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Phytotoxic and genotoxic effects of ZnO nanoparticles on garlic (*Allium sativum* L.): A morphological study

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Abstract

The effects of zinc oxide nanoparticles (ZnO NPs) on the root growth, root apical meristem mitosis and mitotic aberrations of garlic (*Allium sativum* L.) were investigated. ZnO NPs caused a concentration-dependent inhibition of root length. When treated with 50 mg/L ZnO NPs for 24 h, the root growth of garlic was completely blocked. The 50% inhibitory concentration (IC_{50}) was estimated to be 15 mg/L. The mitosis index was also decreased in a concentration- and time-dependent manner. ZnO NPs also induced several kinds of mitotic aberrations, mainly consisted of chromosome stickiness, bridges, breakages and laggings. The total percentage of abnormal cells increased with the increase of ZnO NPs concentration and the prolongation of treatment time. The investigation provided new information for the possible genotoxic effects of ZnO NPs on plants.

Keywords: *Garlic (Allium sativum L.), ZnO nanoparticles, root growth, mitotic index, chromosomal abnormalities*

Introduction

Nowadays, metal oxide nanoparticles (NPs) have received much attention and have been applied in a wide range of fields, especially because of their semiconducting, optical, piezoelectric and ultraviolet (UV)-shielding properties. ZnO NPs, one of the most widely used metal oxide nanoparticles, have been used in a large variety of commercial products, such as electronic devices, transparent UV-protection films, chemical sensors, UV-filters in sunscreens, as well as textiles (Meulenkamp 1998; Serpone et al. 2007; Becheri et al. 2008). However, with the increased presence of ZnO NPs in commercial products, there is clearly a growing public concern about the toxicological and environmental effects of ZnO NPs. Unfortunately, toxicological studies carried out in the last 10 years have shown that ZnO NPs had potential health and environmental risks. ZnO NPs pose serious toxicity to bacteria, *Daphnia magna*, freshwater micro alga, mice, and even to human cells (Brayner et al. 2006; Franklin et al. 2007; Heinlaan et al. 2008; Wang et al. 2008; Sharma et al. 2009; Lin et al. 2009).

To date, although investigations about the toxicological effects of NPs continue to increase with time, only a few studies have been conducted to assess the effects of ZnO NPs on plants (Lin and Xing 2007; Lin and Xing 2008; Stampoulis et al. 2009; López-Moreno et al. 2010). Lin and Xing (2007) examined the toxicity of five types of NPs (multi-walled carbon nanotubes, aluminum, alumina, zinc, and zinc oxide) on seed germination and root growth of six higher plant species (radish, rape, ryegrass, lettuce, corn, and cucumber) and showed that seed germination was in general not affected in most cases while root elongation was inhibited. The IC_{50} of ZnO NPs was estimated to be near 50 mg/L for radish, and about 20 mg/L for rape and ryegrass. The authors concluded that the inhibition occurred during the seed incubation process rather than seed soaking stage. In a later study, the same authors (Lin and Xing 2008) reported the cell internalization and upward translocation of ZnO NPs in ryegrass. In the presence of ZnO NPs, the biomass of ryegrass was significantly reduced, root tip shrank and root epidermal and cortical cells were highly vacuolated or collapsed. ZnO NPs were greatly adhered onto the root surface and individual NPs

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were observed present in the apoplast and protoplast of the root endodermis and stele. Only few or no dissociated Zn^{2+} ions were translocated into rye-grasses that were exposed to ZnO nanoparticles. The authors also showed evidence that the phytotoxicity of ZnO NPs was not directly correlated with their limited dissolution in the bulk nutrient solution or rizosphere. Stampoulis et al. (2009) studied the effects of five types of NPs (multi-walled carbon nanotubes, Ag, Cu, Si, and ZnO) on germination, root elongation, and biomass of zucchini and found that seed germinations were little impacted by these nanoparticles. This observation agreed with that of Lin and Xing (2007). ZnO treatments also reduced the zucchini biomass by 78–90% relative to the controls. Unlike Lin and Xing (2007), who found that ZnO NPs at 2000 mg/L dramatically reduced the root growth of all five species used, Stampoulis et al. did not find a statistically significant impact of ZnO NPs on the root elongation. The difference, on the opinion of Stampoulis et al. (2009) may be due to the higher particle concentration used by Lin and Xing, the different plant species used, and also the high replicate variability within their ZnO treatments. López-Moreno et al. (2010) showed evidence in ZnO NPs-treated soybean that seed germination was not significantly reduced, while an inverse U-shape response was observed in root growth, with maximum root length at 500 mg/L (30% longer than control) and a minimum at 4000 mg/L (40% shorter than control). It has also been reported that ZnO NPs could cause significant effect on the genetic stability of soybean.

