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Comparative Analysis Of Morphophysiological And Genetic Traits Of Triticum Vulgare Germinants After Exposure To Metal Nanoparticles.

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ABSTRACT

We studied the effect of Cu and Ni nanoparticles on the plant Triticum vulgare. Observing the germination energy, we faced a decrease 16.7 times for Ni˚ and total suppression for Cu. These nanoparticles reduced the growth of seedlings and affected the placement of lateral roots, which grew like a "goose-paw". The total sum of reactive oxygen species ended up with an increase in the roots after exposure to Cu˚ (up to 35.8% compared to the negative control). Expressed statistically significant (in comparison with the control ($P < 0.05$)) accumulation of HO• when Cu was applied at a dose of 0.05 and 0.1 M (up to 8.5 and 13.6%). The cell viability and proliferation assays indicated that the toxic effects of nanoCuwas maximum after 24 of exposure and that accounted 89% in comparison tocontrol. The presence of Ni˚ in the medium resulted in a smoother formazan reduction after 24 hours of incubation (up to 56% relative to control). Thus, we obtained data indicating a decrease in viability of root cells under the action of nanometals, with the form of degradation of DNA molecules having either necrotic, unsystematic (in case of Cu) or internucleosomal apoptotic character (Ni).

Keywords: Triticum vulgare, nanoparticle, nickel, copper, cell viability, DNA damage.

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INTRODUCTION

Among the wide range of nanoparticles (NP), copper and nickel NP attract considerable interest since they have been leading in manufacturing for long and are among the five most used by industrial enterprises around the world (Feldblum, 2013).Despite the fact that nanopowders are produced indoors, industrial enterprises should be considered as an active source of powder particles accused of entering the environment. At each stage of the implementation of the production chain such as explosion, cooling, and passivation there are losses during spraying. Once having got into air and water, dispersed NP can form time-stable aerosols (El-Temsah, 2010) and aggregates (Lu et al., 2002), which, through various mechanisms, can enter the soil and other components of the ecological system (Lin et al., 2004). By means of sorption, NPs are actively absorbed by plants, which are a food source for almost all bioobjects (Da Silva et al., 2006; Buzea et al., 2007). Due to the fact that nano-scale metals have more reactivity and surface area than metals in the macroform, they make easier chemical transformations (Lin et al., 2004) and are actively absorbed by plants (Pan et al., 2007).In this regard, the issues of biological and environmental risks of using low-frequency metals are important in predicting the effectiveness and safety of nanotechnology implementation.NPs are easily digested and accumulated in plants, influencing their further fate and transport in the environment (Ma et al., 2013). The interaction of plants with NPs mainly leads to phytotoxicity, especially at high concentrations, for example, in tomatoes, cabbage, carrots, peas and lettuce leaves. In some works, it is pointed out that the influence of NP also contributes to the growth of plants (Tripathi et al., 2017).Such apparent differences in the effect of nanoparticles on plants can be explained by the properties and concentration of metals, the type of plants, their age and physiological location, exposure time, etc.

(Stampoulis et al., 2009).Metal ions belonging to the group of Cu, Cd, Hg, Ni and Zn cause toxicity in living systems by binding to important cellular components such as DNA or sulfhydryl, carboxyl or imidazole groups of proteins, thereby modifying their functioning (Rico et al., 2011). The transition metal – nickel – exists in five oxidation states, which determines its toxicity (Munoz, Costa, 2012).The resistance of plants to the action of NP metals is largely determined by the intensity of oxidative reactions occurring in plant tissues under the action of this type of toxicant and the ability of the plant's antioxidant system to withstand oxidative stress.Many of metal NP induce the formation of active forms of oxygen (ROS), which leads to a general change in the redox status of the cell. Thus, metals of variable valence can release ions Cu^{2+} and Cu^{+}, which leads to an increase in the total pool of ROS. In turn, an excessive amount of ROS, exceeding the adaptive capacity of the organism, contributes to mechanical damage to the cell wall and/or membrane (Ma et al., 2010). These phenomena lead to oxidative stress up to unsystematic damage to DNA and development of necrosis.Numerous experimental studies and a number of review articles have been devoted to the results of studying nanometals on plant organisms (Faisal et al., 2013; Masarovicova et al., 2013). It should be noted that the available literature gives us a large number of disparate studies, which are assigned to assessment of the yield and the main growth indices of plants in response to the action of nanometals (Dimkpa et al., 2012).It is known that some of the NP metals, especially divalent metal oxides (Keller et al., 2010), are capable of releasing toxic ions from their colloidal matrix and stimulating production of reactive oxygen species (ROS) (Ishino et al., 2015). To date, the specific mechanisms underlying the probable damaging effect on cells of a wide range of concentrations of nanometals have not been discovered.

