Progress in Biological Sciences
Vol. 6, Number 1, Winter / Spring 2016/75-83

Nano-Metal oxides induced sulforaphane production and peroxidase activity in seedlings of *Lepidium draba* (Brassicaceae)

Maryam Aminizadeh[1], Ali Riahi-Madvar[2]*, Mehdi Mohammadi[1]

[1] *Department of Biotechnology, Faculty of Science and Modern Technology, Graduate University of Advanced Technology, Kerman, Iran.*

[2] *Department of Biotechnology, Institute of Science and High Technology and Environmental Sciences, Graduate University of Advanced Technology, Kerman, Iran.*

Received: October 15, 2015; Accepted: June 7, 2016

ABSTRACT

Effects of different concentrations [0, 1, 5, 10, 20 and 40 mgL^{-1}] of Fe_3O_4 and CuO nano-particles (nFe_3O_4 and nCuO) were investigated on sulforaphane (SFN) production level in 7-day-old seedlings of *Lepidium draba* at different time intervals (8 and 16 hrs). According to the results, the influence of the particles on SFN content depends on nano-particle (NP) concentrations, time of treatment as well as chemical nature of NPs. The SFN content was significantly increased in treated seedlings with 5 mgL^{-1}nCuO and all nFe_3O_4 concentrations as well as nFe_3O_4-treated callus after 8 hrs. However, by the increasing treatment time to 16 hrs, no significant changes on the SFN content were seen compared to the control. Furthermore, activity of peroxidase was also significantly promoted in treatment with both NPs (especially at higher concentrations) after 8 hrs and drastically decreased after 16 hrs. On the other hand, seed germination as well as the root and shoot length (except root length in treatment with nFe_3O_4) decreased compared to the control when seed germinated and plant growth in presence of both NPs for 7 days. Totally, these observations can be attributed to induce oxidative stress by NPs as a subsequence of their uptake by the plant. The increment in production of the phytochemicals through nano-metals treatment (nano-elicitation), opens an opportunity for induction of beneficial phytochemical content.

Keywords: CuO nano-particles; Fe_3O_4 nano-particles; Glucoraphanin; Nano-elicitaion.

* **Corresponding author:** a.riahimadvar@kgut.ac.ir

Introduction

Nano-materials that are aggregation of atoms or molecules with at least one dimension of 100 nm or less, are increasingly being used in broad fields of electronics (1), energy (2), medicine and life sciences (3). Recently, the use of nanotechnology has been a tremendous interest in agriculture in order to deliver water, fertilizers and herbicides to plants more efficiently (4). However, the positive or negative effects of NPs have been reported on growth of different plant species (5, 6). While, the inhibitory effects of several nano-oxide materials including Cu, Al, Si, Fe and Zn have been shown on development of plant growth (6, 7), plenty of literatures have been focused on toxicity effects of these particles which was revealed particularly at higher concentrations. Although, the exact mechanism of nano-toxicity has not been fully understood on plant, oxidative stress has been addressed as the possible routes of NPs-induced toxicity after absorption (8, 9). In oxidative stress, Reactive Oxygen Species (ROS) including superoxide anion, hydroxyl radicals and hydrogen peroxide are the main radicals that injure cells by damaging the cell membrane, nucleic acids and proteins (10). Additionally, the elicitor function of nanoparticle (silver NP) has been recently reported by several researchers in order to increase phytochemicals in plants (11, 12). They showed elicitation by the NP, mediated through ROS formation.

Whitetop (*Lepidium draba* L.), a perennial plant of the Brassicaceae family, contains two major types of glucosinolates: glucoraphanin and glucosinalbin (13). When the plant (Brassicaceae spp.) tissue is damaged, glucosinolates comes into contact with myrosinase (β-thioglucoside glucohydrolase, EC 3.2.3.1) and hydrolyse to glucose and unstable intermediate: aglycone (14). The resulting intermediate rearranges and converts to several compounds such as isothiocyanates, thiocyanates and nitriles depending on environmental conditions (15). Sulforaphane as the most important isothiocyanate, which can be produced through myrosinase catalysed of glucoraphanin (16). It has recently attracted researcher's interest due to its anticarcinogenic and anticancer activities (17, 18).

So far, several elicitors have been applied to induce glucosinolate biosynthesis or derivatives in different species of Brassicaceae (19-21). As far as we know, no report has ever been published on production level of SFN by the use of nano-elicitation as well as using nano-metal oxide as elicitor.

The aim of this study was to examine the influence of different concentrations of nFe_3O_4 and nCuO on SFN production and H_2O_2 scavenger enzymes activity. Furthermore, the effects of these particles were analysed on some morphological properties of seedlings of *L. draba*.

Materials and Methods

Metal oxide nano-particles were purchased from NaBond Technology Inc. Their characteristics as reported by the commercial agent were as follows:

- Fe_3O_4; purity >99.2%, diameter 60 nm and surface area 55 m^2g^{-1}.
- CuO; purity >99.2%, diameter 60 nm and surface area 55 m^2g^{-1}.

