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Original Research

Humic acid reduces the CuO and ZnO nanoparticles cellular toxicity in rapeseed (*Brassica napus*)

Afsaneh Yarmohammadi[1], Mahmud Khoramivafa[2*], Saeid Jalali Honarmand[2]

[1] Department of Agronomy and Plant Breeding, Razi University, Iran
[2] Former student of Agroecology, Department of Agronomy and Plant Breeding, Razi University, Kermanshah, Iran

Correspondence to: khoramivafa@razi.ac.ir

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Abstract: Concerns about nanoparticles environmental pollution risk have been increased globally due to an increase in the production of nanoparticles in recent years and their use in diverse cases. The purpose of this experiment was to study the alleviation effect of humic acid on nanoparticles toxicity in greenhouse conditions. Thus two separate experiments were conducted at the rosette growing stages of rapeseed in a factorial experiment as a completely randomized design with three replications. The first factor was copper and zinc oxide nanoparticle in five concentrations of 0, 500, 1000, 1500, 2000 $mg.L^{-1}$ in each of experiments and the second factor was humic acid in two concentrations of 0 and 100 $mg.L^{-1}$ in both experiments. The results showed that simultaneously application of humic acid and the nanoparticles resulted in increasing of chlorophyll, protein contents, and antioxidants enzymes activity. For example, the maximum activity of catalase was 170.72 and 296.82 $\mu mol.min^{-1}.mg^{-1}$ proteins when CuO nanoparticle was utilized alone and together with humic acid respectively. Also increasing the concentration of CuO nanoparticle reduced protein content from 2.44 to 1.88 ($mg.gr^{-1}$ Fresh leaf weight), while its range was 2.86 and 2.49 ($mg.gr^{-1}$ Fresh leaf weight) when adding the humic acid. Transmission electron microscopy images of root tissue confirm the decreasing of nanoparticles entrance to plant cell and tissue by humic acid. In general, application of humic acid alleviated the nanoparticles toxicity, due to the high adsorption capacity that is able to get out the metals from plants or like-hormonal activity probably.

Key words: Environmental pollution; Oilseed; Organic matter; Like-hormonal activity.

Introduction

It is expected that nanoparticles (NPs) can be accidentally or incidentally released into the environment due to a wide range of their applications (1, 2). The NPs potentially hazardous effects have been lead to the increasing amount of research on ecotoxicity. However, studies on the toxicity of NPs are still emerging and basically evidence several negative effects on the growth and development of plants (3, 4). Copper oxide (CuO) and Zinc oxide (ZnO) NPs are mostly used as a UV light scattering additive in cosmetics such as sunscreens, kinds of toothpaste and beauty products. Also, ZnO and CuO NPs are widely used in rubber manufacture, production of solar cells, chemical fibers, electronics and textiles. (5, 6). Then, this high volume uses of ZnO and CuO NPs in many used industries that will release them to many environmental ecosystems will confirm the need to investigate their effects on living organisms, including plants. ZnO and CuO NPs have also included in a list of 14 representatives manufactured NPs for testing by the organization for economic cooperation and development (7).

Humic acid as a natural organic matter (NOM) is a mixture of various organic compounds derived from the residual components of plants and animals, and it has a weak acid pH range of 3.8 to 5 (8). There are limited studies on the interaction between humic acid and NPs (9). For example, it was reported that the TiO_2 NPs has been aggregated by NOM (10). NOM had a large influence on the amount of CeO_2 by inhibiting the sorption of NPs to roots (11). There has been also reported that humic acid plays an important role in reducing the toxicity of NPs and even more effective than folic acid in the stability and sustainability of NPs (12).

Plants play a critical role in the destiny and move of NPs in the environment through plant uptake and bioaccumulation. Even though scientific investigation on plant uptake and accumulation of NPs is still in its basic stage, some new publications have been added to the literature in the past few years yielding new advances in the area of NPs toxicology and uptake by plants (13). Rapeseed (*Brassica napus*) is the third source of vegetable oil after palm and soybeans and is an important source of food and pharmaceutical in the world (14-16). Rapeseed is documented as fast-growing metal-accumulator species, constituting a good candidate for induced phytoextraction (17-19). The condition of rapeseed plantation in Iran is such that the forecast for harvesting in 2019 is about 180 thousand hectares and production is more than 300 thousand tons. As a result, the study of reducing the toxicity of nanoparticles is a priority for theoretical research the organic matter, such as humic acid, on rapeseed. Although there are no specific testing guidelines for nanotoxicity, so the United States Environmental Protection Agency (EPA) guidelines for

chemical testing are frequently followed. Phytotoxicity assays generally use plants recommended by these guidelines; these are mostly crop species and include both monocotyledonous and dicotyledonous plants. Economically or ecologically important non-crop species are also studied (20).

The purpose of this experiment was to investigate the effect of humic acid on the biochemical activity of rapeseed plant under conditions of application of copper oxide and zinc oxide nanoparticles. The study about the effect of humic acid on reducing the toxicity of copper and zinc oxide nanoparticles in the rosette by investigating the activity of antioxidant enzymes, protein, and proline content and it was also investigated the accumulation of copper and zinc oxide nanoparticles, in the root tissue of rapeseed, in the presence of humic acid, the rapeseed rosette growing stage.