In this regard, studying mechanisms of plant adaptation to structurally different nanometals, taking into account physiological and biochemical parameters, is relevant for a more complete understanding of the adaptive capabilities of organisms under the impact of technogenic nanomaterials.However, the potential impact on the environment and living organisms is still poorly understood, and appropriate methodologies are needed to identify NP interactions with cellular components in order to gain a better understanding and define guidelines for their safe use (Smita et al., 2012).In this regard, studyingchanges of some physiological and biochemical parameters as a result of plant adaptation mechanisms tostructurally different nano-metals isrelevant for a better understanding of organism’s adaptive capacities of organisms under conditions of man-made nanomaterials.

Thus, the purpose of thisworkis comprehensive studying the physiological and biochemical mechanisms of stability and/or sensitivity of Triticum vulgaregerminants to metal nanoparticles.

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MATERIALS AND METHODS

Chemical substances and substrates

Ni powders (57 ± 1.15 nm) were obtained by the method of conductor electric burst in the atmosphere of argon or air in "Advanced powder technologies LLC" (Tomsk), Cu powders (54±2.06 nm) were derived by means of the thermal decomposition method in a nitrogen plasma in LLC "Plazmoterm" (Moscow). Material testing of preparations included: scanning electron microscopy, transmission microscopy, and atomic-force microscopy using following devices: LEX T OLS4100 T, JSM-740, JEM-2000FX ("JEOL", Japan). The analyzers Brookhaven 90Plus/BIMAS and Photocor Compact ("Photocor", Russia) were used for measurement of average hydrodynamic particle diameter and ζ-potential (by the method of Dynamic Light Scattering). The samples were intensively pipetted and treated in an ultrasonic bath ("Sapphire TTTS", Russia) at frequency of 35 kHz within 30 minutes to maintain the needed dispersity of NP suspensions.

Test organisms

Triticum vulgareVill(wheat) plants were used in the study.

Wheat seeds were first disinfected with 0.01% $KMnO_4$ solution within 10 minutes and washed with distilled water three times for the analysis. Then they were soaked between two layers of moistened gauze and left for three h at temperature of 37°C. Next the swollen seeds were transferred onto moist filter paper substrates in Petri dishes (d = 9 cm) and couched in a climatic chamber ("Agilent", the United States) with 12-hour light, temperature of 22±1°C and humidity of 80±5% within 48 h. At the same time, the nutrient solution was not added to avoid probable interaction of ions in the medium, which could complicate the result interpretation. Thenfor relative growth synchronization equally germinated seeds (20 pieces) were carried on wet filter paper in individual plastic cups, and 5 ml of NP metal suspensions at concentrations ranging from 0.0125 to 1 M were added. Next the samples were left to sprout for 48 hours, after that the length of the first leaf and the tap root of ten germinants was measured. Percent of germinating energy was calculated on the third day (GOST 12038-84) in the test where the seeds were pipetted with 5 ml NP metal suspensions immediately after soaking. Based on the results of the morphometric parameters wecalculated the tolerance index (TI), which is apercentagedratio of the effective parameter of the experimental plants to that ofthe control group plants (Wilkins, 1978). Then wetook three germinants of each variant for further measurements.

Content of reactive oxygen species

Levels of intracellular reactive oxygen species (ROS) content were estimated by the fluorescence intensity of 0.25 µmol 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA); 35 µmol H_2O_2 served as a positive control (Pourrut et al., 2011). Hydroxyl radical contents (HO·) were measured using 7-hydroxycoumarin-3-carboxylic acid (7-OHCCA) fluorescence (Gerald & Jamie, 2006) with the Fenton's reagent (50 µmol $CuSO_4$ and 25 mmol H_2O_2) as the positive control(Koul, Gupta, 2013). Generation of superoxide anion radical O2•- was determined by adrenaline oxidation at 347 nm in roots of the germinants(Sirota, 2000), while the positive control was O2•- generation system consisting of 0.05 mmol riboflavin (RF) and 1 mmoltetramethylethylenediamine (TEMED) (Grivennikova&Vinogradov, 2013).

Analysis of cell viability (CV) by changing reductase enzyme activity

Analysis of cell viability (CV) by changing reductase enzyme activity was carried out according to the Protocol of the "Cell counting kit-8 (CCK-8)" manufacturer (WST-8 patent no. 2.251.850, Canada).

Electrophoresis

DNA extraction from the roots of germinants of T. vulgare was performed according to the Protocol of the "DNA-Extran-3" set manufacturer ("Syntol", Russia) with Lyubushkina'smodifications (2011). DNA was extracted from 48-hour germination of seedlings with NP metals at concentrations ranging from 0.0125 to 1 mol. Electrophoresis of the preparations was carried out in 1% agarose ("Helicon", Russia) on Tris/Borate/EDTA buffer (pH = 7.2) in the horizontal camera SE-1 ("Helicon", Russia) at 4-10 V/cm for 40 minutes. To identify the fragment length we took 1 kb DNA marker M12 ("SibEnzyme", Russia) and SM1331

("Thermofisher Scientific", the United States). The gels were photographed in the Gel-Doc ("Bio-Rad", the United States) and analyzed with the "ImageJ" software (National Institutes of Health, the United States).