The researches mentioned above have contributed to our understandings of the effects of ZnO NPs on plants. However, many key issues still remain unresolved concerning the potential toxicology effects of ZnO NPs, especially the effects of ZnO NPs on the chromosomes and mitotic cell divisions of plants. In the present work, we investigated the possible phytotoxic and genotoxic effects of ZnO NPs on garlic.

Materials and methods

Plant material

Garlic (*Allium sativum* L., China origin 55/70 mm CAT.1) was purchased from a local market. Healthy and equal-sized garlic cloves were selected. The bulbs had not initiated the formation of root growth. Before commencing the experiment, the dry scales of the bulbs were removed. It was confirmed that the root growth and MI of different mass of bulb from different garlic had almost no significant differences in a preliminary study.

Synthesis of ZnO nanoparticles

ZnO NPs was prepared via sol-gel method (Sakohara et al. 1998). The process was as follows: 0.1 M ethanol solution of LiOH was slowly added to 0.12 M ethanol solution of $Zn(CH_3COO)_2 \cdot 2H_2O$ kept at 0°C. The mixed solution was then hydrolyzed in an ultrasonic bath at about 0°C for 25 min before a certain amount of hexane was added into the colloidal solution. White precipitate was obtained, centrifuged and washed with de-ionized water three times to wash out the ethanol, and was then dried in a vacuum condition to obtain ZnO quantum dot powders. The transmission electron microscopy (TEM) image of ZnO NPs was taken with Hitachi H-8100 IV transmission electron microscope operating at 200 kV. The crystal structure of ZnO NPs was characterized using X-ray diffraction (XRD) method with Cu Ka radiation (λ = 0.15406 nm) (Shimadzu XRD-6000).

ZnO NPs were suspended directly in de-ionized water (DI-water) and dispersed by ultrasonic vibration (100 W, 40 kHz) for 40 min (Lin and Xing 2007). The pH of the suspensions was measured. The concentration of free Zn^{2+} in the supernatants of NPs suspensions after centrifugation (3,000 *g* for 1 h) was determined by atomic absorption spectrometer (SpectrAA 220FS, Varian). Zn^{2+} reference solution was prepared by dissolving $Zn(CH_3COO)_2{\cdot}2H_2O$ in DI-water.

Root growth

The bulbs of garlic were allowed to germinate in DI-water until the radicals had elongated to 2.0 cm in length. The seedlings were selected for uniformity in size and shape. Twenty-five seedlings (five seedlings per treatment) were directly placed in ZnO NPs suspensions (10, 20, 30, 40, 50 mg/L) or Zn^{2+} reference solutions (0, 0.5, 1, 1.5, 2 mg/L) for 24 h. At the end of each treatment, the seedlings were washed carefully with DI-water, and transferred to beakers with DI-water and allowed to germinate at room temperature of 22 ± 1°C. Controlled seedlings were placed in DI-water. The roots were protected from direct sunlight in order to minimize fluctuation of the rate of cell division. After 24 h, the root length of the treated seedlings and the controls were measured.

TTC viability tests for root tips

We used 2, 3, 5-triphenylte trazolium chloride (TTC) as a histopathologic stain for testing the viability of root tips. The test was as follows: 5 mL of 0.5% solution of TTC was added to test tubes containing 10 root tips, the temperature was kept at 35 ± 1°C.

After 5 h in the dark, the TTC solution was removed with a syringe and root tips were thoroughly rinsed with distilled water and then examined. The red-colored root tips were considered to be viable and others were non-viable or dead.