Materials and Methods

Nanoparticles solution set up

CuO and ZnO NPs are commercially purchased from Iranian Nanomaterials Pioneers Company. The nanoparticles were suspended directly in distilled water and dispersed by ultrasonic vibration (100 W, 40 kHz) for 30 minutes and the temperature of 50 ℃. Small magnetic bars were placed in the suspension for stirring before use, to avoid aggregation of the particles (21). Table 1 and Figure 1 illustrate the prominent properties of nanoparticles used.

Nanoparticles solution set up

The nanoparticles were suspended directly in distilled water and dispersed by ultrasonic vibration (James Products Ultrasonic Model Heating, 100 W, 40 kHz) for 30 minute and the temperature of 50 degrees Celsius. Small magnetic bars were placed in the suspension for stirring before use to avoid aggregation of the particles (21). Table 1 and Figure 1 illustrate the prominent properties of nanoparticles use.

Pot experiment

The experiment was carried out in a greenhouse of agricultural and natural resources campus of Razi University of Kermanshah as a factorial based on a completely randomized design with three replications in 2016 on rapeseed plant (*Brassica napus* L.), Okapi cultivar.

The factors include copper oxide nanoparticles (CuO) and zinc oxide (ZnO) in five concentrations of 0, 500, 1000, 1500, 2000 $mg.L^{-1}$ and humic acid in two concentrations of 0, 100 $mg.L^{-1}$. Humic acid consumed by the company (EURO SOLLDS) commercial HURO HUMIC as a water-soluble granule with 68% Humic acid, 10-30% Folic Acid, 7.5% Potassium Oxide, 12-22% Organic Nitrogen.

To prepare the soil of the pots, a compound sample was prepared from four fields of a research farm that did not undergo a few years. Table 2 shows some physical and chemical properties of the soil.

In order to prepare seedlings, seeds were cultivated in tray transplant (3×3×3 cm) containing cocopeat, and after 27 days (double leaf stage), seedlings produced in dark pots with a diameter of 10 cm and height of 13 cm were transmitted. All the pots were irrigated until field capacity with deionized water, and three seedlings of rapeseed were planted in each pot.

After seedlings establishment, 200 ml of each of the copper oxide and zinc oxide nanoparticles treatments, humic acid was added to each pot separately.

Samples were taken at the rosette stage for physiological, biochemical activities. In order to prevent damage to the samples, they were quickly frozen in liquid nitrogen and stored at -70 °C until they were placed in an aluminum foil.

Assessment of antioxidant enzymes activity

0.5 g of leaf samples were first crushed in a porcelain mortar, then two ml of extraction buffer was added. The obtained mixture was centrifuged in an Eppendorf tube for 15 minutes, with a round of 13000 centrifuges. Then, the upper phase was used to read out the amount of leaf soluble proteins and the activity rate of antioxidant enzymes.

To measure the rate of activity of the ascorbate peroxidase, 50 µL of extracted extract was mixed with one milliliter of ascorbic peroxidase measurement solution. Then its absorbance at 290 nm wavelength was measured by a spectrophotometer (Biotek Power Wave xs2) after one minute, (22). To calculate the rate of the peroxidase enzyme activity, 33 µL of the diluted enzyme extract was mixed with 1 mL of peroxidase substrate and read for 20 minutes at intervals of 30 seconds in a wavelength of 470 nm (23). The activity of catalase enzyme activity was measured by the following methods; 500 µL of enzyme extract diluted with 1 mL of Phosphate-buffered saline 100 mM (pH =7) mixture and the reaction was started by adding 500 µL of 60 mM Hydrogen peroxide solution. After a certain time, the reaction completed using a 2 mL indicator dichromate (5%) acetic acid (3: 1). The test tubes were quickly placed in the boiling water bath for 15 minutes. The readings

Table 1. Some physical properties of CuO and ZnO nanoparticles.

Nanoparticle	Property		
	Purity (%)	Particle size (nm)	Color
CuO	0.99	40	Black
ZnO	0.99	10-30	White

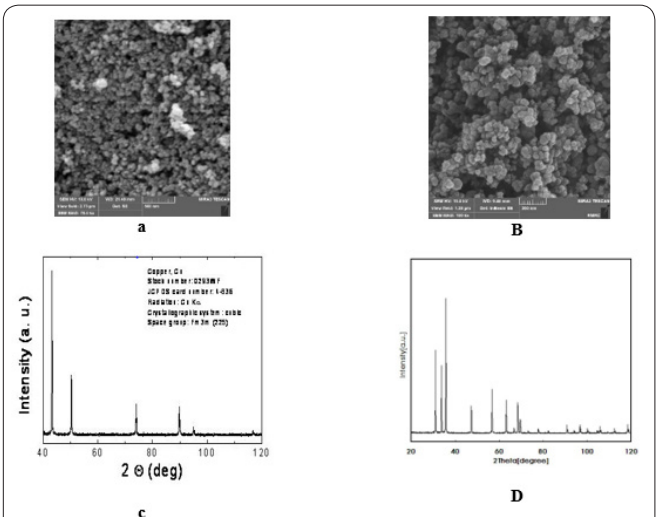

Figure 1. The nanoparticles scanning electron microscope (SEM) (a and b) and images of X-Ray (c and d). a and c, are related to CuO, b and d are related to ZnO.

were taken using a spectrophotometer in an absorption spectrum of 570 nm (24). For measuring the rate of superoxide dismutase enzyme activity, first, 50, 100, 150, 200 µL of the extracted extracts were extracted with a final solution of 200 µL extracted into a solution and subjected to 4 mL of superoxide dismutase solution containing 50 mM Buffer solution of Monopotassium phosphate) Was mixed with (pH =7), 75 µM Nitro blue tetrazolium chloride, 13 mM L-Methionine, 0.1 mM EDTA and 2 mM Riboflavin. Then two extracts without extracts containing 200 µL extraction buffer were used as control and blanc. Extraction extracts were added and the superoxide dismutase solution was added to Kot. To react, the mixture was placed in a light chamber for 15 minutes. Then, the absorbance of the solution was read on a spectrophotometer in a wavelength of 560 nm (25).

