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Effect of nano- and crystalline metal oxides on growth, gene- and cytotoxicity of plants *in vitro* and *ex vitro*

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Keywords: chromosomal aberrations, nanoparticles, root mitotic index, tissue culture.

Summary. Results of the research on the phytotoxic, cytotoxic and genotoxic effects revealed in common wheat (*Triticum aestivum* L.) plants germinated from seeds treated with nanooxide iron (Fe_2O_3 NP) and crystalline iron oxide (Fe_2O_3 cr.) are described. Stimulating effects of the majority of their studied concentrations on cell division and tissue growth were demonstrated. Effects of Fe_2O_3 NP and Fe_2O_3 cr. also have been studied on growth and development of the tomato (*Lycopersicon esculentum* L.) tissues cultivated *in vitro.* It has been shown that Fe_2O_3 NP and Fe_2O_3 cr. induced both inhibitory and stimulating effects depending on the concentrations applied.

Влияние нанооксидов и кристаллических оксидов железа на рост, генотоксичность и цитотоксичность растений *in vitro* и *ex vitro*

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Ключевые слова: корневой митотический индекс, культура тканей, наночастицы, хромосомные аберрации.

Аннотация. Исследована цитотоксичность, фитотоксичность и генотоксичность наночастиц (Fe_2O_3 NP) и кристаллических препаратов оксида железа (Fe_2O_3 cr.), выявляемая в экспериментах с растениями мягкой пшеницы (*Triticum aestivum* L.) после обработки этими препаратами ее семян. Показано стимулирующее влияние большинства концентраций суспензий оксидов железа на деление клеток и рост тканей. Также изучено влияние Fe_2O_3 NP и Fe_2O_3 cr. на рост и развитие растений томата (*Lycopersicon esculentum* L.) *in vitro* культуре тканей. Показано, что Fe_2O_3 NP и Fe_2O_3 cr. оказывали стимулирующие и ингибирующие воздействия в зависимости от концентрации.

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Introduction

In recent years biological effects induced in plants by nanoparticles (NP) are of a wide interest due to the industrial technology development and the nanoparticle emission incoming into the environment together with the industrial waste, production and materials (Siddiqui et al., 2015). Application of nanoparticles is quite promising in plant biotechnology, where their antibacterial and stimulating effects are confirmed by several researchers (reviewed by Kim et al., 2015).

Considerable expectations are being placed on the preparations developed on the basis of nanotechnology and designated for the agriculture and crop production, especially for plant growth stimulating (Godimchuk, Ikhalainen, 2013). Application of NP-based preparations may also contribute in the increasing of plant resistance to adverse environmental conditions. A number of chemical elements including iron, cobalt, copper, manganese etc. is known to stimulate plant growth and development, but it would be a wrong idea to assert this in every separate case (Vinogradov, 2010).

Nanoparticles have an ability to penetrate unhindered into seed cells actively influencing enzyme system thus increasing germination (Nazarova et al., 2012; Kolesnikov et al., 2016). Studies on the interaction of nanoparticles with plants have revealed both positive and negative effects related with the germination energy and root length (Lei et al., 2005; He et al., 2011) as well as with the influence inhibiting these processes (Barrena et al., 2009; Seeger et al., 2009; Ghodak et al., 2011).

Iron nanooxide is widely used for the magnetic media manufacturing and it is also comprised in the compositions of various preparations for the agriculture and crop production. Nanopowder of iron oxide Fe_2O_3 is applied as an effective adsorbent for heavy metals, for example, chromium from different environment and used as a contrast MRI-agent in clinics. Besides, it is applied in the producing pig-iron by the blast-furnace method, producing of ammonia, ceramics, mineral paints, glass and steel, in the food industry and for the production of modern building materials.

Results of the study on the rice plant development have revealed that growth rate of rice roots was significantly increased under the influence of 500, 1000 and 2000 mg L^{-1} Fe_2O_3 NP suspensions (Alidoust, Isoda, 2013).