Mitotic division

The germinated seedlings with actively growing roots (2.0–2.5 cm in length) were placed in the ZnO NPs suspensions (10, 20, 30, 40, 50 mg/L) or Zn^{2+} reference solutions (0, 0.5, 1, 1.5, 2 mg/L) for 8, 16 and 24 h. After each interval of treatment, the roots were cut and fixed immediately in a freshly prepared mixture of absolute ethyl alcohol and glacial acetic acid (3:1 (v/v)) for 24 h, then stored under refrigeration in 70% ethyl alcohol. Cytological preparations were prepared through the Feulgen squash technique. Five tips per treatment were evaluated and at least 300–400 cells per tip were scored. The mitotic index (MI) was calculated as the percentage of the number of dividing cells to the total number of cells scored. Abnormal cells were counted during mitosis process.

Statistical analysis

The statistical analysis was performed using the procedure of Analysis of Variance (ANOVA) with Microsoft Excel. Data were expressed as mean ± standard error (SE). The statistical significance of the differences among values (mitotic index, and total abnormalities) in the treated samples and the control was evaluated by means of the *t*-test. Statistical significance was assumed at $P < 0.05$.

Results

Figure 1 presents the XRD pattern of the ZnO NPs. All of the diffraction peaks could be indexed to the hexagonal wurtzite structure of ZnO. The TEM image of ZnO NPs is shown in Figure 2. As can be seen, the ZnO particles were spherical and well dispersed; the average diameter was about 4 nm. The pH values were 6.5–7.5 in the ZnO NPs suspensions.

The effects of ZnO NPs on the root growth of garlic varied with concentration when compared with the control roots, as shown in Figure 3. The inhibition of root growth increased with the increase of concentration of ZnO NPs. Before the ZnO NPs treatment, the average root length was about 2.1 ± 0.4 cm (mean ± SE), after the treatment, the control seedling root length was 4.3 ± 0.1 cm, while the root lengths were 3.3 ± 0.1 cm, 2.9 ± 0.1 cm, 2.6 ± 0.1 cm, 2.5 ± 0.1 cm, 2.2 ± 0.1 cm at ZnO NPs concentrations of 10, 20, 30, 40, 50 mg/L, respectively. It was found

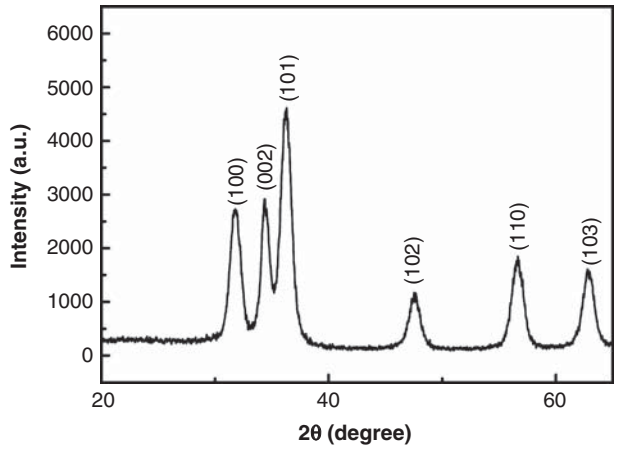

Figure 1. X-ray diffraction spectra of ZnO NPs synthesized with sol-gel method.

that in the 50 mg/l concentration of ZnO NPs suspensions, root growth was completely inhibited. The 50% root growth inhibitory concentration (IC_{50}) was estimated to be about 15 mg/L. The effects of ZnO NPs on the morphology of the roots were also found in the experiment. The root morphology was nearly normal for all the 6-h treatments, but for 24 h, the root morphology showed an obvious difference in its appearance. The root tips became fragile, thinner, and turned to brown in color.

The TTC tests showed that the effects of ZnO NPs on root tips varied with both concentrations applied and time of treatment (Figure 4). For 2-h treatment, all root tips were colored red. The root tips treated with 10, 20, 30 and 40 mg/L ZnO NPs for 4 h and with 10, 20 and 30 mg/L for 8 h were colored red. For 16 and 24 h exposures, root tips were not colored except for control roots and those treated with 10 mg/L ZnO NPs.