Protein content measurement

To measure the protein content, 20 µl of the extract was diluted in 80 µl of extraction buffer, and five ml of the Bradford indicator were added to it, then vertex, and its absorbance was read at 595 nm after five minutes (26). Standard Bovine Serum Albumin Serum (BSA) was used to draw the standard curve: First, 10 mg of BSA was dissolved into 10 ml of extraction buffer (This solution was considered as 100% solution). To make a 50% solution, 5 ml of 100% solution was mixed with 5 ml of extraction buffer. Next, five milliliters of 50% solution was mixed with 5 ml of extraction buffer (25% solution). To make a solution of 12.5%, 5 ml of 25% solution was mixed with 5 ml of extraction buffer and extraction buffer was used for zero solution. In the next step, from each test tube, 20 µl of solution was removed and diluted in 80 µl extraction buffer. Within each of the tubes, five milliliters of the colored reagent was mixed and vertex cooled well and after five minutes, the samples were placed on a spectrophotometer and the absorbance was read at 595 nm. Then, according to the optical absorption values and the soluble concentrations of the standard protein curve, they were drawn.

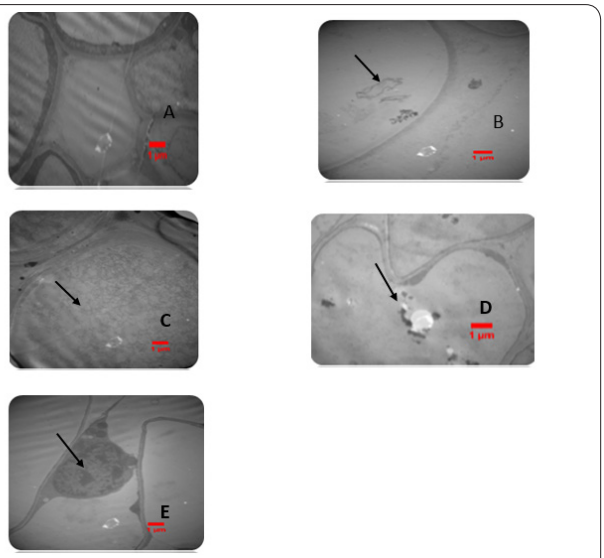

Figure 2. Microscopic analysis of rapeseed root in control plants (A), treated with ZnO nanoparticle (B), ZnO nanoparticle with humic acid (C), CuO nanoparticles with humic acid (D) and CuO nanoparticles (E). Arrows show the nanoparticles accumulation in cells.

Proline content measurement

To measure the proline concentration of 0.5 g of leaf, each sample was placed in 10 ml of aqueous sulfosalicylic acid solution (3%) and the resulting mixture was completely homogenized in a porcelain mortar. The homogenized mixture was then straightened with Whatman paper No.2 filter. In the next step, two ml of this solution were mixed with 2 ml of Nin Hydrine and two ml of acetic acid was added to each tube. Then, the samples were placed in an ice bath for a few minutes at a temperature of 100 ° C for 1 hour in a bath and then immediately removed from the bath. After this step, four ml of toluene were added to each tube and the samples were mixed together for 20 to 15 seconds until a completely uniform solution was obtained during this experiment; the two supernatant and bottom phases were completely detectable in the tube. The supernatant phase was used to determine the proline concentration in a spectrophotometer (Bio Tek Power Wave XS2) in a 520 nm wavelength range (27).

Trace of nanoparticles via Transmission electron microscope (TEM) analysis in root tissue

To investigate the influence of zinc oxide nanoparticles, copper oxide and humic acid in root cells as the first tissue exposed to nanoparticles. After two weeks, root samples were prepared for microscopic analysis in the Institute of Biochemistry and Biophysics (IBB), the University of Tehran and TEM imaging was taken at Iran Nanotechnology laboratory using a Transmitted Electron Microscope (TEM) (AM model, 208, Phillips, Netherlands). Primary fixation of root samples was performed by glutaraldehyde 2.5% for one hour at 4 ° C. Then, it washed twice with sodium cocadilat 0.1%; the first time for 10 minutes, and the second time washed for three hours. The secondary fixation was accomplished with azio tetraoxide 1% for one hour. Finally, the dehydration step was performed with ethanol 25, 50, 70, 90, 100% for 10 to 15 minutes. The sample was placed in propylene oxide for 20 minutes, and then they were placed in an Araldite resin for two to four hours. The molded sample was stored at 60 ° C for two to three days and then the resin block was prepared. The resin molded sample was cut by a cutting ultra-microtome machine (model OMU 3, Richart Inc, Austria) with a cutting edge of 70-100 nm. Finally, the staining of cut samples was done by purely acetate 2% and lead citrate 1%.

Data analysis

Data analysis of the experiment was performed by SAS software (9.2) and charts were plotted with Excel (2013). In order to compare the averages, the least significant difference test (LSD) was used at the probability level of 5%.