Seeds of *L. draba* were collected from Kerman province (Iran) in the end of May and early June 2012. Acetonitrile was HPLC grade and sulforaphane standard were purchased from Sigma Chemical Co. All other chemicals used were analytical grade and obtained from Merck.

Seed Culture and Plant Growth

The seeds were surface-sterilized for 15 min in NaOCl solution (3.0%), and were washed thoroughly using sterile distilled water. Solidified basal media of MS (22) containing 1.0% agar was used as a medium for seed cultivation. Around 40 disinfected seeds with about 5.0 mm spacing between them, were placed on the surface of the medium in 10 cm Petri dishes. The seeds were grown under a 16-hour light and 8-hour dark photoperiod in a germinator at controlled temperature of 28±2 ºC and relative humidity of 60-65%.

Elicitor Preparation and Seedling Treatment

Different concentrations of NPs (0 as control, 1, 5, 10, 20, 40 mgL^{-1}) were suspended in deionized water (pH 5 ± 0.2) separately (without dilution). After incubation

period of 7 days the seedlings were harvested and rinsed with sterile distilled water and subjected to different NPs concentrations. In brief, around 40 seedlings were transferred in 250 mL Erlenmeyer flasks containing 100 mL of the solution. The flasks were shaken at 100 rpm on an orbital shaker for 8 and 16 hrs at room temperature. In order to remove the particles from seedlings surface, the treated seedlings were rinsed several times with distilled water and immediately frozen in liquid nitrogen, and kept at − 80°C.

Callus Induction

Calli were induced from cotyledon explants of in vitro grown 7-days-old seedlings of *L. draba*. The seedlings were grown as mentioned above. The explants were proliferated in solidified basal media of MS supplemented with different hormone (3 mgL^{-1} of BAP, 0.5mgL^{-1} of NAA and 0.2 mgL^{-1} of 2,4-D). Culture vessels were transferred to darkness at controlled temperature of 26±2°C, allowing growth of the calli. The 7-days-old calli were sub-cultured in a fresh medium containing similar hormone concentration as above.

Cell suspension culture medium was similar to the medium for callus induction experiments without addition of agar. Proper callus pieces were transferred to Erlenmeyer flask containing 50 ml of MS medium suspension with equal amounts of callus. Flasks were put on a shaker with speed of 100 rpm and temperature of 25°C for 48 hrs. Finally, different concentrations of nano-Fe_3O_4 (0 as control, 1, 5, 10, 20, 40 mgL^{-1}), were added to the cell suspension for 8 hrs. At following, the cells were harvested and the SFN content was measured as described below.

SFN Determination and Quantification

Approximate SFN concentration of the seedlings and calluses under treatment with NPs was calculated as described by Liang and co-workers (23), using L-sulforaphane as a standard. In brief, 0.5 g of the fresh tissue was crashed with a mortar and pestle and then mixed with 1.0 mL of acidic water (pH=5). The homogenate was incubated for 2 hrs at 42°C, and then acetonitrile (5 mL) was added to the mixture and sonicated for 3 mins. The resulting mixture was centrifuged for 10 mins at 10000 rpm and 4.0°C. At following, the supernatant was passed through the 0.2 µm syringe filter prior to loading onto the reversed phase C_{18} column (25×0.46 cm) of High Performance Liquid Chromatography (HPLC, Agilent 1100 series) system.

In order to separate SFN, HPLC condition was as follows: mobile phase solvent acetonitrile/H_2O (65/35 v/v), flow rate of 1.0 mL/min at 25ºC and run time for each analysis was 14 mins which continued by holding for 6 min to re-equilibrate the column for the next injection. The SFN peak in the seedlings was recognized by comparing the retention time with SFN reference standard at 254 nm.

Antioxidant Enzymes Assay

Protein extraction procedure: Fresh tissue (0.5 g) was grinded in 5 mL of 50 mM phosphate buffer (pH=7.5) containing 0.5 mM EDTA, 1% polyvinylpyrrolidone and 0.1 mM PMSF. The homogenate was centrifuged for 15 mins at 12000 rpm and 4°C. Protein approximate concentration in seedling crude extracts was calculated according to the Bradford method, with bovine serum albumin as a standard (24). The supernatant was stored at 80°*C* until used for enzyme activity assay. All spectrophotometric analyses were conducted using an ultraviolet-visible spectrophotometer (Varian cary50, Australia). The experiments were separately repeated in triplicate.

Peroxidase (POD) assay: The POD (EC 1.11.1.7) activity was assayed according to the method of Plewa et al. (25). The reaction was followed by adding 30 µL of the enzyme extract to the mixture of 2.77 mL of 50 mM phosphate buffer (pH=7.0), 100 mL guaiacol 4% and 100 mL H_2O_2 1%. The activity was calculated as alteration in absorbance at 470 nm against mg of protein.