Statistical analysis

Laboratory experiments were performed in 3-fold biological replicates, analytical determination for each sample - in triplicate. The significance of the differences between the analyzed samples was calculated arithmetic averages and their standard errors. The differences were significant with an error probability of P≤0.05. The results were processed using the computer software Statistica for Windows 10.0 and Microsoft Office Excel 2010.

RESULTS AND DISCUSSION

Comparative analysis of morphophysiological traits of Triticum vulgare germinants after exposure to metal nanoparticles

The integral estimate of TI testing results on germinative energy (E) and morpho-metric characteristics of T. vulgaregerminants after 48-hour exposure to NP metals showed low plant stability to Cu˚ and Ni˚ (table 1). Counting the germinative energy (E) revealed a significant measure reduction ($p < 0.05$) after adding NP of Cu˚ and Ni˚ into the germinant medium. Also, the most expressed effectin percentage for germination was observed for Cu° NPs: in the presence of the metal germinant sprouting inhibition occurred in a sharp, much greater (comparison with controls) form (in 44 times) with its full suppression at concentrations of 0.5 and 1 Mol. However, in the presence of Ni° NPs (from 0.1 to 1 M) E was 16.7 times below than the control ($p < 0.05$).

Detailed metric analysis of T. vulgaregerminants after 48 hours of exposure to NP metals and their oxides showed that use of Cu and Ni NPs helped to reduce the germinant growth which gave stronger effects with metal concentration increasing in the medium. Especially for Cu NPs that noticeably suppressed the first leaf growth up to 11 times, and the root - up to 19 times in comparison to the control ($P \leq 0.05$). At the same time, compared to the control Ni° NPs reduced root growth to 7.4 times, and the first leaf growth up to 5.3 times ($P < 0.05$).

We also found that Cu and Ni influenced even on lateral root initiation, which is usually resistant to the action of most metals process (Dimkpa et al., 2012). So, NiNPs reduced number of lateral roots to 3.4 ± 1.02 pcs. (versus 4.6 ± 0.11 pcs. in the control), Cu NPs increased to 6.8 ± 0.17 pcs. Reduction of adventitious root is probably a common manifestation of specific adaptive plant reaction to HM exposure (Dimkpa et al., 2012) or to lack of aeration (Chirkova, 2002). It is interesting to note that plants grown in the presence of Ni and Cuwere characterized ofmore compact root system development compared with intact samples. In versions with Cubranching type of "silverweed cinquefoil" and sprouting of "dwarf" plant wereobserved. Similar results on the impact on growth and morphological parameters of roots were shown in both the ion (Feigl et al., 2013) andnano-forms of copper (Nair & Chung, 2014; Deryabina, 2015).

Table 1: The value of the upper limit of the tolerance index, TI (%) as of seed germinative energy and growth indicators of T. vulgare after 48-hour exposure to metal NPs

Metal NP concentration, Mol		TI as of germinative energy, %	Metal NP concentration, Mol		TI as of morpho-metric characteristics, %	
					Leaf	Root
Cu	0.0125	46.2*	Cu	0.0125	17.2*	6.4*
	0.025	25.1*		0.025	21.0*	10.1*
	0.05	4.2*		0.05	15.4*	5.2*
	0.1	2.3*		0.1	6.8*	5.8*
	0.5	-		0.5	–	–
	1	-		1	–	–

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Ni	0.0125	76.3	Ni	0.0125	45.0	32.51*
	0.025	71.7		0.025	37.7*	39.2*
	0.05	69.4		0.05	30.1	20.6*
	0.1	26.9*		0.1	38.9*	26.4*
	0.5	5.8*		0.5	22.5	23.6*
	1	7.0*		1	16.5	20.2*
N o t e - * is for variants that are reliably different from the control (P≤0.05)						

So, based on the growth characteristics of T. vulgare we calculated TI, which was, in general, less for root system than for leaves. This seems quite logical since the root is the "primary target" for action of many metals.

Studying pro-/antioxidant status of Triticum vulgare germinants after exposure to metal nanoparticles

The wheat root growth change after adding Cu and Ni gave interest in a more accurate analysis of their recent prooxidant effects. So, fluorimetric DCFH-DA measurement allowed to state fair ($p<0.05$), directly proportional increase of total ROS amount in roots after exposure to Cuin comparison with the negative control (up to 35.8%). In general, the level of ROS intest samples was 39% lower relative to the positive control (35 µm H_2O_2). These results are consistent with microscopy ofgerminant root apex, according to which the most expressed fluorescence was recorded in the zone of calyptra after exposure to Cu(Figure 1).