Results

Microscopic analysis

Figure 2, presents the electronically micrographs of TEM electron microscope achieved rapeseed root in treated plants with a concentration of 2000 $mg.L^{-1}$ ZnO and CuO nanoparticles, alone and or together with humic acid, compared with the control plants. There is

recognizable the rupture of the cell membrane and nanoparticles accumulation inside the cell, compared with control clearly (Figure 2a and 2b). It seems that humic acid has been controlled the nanoparticles entrance to cells and a lower amount of nanoparticles accumulated consequently (Figure 2c and 2d).

The activity of antioxidant enzymes

The activity of all four studied enzymes (catalase, ascorbate peroxidase, peroxidase and superoxide dismutase) were affected by humic acid, nanoparticles and their interaction ($P<0.05$) (Table 3). In this respect, addition the humic acid to growth mediums followed by a synergistic effect on the activity of enzymes for both ZnO and CuO nanoparticles. For example, the maximum activity of catalase was 170.72 $\mu mol.min^{-1}.mg^{-1}$ proteins at what time CuO nanoparticle was utilized alone, while it was 296.82 $\mu mol.min^{-1}.mg^{-1}$ when CuO nanoparticle and humic acid were applied at the same time. Also, the maximum activity of catalase was 279.5 and 323. $\mu mol.min^{-1}.mg^{-1}$ proteins in ZnO nanoparticle and ZnO together with humic acid growth mediums, respectively (Table 2). This trend was observed similarly for other studied enzymes between 1.3 to 3 times (tables 4 and 5).

Protein

Based on the results, the nanoparticles × humic acid influenced the content of soluble protein significantly (Table 3). Results showed that both nanoparticles reduced the content of soluble protein, while its reduction showed a milder trend when nanoparticles were utilized with humic acid concurrently. In fact, humic acid-regulated the nanoparticle effects on protein reduction. For example, increasing the concentration of CuO nanoparticle (500 to 1500 $mg.L^{-1}$) reduced protein content from 2.44 to 1.88 ($mg.gr^{-1}$ Fresh leaf weight), while the protein contents were 2.86 and 2.49 ($mg.gr^{-1}$ Fresh leaf weight) in the same nanoparticles concentration when adding the humic acid (table 4 and 5).

Proline

The nanoparticles increased proline content significantly and use humic acid resulted in the synergistic effect on both ZnO and CuO performance (Table 4 and 5). Related to ZnO nanoparticle, the most recorded proline

Table 2. Some physical and chemical properties of the utilized soil in pots.

Cu	Zn	Fe	Mn	K	P	Soil Texture	OC (%)
$mg.kg^{-1}$							
1.2	0.68	5.24	4	356	6.6	Silty-Clay	0.38

Table 3. Analysis of variance for Activity of antioxidant enzymes, the content of soluble protein and proline under ZnO and CuO NPs in rapeseed.

SOV	Df	MS					
		Catalase	Ascorbate peroxidase	Peroxidase	Superoxide dismutase	Protein	Proline
Humic acid (H)	1	4396.87**	2.74*	1938.8*	967.82**	0.144ns	674.97**
Copper oxide (C)	4	19677.78**	3.29**	10065.8**	2324.46**	1.633**	442.53**
H × C	4	7945.68**	0.57*	760.9*	300.04*	0.239*	90.45**
Error	20	365.49	0.192	247.27	29.31	0.044	8.64
CV(%)	-	10.47	10.04	9.09	9.53	8.58	8.41
Humic acid (H)	1	6026.25*	1.27*	28296.2**	1171.06*	0.0187ns	439.18**
Zinc oxide (Z)	4	30620.6**	7.24**	22279.5**	11250.4*	3.48**	779.8**
H × Z	4	1918.85*	0.92*	5147.1**	1452.2*	0.25*	63.73**
Error	20	622.91	0.102	126.13	35.47	0.035	10.21
CV(%)	-	11.67	8.43	5.83	9.53	7.70	9.51

* and ns respectively show a meaningful and non-significant at the probability level of 0.05.

Table 4. Mean comparisons of antioxidant enzymes activity, the content of soluble protein and proline in humic acid× copper oxide.

Humic acid $mg.L^{-1}$	Copper oxide $mg.L^{-1}$	Catalase	Ascorbate peroxidase	Peroxidase	Superoxide dismutase	Protein ($mg\ gr^{-1}$ Fresh leaf weight)	Proline ($\mu mol.\ gr^{-1}$ leaf dry weight)
		($\mu mol\ min^{-1}mg$ protein)					
0	0	110.38	2.38	122.04	37.43	3.42	24.52
0	500	141.92	3.9	135.96	37.95	2.44	25.93
0	1000	148.5	4.1	177.05	54.56	2.36	30.35
0	1500	149.31	4.43	189.98	58.72	1.88	32.98
0	2000	170.72	4.49	198.87	66.88	1.78	37.21
100	0	58.83	3.03	99.43	28.19	3.04	21.36
100	500	231.99	4.56	171.88	41.18	2.86	37.67
100	1000	254.4	4.76	195.74	70.98	2.24	39.66
100	1500	261.62	5.12	213.79	83.35	2.49	44.45
100	2000	296.82	5.87	223.45	88.63	1.95	55.17
LSD		32.56	0.74	26.78	9.22	0.35	5.008

Table 5. Mean comparisons of antioxidant enzymes activity, the content of soluble protein and proline in humic acid× zinc oxide.

