A study on the biological activity of biosynthesized nanoparticles of metal oxides

A M Korotkova¹,², O B Polivanova³, I A Gavrish¹, M Y Koroleva⁴, E N Baranova⁵, and S V Lebedev¹,²

¹Federal Research Centre of Biological Systems and Agro-Technologies of the Russian Academy of Sciences, 29, 9 Yanvarya str., Orenburg, 460000, Russia
²Institute of Bioelementology, Orenburg State University, build. 16, 13, Pobedy ave., Orenburg, 460018, Russia
³Department of Genetics, Biotechnology, Breeding and Seed Russian State Agrarian University – Moscow Timiryazev Agricultural Academy, 49, Timiryazeva str., Moscow, 127550, Russia
⁴Institute of Modern Power Engineering and Nanotechnology, D. Mendeleev University of Chemical Technology of Russia, 9, Miusskaya sq., Moscow, 125047, Russia
⁵Laboratory of Cell Biology, All-Russian Research Institute of Agricultural Biotechnology, 42, Timiryazevskaya str., Moscow, 127550, Russia

E-mail: anastasiaporv@mail.ru

Abstract. There was synthesized a number of nanopowders of metals oxides (Fe₂O₃, Co₃O₄, ZnO, and CeO₂) in the aqueous extract from Petroselinum crispum leaves. There was performed the physicochemical qualification of the synthesized nanomaterials by the methods of UV spectrophotometry and the scanning electron microscopy. SEM-visualization showed CeO₂ powders had a spherical morphology (from 14 nm to 500 nm), Fe₂O₃ had a plate structure (more than 80 nm), and Co₃O₄ was presented in small cubic particles with sharp edges (from 20 to 100 nm) stuck together in large units (~ 1 µm), ZnO was characterized by a spherical, oval and hexagonal structure (from 60 to 160 nm). The analysis of cytotoxicity of the biosynthesized powders was carried out as counting dead cells in the roots of T. vulgaré stained with the vital dye Evans blue after 14 days of exposure with a preparation in a wide range of concentrations (from 10⁻¹ to 10⁻⁵ M). The results showed a dose-dependent increase in cell death at a greater extent on the apex of the root compared to the basal area. A remarkable (over 50%) decrease in the cell viability was recorded after the treatment of plants with Fe₂O₃ (more than 80%), 10⁻¹ and 10⁻² M Co₃O₄ (165 and 70%, respectively). However, a slight increase of viable cells was observed after the treatment with ZnO and CeO₂ in dilutions of 10⁻⁴ to 10⁻⁵ M (by 8% compared to the intact samples). In general, the cell viability of the seedling roots of T. vulgaré after the exposure to the biosynthesized nanomaterials increased as follows: Fe₂O₃<Co₃O₄<CeO₂<ZnO.

1. Introduction
Since plants have a great potential for over-cascade and biological reduction of metal ions, the prospects of their application to the synthesis of biocompatible nanoparticles of metals (NPM) are obvious [1].
The presence of natural reducing metabolites in extracts of plants (polyphenols, flavonoids, alkaloids, terpenoids, phenolic acids, carbohydrates, and proteins) facilitates not only reduction of metal ions to metal nanoparticles (NPM) of various sizes and shapes, but also leveraging the stability and bioavailability of the latter [2].

The biological (or “green”) synthesis of nanoparticles using plants and plant extracts is an attractive alternative to the traditional chemical synthesis and to more advanced techniques of the cultivation and the isolation required for many microorganisms [3]. In addition, the plant-based NPM biosynthesis is a relatively simple process that can easily be embedded for the large-scale production of NPM [4].

The antibacterial activity of biogenic ZnO, Fe₂O₃, and CeO₂ has been widely researched [5-7]. And just few works investigated the biological properties of the biosynthesized nanoparticles on plant cells [3], many of them are contradictory and do not reflect the complex dynamics of the effects. In accordance with the desire to achieve more understanding in this respect, we studied the outcome of the impact of metal oxide nanoparticles obtained by the method of “green synthesis” on the viability of one economically important plant – wheat.

2. Materials and Methods

2.1 Extract preparation

Fresh leaves of Petroselinum crispum were washed under running water, then twice with distilled water; dried on filter paper to remove moisture from the surface of the leaves. Next, raw materials were ground to powder in liquid nitrogen, distilled water at a ratio of 1:2.5 (by mass) was added, the mixture got stirred and boiled for 30 min at a temperature of 90°C. Then, the extract was percolated through two layers of cheesecloth and centrifuged for 15 min at 15000 g, the supernatant was passed through a 0.45-μm Millipore filter [8].

2.2 Synthesis of zinc oxide nanoparticles

The original vegetable extract was diluted two times (20 ml) and 2 g of Zn(NO₃)₂·6H₂O were added; the synthesis was carried out with constant stirring at 60-80°C for 8 h before sedimentation and liquid evaporation. The formed precipitate was washed with distilled water (two times) and alcohol (one time) by centrifugation at the maximum speed for 10 to 15 min. The precipitate was dried at 100°C for 3 h then annealed in the muffle furnace at 500°C for 2 h.