Treatment of spring rapeseeds with Fe-NP in concentration 0.03 kg ha^{-1} led to the increase of germination by 2–3 %. Application of NP positively influenced the content of water-soluble polysaccharides in the aboveground parts of plants (Churilov et al., 2010).

Stimulating effect of iron nanopowder was shown in the studies on accumulation of the ascorbic acid and carotene in *Potentilla anserina* and *Polygonum aviculare* plants. In addition, treatment of seeds of these plants with Fe NP increased their germination and yield by 25–32 %. Ascorbic acid content in the pants was increased by 24–37 %, carotene – by 23–50 %, carbohydrates – by 23–50 % depending on the plant species and longevity of vegetation period. Positive effects of Fe NP treatment were observed in sunflower, corn, spring wheat: field germination was increased by 8, 12.6 and 10.5 % respectively; leaf blade square and photosynthesis intensity were increased by 15–25 % (Churilov et al., 2010).

In consequence, there was a considerable increase of the yield of these plants (Mahmoudi et al., 2012). Nanoparticles contained in the cultivation media in the concentration range from 26 to 6.25 nmoles induced growth stimulation of wheat (*Triticum aestivum* L.) seedlings (Deryabina, 2015).

Cytotoxic effects of iron oxide NPs are not studied enough, but it is known that they are not citotoxic for human in concentrations below 100 mg L^{-1} (Auffan et al., 2006; Karlsson et al., 2008) although they induce oxidative stress in mice macrophages (Stroh et al., 2004).

In the present work we have investigated influence of Fe_2O_3 NP and Fe_2O_3 cr. on the plant growth and development. We have evaluated phyto-, cyto- and genetoxicity of Fe_2O_3 NP and Fe_2O_3 cr. on wheat (*T. aestivum*) seeds. In addition, we have studied influence of these compounds on growth and development of tomato (*Lycopersicon esculentum*) plants cultivated *in vitro*.

Materials and methods

Seeds of wheat were treated during 24 hours at 25 °C with suspensions of Fe_2O_3 nanoparticles (Fe_2O_3 NP, < 50 nm, Sigma-Aldrich, USA) and Fe_2O_3 crystalline preparations (Fe_2O_3 cr., fraction 5–10 μm). Both Fe_2O_3 NP and Fe_2O_3 cr. were applied in concentrations of 20, 40 and 80 mg L^{-1}. Blank plants were treated with distilled water.

Germination of seeds were performed in Petri dishes (20 seeds per dish) in darkness at 30 °C. *In vitro* cultivation of *L. esculentum* plants was performed using a standard method (Butenko, 1999).

Vegetation parameters of wheat plants were evaluated measuring the germination energy and root

growth rate. Germination energy was determined on the 4-th day as $\boldsymbol{B} = \boldsymbol{a}\,\boldsymbol{b}^{-1}$ 100 %, where ***a*** is a number of germinated seeds and and ***b*** is a total number of seeds.

Genetoxicity of Fe_2O_3 NP and Fe_2O_3 cr. was evaluated using anaphase method of analysis of chromosomal aberrations, which were revealed in the root meristem of germinated seeds. The following aberrations were considered calculating their percentage per total number of aberrations: single chromosomal/chromatid fragments, multiple fragments, chromosome/chromatid bridges, multiple aberrations, chromosome lagging. Fixation, staining and making preparation for the light microscopy were described previously (Ham, Cormac, 1982).

Cytotoxicity was evaluated in wheat root meristem measuring mitotic index by a standard method (Grif, Machs, 1996).

In addition, presence of pinpoint aggregations of iron compounds was tested histochemically using Perls's method **(**Korzhevsky, Gilyarov, 2010**).**

Testing of statistically significant difference between the studied parameters of treated and blank (untreated) plants (done by ANOVA test) and evaluating of mean ± SD at $p < 0.05$ were performed using Microsoft Excel 2007.