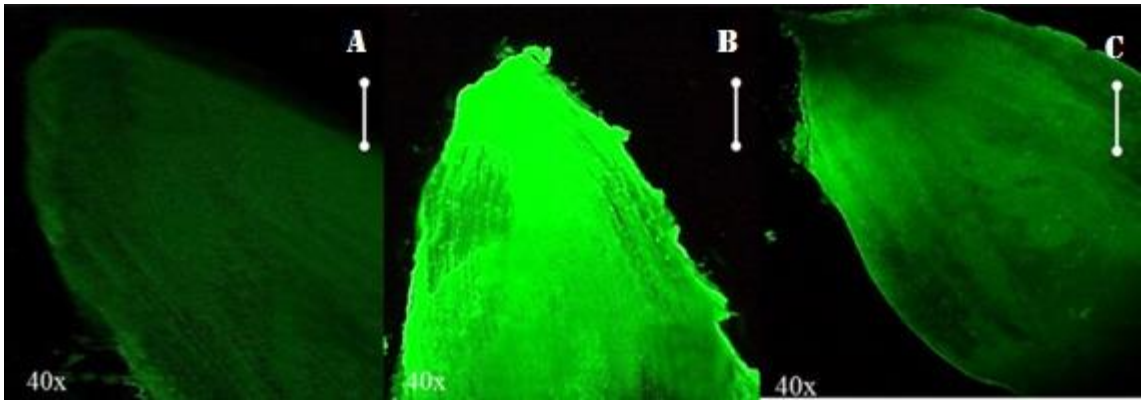

Figure 1: DCF fluorescence at a distance of 0.5 cm from the apex in the negative control (A) and after exposure to 0.1 m Cu° (B) and Ni° (c), 40x zoom(label size 50 µm)

NP metal influence on HO· radical generation in the root part of T. vulgare consisted in increasing of the 7-OHCCA fluorescent signal intensity. So, most expressed statistically significant accumulation ofHO·content compared to the controls ($p < 0.05$) was recorded when exposed to Cuin dose of 0.05 and 0.1 M (up to 8.5 and 13.6%, respectively).In spite of this, the level of the radicals was (8.7%-12%) lower than that after handling the plants withFenton's reagent.At the same time, the presence of Ni° in the medium did not substantially affect HO· radical generation (less than 3%).

In addition, it was found that the rate of $O_2^{\bullet-}$ generation progressively escalated after exposure to Ni (from 52 to 68% in comparison to the negative control). Against this background, Culesser increased $O_2^{\bullet-}$ production (up to 12.5%) ($p< 0.05$), which is consistent with the generation dynamics of this radical shown on the example of Arabidopsis thaliana (Nair&Chung, 2014). It is worth noting that the amount of thisparameter was more than 2 times lower than in plants grown in the$O_2^{\bullet-}$ generating system, which is clearly visible in the spectrum at 347 nm (Figure 2).

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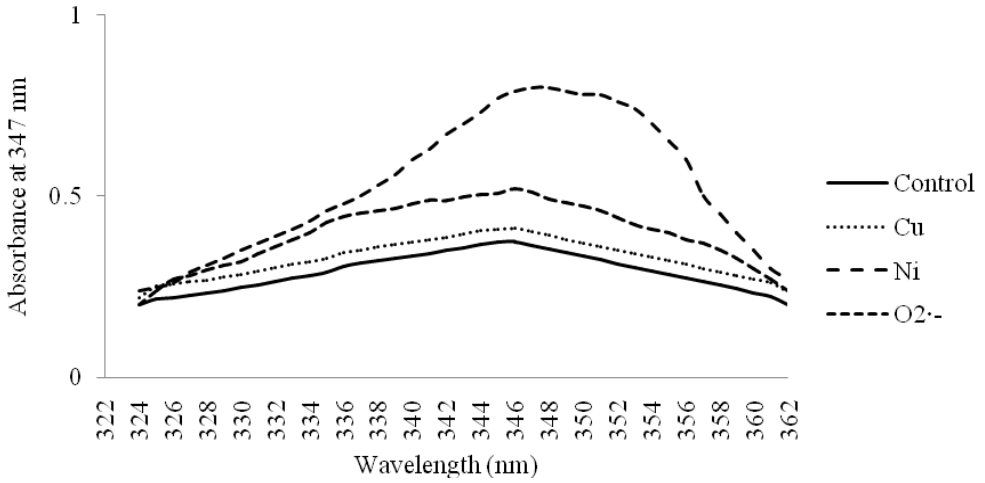

Figure 2: Absorption spectra of adrenaline oxidation products at 347 nm in roots of T. vulgaregerminants after 48-hour exposure to 0.1 M Cu° and Ni° NPs: the control is distilled water, $O_2^{\cdot-}$means generation system RF+TEMED (the measurements were carried out in 3-fold biological replicates)

During the research, we also observed an intense 7-OHCCA fluorescent signal ofHO· radicals after germination T. vulgare with nano-Cu (Figure 3). Compared with the controls, most expressed statistically significant ($p < 0.05$) accumulation of $HO^{\bullet}$ in plants' root parts was noted upon exposure to NP Cu° in the dose of 0.05 and 0.1 M (8.5% and 13.6%, respectively). Despite this, the level of radicals was (8.7%-12%) lower than that after handling plants withFenton's reagent. Presence of CuO and Ni in the medium didn't materially affect HO· radical production (accumulation less than 3%).

