The effects of CuO nanoparticles on wheat seeds and seedlings and Alternaria solani fungi: in vitro study

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Abstract. The paper analyses the influence of copper oxide nanoparticles (CuO NPs) dispersions (0.01…1 g/L) on seed germination capacity, development and biochemical status of wheat seedlings. Also the paper presents the results of the in vitro study of effect produced by CuO NPs (0.001…0.1 g/L) on Alternaria solani fungi. The most pronounced combined positive effect on the studied parameters has been observed at 0.01 g/L concentration of copper oxide nanoparticles. Improvement of germination capacity by 14.5 per cent and a twofold increase in the root and stem length compared to the control group have been recorded at this concentration. At higher NPs content in the dispersion stimulation was combined with toxic effects (decrease of root length). A slight impact of NPs on the activity of antioxidant system enzymes has been registered at concentrations of 0.01 g/L and 1 g/L. CuO NPs at 0.001...0.1 g/L concentrations inhibited Alternaria solani mycelium development and spore germination by 34-50 per cent, but the ionic solution of CuCl₂ ∙ 6H₂O displayed higher efficacy. The obtained results can be used for development of new micronutrient fertilizers and crop protection agents based on copper nanoparticles.

1. Introduction
Effective micronutrient fertilizers and crop protection agents play an extremely important role in obtaining high crops. The existing soluble metal salts-based preparations possess several drawbacks making them a constant source of environmental hazard as they require high application rates and they are quickly washed out from the soil. Unlike most modern growth stimulants and crop protectors, agrochemical agents based on nanoparticles of biogenic elements combine sufficient efficacy with comparatively low application rates thus reducing the adverse environmental impact.

Copper is an important micronutrient element essential for plant growth and development, it contributes to various important physiological processes acting as an oxidation-reduction reaction catalyst in mitochondria, chloroplasts and cell cytoplasm [1] or as electron transport in plant breathing processes [2]. At the same time a number of studies prove germicidal effect of copper nanoparticles comparable to that of silver NPs [3-5]. The authors of [6] have shown that copper can disrupt cell functions in several ways simultaneously thus decreasing the chances of resistance development by microorganisms. On the one hand, copper NPs have an ability of killing bacteria by destroying their cell walls and membranes. On the other hand, it is assumed that copper NPs penetrating into microorganism cells cause damage by interacting with phosphorus-containing and sulfur-containing compounds, such as deoxyribonucleic acid (DNA), due to high affinity of copper nanoparticles to...
compounds of this type. Bouson et al. have studied antifungal activity of copper benzene tricarboxylate against *Candida albicans*, *Aspergillus niger*, *Aspergillus oryzae* and *Fusarium oxysporum*. It has been shown that the agent effectively inhibits *C. albicans* growth rate while also having a pronounced effect on spore germination in *A. niger*, *A. oryzae* and *F. oxysporum* [7].

However, impacts of CuO NPs on widely used plants and common phytopathogenic microorganisms lack sufficient study. In most of the known works, the effect of nanoparticles on plants or on fungi is investigated separately. But nanoparticles are very variable in their physicochemical properties, which leads to contradictory results in various studies. Therefore, in the present study we analyses the influence of copper oxide nanoparticles on seed germination, on morphometric and biochemical parameters of wheat seedlings as well as on *Alternaria solani* development in vitro.

2. Materials and methods

2.1. Nanoparticles

CuO nanoparticles (NPs) were synthesized by the simple and convenient chemical precipitation method [8].

The crystalline phase of the nanoparticles was identified by X-ray powder diffraction (XRD) analysis (Diffray, Russia). The presence of elements was confirmed through Energy dispersive spectrum (EDS) (EDX SSD X-MAX, JAPAN). Scanning electron microscope (SEM- JEOl, JSM—6610 LV, JAPAN) technique were used to figure out the dimension and the form of nanoparticles. Nitrogen adsorption isotherms of the synthesized nanoparticles were analyzed by using a Nova 1200e analyzer (Quantachrome Instruments, USA). The surface area of the powder was calculated using the Brunauer-Emmette-Teller (BET) method.

CuO NPs were a monoclinic crystalline phase. The EDX spectra is revealed in a presence of copper and oxygen in the sample powder. The average size of CuO NPs according to electron microscopy was 50 nm. The specific surface area of the nanoparticles was 18.9 m²/g.