Results

Data obtained on the evaluation of phytotoxicity of Fe_2O_3 NP and Fe_2O_3 cr. for wheat (*T. aestivum*) seeds have shown clear pronounced patterns of action of these compounds. They both stimulated root growth, but in different degree. Thus, the maximum distinction in root lengths between treated with Fe_2O_3 NP plants and blank plants (70 ± 6 and 47 ± 4 mm respectively) was observed at 6-th day of cultivation in the experiments with 20 mg L^{-1} Fe_2O_3 NP. At that time, root lengths of plants treated with 40 and 80 mg L^{-1} were 50.5 and 66.3 mm (against the same blank values: 47 mg L^{-1}). Fe_2O_3 cr. also caused elevated root growth at the 6-th day of cultivation, but the maximum effect was observed in the experiments with 80 mg L^{-1}. At the 3-rd day of cultivation the maximum stimulating effect of Fe_2O_3 NP **(**45 ± 4 mm) was observed at 20 mg L^{-1} whereas in the experiments with Fe_2O_3 cr. it was observed to be 42 ± 3 mm at 80 mg L^{-1} (blank values were 32 mm).

Significant decrease in the germination rate of wheat seeds was observed after exposure of seeds to 40 mg L^{-1} Fe_2O_3 cr. Stimulating effects on germination were observed only at 20 mg L^{-1} Fe_2O_3 cr. Germination energy of Fe_2O_3 NP- and Fe_2O_3 cr.-treated seeds at all concentrations was not changed comparing to blank seeds.

In addition, we have obtained histological data demonstrating that Fe_2O_3 NP may possibly penetrate wheat root meristem cells (fig. 1a and 1b) as far as iron was revealed in them. However, we do not exclude solving (chemical transformation) of iron in soil with the subsequent penetration of its water soluble form in cells and with the further sedimentation.

