 wt ppm (+118.6 %).

The dependence of the root weight on the increase in copper NP dosage is dome-like. The peak in weight of both 3-day (+69.7 %) and 7-day roots (+44.2 %) was recorded at a concentration of 0.5 wt ppm.
Figure 4. The weight of white mustard seedlings treated with NP Cu 35-60 nm: a) stem weight (3 days); b) root weight (3 days); c) stem weight (7 days); d) root weight (7 days).

Thus, in the range of concentrations from 0.01 to 5.0 wt ppm a direct correlation between the activity of nanoparticles and their concentrations is observed. The peak in NPs activity appeared at 10 wt ppm. At higher concentrations the dose-effect dependence was not manifested.

One more experiment was performed for a more detailed study of the effect of the ultra-low dose (ULD) of copper nanopowder. The results of this experiment are presented in figures 5 - 7.

According to the experimental data, copper nanoparticles increase germinating energy at concentrations of 0.25 wt ppm by 3 %, while at concentrations of 2.5 wt ppm, 4.0 wt ppm, 5.0 wt ppm and 6.0 wt ppm the increase is 11 % as compared to the control.

Figure 5. Germinating energy of white mustard seeds treated with NP Cu 35-60 nm.
The dependence of the length of 7-day seedlings became more pronounced with increase in the dose and the length values reached their maximum at 5.5 wt ppm, exceeding the control by 43.4%. The dependence of the length of roots had several peaks and significantly exceeded the control at doses of 2.5 wt ppm (+20.1%), 3.5 wt ppm (+22.1%) and 5.5 wt ppm (+27.3%).

Figure 6. The length of white mustard seedlings treated with NP Cu 35-60 nm.

In this experiment the total weight of all stems and roots of the seedlings from a whole Petri dish was measured and the mean was calculated. From figure 7 one can see that the effect of ULD with regard to the mass of mustard seedlings is most demonstrative at the initial concentrations from 0.1 to 6.5 wt ppm, while at 7.0 wt ppm this dependence disappears sharply. Further increase in the dose produced negligible effect on this parameter. The peaks in the parameter values were observed at the following concentrations: 0.1; 0.5; 1.5; 3.0; 4.0 and 5.5 wt ppm.

Figure 7. Weight of white mustard seedlings treated with NP Cu 35-60 nm.

The graph of the root mass versus the dose of copper NPs is somewhat different. At first, the index rises sharply at 0.1 wt ppm (+66.7%), then the graph takes the dome-like shape reaching its peak at 5.5 wt ppm where it exceeds the control by almost 3.7 times (+273.3%). These results confirm the dose dependence at concentration of 5.5 wt ppm and its absence at concentrations close to 10 wt ppm.

In set 2 of experiments the effect of suspension of copper nanopowders with a particle size of 200 nm was studied. Figures 8 and 9 represent the results.

L.A. Blumenfeld [25] expressed the idea of parametric resonance as a possible mechanism for the action of ultra-low concentrations of biologically active substances at the cellular and subcellular
levels. He believes that parametric resonance occurs when the timing of parameters, triggered by biologically active substances of intracellular processes and the characteristic time of the substance approach to the target. As a result of binding of the active substance to its target, the enzyme (receptor) enters a conformationally non-equilibrium state, which at a certain stage of relaxation ensures its maximum activity. In the framework of these ideas, the observed decrease in enzyme activity with increasing the dose of the active substance is also explained. The ideas on the allosteric interaction of catalytic centres in an enzyme molecule are considered in some scientific works [26,27]. If an enzyme or receptor contains several centres with different affinities for the substrate, and when low doses of the preparation are administered, its molecules predominantly bind to the highly efficient centre of the enzyme. When increasing doses, the second enzyme centre takes effect. It interacts allosterically with the first centre, lowering its affinity for the substrate, and then all the molecules that were associated with the first centre go off of it. They can contact it again only after the concentration of the preparation approaches the value of the dissociation constant of the ligand complex with the first centre, reached under the influence of the second centre. Consequently, a biological system that is affected by MD may react to the first, fastest single molecules, and not to their stationary concentrations (the moment of the first attainment).

To establish the dependence of NP activity on particle size, the effect of copper nanopowder 200 nm in size was studied. Figure 8 shows that such particles affect the growth of plants in a lesser degree. It is possible that, due to their large size, they penetrate into the seeds only partially and partially interact with cell organelles. However, with an increase in their concentration, the growth of the aerial parts of mustard increased by 12 % as compared with the control, while the underground part growth increases by 30 %. It is possible that in the process of bioaccumulation some changes occur in cells leading to increased permeability.