Catalase (CAT) assay: CAT (EC 1.11.1.6) activity was measured according to Dhindasa method (26). The assay mixture consisted of 30 µL of the enzyme extract, 2.87 mL of 50 mM phosphate buffer (pH=7.0), and 30 µL of 15 mM H_2O_2. The decline in absorbance at 240 nm was measured following the decomposition of H_2O_2. One unit of CAT is defined as

the amount of enzyme that decomposes 1 µM H_2O_2 in 1 min and the activity was presented in unit against mg of protein.

Morphological Studies

Analysis of morphological properties was conducted on the seeds that germinated and grew for 7 days in the presence of various concentrations (0, 25, 50, 100, 200, 500 and 1000 mgL^{-1}) of metal oxide nano-particles. The disinfection and growth condition were similar as mentioned above.

Seed germination: The germination (the least length of the radicals was around 1 mm) was begun after 48 hrs and continued until 4th Day. Seed germination was presented as the percentage of the number of germinated seeds on 4th day.

Measurement of shoot and root elongation: After an incubation period of 7 days, 20 seedlings were harvested from the medium randomly. The root and the shoot length were measured using ruler and were presented in millimetres.

Statistical Analyses

Experiments were conducted in completely random designs. Statistical analyses were done using windows-based SPSS 21.0 (SPSS Inc.). Statistical significance of differences between treatments was determined by one-way ANOVA method with Duncan's multiple range tests at the 95% confidence level. Values are means ± standard deviation (SD) of at least three separate experiments with three replicates for each treatment.

Results

The effects of NPs on SFN content

The retention time of SFN was about 4 min after injection into the column as revealed for standard SFN (data not shown). Treatment of seedlings with the NPs led to significant increase in SFN content compared to the control after 8 hrs treatments, but by increasing the treatment time, no significant change in SFN content was revealed. As shown in Figure 1A, the SFN content was significantly promoted with all concentrations of nFe_3O_4 compared to the control. While in nCuO-treated seedlings, the SFN content level was similar to the control at the lowest concentration (1 mgL^{-1}), and its content elevated at higher concentrations that was significant at level of 5% at 5 mgL^{-1} (Fig. 1B). Furthermore, whilst the SFN content was affected more with different concentrations of nFe_3O_4 after 8 hrs, its content was examined in *L. draba* cell suspension with this NP, and showed that this particle also induced the SFN production level in calli up to 10 mgL^{-1} concentration (Fig. 2).

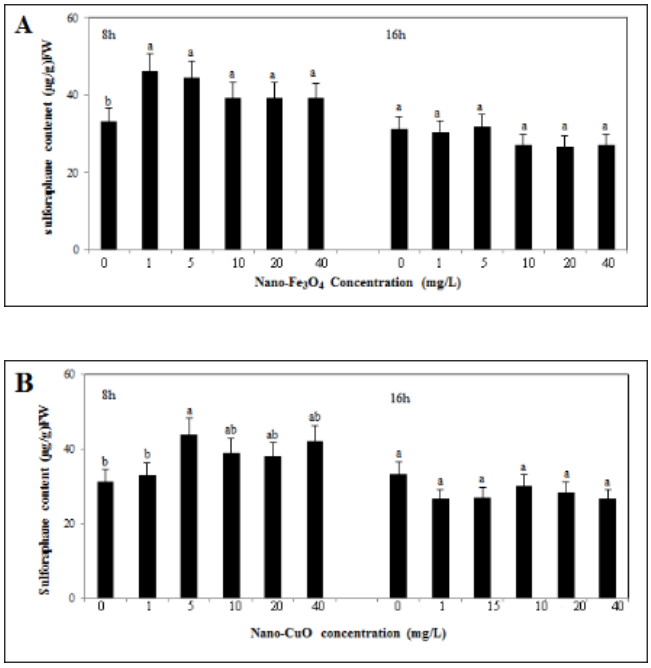

Figure 1. SFN content in *Lepidium draba* seedlings that were treated for 8 and 16 hrs with different concentrations of nFe_3O_4 (A) and nCuO (B). Signs with different letters are significantly different at p≤0.05, according to Duncan's multiple range tests.

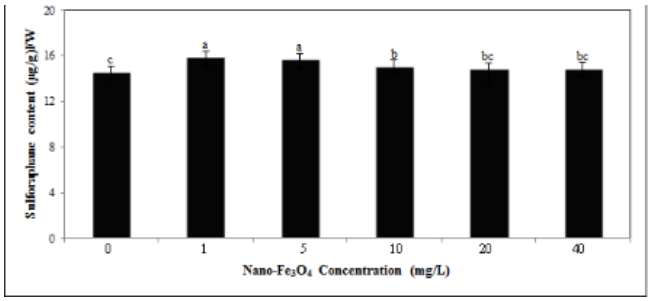

Figure 2. SFN content in cell suspension that were treated for 8 hrs with different concentrations of nFe_3O_4. Signs with different letters are significantly different at p≤0.05, according to Duncan's multiple range tests.