A detailed characterization of these nanoparticles was given by us in a previous article [9].

2.2. Nanoparticles suspensions

CuO NPs suspensions were prepared in distilled water. Quantities of nanoparticles were weighing on a ViBRA HT scales (Shinko Denshi, Japan) with an accuracy ±0.0001 g, poured into prepared dispersion medium and stirred for 20 sec with a glass rod. Well-mixed suspensions were processed for 5 min in an ultrasonic bath Ultrasonic Cleaner CD-4800 (Codyson, China) (70 W, 44 Hz, volume 1.4 L). The initial copper concentration in every solution was 1 g/L, the working suspensions were prepared by diluting the initial solution with distilled water to obtain 0.1 g/L and 0.01 g/L nanoparticles concentrations for wheat treatment and 0.1…0.001g/L concentrations for the microbiological experiment.

2.3. Comparison solutions

Solutions of Cu(OH)₂, Cu(metal), CuCl₂·6H₂O, CuO(bulk) (Sigma-Aldrich, ACS grade) with the equal copper concentrations were used for comparison in the wheat experiment while in the *Alternaria solani* experiment only CuCl₂·6H₂O solution was used for comparison.

2.4. Germination

Studies were carried out on winter wheat seeds (*Triticum aestivum* L., 1753), variety “Prestige”. Healthy seeds were surface sterilized in 10% sodium hypochlorite solution for 10 min and washed thoroughly with sterilized double distilled water [10]. The experiments were carried out in laboratory conditions under daylight at relative humidity of the air 80±5% and temperature 20±2°C. The seeds were sown in petri plates on moist filter paper, 30 seeds per each plate. For pre-seeding treatment 1%
solution of potassium permanganate was used. The NPs suspensions were used for wetting the paper filters. Distilled water was used for control.

The seeds were watered when necessary. Germination capacity and morphometric characteristics of the seedlings (root and stem length) were assessed on the seventh day after the onset of the experiment. Speed of germination was determined using the method suggested by McGuire (1962) [10]. The plants were washed and root was separated from shoot. Then the length of root and shoot was measured in centimeter (cm) using scale [11, 12].

Each experiment and assessment was carried out for at least three times. The analysis of statistically significant differences within the treated groups was carried out by means of analysis of variance (ANOVA) for a single factor with further application of Tukey’s multiple analysis of variance with a family error rate of 0.05. In some cases, the least significant difference was computed (multiple t tests without α adjustment).

2.5. Determining of peroxidase and catalase activity

The activity of peroxidase was determined by means of common spectrophotometric technique [13], based on the assessment of the velocity of chemical reaction of benzidine (4,4′-diaminobiphenyl) oxidation in the presence of hydrogen peroxide and peroxidase. 200-300 mg of raw plant material was ground in a cold porcelain mortar with a cold pestle with 0.5 ml of acetate buffer (pH 5.0). The obtained homogenate was centrifuged for 5 min at 12,000 g and cooled down to 4 °C. 0.98 ml of 0.2 M acetate buffer (pH 5.0), 0.5 ml of 0.01 % solution of benzidine hydrochloride, 0.02 ml of plant extract, 0.5 ml of 0.3 % of hydrogen peroxide were put into the spectrophotometer cuvette. 1.48 ml of 0.2 M acetate buffer (pH 5.0), 0.5 ml of 0.01 % solution of benzidine hydrochloride, 0.02 ml of plant extract were put into the control cuvette. Optical density was measured at the wave length of 590 nm every second for 120 seconds. The activity of peroxidase (per gram of the dry weight of the vegetable material) was calculated in relative units by means of the formula (1):

\[ A = \frac{AD\cdot V\cdot X}{(T\cdot L\cdot m\cdot \Delta m)} \]

where \( A \) – enzyme activity, \( AD \) – change of optical density during the reaction \( T \); \( V \) – total volume of the raw material, ml; \( X \) – correlation between the volume of the raw material put into the cuvette and the cuvette volume; \( T \) – time of reaction, s; \( L \) – optical path length of the cuvette, cm; \( m \) – weight of vegetable material, g; \( \Delta m \) – the difference between the weight of dry vegetable material and the weight of raw vegetable material.