2.3 Synthesis of cerium oxide nanoparticles

0.862 g Ce(NO₃)₃·6H₂O were added to the supernatant of the aqueous extract (20 ml), heated up with stirring for 6 h at 80-90°C, the sediment was separated by centrifugation at 10000 rpm for 10 min, multiply washed with deionized water in order to remove uncoordinated biomolecules from the extract. The obtained residue of NPM was dried at 60°C for 6 h and burned in the muffle furnace at 500°C for 2 h [9]. Changing color from bright yellow to cloudy yellow proved the formation of NPs CeO₂.

2.4 Synthesis of iron oxide nanoparticles

The extract diluted with distilled water (1:3) in the amount of 9 ml was mixed with 1 ml of 0.1 M FeCl₃ and intensely stirred at the room temperature; the reaction mixture was centrifuged to separate the sediment in 24 h. Next, the residue was washed with alcohol (one time) and distilled water (two times) by centrifugation at the maximum speed for 10 to 15 min and dried at 50°C for 12 h [10].

2.5 Synthesis of cobalt oxide nanoparticles

The vegetable extract was diluted with distilled water (1:3) and in the amount of 90 ml was mixed with 10 ml of 1 M CoNO₃·6H₂O with stirring and heating up at 90°C until the sediment emerged, then the temperature was lowered to 60°C and heated for 90 min, settled for 24 h at the room temperature. Next,
the residue was washed with alcohol and distilled water by centrifugation at the maximum speed for 10 to 15 min, dried at 60°C for 24 h and annealed at 500°C for 2 h [11].

2.6 Characterization of nanoparticles
The obtained particles were resuspended in distilled water in an amount of 1 g/l, the suspension was treated in an Elmasonic ultrasonic bath for 15 min, and characterized using UV/vis-spectrophotometry and scanning electron microscopy (SEM).

2.7 UV-spectrophotometry
A suspension of 0.1 M NPs and a solution of the salt were analyzed spectrophotometrically in the wavelength range of 200-600 nm in quartz cuvettes with an optical path length of 1 mm. Water was used as a reference solution. The efficiency of the synthesis of NPs was analyzed by integrating the UV-spectra of the salt and the supernatant. The supernatant was obtained by mixing the salt and the extract and then filtering the mixture through a filter (0.22 μm).

2.8 SEM
The nanoparticles were deposited onto a double-sided adhesive carbon tape (2SPI, USA) and examined with a Zeiss Merlin microscope equipped with Gemini II Electron Optics (Zeiss, Oberkochen, Germany). The measurements were carried out at accelerating voltage of 1-5 kV and probe current 25-80 pA without any conductive coating on the sample surface.

2.9 Evaluation of the biological activity of nanoparticles
Wheat seeds of Triticum vulgare were used as the object of the research. Previously disinfected seeds were sprouted in a climatic chamber (“Agilent”, the United States) and subsequently treated with suspensions of Fe₂O₃, Co₃O₄, ZnO, or CeO₂ NPs in five concentrations (from 10⁻¹ to 10⁻⁵ M) for 7 and 14 days. The germination technique consisted of consecutive actions that allowed to study the impact of NPM at early stages of the development and to avoid external contamination of the sprout by metals. The detailed methodology of qualification was described in our previous works on nanomaterials obtained by physical and chemical methods [7, 10, 12]. Plants treated with 50 μm H₂O₂ were used as positive control.

At the end of the incubation, there was held the microscopy analysis of the apical and basal parts of the seedling roots. The roots were cut off the stems and placed in the dye 0.025% Evans blue for 15 min and then washed with distilled water and divided into segments – the apical (0 to 1 cm from the apex) and the basal root segments (5-mm sized). Microscope slides were visualized in the light microscope mode (“Micromed-3”, Russia) and the number of total and stained (dead) cells was calculated, and, on this basis, the cell viability was determined according to the formula: cell viability = (1 - (number of stained cells/total number of cells)) • 100%.

All experiments were performed in three replicates; the data were processed by methods of variational statistics using Microsoft Excel and Statistica V8.

3. Results and Discussion
Suspensions of the synthesized nanoparticles exhibited the light absorption in the UV spectrum. According to the UV-spectrum of NP CeO₂ suspension, Ce⁴⁺ (Ce(NO₃)₃) transited into Ce⁴⁺ (CeO₂) in the form of 303 nm peak at 298 nm (Maqbool et al., 2017) (Figure 1). NPs ZnO were characterized by a smooth peak at 366 nm (Figure 2), which is consistent with data by Manokari and Shekhawat (2017). NPs Fe₂O₃ became identified by a peak at 292 nm (Figure 3), which corresponds to data by Chandra and Khan (2017). NPs Co₃O₄ were defined with a peak at 370 nm (Figure 4).
Figure 1. UV-spectra of nanoparticles CeO$_2$ synthesized in the aqueous extract from *P. crispum* leaves.

Figure 2. UV-spectra of nanoparticles ZnO synthesized in the aqueous extract from *P. crispum* leaves.
Figure 3. UV-spectra of nanoparticles Fe₂O₃ synthesized in the aqueous extract from *P. crispum* leaves.