Antioxidant Enzymes activity

The results showed that evaluation of CAT activity was similar to the control in nFe_3O_4-treated seedlings and in nCuO-treated seedlings except at treatment with 10 mgL^{-1} nCuO concentration that its activity significantly increased after 8 hrs (Figs. 3A, B). But the POD activity significantly increased in nFe_3O_4-treated seedlings at concentrations more than10 mgL^{-1} (whereas its activity reduced up to 5 mgL^{-1} concentration) and in treatment with 10 and 20 mgL^{-1} of nCuO after 8 hrs (Figs. 4A, B). In contrast, the CAT and POD activities tended to decrease by the increase treatment time to 16 hrs.

Morphological characteristics

To assay toxicity effects of these particles on *L. draba* plant, seed germination rate and root and shoot growth were measured. As shown in Figure 5 the rate of seed germination was significantly reduced to 60% of the control level in treatment with all concentrations of both NPs. The root growth was significantly promoted by the Fe_3O_4 NPs at the lowest concentration (25 mgL^{-1}) and thereafter decreased as the level of control (except at 200 mgL^{-1} that decrease of root growth was significant compared to the control) (Fig. 6A).

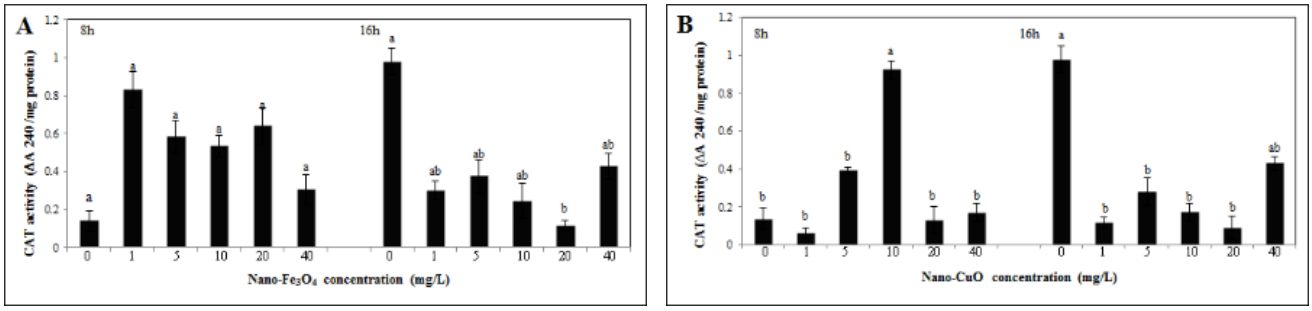

Figure 3. CAT activity in *Lepidium draba* seedlings that treated for 8 and 16 hrs with different concentrations of nFe_3O_4 (A) and nCuO (B). Data are presented as the means ± SD (n=3). Signs with different letters are significantly different at $p \leq 0.05$, according to Duncan's multiple range tests.

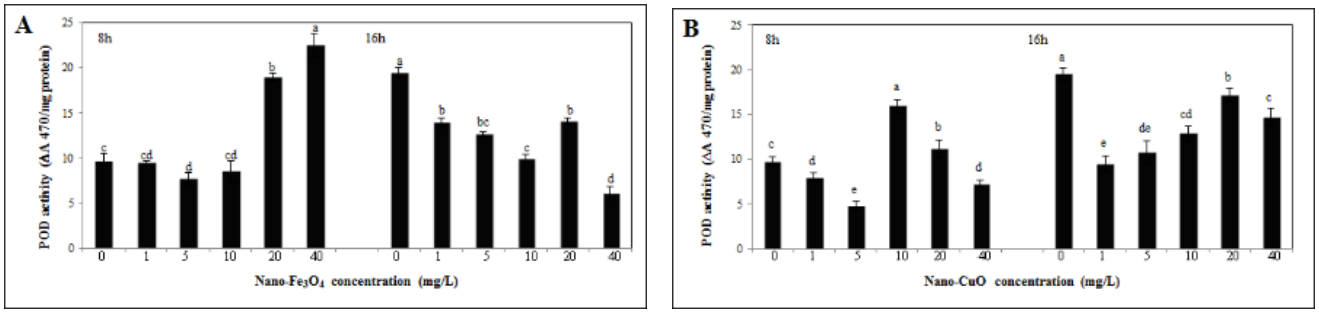

Figure 4. POD activity in *Lepidium draba* seedlings that treated for 8 and 16 hrs with different concentrations of nFe_3O_4 (A) and nCuO (B). Data are presented as the means ± SD (n=3). Signs with different letters are significantly different at $p \leq 0.05$, according to Duncan's multiple range tests.