![Figure 8](image)

**Figure 8.** Length of white mustard stems (a) and roots (b) treated with NP Cu 200 nm.

Analyzing the length of 7-day mustard seedlings we can say that almost no dose-effect dependence was observed. The length of the treated seedlings exceeded that of the control group only at very high concentrations (100-1000 wt ppm). Thus, there is no sense in talking about the effect of ULD, since the number of nanoparticles was limited and about the same. This dependence appears again when analyzing the weight of 7-day seedlings as represented in figure 9. Discontinuities in the parameters were observed at the doses of 0.01, 1.0 and 10.0 wt ppm. This is apparently due to the fact that the manifestation of the dose-effect dependence requires long-term interaction of the living system and particles of 200 nm in size, which increases their number in the cell and its effect on its structures. Perhaps a longer experiment (10-15 days) would show a completely different effect of LD.
According to the results of the experiment, we can conclude that the size of nanoparticles has a very large impact on both their biological activity and the manifestation of the effect of low doses.

The pore diameter in cell walls is 5–20 nm, which determines their ability to fractionate particles. Therefore, only NPs and their agglomerates, whose size is smaller than large pores, can presumably penetrate into the cells and reach the plasma membrane. But the throughput of cells can vary [28,29], since new pores can form under the action of NPs. Therefore, nanoparticles 40–60 nm in size are biologically active and comply with the “low dose” effect.

The particle size of 200 nm limits their access to cell organelles, but long-term interaction and an increase in their number contribute to bioaccumulation and they become able to influence the cell structure.

The cell membrane is coated with many receptors that, through extracellular binding to a specific ligand (or nanoparticles), transmit a signal into the cellular space [30]. This signal can serve as the beginning of the launch of many biochemical reactions. It can also lead to internalization of the ligand (and nanoparticles) through endocytosis. The potential pathway of NP penetration through the double lipid layer of the cell membrane can be represented as it follows from [31]. During endocytosis the plasma membrane forms a hole structure, surrounding NPs, and drawing them into the cell. NPs can also enter the cell using proteins and ions as transporters [32–34]. As NPs enter the cell, they interact with organelles (for example, the endoplasmic reticulum, the Golgi complex and the endolysosome system) and are drawn into metabolic processes [35].

4. Conclusion

It is possible to distinguish two main factors that influence the formation of the properties of nano systems. These are the change in the thermodynamic state of nano systems as compared to the classical one and the appearance of quantum-size effects with a decrease in the characteristic dimensions of structural elements. In general, starting from a certain point nano systems produce the so-called size effect, i.e. dependence of the properties of nano systems on the size of nanoparticles. According to Yamakoshi Y. and others [35], the size effect is observed when the size of the microstructure block coincides with a certain critical length characterizing the phenomenon (the mean free path of electrons and phonons, the size of magnetic domains, the critical radius of the dislocation loop, etc.), which is characteristic of the nanoparticle structure.

Based on the results and summarizing the obtained data, we can draw the following conclusions:

1. For copper nanopowder obtained by chemical synthesis the effect of low doses is most pronounced when the particle size is in the range between 35 and 60 nm. Both in the first and in the second experiment, the dose-effect relationships had their own characteristics. The most active concentrations for germinating energy were 0.5 and 5.0 wt ppm, those for the root length were 0.01, 0.1 and 1.0 wt ppm, those for the mass of 3-day sprouts were 0.05 and 5.0 wt ppm, those for the mass
of 7-day sprouts were 0.1 and 1.0 wt ppm and that for the root mass was 0.5 wt ppm. And the concentrations differed from each other most often by 10, less often by 100 times.

2. For copper powders with particle size of 200 nm a significant increase in the length of seedlings is shown only at the maximum concentration, which is indicated in the absence of a dose-effect. But when analysing the weight of 7-day seedlings, this dependence re-appears. Slight peaks in parameters are observed at 0.01, 1.0 and 10.0 wt ppm.

The obtained results can be used in the development of copper nanoparticles-based preparations for pre-sowing treatment of seeds.