The results also showed that the rate of $O_2^{\bullet-}$ production directly proportional increases after T. vulgare exposure to Ni°. The rise is 52-68% higher than the negative control (Figure 4). At the same time, Cu° increased $O_2^{\bullet-}$ production in 12.5% lesser extent. It is worth noting that the amount of this parameter was more than 2 times lower than of plants sprouted in $O_2^{\bullet-}$ generation system medium. Rise of $O_2^{\cdot-}$ and H_2O_2 content after Arabidopsis thaliana exposure to nano-Cu was also postulated in the recent work (Nair, Chung, 2014).

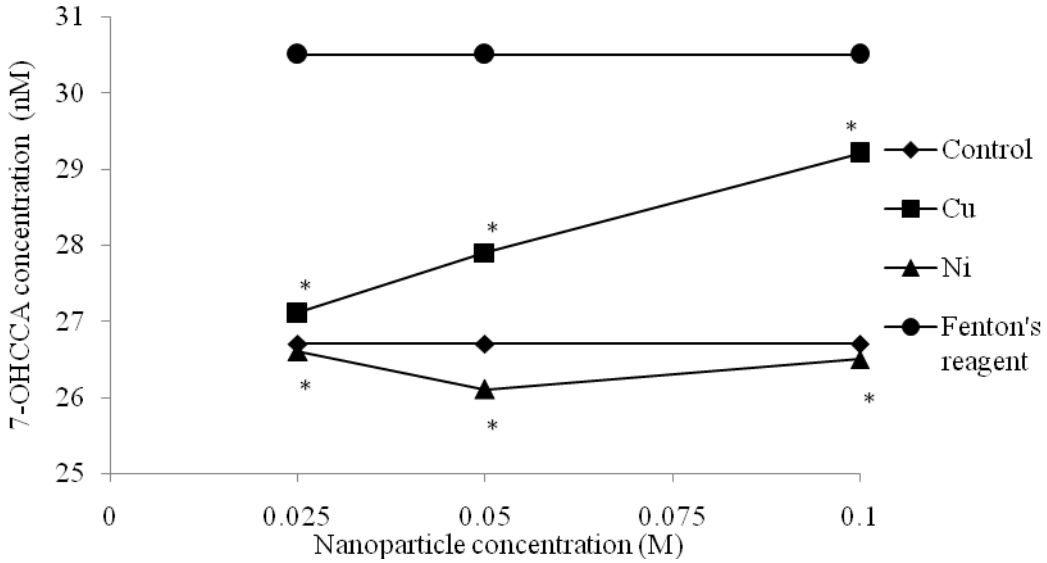

Figure 3: Hydroxyl radical $HO^{\bullet}$ content via 7-OHCCA fluorescence in cells of the T. vulgare germinant roots after 48-hour exposure to Cu and Ni NPs: (*) means the variant is reliably different from the controls ($p \leq 0.05$)

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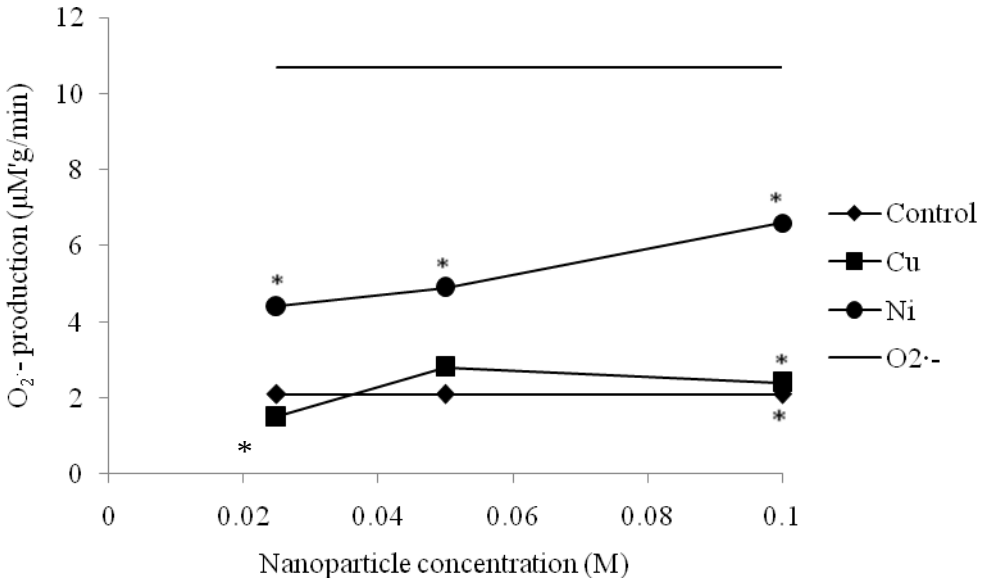