Humic acid mg.L^{-1}	Zinc oxide mg.L^{-1}	Catalase	Ascorbate peroxidase	Peroxidase	Superoxide dismutase	Protein (mg.gr^{-1} Fresh leaf weight)	Proline (µmol. gr^{-1}leaf dry weight)
		(µmol min^{-1}mg protein)					
0	0	126.92	2.6	118.1	41.18	3.89	21.76
0	500	163.22	3.09	140.47	41.74	2.91	24.51
0	1000	212.32	3.93	171.7	60.02	2.15	25.6
0	1500	216	4.04	179.21	64.59	1.88	30.36
0	2000	279.5	4.31	200.04	73.57	1.56	40.6
100	0	91.66	1.9	96.47	31.01	3.11	25.6
100	500	208.57	3.2	162.94	45.3	2.99	29.93
100	1000	250.8	4.54	261.34	78.08	2.32	30.46
100	1500	265.34	4.65	295.17	91.68	2.02	47.62
100	2000	323.31	5.76	300.7	97.49	1.7	57.38
LSD		42.50	0.54	19.12	10. 14	0.32	5.44

content was 40.6 µmol.gr-1 dry weight, while it uprose to 57.38 µmol.gr-1 dry weight, when ZnO nanoparticle accompanied by humic acid. Also, the approximating condition was observed in the simultaneous presence of humic acid and CuO, where the proline content showed increasing by the 1.48 compared to condition with no humic acid.

Discussion

TEM images show damage to the cell wall as compared to the control group. Probably the cause of this damage is the high accumulation of nanoparticles of zinc oxide and copper and the formation of oxidative stress, and as a result, inhibits the growth of rapeseed. After the entrance of nanoparticles into cells, they are transmitted from one cell to another via plasmodesma. It is also possible that nanoparticles lead to pore enlargement or the induction of new pores in the cell wall, which in turn increases the absorption of nanoparticles. Nanoparticles can be transmitted through the carrier proteins or ions channels in the cytoplasm and linked to different cytoplasmic organs and interfere with metabolic processes (28). The uptake of silver nanoparticles from the media was also confirmed through transmission electron microscopy of tissues from treated *Brassica juncea* seedlings (29).

The interaction between humic acid carboxylic groups occurs with nanoparticles, and the changes in the surface of the nanoparticles with humic acid significantly reduce the total surface of the nanoparticles, resulting in a reduction in toxicity (30). There was also observed the effects of natural organic matter on reducing the toxicity of heavy metals such as copper, zinc, cadmium, and lead on potatoes (31).

Humic acid can change nanoparticles toxicity and mobility in different environments by binding to various biotic toxins, so the interaction of nanoparticles and humic acids is of particular interest from the environmental point of view. Since natural organic matter acts as a carbon source, it can also increase the soluble carbon content of the soil. The metal carbon-soluble organic carbon forms the metal-carbon compound, which reduces the absorption of these elements by plants and even microorganisms. In fact, one of the reasons for the growth and activity of plants in soils infected with metals due to the addition of organic matter is the increase of soluble organic carbon which creates an environment suitable for plant growth (32, 33). On the other hand, natural organic materials are more suitable than other metal adsorbents, due to their low cost, high abundance and the presence of functional groups such as hydroxyl, carboxyl, and phenol (34, 35).

In general, studies have shown that humic acid reduces the toxicity of metals in two different ways: first, the amount of free metal ion decreases due to the metal exchange reactions and the formation of total metal-metal reduce the bioavailability of metals, and second, the humic acid adsorbed at the root surfaces protects the cells from free metal ions (35). Another study reported that the use of vermicompost reduced the cadmium content by about 70% and also affected plant toxicity (36).

Similar to the results of this experiment, an increase in the activity of catalase enzyme has been reported in the treatment of metallic NPs of CeO_2 (11) and ZnO in buckwheat (*Fagopyrum esculentum*) (37). It has also been documented the increased activity of the enzyme peroxidase due to the application of TiO_2 NPs in spinach (*Spinacia oleracea*) (38). In respect with an increase in the activity of the enzyme superoxide dismutase, reports are presented, such as the effect of ZnO on increasing the activity of ascorbate peroxidase as a result of nanoparticle treatment (39).

Under non-stress conditions, there is a balance between the production of active oxygen species and the capacity for sweeping these compounds by an antioxidant defense system (enzymatic and non-enzymatic). However, under stress conditions, the production of active oxygen species increased their capacity to sweep them by the antioxidant defense system. As a result, oxidative stress occurs, so changing the capacity of the antioxidant defense system is essential to counteract oxidative stress. Although superoxide dismutase acts at the front of defense against active oxygen species, its product, hydrogen peroxide, is still toxic and should be removed from the cell. Some enzymes such as catalase contribute to the removal of hydrogen peroxide in the cell (40). The effect of humic acid on increasing the activity of catalase enzyme activity was reported in rice

(*Oryza sativa*) (41) and maize (*Zea mays*) (42).