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References

[1] Burlakova E B and Khokhlov A P 1985 Biological membranes 2 557
[2] Burlakova E B 1994 News of RAS 64 1 5 425
[3] Ashmarin I P, Karazeeva E P and Lelekov T V 1999 Rus. Chem. J. 5 21
[4] Rajput V D, Minkina T, Suskova S et al 2018 Effects of Copper Nanoparticles (CuO NPs) on Crop Plants: a Mini Review BioNanoSci. 8 36 https://doi.org/10.1007/s12668-017-0466-3
[5] Voronkov M G, Dolmaa G, Tserenpil С, Ugtakhbayar О and Chimidtsogzol A 2005 Stimulating the germination of barley seeds in micromolar aqueous solutions of silatran and cresacin Report RAS 404 562-564
[6] Burlakova E B, Boykov P Ya, Panina R I and Karcev V G 1996 The bimodal effect of picolinic acid derivatives on the germination rate of wheat and peas Izv. RAS. Ser. Biol. 1 9-45
[7] Zaytsev S V, Efkanov A M and Sazonov L.A. 1999 Chem. J. 5 28
[8] Bogatyrenko T N, Redkozubova G P, Konradov A A, et al 1989 Biophysics 34 26 327
[9] Veselovskiy V A, Veselova T V and Chernavskiy D D 1999 Rus. Chem. J. 5 21
[10] Davis J M and Svendsgaard D J J 1990 Toxic and Env. Health 30 71
[11] Burlakova E B, Konradov A A and Maltsev E L 2003 Chemical Physics 22 2 21-40
[12] Veselova T V, Veselovskiy V A and Chernavskiy D S 1993 Stress in plants (Biophysical approach) Moscow Publ. House of Moscow University
[13] Gorskhova T A, Mishkina P V, Guryanov O P and Chemikosova S B 2010 Biochemistry 75 2 196-213
[14] Polischuk S D, Nazarova A A, Kutskir M V, Churilov D G, Ivanycheva Y N, Kiryshin V A and Churilov G I 2015 Mod. Appl. Science 9 6 354-364
[15] Polischuk S.D., Churilov G.I., Churilov D.G., Borychev S.N., Churilova V.V. 2018 Int. J. Nanotechnol. 7 4.36 231-236
[16] Samoylova M V, Churilov D G, Nazarova A A, Polischuk S D and Byshov N V 2017 Nano Hybrids and Composites 13 91-95 DOI 10.4028 /www.scientific.net /NHC.13.91.
[17] Churilov G I 2009 Herald of SamSU, Nat. Sc. Series 6 72 206-212
[18] Churilov G I 2010 Ecological and biological effects of nanocrystalline metals. Dis. Dr. of Biol. Sc., Balashikha
[19] Folmanis G Eh and Kovalenko L V 1999 Ultradispersed metals in agricultural production Moscow, IMET RAS
[20] Polischuk S, Fadkin G, Churilov D, Churilova V and Churilov G 2018 Forestry, IOP Conf. Series: Earth and Environmental Science 226 012020 doi:10.1088/1755-1315/226/1/012020
[21] Churilov G I, Polischuk S D, Kutskir M V, Churilov D G and Borychev S N 2015 Activators of Biochemical and Physiological Processes in Plants Based on Fine Humic Acids. IOP Conf. Ser.: Mater. Sci. Eng. 98
[22] Churilov G I, Polischuk S D, Kuznetsov D, Borychev S N, Byshov N V and Churilov D G 2018 Int. J. Nanotechnol. 15 4 258-279
[23] Dzidziguri Eh L, Kuznetsov D V, Lyovina V V and Sidorova E N 2000 Perspective materials 6
87–92

[24] Novakova A A, Kiseleva T Y, Lyovina V V, Kuznetsov D V and Dzidziguri Eh L 2001 J. of Alloys and Compounds 317 423-427
[25] Blumenfeld L A, Grosberg A Yu and Tikhonov A N 1991 Chem. Phys. 95 10 7541
[26] Blumenfeld L A 1993 Biophysics 1 129
[27] Burlakova E B, Konradov A A and Khudyakov I V 1990 Izvestiya AS, Ser. Biol. 2 184
[28] Nel A E, Mädler L, Velegol D, Xia T, Hoek E M V, Somasundaran P, Klaessig F, Castranova V and Thompson M 2009 Nature Materials 8 543-557
[29] Auffan M, Rose J, Bottero J-Y, Lowry G V, Jolivet J-P and Wiesner M R 2009 Nanotechnology 4 634–641
[30] Oberdorster G, Stone V and Donaldson K 2007 Nanotoxicology 1 2-25 DOI: 10.1080/17435390701314761.
[31] Jia G, Wang H F, Yan L, Wang X, Pei R J, Yan T, Zhao Y L and Guo X B 2005 Env. Sc. and Techn. 39 1378-1383.
[32] Boldyreva A A 1987 Biochemistry of membranes. Edocytosis and exocytosis. Moscow 2 p. 95
[33] Bulychev A G 1986 Cytology 28 4 387-402
[34] Sergeev P V, Shimanovskiy N L and Petrov V I 1999 Receptors of physiologically active substances Moscow, Volgograd, Seven Winds.
[35] Yamakoshi Y, Umezawa N, Ryu A, Arakane K, Miyata N and Goda Y 2003 J. Am. Chem. Soc. 125 12803-12809