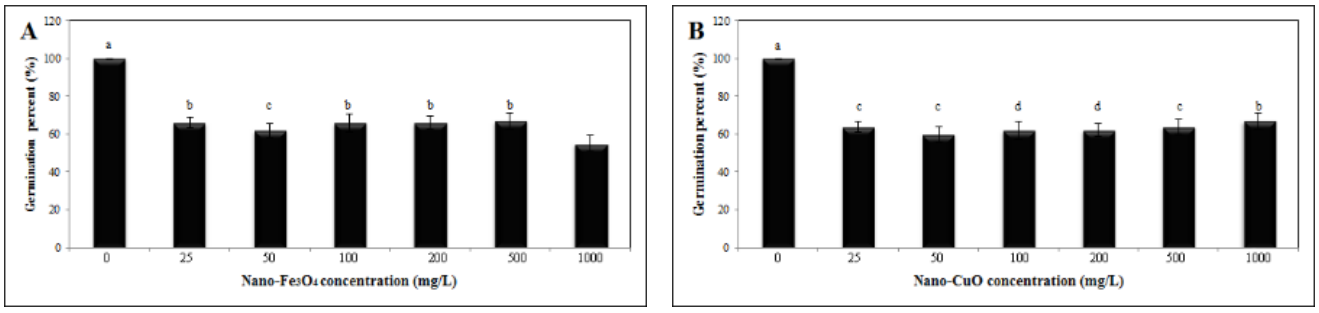

Figure 5. Seed germination of the *Lepidium draba* in presence of different concentrations of nFe_3O_4 (A) and nCuO (B). Data are presented as the means ± SD (n=3). Signs with different letters are significantly different at $p \leq 0.05$, according to Duncan's multiple range tests.

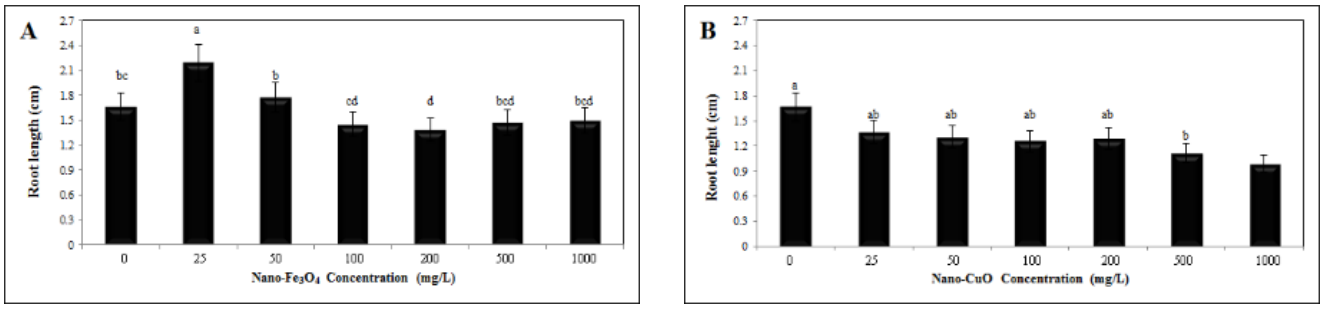

Figure 6. Root length of the treated *Lepidium draba* seedlings with different concentrations of nFe_3O_4 (A) and nCuO (B). Data are presented as the means ± SD (n=3). Signs with different letters are significantly different at $p \leq 0.05$, according to Duncan's multiple range tests.

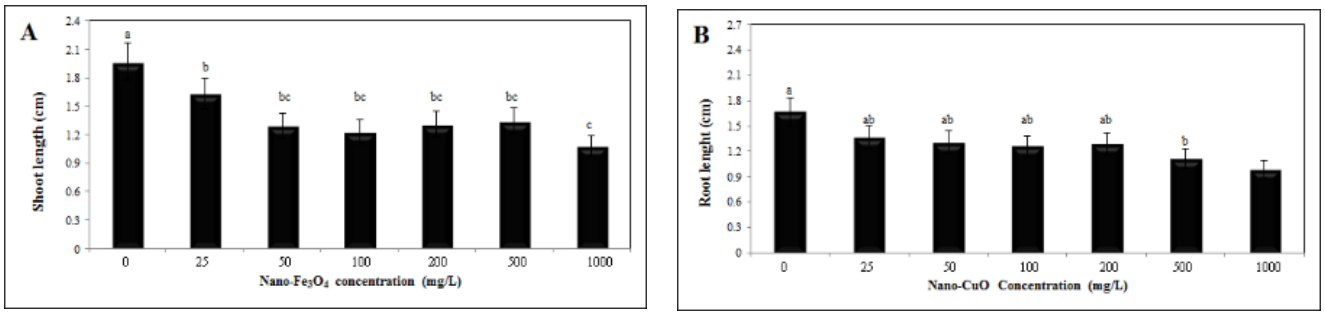

Figure 7. Shoot length in *Lepidium draba* seedlings that treated with different concentrations of nFe_3O_4 (A) and nCuO (B). Data are presented as the means ± SD (n=3). Signs with different letters are significantly different at $p \leq 0.05$, according to Duncan's multiple range tests.

The shoot growth was drastically inhibited as the manner increase of Fe_3O_4 NPs in media (Fig. 7A). The root and shoot length reduced at the presence of all CuO NPs concentrations that were significant at the level of 5% at concentrations higher than 200 mgL^{-1} (Figs. 6B, 7B).