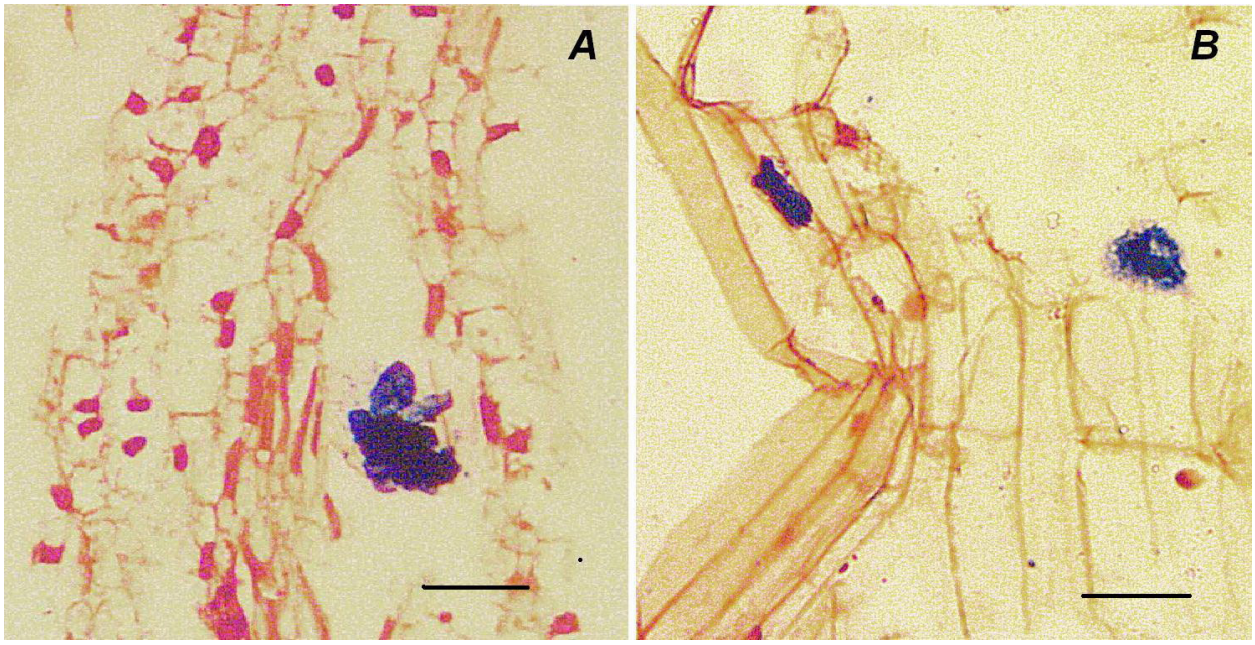

Fig. 1. Fe-clusters in histological section of wheat tissues (staining by Pearls) germinated from Fe_2O_3 NP-treated seeds; (a) – root parenhyma, (b) – leaf mezophyll. Bars 100 mm.

Results of the analysis of Fe_2O_3 NP and Fe_2O_3 cr. cytotoxicity are shown in Table 1. All the studied Fe_2O_3 NP concentrations induced a significant increase (compared to blank plants) of mitotic index (MI) in wheat root meristem and its maximum increase was achieved at 20 mg L^{-1}. Fe_2O_3 cr. applied in the same concentrations also led to the MI increase, but it was weakly pronounced and demonstrated tendency to significance only at concentration of 40 mg L^{-1}.

Table 1

Mitotic index in wheat (*Triticum aestivum*) root meristem influenced by metall oxide nanoparticles and crystalline metal oxides of different concentrations

№	Tested iron oxides of different concentrations, mg L^{-1}	Mitotic index, mean ± SD, $p < 0,05$
1.	Blank (distilled water)	4.8 ± 0.67
2.	Fe_2O_3 NP, 20	8.5 ± 0.8**
3.	Fe_2O_3 NP, 40	7.04 ± 0.8**
4.	Fe_2O_3 NP, 80	6.4 ± 0.92*
5.	Fe_2O_3 cr., 20	5.07 ± 0.69
6.	Fe_2O_3 cr., 40	6.4 ± 0.92*
7.	Fe_2O_3 cr., 80	4.9 ± 0.68

** – statistically significant difference in mitotic index at $p < 0.05$ compared to blank plants; * – tendency to significancy of MI compared to blank plants.

Thus, the obtained data demonstrate an effect of stimulating cell divisions and tissue growth at the most of the Fe_2O_3 NP and Fe_2O_3 cr. concentrations applied.

Results of the study of the mutagenic activity of Fe_2O_3 NP and Fe_2O_3 cr. evaluated by the percentage of anaphases with chromosomal aberrations in wheat root meristem cells are shown in Table 2. The obtained data demonstrate that Fe_2O_3 NP ability to induce aberration level increases with increasing its concentration. At 40 and 80 mg L^{-1} Fe_2O_3 NP this ability is significantly higher compared to blank experiments. The highest percentage of chromosomal aberrations was revealed at 80 Fe_2O_3 NP mg L^{-1}. In general, all the studied concentrations of Fe_2O_3 NP induce the elevated rate of chromosomal aberrations compared to blank experiments.

Table 2

Level of chromosomal aberrations in wheat (*Triticum aestivum*) root meristem influenced by metall oxide nanoparticles and crystalline metal oxides of different concentrations

№	Tested iron oxides of different concentrations, mg L^{-1}.	Level of anaphases with chromosomal aberrations, %, mean ± SD, $p < 0.05$
1.	Blank (distilled water)	2.42 ± 0.37
2.	Fe_2O_3 NP, 20	12.2 ± 1.07**
3.	Fe_2O_3 NP, 40	11.1 ± 0.9**
4.	Fe_2O_3 NP, 80	15 ± 1.12**
5.	Fe_2O_3 cr., 20	4.8 ± 0.67*
6.	Fe_2O_3 cr., 40	4.25 ± 0.63*
7.	Fe_2O_3 cr., 80	5 ± 0.68*

** – statistically significant difference in chromosome aberration level at $p < 0.05$ compared to blank plants; * – tendency to significance of anaphase values compared to blank plants.