The activity of catalase was determined by means of the spectrophotometric technique [14], as in the assessment of the velocity of the chemical reaction of decomposition of hydrogen peroxide with catalase. 250 mg of raw plant material was ground in a cold porcelain mortar with 0.5 ml of extraction buffer solution (50 mM phosphate buffer, pH = 7.0). The obtained homogenate was centrifuged for 5 minutes at 12,000 g and cooled down to 4 °C. 2.95 ml of 50 mM phosphate buffer (pH = 7.0), 30 µl of extract, 20 µl of 0.6 M hydrogen peroxide were put into the spectrophotometer cuvette. 2.95 ml of 50 mM phosphate buffer (pH = 7.0), 30 µl of extract were put into the control cuvette. Optical density was measured at the wave length of 240 nm every second for 120 seconds. The activity of catalase in relative units as per one gram of the dry weight was determined by means of the formula (1) [12].

The activity of enzymes was measured in three biological and analytical repetitions.
2.6. Microbiological experiment

*Alternaria solani* isolated into pure culture according to [15] was used in the experiment. All the isolated fungal isolates were maintained on potato sucrose agar medium at 20°C for 7 days. To induce sporulation, cultures were transferred on 23-25°C for 6 days on potato sucrose agar medium at natural day light with 16 h/day light. Specifically, fungal cultures were flooded with sterile water and conidia were gently dislodged with a glass plate. Mycelial and conidial suspensions were filtered through two layers of cheesecloth. Spore density was counted using a haemocytometer and adjusted to 1 · 10³ conidia per mL [16].

To study the effect of nanoparticles on *Alternaria solani in vitro* development (mycelium growth, sporulation intensity) 0.1…0.001 g/L of nanoparticles was introduced into agar medium with further fungal spore inoculation. After 7 days of incubation at 23-25°C the diameters of the colonies were measured.

To study the effect of nanoparticles on *Alternaria solani* spore germination 0.1 ml of spore suspension was introduced into 10 ml of liquid media, with nanoparticles concentrations of 0.1…0.001 g/L. The spores were incubated for 24 hours at 23-25°C. In each variant the amount of ungerminated spores expressed as a percentage in relation to the control batch was calculated according to Abbott’s formula [17].

The experiments were performed in three analytical replications.

3. Results

3.1. The effect of copper oxide on wheat

The analysis of CuO NPs impact on wheat seeds germination has shown that higher concentrations of nanoparticles in the solutions lead to increase in the value of the studied parameter by 14%, 15% and 18% correspondingly, compared to the control (Figure 1). The most pronounced effect was observed when copper hydroxide suspension was added to the growth media - germination capacity increased by 24% at the lowest concentration, by 15% at 0.1 g/L and by 13% at 1 g/L. Metallic copper suppressed germination by 10% at concentration of 0.1 g/L and stimulated it by 10% at concentration of 1 g/L. The influence of copper oxide in bulk on wheat seeds germination was negligible. For CuCl₂·6H₂O stimulating effect was observed at concentration of 0.01 g/L (+13%) while at 1 g/L inhibition of seed germination capacity (-20%) was registered.

![Figure 1](image-url)  
*Figure 1.* The effect of cupriferous material on wheat germination capacity (hereinafter: a) bar is mean, vertical line is standard error of the mean, and asterisk is significant difference from the control  
b) “concentration” on x axis means concentration of Cu (dry matter)).
The assess of copper containing substances influence on morphometric characteristics of seedlings (Figure 2 a, b) has shown that the maximal growth resulted from copper oxide samples with nano- and micro-sized particles at the lowest concentration (Figure 2 a). CuCl₂·6H₂O at the minimal concentration produced the most significant inhibiting effect of the stem growth, in this case the treated plants were more than 5 times shorter than the control. At concentration of 0.1 g/L the influence of both copper oxide and copper hydroxide on the studied characteristic was negligible. Metallic copper and CuCl₂·6H₂O at a medium concentration affected the seedlings growth adversely. The stems of the treated seedlings were 2.2 - 3 cm, i.e. almost twice, shorter than in the control. At concentration of 1 g/L the worst root development was observed in the plants treated with copper hydroxide and CuCl₂·6H₂O, with the stem length decrease by 3 and 2 cm correspondingly. It should be noted that the inhibiting effect of CuCl₂·6H₂O weakened when the substance concentration in the growth media increased. The maximal concentrations of copper oxide nanoparticles, metallic copper and copper oxide (bulk) had a moderate stimulating effect on the stems growth (+1.3 - 1.5 cm).