The effect of humic acid on the increase in the amount of ascorbate peroxidase enzyme in the *Borago officinalis* has also been reported (43). In addition, increasing the activity of catalase, peroxidase and superoxide dismutase enzymes has been observed following the use of humic acid (44), which were consistent with the findings of this experiment

Along with the direct effects of humic acid mentioned above, other proposed mechanisms for the effects of humic acid are: the production of indoleacetic acid, cytokines with tryptophan amino acids and adenosine secreted from the roots, hydrolysis of the precursor of ethylene and the production of hormonal and quasi-hormonal substances, due to the reaction of nitrites resulting from respiration of nitrates with ascorbic acid. Therefore, in addition to having a direct impact on the mechanism of production of the plant growth regulator, it also indirectly influences the control of the activity of antioxidant enzymes in plants (45).

The decrease in the content of soluble protein under trace element stress has also been reported in lemna (46) and beans (47) plants. It may be because of enhanced protein degradation process as a result of increased protease activity, which is found to increase under stress conditions. It is also likely that these heavy metals may have induced lipid peroxidation and fragmentation of proteins due to toxic effects of reactive oxygen species, which led to reduced protein content (48). On the other hand, in addition to the direct effects of humic acid in reducing the entry of NPs into the plant, it generally acts as auxin and cytokinin growth regulators and increases the tolerance to different stresses, increasing the antioxidant enzyme activity of the plant (12).

Similar to our results, the increased proline content by copper oxide nanoparticles has been reported in *Arabidopsis thaliana* (49) and carrot (50) or by zinc oxide nanoparticles in *Brassica juncea* (51). Also, the effect of humic acid on increasing proline content in this experiment was consistent with some researchers' reports (43, 52, 53). Increasing the amount of proline in stress conditions is one of the criteria for tolerance in plants, and proline can play a protective role for proteins and enzymes under stress conditions (54). Several reasons have been suggested for proline accumulation in the plant during stress, due to the effect of the regulation of the acidic acid on optical processes in the proline metabolism, and some of them because of the presence of high energy compounds derived from photosynthesis which stimulates proline synthesis (55). In addition, the increase in degradation enzymes in tensions increases the amount of proline in the plant. The increase of proline during stress may result in the decomposition of proteins and reduce their use due to plant growth retardation (56). As a result, during prolonged stress, proline production increases to keep the plant from being toxic, and under glutamate stress conditions, which is the precursor of chlorophyll and proline synthesis, it is progressing to proline production. Totally, four reasons for increasing proline accumulation due to stress have been suggested: (1) stimulating its synthesis from glutamic acid, (2) reduce its exports through Phloem (vessel), (3) preventing its oxidation during stress, (4) destruction and disruption of the protein synthesis process (57).

The reason for the increase of proline content by treating humic acid is attributed to cases such as complex formation with heavy metals, as well as activating the antioxidant system, the cytokine hormone balance, proline accumulation (58, 59).

The results of this experiment showed that ZnO and CuO nanoparticles toxicity effects revealed as decreasing of leaf soluble protein content and increasing the activity of antioxidant enzymes. Meanwhile, the results show that humic acid caused plant tolerance promotion to nanoparticles toxicity as a result of increasing the activity of antioxidant enzymes via reducing infiltration of nanoparticles, and hormone-like properties. Although the nanoparticles performance in plants are obscure, and there are proposed the various mechanism that nanoparticle how the effect on plant system, while plants are one of the most important pathways for entrance and accumulation of nanoparticles in the food chain, especially in exceedingly utilization of nanoparticles in several industrials. Then, the study of lowering approaches of this trend such as humic acid and other natural organic matter application can play an important role in human and environment health.

References

1. Moradi S, Khaledian S, Abdoli M, Shahlaei M, Kahrizi D. Nano-biosensors in cellular and molecular biology. Cellular and Molecular Biology (Noisy-le-Grand, France) 2018; 64(5):85-90.

2. Ansari F, Kahrizi D. Hydrothermal synthesis of highly fluorescent and non-toxic carbon dots using Stevia rebaudiana Bertoni. Cellular and Molecular Biology (Noisy-le-Grand, France) 2018; 64(12):32-36.

3. Gottschalk F, Lassen C, Kjoelholt J, Christensen F, Nowack B. Modeling flows and concentrations of nine engineered nanomaterials in the Danish environment. Int J Environ Res Public Health 2015; 12(5):5581-5602.

4. Parveen A, Rizvi S, Gupta A, Singh R, Ahmad I, Mahdi F, et al. NMR-based metabonomics study of sub-acute hepatotoxicity induced by silica nanoparticles in rats after intranasal exposure. Cellular and Molecular Biology (Noisy-le-Grand, France) 2012; 58(1):196-203.

5. Bondarenko O, Juganson K, Ivask A, Kasemets K, Mortimer M, Kahru A. Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells in vitro: a critical review. Arch Toxicol 2013; 87(7):1181-1200.

6. Vicario-Parés U, Castañaga L, Lacave JM, Oron M, Reip P, Berhanu D, et al. Comparative toxicity of metal oxide nanoparticles (CuO, ZnO and TiO 2) to developing zebrafish embryos. J Nanopart Res 2014; 16(8):2550.

7. Hernandez-Viezcas J, Castillo-Michel H, Servin A, Peralta-Videa J, Gardea-Torresdey J. Spectroscopic verification of zinc absorption and distribution in the desert plant Prosopis juliflora-velutina (*velvet mesquite*) treated with ZnO nanoparticles. Chem Eng J 2011; 170(2):346-352.

8. Nikbakht A, Kafi M, Babalar M, Xia YP, Luo A, Etemadi N-a. Effect of humic acid on plant growth, nutrient uptake, and postharvest life of gerbera. J Plant Nutr 2008; 31(12):2155-2167.