There was only the tendency to significance revealed in experiments with Fe_2O_3 cr. that suggests to the low (insufficient) difference between treated and untreated (blank) plants. The highest level of chromosome aberrations was observed at 80 mg L^{-1} Fe_2O_3 cr. A clear dependency between the chromosome aberration level and the concentrations applied was not found.

Analysis of chromosome aberration spectrum has shown that the extra chromosomal fragments, chromatide bridges and chromosome lags were observed most frequently.

Studies on Fe_2O_3 NP and Fe_2O_3 cr. action influencing growth and development of tomato plants cultivated *in vitro* included evaluations of germination energy measured at 14-th day, changes in stem length at 14-th, 21-st and 28-th days of cultivation, visual determination of the number of true leaves and determination of root lengths.

There were no changes observed in germination energy in the experiments with Fe_2O_3 NP and Fe_2O_3 cr. Increase in germination energy was found to be up to 80 % after application of 10 mg L^{-1} Fe_2O_3 cr. Both Fe_2O_3 NP and Fe_2O_3 cr. in concentrations 60, 100 and 150 mg L^{-1} induced the decrease of germination energy, which was the most significant at 150 mg L^{-1} Fe_2O_3 NP (Table 3).

Table 3

Germination energy of tomato (*Lycopersicon esculentum*) seeds measured at 14-th day of cultivation influenced by metall oxide nanoparticles and crystalline metal oxides of different concentrations

Metal oxide type (nano- or crystalline) at concentration, mg L^{-1}	Germination energy of seeds, %
Fe_20_3 NP, 10	75 ± 6.1
Fe_20_3 cr., 10	74.8 ± 6.2
Fe_20_3 NP, 40	81.0 ± 6.8*
Fe_20_3 cr., 40	75.2 ± 6.0
Fe_20_3 NP, 60	73.3 ± 5.9
Fe_20_3 cr., 60	68.8 ± 4.8
Fe_20_3 NP, 100	65.0 ± 4.7
Fe_20_3 cr., 100	65.4 ± 5.0
Fe_20_3 NP, 150	59.1 ± 4.6*
Fe_20_3 cr., 150	65.5 ± 5.6
Blank (distilled water)	74.9 ± 6.0

* – statistically significant difference in percentage of anaphases with chromosomal aberrations at $p < 0.05$ compared to blank plants.

Data obtained on changes in tomato plant steam length at 14-th, 21-st and 28-th days of cultivation are shown in Table 4. The most pronounced inhibition of steam growth was observed at 28-th day in the experiments with 150 mg L^{-1} Fe_2O_3 NP. Stimulation of steam growth was observed at 14-th, 21-st and 28-th days with 40 mg L^{-1} concentrations of Fe_2O_3 NP and Fe_2O_3 cr.

Table 4

Lycopersicon esculentum steam lenght at 14, 21 and 28 days of cultivation after passage influenced by metall oxide nanoparticles and crystalline metal oxides of different concentrations