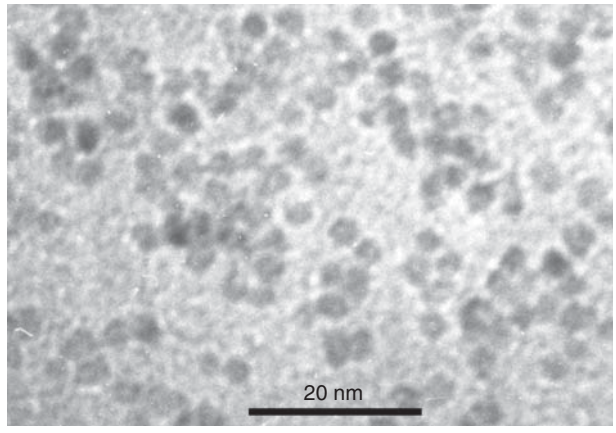

Figure 2. TEM image of ZnO NPs synthesized with sol-gel method.

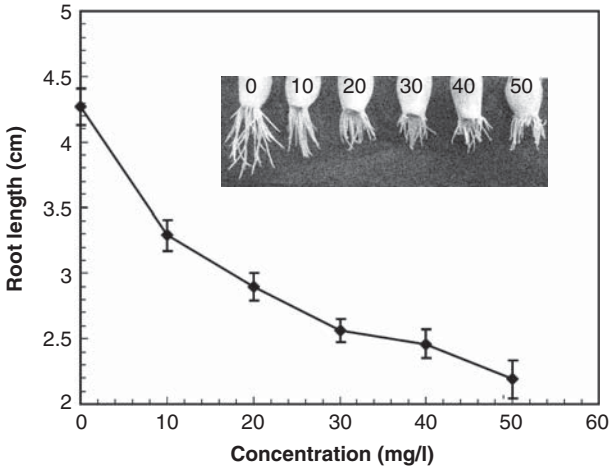

Figure 3. Effects of different concentrations of ZnO NPs on root length. The values were given as mean ± SE (standard error) of triplicate samples with 10 seeds each.

The influence of ZnO NPs on mitotic activity expressed as MI is given in Figure 5. Cells treated with ZnO NPs of higher concentrations (30, 40 and 50 mg/L) showed significant reduction in the frequency of mitotic index during all durations compared to those of control sets ($P < 0.05$, $P < 0.01$). The MI was remarkably reduced in roots treated with 50 mg/L ZnO NPs for 24 h, which was a minimum value of 3.91 ± 0.78%. This decrease in mitotic index was found to be statistically highly significant ($P < 0.01$) at all concentration for 24-h treatment.

Table I shows the percentage of abnormal cells for different treatments with ZnO NPs suspensions. The treated roots, compared with controls, had a

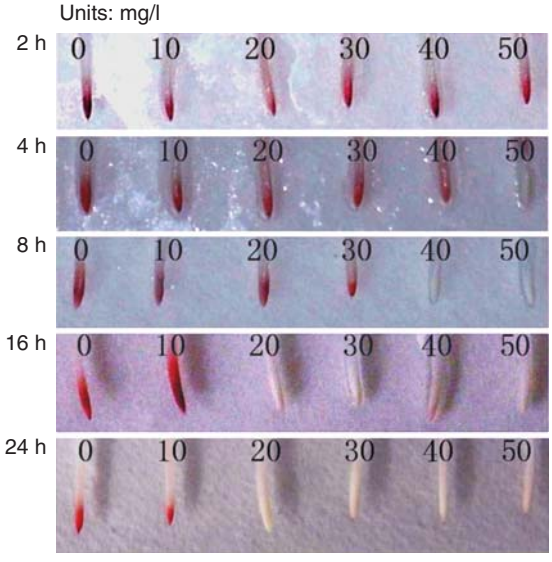

Figure 4. TTC tests for different concentrations and treatment time of ZnO NPs. This Figure is reproduced in color in the online version of *Nanotoxicology*.