9. El-Hak SG, Ahmed A, Moustafa YJJHSOP. Effect of foliar application with two antioxidants and humic acid on growth, yield and yield components of peas (*Pisum sativum* L.). J Hortic Scie Ornam Plant 2012; 4(3):318-328.

10. Wang H, Ye Y, Qi J, Li F, Tang Y. Removal of titanium dioxide nanoparticles by coagulation: effects of coagulants, typical ions, al-

kalinity and natural organic matters. Wat SciTech 2013; 68(5):1137-1143.
11. Schwabe F, Schulin R, Limbach LK, Stark W, Bürge D, Nowack B. Influence of two types of organic matter on interaction of CeO_2 nanoparticles with plants in hydroponic culture. Chemosphere 2013; 91(4):512-520.
12. Zhang X, Schmidt R. Hormone-containing products' impact on antioxidant status of tall fescue and creeping bentgrass subjected to drought. Crop Sci 2000; 40(5):1344-1349.
13. Ma X, Geiser-Lee J, Deng Y, Kolmakov A. Interactions between engineered nanoparticles (ENPs) and plants: phytotoxicity, uptake and accumulation. SciTotal Environ 2010; 408(16):3053-3061.
14. Kakaei M, Kahrizi D. Study of seed proteins pattern of brassica napus varieties via sodium dodecyl sulfate polyacrylamid gel electrophoresis. International Research Journal of Biotechnology 2011; 2(1):026-028.
15. Chaghakaboodi Z, Kahrizi D, Zebarjadi A. Heritability and genetic advance in rapeseed (Brassica napus L.). Iranian Journal of Genetics and Plant Breeding 2012; 1(2):16-21.
16. Zirgoli MH, Kahrizi D. Effects of end-season drought stress on yield and yield components of rapeseed (Brassica napus L.) in warm regions of Kermanshah Province. Biharean Biologist 2015; 9:133-140.
17. Ashraf M, McNeilly T. Salinity tolerance in Brassica oilseeds. Crit Rev Plant Sci 2004; 23(2):157-174.
18. Zaier H, Ghnaya T, Rejeb KB, Lakhdar A, Rejeb S, Jemal F. Effects of EDTA on phytoextraction of heavy metals (Zn, Mn and Pb) from sludge-amended soil with *Brassica napus*. Bioresour Technol 2010; 101(11):3978-3983.
19. Kahrizi D, Alaahvarand T. Estimation and interrelationships of genetic variability parameters of some morpho-phenological traits in spring rapeseed (Brassica napus L.). Asian Journal of Biological Sciences 2012; 5(7):358-364.
20. Miralles P, Church TL, Harris AT. Toxicity, uptake, and translocation of engineered nanomaterials in vascular plants. Environ Sci Technol 2012; 46(17):9224-9239.
21. Adhikari T, Kundu S, Biswas AK, Tarafdar JC, Rao AS. Effect of copper oxide nano particle on seed germination of selected crops. J Agric SciTechnol 2012; 2(6A):815.
22. Nakano Y, Asada K. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 1981; 22(5):867-880.
23. Chance B, Maehly A. Assay of catalases and peroxidases. Methods Enzymol 1955,2:764-775.
24. Sinha AK. Colorimetric assay of catalase. Anal Biochem 1972; 47(2):389-394.
25. Beauchamp C, Fridovich I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 1971; 44(1):276-287.
26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72(1-2):248-254.
27. Bates L, Waldren R, Teare I. Rapid determination of free proline for water-stress studies. Plant Soil 1973; 39(1):205-207.
28. Navarro E, Baun A, Behra R, Hartmann NB, Filser J, Miao A-J, et al. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. Ecotoxicol 2008; 17(5):372-386.
29. Sharma P, Bhatt D, Zaidi M, Saradhi PP, Khanna P, Arora S. Silver nanoparticle-mediated enhancement in growth and antioxidant status of *Brassica juncea*. Appl Biochem Biotechnol 2012; 167(8):2225-2233.
30. Van Hoecke K, De Schamphelaere KA, Ramirez–Garcia S, Van der Meeren P, Smagghe G, Janssen CR. Influence of alumina coating on characteristics and effects of SiO2 nanoparticles in algal growth inhibition assays at various pH and organic matter contents. Environ Int 2011; 37(6):1118-1125.
31. Angelova V, Ivanova R, Pevicharova G, Ivanov K, editors. Effect of organic amendments on heavy metals uptake by potato plants. 19th World Congress of Soil Science, Soil Solutions for a Changing World; 2010.
32. Han D-H, Lee J-H. Effects of liming on uptake of lead and cadmium by *Raphanus sativa*. Arch Environ ContamToxicol 1996; 31(4):488-493.
33. Hanc A, Tlustos P, Szakova J, Habart J. Changes in cadmium mobility during composting and after soil application. Waste Manag 2009; 29(8):2282-2288.
34. Jadia CD, Fulekar MH. Phytoremediation: The application of vermicompost to remove zinc, cadmium, copper, nickel and lead by sunflower plant. Environ Eng Manag J 2008; 7(5).
35. Koukal B, Gueguen C, Pardos M, Dominik J. Influence of humic substances on the toxic effects of cadmium and zinc to the green alga *Pseudokirchneriella subcapitata*. Chemosphere 2003; 53(8):953-961.
36. Park JH, Lamb D, Paneerselvam P, Choppala G, Bolan N, Chung J-W. Role of organic amendments on enhanced bioremediation of heavy metal (loid) contaminated soils. J Hazard Mater 2011; 185(2):549-574.
37. Lee S, Kim S, Kim S, Lee I. Assessment of phytotoxicity of ZnO NPs on a medicinal plant, *Fagopyrum esculentum*. Environ Sci Pollut Res 2013; 20(2):848-854.
38. Hong F, Zhou J, Liu C, Yang F, Wu C, Zheng L, et al. Effect of nano-TiO_2 on photochemical reaction of chloroplasts of spinach. Biol Trace Elem Res 2005; 105(1-3):269-279.
39. Kumari M, Khan SS, Pakrashi S, Mukherjee A, Chandrasekaran N. Cytogenetic and genotoxic effects of zinc oxide nanoparticles on root cells of *Allium cepa*. J Hazard Mater 2011; 190(1):613-621.
40. Mittler R. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 2002; 7(9):405-410.
41. García AC, Santos LA, Izquierdo FG, Sperandio MVL, Castro RN, Berbara RLL. Vermicompost humic acids as an ecological pathway to protect rice plant against oxidative stress. Ecol Eng 2012; 47:203-208.
42. Cordeiro FC, Santa-Catarina C, Silveira V, de SOUZA SR. Humic acid effect on catalase activity and the generation of reactive oxygen species in corn (*Zea mays* L.). Biosc Biotechnol Biochem 2011; 75(1):70-74.
43. Heidari M, Miri HR, Minaei A. Antioxidant enzymes activity and biochemical components of borage (*Borago officinalis*) in response to water stress and humic acid treatment. Environmental Stresses in Crop Sciences 2014; 6(2):159-170.
44. Kesba HH, El-Beltagi HS. Biochemical changes in grape rootstocks resulted from humic acid treatments in relation to nematode infection. Asian Pac J Trop Biomed 2012; 2(4):287-293.
45. Khan A, Khan M, Hussain F, Akhtar M, Gurmani A, Khan S. Effect of humic acid on the growth, yield, nutrient composition, photosynthetic pigment and total sugar contents of peas (*Pisum sativum* L.). J Chem Soc Pakistan 2013; 35(1):206-211.
46. John R, Ahmad P, Gadgil K, Sharma S. Effect of cadmium and lead on growth, biochemical parameters and uptake in *Lemna polyrrhiza* L. Plant Soi Environ 2008; 54(6):262.
47. Bhardwaj P, Chaturvedi AK, Prasad P. Effect of enhanced lead and cadmium in soil on physiological and biochemical attributes of *Phaseolus vulgaris*. J Nat Sci 2009; 7(8):63-75.
48. John R, Ahmad P, Gadgil K, Sharma S. Heavy metal toxicity: Effect on plant growth, biochemical parameters and metal accumulation by *Brassica juncea* L. Int J Plant Prod 2012; 3(3):65-76.
49. Prakash M, Nair G, Chung IM. Impact of copper oxide nanopar-