Concentration and metal oxide type (nano- or crystalline)	Steam length at14-th day after passage, mean ± SD	Steam length at 21-st day after passage, mean ± SD	Steam length at 28-th day after passage, mean ± SD
Fe_20_3 NP, 10 mg L^{-1}	8.3 ±	10.5 ±	15.0 ± *
Fe_20_3 cr., 10 mg L^{-1}	8.2 ±	10.0 ±	15.2 ± *
Fe_20_3 NP, 40 mg L^{-1}	10 ± *	12.1 ± *	17.8 ± *
Fe_20_3 cr., 40 mg L^{-1}	9 ± *	12.0 ± *	17.5 ± *
Fe_20_3 NP, 60 mg L^{-1}	8.3 ±	10.1 ±	16.1 ±
Fe_20_3 cr., 60 mg L^{-1}	8.2 ±	9.8 ±	16.3 ±
Fe_20_3 NP, 100 mg L^{-1}	7.6 ±	9.0 ± *	12.2 ± *
Fe_20_3 cr., 100 mg L^{-1}	8.0 ±	9.5 ±	12.5 ± *
Fe_20_3 NP, 150 mg L^{-1}	7.1 ± *	8.6 ± *	11.0 ± *
Fe_20_3 cr., 150 mg L^{-1}	7.3 ± *	8.7 ± *	11.0 ± *
Blank (distilled water)	8.0 ±	10.0 ±	16.5 ±

* – statistically significant difference of steam lenght at $p < 0.05$ compared to blank plants.

Root lengths of tomato seeds were measured at 28-th day of cultivation in the presence of Fe_20_3 NP and Fe_20_3 cr. The obtained data are shown in Table 5.

The plants cultivated *in vitro* in the presence of the highest concentrations of Fe_2O_3 NP (100 and 150 mg L^{-1}) have the minimal root lengths. There were no significant changes of root lengths of plants treated with other concentrations compared to blank plants.

Table 5

Root lengths of *Lycopersicon esculentum* seeds at 28-th day of cultivation in the presence of Fe_20_3 NP and Fe_20_3 cr.

Concentration and metal oxide type (nano- or crystalline) at concentration, mg L^{-1}	Root length, mm, mean ± SD
Fe_20_3 NP, 10	18.2
Fe_20_3 cr., 10	17.5
Fe_20_3 NP, 40	17.6*
Fe_20_3 cr., 40	16
Fe_20_3 NP, 60	17
Fe_20_3 cr., 60	16*
Fe_20_3 NP, 100	12*
Fe_20_3 cr., 100 mg	12.5*
Fe_20_3 NP, 150 mg	10*
Fe_20_3 cr., 150 mg	9.6*
Blank (distilled water)	19

* – statistically significant difference in root lenght at $p < 0.05$ compared to blank plants.

Discussion

Previous researches carried out mainly with animals have shown genetoxicity of titanium and zinc oxide NP whereas there are no data on iron oxide NP genotoxicity for plant cells as well as for animal cells. The direct mechanisms of metal oxide genotoxicity (for Ti, Zn, Ag, Al) are based on DNA strand breaks revealed in most cases by molecular methods (reviewed by Xie et al., 2011; Klien, Godnić-Cvar, 2012). Evidently these mechanisms allow to suppose generation of chromosome aberrations, which, however, are studied by researchers quite rarely. For example, aluminum oxide NP are among a small number of exclusions (Balasubramanyam et al., 2009). Nevertheless, as far as the chromosome aberrations often are the "end point" of DNA damage, they should be studied necessarily. In this context, the main finding of our work is that the genetoxicity of iron oxide nanoparticles was revealed in plants for the first time using the anaphase method of analysis of chromosomal aberrations. However, these studies require to be continued in the future to obtain a detailed information on size effects of Fe_2O_3 NP.

Data obtained in our research on vegetation parameters of plants confirm general previous results from a wide range of various studies (Xie et al., 2011; Klien, Godnić-Cvar, 2012; Vishnu et al., 2018, 2019;) which demonstrate a complicated dose-dependent NP-induced response of plant cells. Our results obtained from *in vitro* studies may be of special interest regarding use the tissue culture method in NP-toxicology researches. They suggest that plant tissue culture may be a convenient and sensitive method to test metal NP toxicity, which is known to have an advantage in high reproducibility.

Author hope that their results will stimulate further researches on toxicity of the metal oxide nanoparticles.

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