Figure 4: Superoxide anion $O_2^{\bullet-}$ generation rate by adrenalin autoxidation in the extract from the T. vulgaregerminant roots after 48-hour exposure to Cu and Ni NPs; (*) means the variant is reliably different from the controls ($p \leq 0.05$)

Summarizing on the study of the pro-oxidants content in T. vulgare leaves, it should be noted that the model plant has a species-specific sensitivity to changes in the content of both Cu° and Ni° of in nano-form. In particular, Ni° stimulates $O_2^{\cdot-}$ production and Cu° does the same with the common pool of ROS, including H_2O_2 and HO·.

The achieved results serve as additional proof of the existence of selectivity in activation of varying plant antioxidative system reactions determined by the nature of nano-material. However, the change in the ROS level in the presence of Ni° and Cu° can be attributed to the non-specific plants' response, so far as analogous changes are specific for different kinds of plants' stress and in most cases require further research. In this aspect, the main "target" for NP metal action turned out to be the root system of plants that determined interest in discovering mechanisms for phytotoxicity, with emphasis on the study of cellular damage in this exact part of the plant.

Viability of Triticum vulgare germinant roots after exposure to metal nanoparticles

The previously discovered picture of the typical oxidation-reduction disbalance can cause cell proliferation change (Higuchi, 2004). Therefore, we studied cell viability (CV) via formazanoutput from water-soluble salt of tetrazolium (WST). WST-test results demonstrated direct dependence of T. vulgare germinant rootCVon NP content and time of NP incubation (Figure 5).

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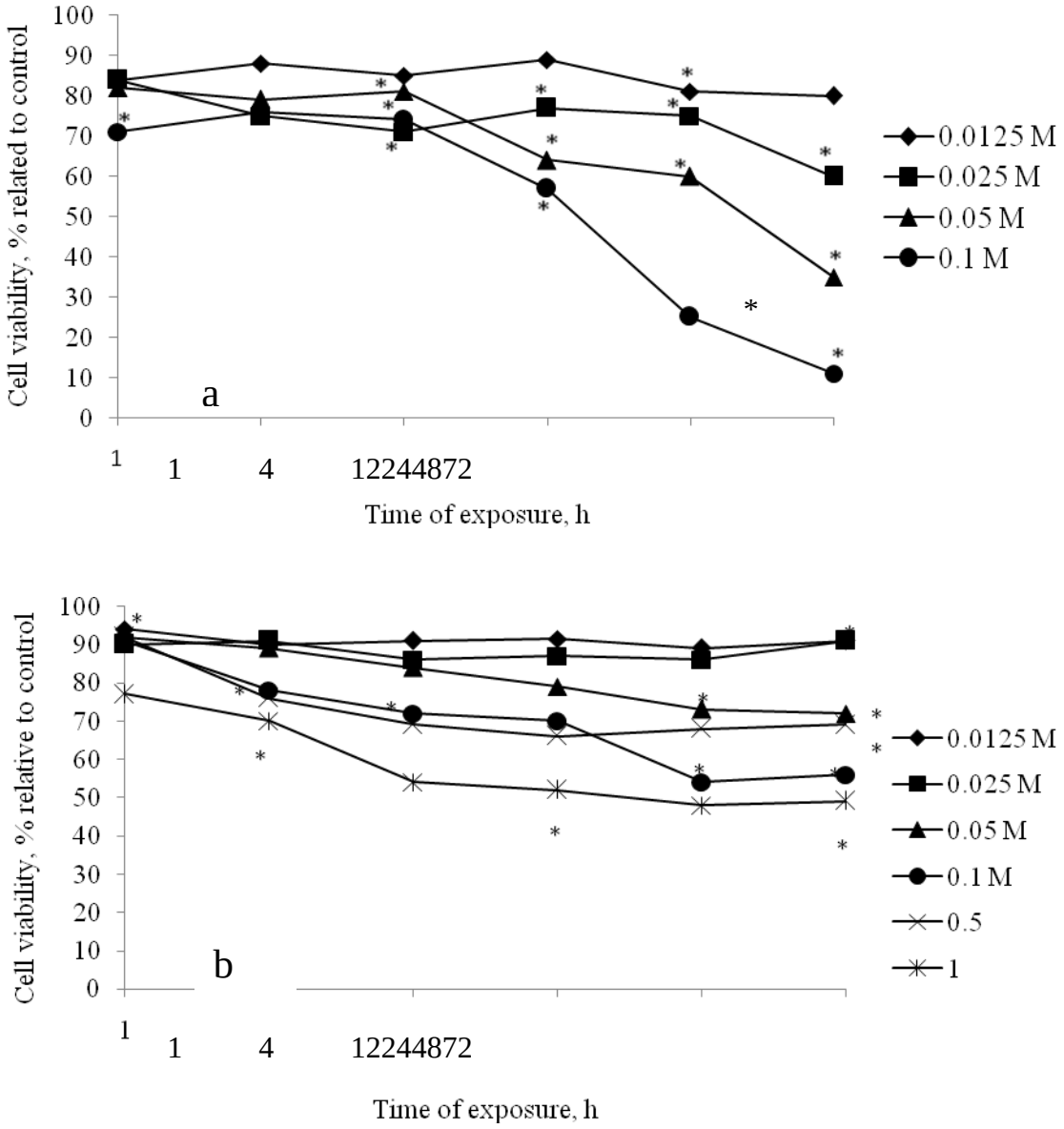