ticles exposure on *Arabidopsis thaliana* growth, root system development, root lignification, and molecular level changes. Environ Sci Pollut Res Int 2014; 21(22):12709.

50. Szafrańska K, Cvikrová M, Kowalska U, Górecka K, Górecki R, Martincová O, et al. Influence of copper ions on growth, lipid peroxidation, and proline and polyamines content in carrot rosettes obtained from anther culture. Acta Physiol Plant 2011; 33(3):851-859.

51. Rao S, Shekhawat G. Toxicity of ZnO engineered nanoparticles and evaluation of their effect on growth, metabolism and tissue specific accumulation in *Brassica juncea*. J Environ Chem Eng 2014; 2(1):105-114.

52. Ozfidan-Konakci C, Yildiztugay E, Bahtiyar M, Kucukoduk M. The humic acid-induced changes in the water status, chlorophyll fluorescence and antioxidant defense systems of wheat leaves with cadmium stress. Ecotoxicol Environ Saf 2018; 155:66-75.

53. Yildiztekin M, Tuna AL, Kaya C. Physiological effects of the brown seaweed Ascophyllum nodosum) and humic substances on plant growth, enzyme activities of certain pepper plants grown under salt stress. Acta Biol Hung 2018; 69(3):325-335.

54. Vendruscolo ECG, Schuster I, Pileggi M, Scapim CA, Molinari HBC, Marur CJ, et al. Stress-induced synthesis of proline confers tolerance to water deficit in transgenic wheat. J Plant Physiol 2007; 164(10):1367-1376.

55. Serraj R, Sinclair T. Osmolyte accumulation: can it really help increase crop yield under drought conditions? Plan Cell Environ 2002; 25(2):333-341.

56. Siripornadulsil S, Traina S, Verma DPS, Sayre RT. Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae. Plant Cell 2002; 14(11):2837-2847.

57. Hayat S, Hayat Q, Alyemeni MN, Wani AS, Pichtel J, Ahmad A. Role of proline under changing environments: a review. Plant Signal Behav 2012; 7(11):1456-1466.

58. Aydin A, Kant C, Turan M. Humic acid application alleviate salinity stress of bean (Phaseolus vulgaris L.) plants decreasing membrane leakage. Afr J Agric Res 2012; 7(7):1073-1086.

59. Pizzeghello D, Francioso O, Ertani A, Muscolo A, Nardi S. Isopentenyladenosine and cytokinin-like activity of different humic substances. J Geochem Explor 2013; 129:70-75.