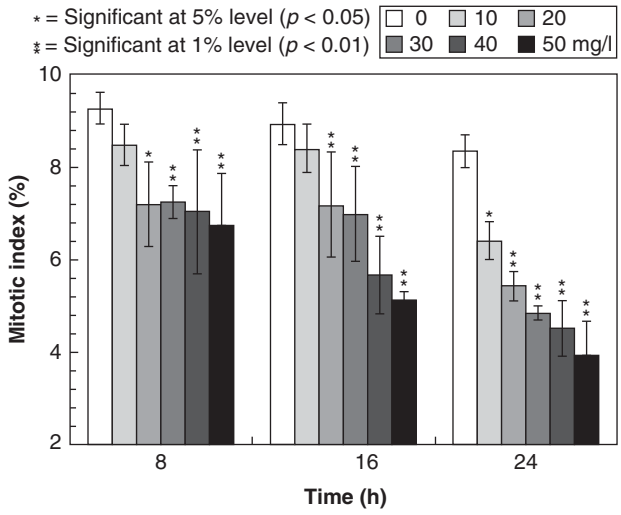

Figure 5. Effect of ZnO nanoparticles suspensions on mitotic index. Vertical bars denote standard error (SE).

statistically significant increase ($P < 0.01$) in the percentage of total abnormal cells with the increase of ZnO NPs concentrations and the increase of treatment time. The highest percentage of abnormal cells, recorded in roots treated with 50 mg/L for 24 h, was 78.5 ± 7.2% compared with that of 5.4 ± 0.9% for controlled set. It can be seen in Table I that ZnO NPs induced different types of mitotic abnormal cells in the roots of garlic. The percentage of some types of abnormal cells increased as the concentration and the duration time of treatment increased. These abnormal cells mainly include stickiness, laggards and chromosome bridges, irregular prophases and C-metaphases as illustrated in Figure 6.

In our study, there were still some dissolved Zn^{2+} ions that remained in the ZnO NPs suspensions. Therefore, in order to check the effects of these Zn^{2+} ions, we also studied the possible toxicity of Zn^{2+} ions generated during the dissolution of ZnO NPs. First, we measured the concentration of Zn^{2+} in the supernatants of NPs suspensions after centrifuging (at 3000 *g* for 1 h), then we tested the toxicity of Zn^{2+} solutions by dissolving $Zn(CH_3COO)_2 \cdot 2H_2O$ in DI-water (Lin and Xing 2007). Concentrations of Zn^{2+} ions in the supernatants (after centrifugation) were 0.353–1.936 mg/L. Therefore, five concentrations of 0, 0.5, 1, 1.5 and 2 mg/L Zn^{2+} solutions were made from $Zn(CH_3COO)_2 \cdot 2H_2O$ to investigate the toxicity of dissolved Zn^{2+} ions. No significant toxic effects were observed on the root growth and the MI of garlic for these Zn^{2+} ions concentrations (Figures 7 and 8).

Discussion

With the increasing applications of nanotechnologies, more and more people are concerned with the

Table I. Types and percentages of mitotic abnormal cells and the percentage of total abnormal cells observed in garlic root tips treated with ZnO NPs. The values were given as mean ± standard error (SE).

Treatment		Types and percentages of abnormal cells						
Time (h)	Concentration (mg/L)	Stick (%)	Brid (%)	Lag (%)	Irreg(%)	C-metaphase (%)	Others (%)	Total abnormal cells (%)
8	Control	–	–	–	4.1	–	–	4.10 ± 1.05
	10	2.2	1.5	–	5.5	–	1.4	10.60 ± 5.68
	20	2.1	2.5	–	6.5	–	1.4	12.5 ± 9.04**
	30	3.5	3.1	–	9.3	1.7	2	19.6 ± 5.08**
	40	5.7	2.7	–	15.1	2.8	5.1	31.4 ± 3.84**
	50	7.4	6.4	1.5	23.3	4.2	8.6	51.4 ± 4.36**
16	Control	–	–	–	3.2	–	–	3.2 ± 1.72
	10	2.2	1.7	–	6.6	1.9	–	14.5 ± 3.84*
	20	5.6	3.4	–	12.5	3.6	–	28.7 ± 3.08**
	30	4.3	4.4	1.2	16.5	4.5	–	35.2 ± 4.50**
	40	6.4	4.8	1.8	20.8	5.4	7.4	46.6 ± 5.49**
	50	10.1	6.5	2.5	29.2	7.5	10.5	66.3 ± 3.93**
24	Control	–	–	–	4.3	1.1	–	5.4 ± 0.9
	10	3.3	2.6	–	9.7	3.8	–	22 ± 3.76**
	20	6.2	2.2	–	17.9	4.1	6.3	36.7 ± 4.20**
	30	8.2	5.2	2.6	19.8	5.3	5.9	47.0 ± 4.70**
	40	10.8	4.3	4.1	24.5	10.4	7.3	61.4 ± 6.36**
	50	12.3	3.5	9.3	31.3	10.6	11.5	78.5 ± 7.16**