Discussion

Lepidium draba, commonly assumed as a noxious weed of the Brassicaceae family, has large amount of SFN precursor (glucoraphanin) (13). Previous study showed that this weed can uptake and accumulate excess amounts of heavy metals such as iron and copper inside its leaves (27). It has been established that these elements can be absorbed effectively under acidic pH condition (28). Therefore, in this study, the treatment condition was conducted under acidic pH (5.8).

Based on the results the SFN production level in 7-days-old seedlings of *L. draba* was affected by the NPs. The influence differs based on the chemical nature of NPs, time of treatment as well as NPs concentration. The positive effect of Fe ions on SFN production level in seedlings of *L. draba* after 8 hrs treatment has been reported (20). Whereas the SFN content in 7-days-old seedlings of *L. draba* was severely increased in the presence of 1 mgL^{-1} Fe^{2+} after 8 hrs treatment, its content decreased at higher concentrations as well as treatment with each concentration of Cu^{2+}. However, the potential elicitation of silver nanoparticles has been addressed for stimulating production of artemisinin in hairy root of *Artemisia annua* L. by Zhang et al. (12). They reported, elicitation effect is attributed to the releasing of dissolved Ag ion and nano-particle nature.

Here, in order to show the occurrence possibility of oxidative stress along with NPs treatment, the activities of the most H_2O_2 sweeping enzyme including CAT and POD were assayed. According to the results, the CAT and POD activities were promoted in treatment with both NPs at some doses after 8 hrs treatment. It may be suggested that the increment activity of the H_2O_2 scavenger enzymes, subsequently of nano-elicitation is a sign of oxidative stress under the treatment conditions.

It has been demonstrated that ROS have dual

function in cells; they either have toxicity effects on cells or act as triggering agent for activation of signalling cascade (specially H_2O_2), which cause inducing gene expression of several defensive system, directly or through mediation by other signalling molecules such as jasmonate and salicylic acid (29, 30). It has been shown that increment glucosinolates content under elicitation with jasmonate and salicylic acid is attributed to their influence on the gene expression of some key enzymes involving in the glucosinolates biosynthesis pathway (31). Our observations are in agreement with the findings reported by Wang et al. (9). They showed SOD activity, which converted superoxide radical to H_2O_2 and O_2, was significantly increased in roots of *Cucurbita mixta* Pangalo under treatment with Fe_3O_4 NPs (30 mgL^{-1}) and its activity decreased at higher concentration (100 mgL^{-1}). They also revealed that CAT activity was meaningfully enhanced in seedling roots of *Lolium perenne* L. when treated with Fe_3O_4 NPs.

Hence, it may be purposed, the enhancement of SFN content in the nano-elicited seedlings attributed to the increment of glucosinolate (glucoraphanin), subsequently uptake or adsorption of the NPs on the surface of plant and then activation of signalling network that induced defined responses through H_2O_2 production. Nevertheless, the diminished activity of CAT and POD after 16 hrs can be introduced as an indicator of negative effect of oxidative stress under treatment conditions.

On the other hand, phytotoxicity effects of these particles were assessed by the measuring seed germination rate and root and shoot growth. As proposed by the U.S. Environmental Protection Agency (32), seed germination and root elongation are the standard indicators of phytotoxicity. The seed germination results are consisted with those reported by Mushtaq (33). They showed that percentage of cucumber seed germination reduced when it was exposed to Fe_3O_4 NPs. Nevertheless, the seed germination of ryegrass and pumpkin were the same as the control in treatment with Fe_3O_4 NP (9).

Increment of root growth in treatment with low concentration of Fe_3O_4 NPs may be due to the dissolved iron ions from particles which serve as nutrients (9). The inhibitory effects of several NPs including nFe_3O_4 and ZnO NPs were shown on seed germination rate as well as root length of *Arabidopsis thaliana* (L.) Heynh. (6). Inhibition of cucumber root growth was also seen in the presence of Fe_3O_4 NPs by Mushtaq (33). It has been established that plant response to NPs is varied depending on the characteristics of NPs, culture media as well as the tested plant species (9, 34, 35).

Until now, the negative effects of NPs have been reported on plant growth but the mechanism of nano-toxicity has not been fully addressed as well as the mechanism of their uptake. According to the selectivity of ion channel and aquaporins for ions and water molecules (with average diameter of 0.28 nm) (9), it seems that NPs passes through plant cell wall (with pore diameter from 2-20 nm) (36) and transport across plant cell membrane leads to damaging them (37). Therefore, in the experimental conditions, the NPs with average diameter of 60 nm may not able to pass through cell wall and reach the plasma membrane without any damage. Damage to the membrane cells through elicitation with silver nanoparticles has been recently shown by Ghanati and Bakhtiarian (11).