Figure 5: Cell viability of the T. vulgare germinant root cells after exposure to Ni (a) and Cu NPs (b): the indicator is calculated by the formazan content: asterisk (*) means the variant is reliably different from controls (P ≤ 0.05) (the measurements were carried out in 3-fold biological replicates)

Cu NPs turned out to be the most toxic; they caused unstable and sharp decrease in the formazan output immediately after the start of exposure (1 h). The effect became to manifest especially clearly after 24 h exposure and reached 89% compared with intact plants. We assume that such repression of reductase enzyme activity presents an early response to the metal. On the contrary, the presence of Ni in the medium resulted in more smooth reduction of the formazan content after 24 h of incubation (up to 56% compared to the control).

Colligating the received relations we concluded that reliable ($r < 0.05$) cytotoxic effect by NPs in the roots of wheat was recorded after 48 h exposure to metals in doses of 0.05 and 0.1 M.

Influence of metal nanoparticles on DNA damage extent in the roots of Triticum vulgare germinants

Continuing the study of the biological activity mechanism of nanometal, we analyzed their impact on cytogenetic indicators T. vulgare by DNA degradation extent in the apical parts of roots in terms in vivo. The analysis showed direct relation of DNA degradation to the dose and composition of NPs. So, adding Ni˚ NP into

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germination medium for 48 hours resulted in increase of mobile nucleotides from 60 to 79% compared to the control. It is essential that chromatin degradation on electrophoretograms displayed in the form of apoptotic “staircase” (“ladder”) consisting of multimers, sized divisibly 180-200 bp.In turn, in cases of Cuimpact electrophoretic picture changed qualitatively: specific stripes of DNA fragments with fixed length 10000-3000 bpal most disappeared; they disintegrated in to mobile fragments of irregular length forming a united “smear” (Higuchi, 2004). With that, the fragments less than 3000 bp increased in number compared to inoculated samples. We observed rise from 58.5 to 65.8% in cases with Cu, respectively. Such degradation of molecules can beconfirmedby temporally calculated linear profiles, on which a sharp transition of the bulk of the fragments into the area less than 300 bpNP and formation of typical ladder-like group of peaks ebbing in area becomes clearly visible in the case of nickel (Figure 6).