The values were given as mean ± S.E. * = Significant at 5% level ($P < 0.05$); ** = Significant at 1% level ($P < 0.01$).

potential toxicity of the NPs. Some studies have reported positive, stimulating as well as negative effects of NPs on higher plants. SiO_2 and TiO_2 NPs at low concentrations enhanced nitrate reductase activity in soybean germination and growth (Lu et al. 2002). SiO_2 NPs also enhanced the growth of Changbai larch and the enhancement effect increased with concentrations up to 500 mg/L (Lin et al. 2004). However, most studies indicated toxic effects of NPs on plants. For instance, copper NPs were shown to be toxic to mung bean and wheat, as demonstrated by the reduced seedling growth rate. Mung bean was more sensitive than wheat to Cu NPs (Lee et al. 2008). Ma et al. (2010) analyzed the phytotoxicity of four rare earth oxide NPs (nano-CeO_2, nano-La_2O_3, nano-Gd_2O_3, and nano-Yb_2O_3) on seven higher plant species (radish, rape, tomato, lettuce, wheat, cabbage, and cucumber). A suspension of 2000 mg/L nano-CeO_2 had no effect on the root elongation of six plants, except lettuce. In contrast, 2000 mg/L suspensions of nano-La_2O_3, nano-Gd_2O_3 and nano-Yb_2O_3 severely inhibited the root elongation of all the seven species. A recent work indicated that NPs were not only toxic to plant seedling, but also harmful to plant cells (Kumari et al. 2009). Using Ag NPs and root tip cells of onion (*Allium cepa*), researchers demonstrated that Ag NPs could disrupt cell division process causing chromatin bridge, stickiness and cell disintegration. There were also studies which investigated the phytotoxicity, uptake and effects of ZnO NPs on root growth (Lin and Xing 2007; Lin and Xing 2008).

Despite the rapid progress in the study of phytotoxicity, uptake and accumulation of NPs in resent years, we still face numerous unresolved issues. Therefore, we had focused on the mitotic index and mitotic divisions in garlic root tip cells during mitosis and attempted to develop our knowledge about possible genotoxic effects of ZnO NPs on plants.

The results from the present study showed that root growth of garlic was affected by ZnO NPs, in such a way that the root length was inhibited with the increasing of concentration. At a concentration of 50 mg/L, the root growth was completely inhibited by ZnO NPs. The TTC tests showed that after 2-h treatment, all root tips were colored red. However, after 24 h, root tips were not colored except for control roots and those treated with 10 mg/L ZnO NPs (Figure 4). The tests confirmed that ZnO NPs