According to the results, it may be concluded that the biosynthesis of SFN (a potent anticancer compound) can be stimulated by NPs. Based on the results, it may be speculated that triggering of ROS subsequence of the NPs absorption or uptake, resulted in the activation of signalling network which leads to stimulation of gene expression of many defensive pathways and provides sufficient amounts of SFN substrate. To the best of our knowledge, this is the first report showing the enhancement of a phytochemical in response to Fe_3O_4 and CuO NPs treatment. Therefore, it can be purposed that these NPs may also serve as elicitors (nano-elicitor) for inducing individual phytochemical in plant. Nonetheless, further studies need to be directed to clarify the nano-elicitation, nano-toxicity as well as NPs uptake mechanism. The increment in production and accumulation of the phytochemicals through nano-metals treatment (nano-elicitation), opens an opportunity for induction of novel secondary metabolites in plant together with improvement of the phytochemical accumulation content. These results

also might provide new strategies to large-scale productivity of this compound. Moreover, elicitors have also been applied as effective tools to improve the plants protection against disease and/or pathogen attack (38). Hence, particles with these sizes may be considered as a suitable candidate to develop new phytoprotectants.

REFERENCES

1. Feiner, LF. (2006) Nanoelectronics: Crossing boundaries and borders. *Nat. Nanotechnology*, **1**, 91–92.
2. Hu, L. and Chen, G. (2007) Analysis of optical absorption in silicon nanowire arrays for photovoltaic applications. *Nano Lett.*, **7**, 3249–3252.
3. Barkalina, N., Charalambous, C., Jones, C. and Coward, K. (2014) Nanotechnology in reproductive medicine: emerging applications of nanomaterials. *Nanomed. Nanotech. Biol. Med.*, **10**, 921–938.
4. Ali, M.A., Rehman,I., Iqbal,A., Din,S., Rao,A.Q., Latif,A., Samiullah,T.R., Azam,S. and Husnain,T. (2014) Nanotechnology, a new frontier in Agriculture. *Adv. Life Sci.*, **1**, 129–138.
5. Lu, C., Zhang, C., Wen, J., Wu, G. and Tao, M. (2002) Research of the effect of nanometer materials on germination and growth enhancement of *Glycine max* and its mechanism. *Soybean Science*, **21**, 168–171.
6. Lee, C.W., Mahendra, S., Zodrow, K., Li, D., Tsai, Y.C., Braam, J. and Alvarez, P.J. (2010) Erratum: Developmental phytotoxicity of metal oxide nanoparticles to Arabidopsis thaliana. *Environ. Toxicol. Chem.*, **29**, 1399–1399.
7. Lee, W.M., An, Y.J., Yoon, H. and Kweon, H.S. (2008) Toxicity and bioavailability of copper nanoparticles to the terrestrial plants mung bean (*Phaseolus radiatus*) and wheat (*Triticum aestivum*): Plant agar test for water-insoluble nanoparticles. *Environ. Toxicol. Chem.*, **27**, 1915–1921.
8. Lei, Z., Mingyu, S., Xiao, W., Chao, L., Chunxiang, Q., Liang, C., Hao, H., Xiaoqing, L. and Fashui, H. (2008) Antioxidant stress is promoted by nano-anatase in spinach chloroplasts under UV-B radiation. *Biol. Trace Elem. Res.*, **121**, 69–79.
9. Wang, H., Kou, X., Pei, Z., Xiao, J.Q., Shan, X. and Xing, B. (2011) Physiological effects of magnetite (Fe_3O_4) nanoparticles on perennial ryegrass (*Lolium perenne* L.) and pumpkin (*Cucurbita mixta*) plants. *Nanotoxicology*, **5**, 30–42.
10. Asada, K., and Takahashi, M. (*1987*) *Production and scavenging of active oxygen in chloroplasts.* In: Kyle, D.J., Osmond, C.B., Arntzen C.J.(eds.), Photoinhibition. Amsterdam: *Elsevier*, pp. 227–287.
11. Ghanati, F., Morita, A. and Yokota,H. (2005) Effects of aluminum on the growth of tea plant and activation of antioxidant system. *Plant Soil*, **276**, 133–141.
12. Zhang, B., Zheng, L.P., YiLi,W. and WenWang, J. (2013) Stimulation of artemisinin production in *Artemisia annua* hairy roots by Ag-SiO_2 core-shell nanoparticles. *Curr. Nanosci.*, **9**, 363–370.
13. Powell, E.E., Hill, G.A., Juurlink, B.H. and Carrier, D.J. (2005) Glucoraphanin extraction from *Cardaria draba*: Part 1. Optimization of batch extraction. *J. Chem. Technol. Biot.*, **80**, 985–991.
14. Andreasson, E., Bolt Jorgensen, L., Hoglund, A.S., Rask, L. and Meijer, J. (2001) Different myrosinase and idioblast distribution in *Arabidopsis* and *Brassica napus*. *Plant Physiol.*, **127**, 1750–1763.
15. Bones, A.M. and Rossiter, J.T. (2006) Themyrosinase-glucosinolate system, its organisation and biochemistry. *Physiol. Plantarum*, **97**, 194–208.
16. Fenwick, G.R., Heaney, R.K. and Mullin, W.J. (1983) Glucosinolates and their breakdown products in food and food plants. *Crit. Rev. Food Sci. Nutr.*, **18**, 123–201.
17. Radonic, A., Blazevic, I., Mastelic, J., Zekic, M., Skocibusic, M. and Maravic, A. (2011) Phytochemical analysis and antimicrobial activity of *Cardaria draba* (L.) Desv. volatiles. *Chem. Biodivers.*, **8**, 1170–1181.
18. Liang, H. and Yuan,Q. (2012) Natural sulforaphane as a functional chemopreventive agent: including a review of isolation, purification and analysis methods. *Crit. Rev. Biotechnol.*, **32**, 218–234.