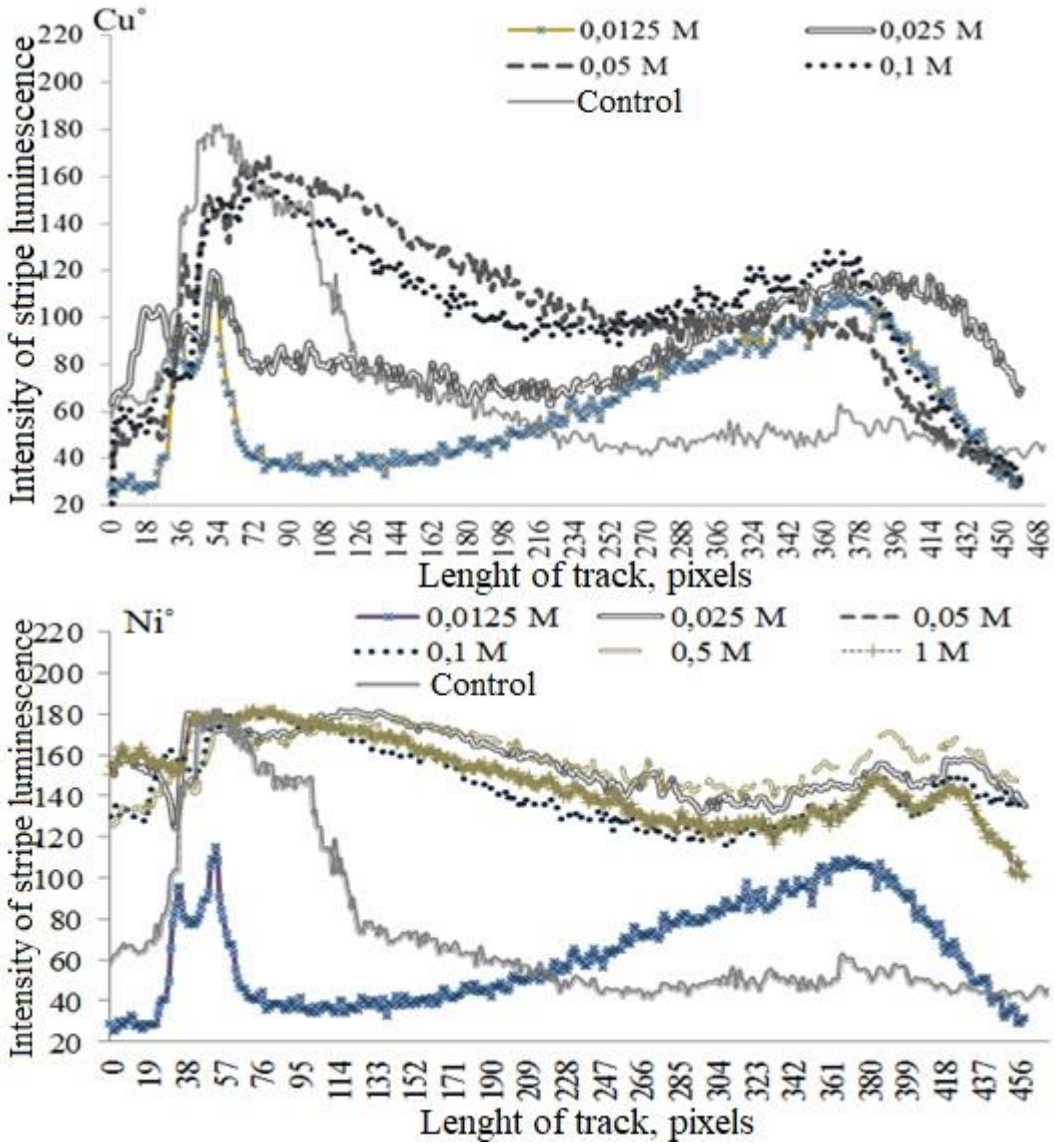

Figure 6: Linear profiles of electrophoretogram stripes of T. vulgare apical root DNA after 48-hour exposure to Cu and Ni NPs (profiles were retrieved from the “ImageJ” software): charts show the intensity of DNA stripe luminescence (x-axis) at each pixel of electrophoretogram track (y-axis)

Cu°-group profile after 1000 bp acquired the appearance of a convex curve with all the analyzed concentrations. It gets well noticeable via further descending of nucleotide peak heights, slightly exceeding thenoise limiter, DNA quickly and unsystem aticallydecays. In turn, DNA-damaging effects became so conspicuous that the total intensity of the electrophoretogramrose, which,on linear profile, looked like a superposition of peaks split into two parts (Figure 6).

Thus, we observed reduction of seed germination, growth indicators and the amount of chlorophyll in the germinant leaves in application of copper and nickel nano-metals. In turn, oxidation-reduction dis balance was registered in roots of germinants, which manifested as an increase in the total pool of ROS, including $HO^{\cdot}$ and $O_2^{\cdot-}$radicals. Despite the action of antioxidants, their protective mechanisms turned out ineffective after incubation with NPs at concentrations over 0.1 M, resulting inDNA structure destruction and CV decrease even with cell death. Probably, after adsorption on the root surface, Ni° NPs caused accumulation of $O_2^{\cdot-}$, "shielding" of germinant root system and the depletion of cell energy resources, which, in turn, led to apoptosis. This was confirmed by a small increase in the activity of caspase-like proteases, apoptotic inter nucleosomal DNA degradation and visualization of single apoptotic cells. At the same time, as the metal of variable (mixed) valence Cu NPs probably emitted $Cu^{2+}$and Cu^{+}ions, and led to an increase in the total pool of ROS. In turn, the excessive ROS, exceeding the adaptive capacities of the organism, contributed to mechanical damage to the cell wall and/or membranes (Ma et al., 2010), resulted in oxidative stress up to unsystematic DNA damage and the necrosis development.

Thus, we have found reduction in T. vulgare germinant stability after exposure to copper and nickel in nano form that is demonstrated by decrease in germinative energy and growth parameters; also, we have discovered the selective accumulation of hydrogen peroxide and hydroxyl radicals in the germinant roots under the action of Cu° NPs and hydroxyl radicals – after handling plants with nano-Ni°; we received data of cell viability reduction in the germinant roots exposed to metal NPs, where the form of DNA molecule degradation may be either necrotic unsystematic (Cu), orinternucleosomal apoptotic (Ni).

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