Figure 4. UV-spectra of nanoparticles synthesized in the aqueous extract from *P. crispum* leaves.
SEM-visualization of CeO$_2$ powders revealed that the particles are highly heterogeneous (Figure 2A), the range of particle sizes was from 14 nm to relatively large particles (greater than 500 nm), consisting of smaller ones that were attached to each other. Such a morphological form of cerium dioxide with a wide particle size distribution was observed previously (Munusamy et al., 2014). Fe$_2$O$_3$ sample had a plate structure (with the length more than 80 nm), both nano-scaled and micro-scaled (B), and Co$_3$O$_4$ was presented by small cubic particles with sharp edges with a diameter from 20 to 100 nm stuck together in large units (~1 µm) (C). ZnO was characterized by a spherical, oval and hexagonal structure with particle sizes from 60 to 160 nm (D).

Death of plant cells like of other living organisms is a natural process that occurs in the early stages of development and continues until organism’s death. Previously, we recorded a higher sensitivity to NPM for the root system as the first target for the toxic effect of metals [7]. The analysis of the cell viability of T. vulgare after 14 days of exposure to the suspensions of the biosynthesized nanopowders showed a dose-dependent increase in cell death with increasing concentrations of the preparations (Figure 3). An especially noticeable negative effect on plant cells was found for Fe$_2$O$_3$; at concentrations of $10^{-1}$ and $10^{-2}$ M, it fully inhibited the seed germination, and at dilutions $10^{-3}$ and $10^{-4}$ M, the number of dead cells in the apical part of the root increased to 80% compared to the control.

The same circumstances showed more than 50% cell death after exposure to 10-1 M CeO$_2$ (84.7% compared to the control), $10^{-1}$ and $10^{-2}$ M Co$_3$O$_4$ (165% and 70%, respectively). It should be noted that damage to the cells after the treatment with $10^{-1}$ M Fe$_2$O$_3$, CeO$_2$, and $10^{-1}$ M Co$_3$O$_4$ was 53, 57 and 127% lower than after exposure to H$_2$O$_2$ (the positive control), respectively. Meanwhile, the degree of cell death after the treatment with small concentrations of ZnO and CeO$_2$ was negligible, and even a significant increase in the cell viability was recorded by 8% compared to the intact samples.

In general, the cell viability of the seedling roots of T. vulgare after exposure to nanomaterials of the researched group increases in a row: Fe$_2$O$_3$<Co$_3$O$_4$<CeO$_2$<ZnO.

Cytotoxic responses of NPM are likely connected with the development of ROS and, therefore, the launch of oxidative stress [11]. Some NPM (including Fe$^0$) obtained by the methods of the plasma chemical synthesis and the conductor electrical explosion allowed us to show the overall chain of the implementation of metabolic effects on the plant of T. vulgare: increase of ROS → redox imbalance → the lipid peroxidation in cellular membranes → damage to DNA molecules → inhibition of growth or death of plants [2, 7, 8, 10, 13].

The realization of the biological effects of the biosynthesized nanomaterials probably took an ion path because it is known that, in acidic media (synthesis of particles was held at pH=3-5), processes of metal ion emission from the NP core do strengthen [14]. Co$_3$O$_4$ found this effect especially pronounced.
due to the large variations in degrees of oxidation (Co$^{2+}$, Co$^{3+}$, and Co$^{4+}$) compared to other transient 3d-elements [15], the ion production took place according to the type of Trojan horse [16]. At the same time, due to unstable stoichiometry on the surface (Ce$^{3+}$/Ce$^{4+}$), CeO$_2$ showed differently directed biological effects: increasing concentrations up to 10^{-5} M lead to an increase in the share of Ce$^{3+}$ (Ce$_2$O$_3$) and in toxic properties [9], and, at the same time, reducing concentrations to 10^{-5} M resulted in an increase of the share of Ce$^{4+}$ (CeO$_2$) and of antioxidant and protective activities [1], which was confirmed by the UV-spectra of metal above.

![Graph showing cell viability of T. vulgare roots](image)

**Figure 6.** The cell viability of *T. vulgare* roots after exposure to metal oxide nanoparticles synthesized in the aqueous extract from *P. crispum* leaves (calculated by the number of cells in the basal and apical parts of the root stained with Evans Blue; * – statistically significant (P≤0.05))
4. Conclusion
The difference in the manifestation of the biological effects of NPM used in this experiment could be linked to the nature of the particle surface pending on the way of synthesis. So, a prominent cytotoxic effect of Fe$_2$O$_3$ and Co$_3$O$_4$ may be attributed to the form of particles as the particles in polygonal, cubic, or plate forms have sharp edges and can cause mechanical damage to the cells [5]. According to SEM-generated images, remaining particles (CeO$_2$, ZnO) were of spherical or oval formation packed in a matrix, which allows their use in the biological implementation. Moreover, normal somatic cells showed less toxicity of biosynthesized CeO$_2$ particles compared to chemical analogs [4]. Of course, the toxicity of the studied NPM might be directly affected by functional groups on their surface (organic phases of the extract like -COOH, -OH, and -NH$_2$ may remain after powder calcination), which was confirmed in the research [6].

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