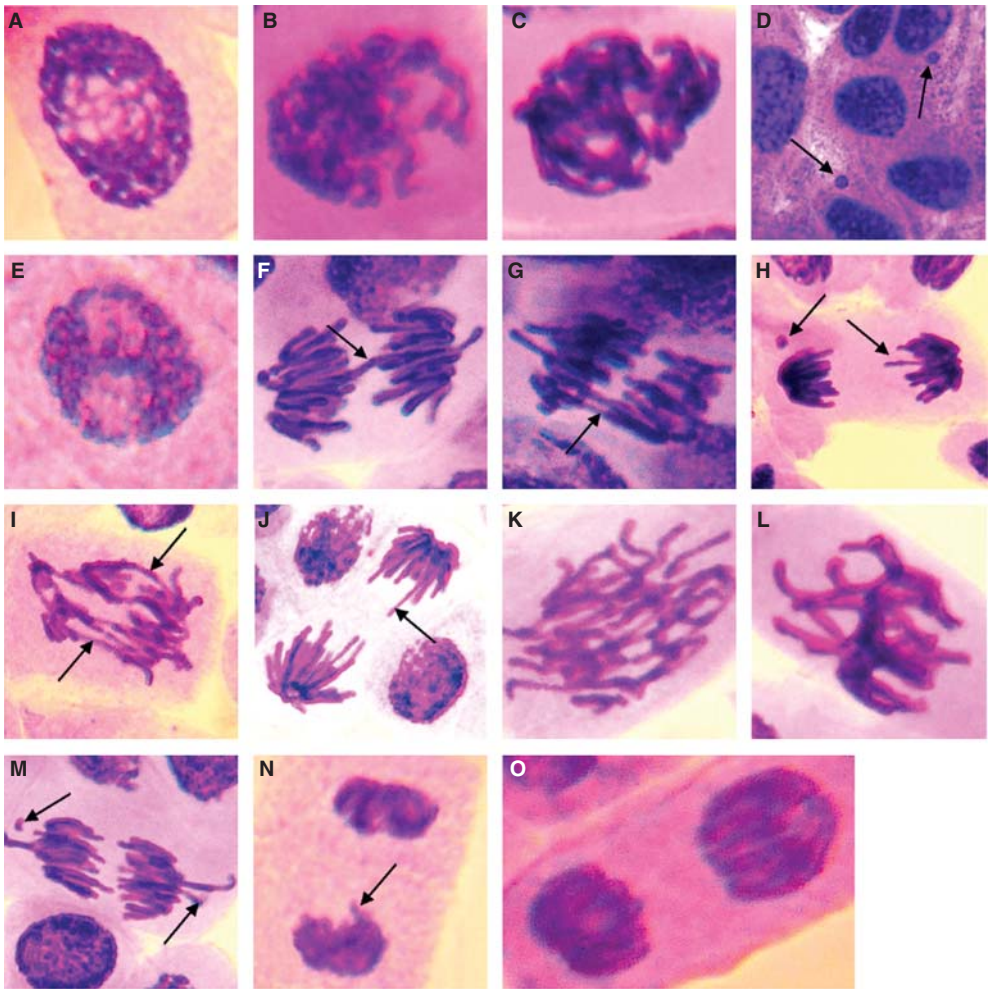

Figure 6. Some types of abnormal cells induced in garlic root tip cells by ZnO NPs. (A) Vacuolated nucleus at prophase (40 mg/L for 16 h); (B) Irregular prophase (50 mg/L for 24 h); (C) Sticky prophase (50 mg/L for 24 h); (D) Micronucleus (50 mg/L for 24 h); (E) Binucleated cells (40 mg/L for 24 h); (F) Single bridge with lagging chromosome (40 mg/L for 24 h); (G) Double bridge with lagging chromosome (50 mg/L for 24 h); (H) Sticky metaphase with micronucleus (40 mg/L for 16 h); (I) Multibridges at anaphase (30 mg/L for 24 h); (J) Lagging chromosome at metaphase (30 mg/L for 24 h); (K) Irregular metaphase (30 mg/L for 16 h); (L) Sticky metaphase (30 mg/L for 8 h); (M) Lagging chromosome with fragment at metaphase (50 mg/L for 16 h); (N) Lagging chromosome at anaphase (40 mg/L for 24 h); (O) Unequally sized nuclei at interphase (40 mg/L for 24 h). This Figure is reproduced in color in the online version of *Nanotoxicology*.

could cause the root tip cells to death. It was also found that the control roots of 24 h were less red than other controls of 2, 4, 8 and 16 h. It might be due to the lack of nutrition in DI water that was not changed in all exposure time. The reduction of root growth and the different morphology of treated roots might be due to the toxicity of ZnO NPs-induced chromosomal aberrations; these aberrations could lead to mitotic arrest and cell death (Datta and Biswas 1985).

The reduction in the rate of mitotic division in garlic was attributed to the mitotic inhibition by ZnO NPs. Such a decrease in mitotic activity indicated that ZnO NPs could interfere with the development of mitosis and cause cytotoxic effects. It might be due to the inhibition of DNA synthesis at S-phase or a blocking in the G_2 phase of the cell cycle (Duan and Wang 1995; Borboa and De la Torre 1996; Sudhakar et al. 2001).

The results in Table I indicate that ZnO NPs induced a wide range of mitotic abnormal cells in the root tips of garlic. An increase of chromosomal aberrations was observed after treatment with ZnO NPs, in direct dependence on the treatment time and concentration. There were statistically significant differences between the control and the treated groups in the percentage of abnormal cells. Stickiness, irregular and C-metaphase were observed with high frequency. The presence of these types of aberration also reflected the toxic effect of ZnO NPs.