![Figure 2](image_url). The effect of cupriferous material on vegetative characteristics of wheat.

As in the case of stem growth, the maximal root growth was observed in CuO and CuO (bulk) variants at the minimal concentration - the root length increased by 2 and 3 times, correspondingly (Fig. 2 b). The rest of the samples in the minimal concentration had negligible effect on the root system development. Increase in copper concentration up to 0.1 g/L resulted in root growth inhibition, especially after copper hydroxide, metallic copper and CuCl₂·6H₂O application, in these cases the roots were more than twice shorter than in the control. Inhibition caused by CuO NPs was less significant, the roots were 1.3 times shorter than the control, while CuO (bulk) displayed no effect at all. At the substance concentration of 1 g/L root growth inhibition has been observed in all the variants. Copper hydroxide had the maximal adverse effect, the root length was reduced by a factor of 4 compared to the control. Treatment with CuO NPs reduced the root length by a factor of 2. And again, the minimal inhibition effect was observed when CuO (bulk) was added to the growth medium.

Multidirectional effects of cuprum nanoparticles on plants ranging from stimulation to inhibition have been described in a number of papers [18-20], while the actual mechanisms of the influence have yet to be studied thoroughly. One of the possible explanations is connected with the impact of nanoparticles on the antioxidant system of plants. This mechanism is often considered as the main one in evaluating the effects of metal based nanoparticles on plants [21-24].

The analysis of CuO NPs influence on biochemical status of the seedlings (Figure 3 a, b) has shown increase in peroxidase and catalase activity, the enzymes activity increasing linearly with the nanoparticles concentration. The minimum concentration of nanoparticles in the growth medium had no significant effect on the studied biochemical parameters.
It is worth noting that CuO NPs at the minimum concentration had the highest combined positive effect. CuO (bulk) also produced considerable stimulating effect on the vegetative organs development, though the germination capacity was lower than in the seeds treated with CuO NPs. Copper hydroxide had the most significant adverse effect on the studied parameters.

3.2. The effect of CuO NPs on Alternaria solani

The analysis of CuO NPs effect on mycelium formation and fungal colonies growth has established that high concentrations inhibit mycelium growth in Alternaria solani (Figure 4). The most significant influence was observed for CuCl₂·6H₂O at a concentration of 0.1 g/L.

CuCl₂·6H₂O at a concentration of 0.1 g/L suppressed germination of more than 65% of Alternaria solani spores compared to the control, thus making it the strongest inhibiting agent among the ones studied in this experiment, while in the control almost all the spores sprouted after 24 hours of incubation. Copper oxide nanoparticles also reduced the amount of
germinated spores, in this case the percentage of sprouting spores varied from 49% to 64% compared to the control (Figure 5).

![Graph showing the effect of cupriferous material on Alternaria solani spores sprouting.](image)

**Figure 5.** The effect of cupriferous material on *Alternaria solani* spores sprouting.

It should be noted that all the studied variants had a considerable suppressing effect both on *Alternaria solani* mycelium development and spores sprouting. The maximal inhibition was observed in the groups treated with CuCl₂·6H₂O, though CuO NPs also displayed a significant antifungal activity which corresponds the results from the study [25], where the authors have shown high efficacy of copper nanoparticles against *Alternaria alternata*, especially in combination with silver nanoparticles. The antimicrobial activity of copper and copper oxide nanoparticles has been described in a number of other papers [26-28].

The obtained results can be explained on the one hand by the fact that copper is an important essential trace element for plants, and on the other hand by the fact that copper compounds, including in nanoform, have proven fungicidal activity. At the same time, in high concentrations, nanoparticles are beginning to show phytotoxicity, probably associated with oxidative stress induction.

4. Conclusions

Thus, we have established positive effect of copper oxide nanoparticles at a concentration of 0.01 on the early stages of wheat ontogenesis. Marginal influence of nanoparticles at concentrations of 0.1 g/L and 1 g/L on the activity of enzymes of plant antioxidant system has also been observed. Suppressing effect of copper oxide nanoparticles at concentrations of 0.001-0.1 g/L on *Alternaria solani* development has been established, though ionic solutions of CuCl₂·6H₂O had a more significant inhibiting activity.

The obtained results can be used for development of new micronutrient fertilizers and crop protection agents based on copper nanoparticles.

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