19. Fritz, V.A., Justen, V.L., Bode, A.M., Schuster,T. and Wang, M. (2010) Glucosinolate enhancement in cabbage induced by jasmonic acid application. *Hortscience*, **45**, 1188–1191.
20. Aminizadeh, M., Riahi-Madvar, A. and Mohammadi, M. (2014) Effects of iron and cupper ions on sulforaphane content and peroxidase activity in *Lepidium draba* seedlings. *Ethno-Pharmaceutical Products*, **1**, 8–14.
21. Mohammadi, M., Riahi-Madvar, A. and Pourseyedi, S. (2014) Elicitors induced sulforaphane production in *Lepidium draba*. *Asian J. Biomed. Pharm. Sci.*, **4**, 64–70.
22. Murashige,T. and Skoog, F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plantarum*, **15**, 473–497.
23. Liang, H.,Yuan, Q.P., Dong, H.R. and Liu, Y.M. (2006) Determination of sulforaphane in broccoli and cabbage by high-performance liquid chromatography. *J. Food. Comp. Anal.*, **19**, 473–476.
24. Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.
25. Plewa, M.J., Smith, S.R. and Wagner, E.D. (1991) Diethyldithiocarbamate suppresses the plant activation of aromatic amines into mutagens by inhibiting tobacco cell peroxidase. *Mutat. Res-fund. Mol. M.*, **247**, 57–64.
26. Dhindsa, R.S., Plumb-Dhindsa, P. and Thorpe, T.A. (1981) Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. *J. Exp. Bot.*, **32**, 93–101.
27. Chehregani, A. and Malayeri, B.E. (2007) Removal of heavy metals by native accumulator plants. *Int. J. Agr. Biol.*, **9**, 462–465.
28. Taiz, L. and Zeiger, E. (2002) Plant physiology. California: *Sinauer*.
29. Maksymiec, W. (2007) Signaling responses in plants to heavy metal stress. *Acta.Physiol.Plantarum*, **29**, 177–187.
30. Maksymiec, W., Wianowska, D., Dawidowicz, A.L., Radkiewicz, S., Mardarowicz, M. and Krupa, Z. (2005) The level of jasmonic acid in *Arabidopsis thaliana* and *Phaseolus coccineus* plants under heavy metal stress. *Plant Physiol.*, **162**, 1338–1346.
31. Glombitza, S., Dubuis, P., Thulke, O., Welzl, G., Bovet, L., Götz, M., Affenzeller, M., Geist, B., Hehn, A. and Asnaghi, C. (2004) Crosstalk and differential response to abiotic and biotic stressors reflected at the transcriptional level of effector genes from secondary metabolism. *Plant Mol. Biol.*, **54**, 817–835.
32. USEPA (1996) Ecological Effects Test Guidelines: Seed Germination/Root Elongation Toxicity Test. http://www.epa.gov/ocspp/pubs/frs/publications/ OPPTS_Harmonized/ 850_Ecological_Effects_Test_ Guidelines/Drafts/index.html.
33. Mushtaq, Y.K. (2011) Effect of nanoscale Fe_3O_4, TiO_2 and carbon particles on cucumber seed germination. *J. Environ. Sci. Health Part A*, **46**, 1732–1735.
34. Nair, R., Varghese, S.H., Nair, B.G., Maekawa, T., Yoshida, Y. and Kumar, D.S. (2010) Nanoparticulate material delivery to plants. *Plant Sci.*, **179**, 154–163.
35. Zhu, H., Han, J., Xiao, J.Q. and Jin, Y. (2008) Uptake, translocation, and accumulation of manufactured iron oxide nanoparticles by pumpkin plants. *J. Environ. Monit.*, **10**, 713–717.
36. Hussain, S., Hess, K., Gearhart, J., Geiss, K. and Schlager, J. (2005) In vitro toxicity of nanoparticles in BRL 3A rat liver cells. *Toxicol. in Vitro*, **19**, 975–984.
37. Soto, K., Carrasco, A., Powell, T., Murr, L. and Garza, K. (2006) Biological effects of nanoparticulate materials. *Mater. Sci. Eng. C*, **26**, 1421–1427.
38. Durango, D., Pulgarin, N., Echeverri, F., Escobar, G. and Quiñones,W. (2013) Effect of salicylic acid and structurally related compounds in the accumulation of phytoalexins in cotyledons of common bean (*Phaseolus vulgaris* L.) cultivars. *Molecules*, **18**, 10609–10628.