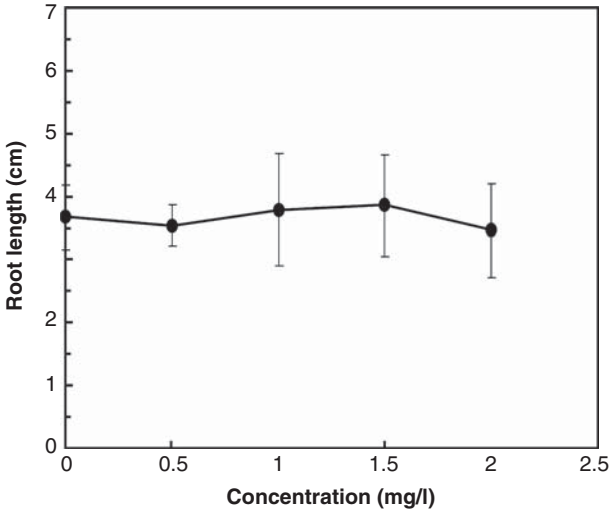

Figure 7. Effects of different concentrations of solution Zn^{2+} on root length. The values were given as mean ± standard error (SE) of triplicate samples with 10 seeds each.

The mechanism of nanotoxicity remains unknown. It might be attributed to two different actions (Brunner et al. 2006): (i) A chemical toxicity based on the chemical composition, e.g., release of (toxic) ions; and (ii) stress or stimuli caused by the surface, size and/or shape of the particles. It was confirmed that solubility of oxide nanoparticles greatly affected the cell culture response (Brunner et al. 2006).

Zn is an essential element for plant growth and development, but above certain concentrations it becomes toxic. Many previous studies reported that Zn^{2+} showed an inhibitory effect on root growth and the cell division (Munzuroglu and Geckil 2002; El-Ghamery et al. 2003). In the present study, 0, 0.5, 1, 1.5 and 2 mg/L of dissolved Zn^{2+} ions (equivalent to the concentrations in the supernatants of ZnO NPs suspensions after centrifugations, respectively) did not show any toxicity to the growth of the root tips of garlic (Figures 7 and 8).

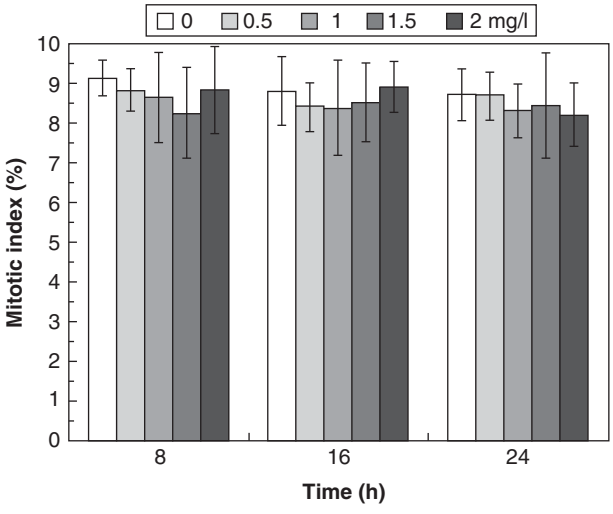

Figure 8. Effects of different concentrations of solution Zn^{2+} on mitotic index. Vertical bars denote standard error (SE).

pH values might also affect root growth. But the pH values of the suspensions were 6.7–7.5 in the present study; this pH range should not have any negative effect on the root growth (Lin and Xing 2007).

In conclusion, the effects of different treatments with ZnO NPs on the root growth, mitosis in root apical meristem and mitotic aberrations were examined in garlic. It was found that the root growth was inhibited, and the mitosis index values were decreased in a concentration- and time-dependent manner. Furthermore, ZnO NPs treatments produced a number of mitotic abnormal cells in dividing cells in root tips. The total percentage of abnormal cells increased with an increase of the ZnO NPs concentration and the prolongation of the treatment time. The results obtained from our investigation provide new information about possible phytotoxic and genotoxic effects of ZnO NPs on plants. Our future investigation will be focused on understanding the exact toxicity mechanisms behind ZnO NPs induced genotoxicity.

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