Effects of alumina nanoparticles on morphological properties and antioxidant system of *Triticum aestivum*

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Abstract

The negative and positive effects of alumina nanoparticles have been reported for various plant species, while the mechanism that brings about these effects has not yet been clearly understood. Here, the effects of different concentrations of nano-scale alumina on growth and enzymatic antioxidant system of wheat seedlings are investigated. The results show while root growth is affected by the nanoparticles (NPs), other morphological properties including seed germination, shoot length, and dry biomass were the same as the control plants' properties. This can be attributed to selective permeability of seed coats which confronts roots with excess in the NPs and low rate in transportation of this material to the shoot. Interestingly, while root elongation was significantly improved in both treatments of 50 and 1000 mg/L nano-scale alumina, length of the roots were measured slightly lower than control in cases of 200 and 500 mg/L treatments. Additionally, the activity of superoxidase dismutase and catalase were elevated for treatments of 200 and 500 mg/L while growth of the roots was apparently decreased and the uptake of aluminum by the roots was more than the uptake for other concentrations. However, due to the potential retarding of alumina nanoparticles on root growth for some plant species, it seems that the activity of antioxidant enzymes which reduces the level of free radicals ($O^{\cdot -}$, H_2O_2) is responsible for reducing phytotoxicity effects of these particles on seedlings. In conclusion, it is proposed that oxidative damage can be introduced as a way of inducing toxicity in plants through the uptake of nanoparticles.

Keywords: Triticum aestivum; alumina nanoparticles; antioxidant enzymes; morphological properties

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Introduction

Nanoparticles are aggregates of atoms or molecules with at least one dimension less than 100 nanometers (Ball, 2002; Roco, 2003). The quantum effects which appear in this size range can drastically modify the physical, chemical and electrical characteristics of NPs as compared to larger particles (Gonzalez-Melendi et al., 2008; Lee et al., 2008). Currently, nanoparticles are produced from a large variety of bulk materials (Brunner et al., 2006), with broad industrial applications including biomedicine and biotechnology (Nasibulin et al., 2000; Salata 2004; Lin et al., 2006; Nel et al., 2006; Yang et al., 2006; Owen and Handy, 2007; Behra and Krug, 2008); hence it is to be expected that these

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particles will find their way into various ecosystems (Owen and Handy, 2007; Behra and Krug, 2008).

Experimental studies have revealed that NPs, due to certain unique properties, exhibit toxicity that is different from toxicity that exists in the same bulk material (Donaldson et al., 1999); and in general higher levels of toxicity occur in the smaller sized particles (Oberdorster, 1996; Battke et al., 2008). Some of the negative effects of nano-scale materials on plant growth have been identified as inhibition in root growth and seed germination (Lin and Xing, 2007; Lee et al., 2010); which varies greatly depending on NPs composition, size, concentration and also plants species (Lin and Xing, 2007). It was reported that root elongation of 5-day-old *Zea mays* seedlings reduced with 2000 mg/L of 60 nm alumina nanoparticles (nAl_2O_3) while under the same conditions no negative effects were observed on the growth of *Raphanus sativus*, *Brassica napus*, *Cucumis sativus*, *Lolium perenne* and *Lactuca sativa*. On the other hand, 2000 mg/L of nano-Zn and nano-ZnO suspensions significantly inhibited root growth in *Zea mays* and terminated the root development of the other five plant species (Lin and Xing, 2007). Phytotoxicity effects of nAl_2O_3 (13 nm) at 2 mg/mL concentration were observed as inhibition in the root length for five plants, namely, *Zea mays*, *Cucumis sativus*, *Glycine max*, *Brassica oleracea* and *Daucus carota* (Yang and Watts, 2005). It was reported that 100 nm size aluminum nanoparticles in the form of Alex and L-Alex with agglomerate size in 1-10 µm range had no adverse effects on the growth of *Phaseolus vulgaris* (with no uptake of Al) and *Lolium perenne* (which absorbed Al and deposited it in the leaves) in the studied concentration range (Doshi et al., 2008). Also, different effects - negative or positive - of nano-scale aluminum (nAl) were observed on root elongation, depending on the plant species (Lin and Xing, 2007).

Aluminum is the third most abundant metal in the earth's crust (about 7%) which is known for its major growth limiting factor in acidic soils (Foy et al, 1978); Recently, the use of nAl has increased significantly in various fields (Novrotsky, 2003; Argonide, 2004). Consequently, an enormous body of scientific literature has been devoted to describing the influence of different aluminum compounds on growth of various plants species, including studies reported on Al (Aniol, 1989; Dong et al., 2002, Ghanati et al., 2005; Jamal et al., 2006), nAl (Lin and Xing, 2007; Doshi et al., 2008) and also nAl_2O_3 (Yang and Watts, 2005; Lin and Xing, 2007; Lee et al., 2010). It should be noted, that the mechanism of interaction (induced toxicity) and the fate of nano-scale materials after being released into the ecosystems (Nel et al., 2006) have not been fully understood yet.

In this study, in order to determine the effects of various concentrations of nano-scale alumina on the growth and the antioxidant system of wheat, morphological properties as well as the activity of some antioxidant enzymes have been analyzed.

Materials and Methods

Preparation of culture media

Nano-scale alumina used in this study was purchased from the PlasmaChem GmbH. Characteristics of the particles were as follows. Average particle size: 40 nm, purity: 99.9 % and surface area >40 (m^2/g) as reported by the commercial agent. Agar culture media (1%) was created utilizing the method of Lee et al*.,* (2008) with little modifications (one layer instead of two layers) were used to evenly disperse the NPs. Nano-scale alumina was suspended in deionized water (pH 7 ± 0.2) and the solution after vigorous shaking was added to autoclaved agar media at different concentrations of 0 (as a control), 50, 200, 500 and 1000 mg/L all of which were prepared separately (without dilution). 20 mL of 1% agar solution containing NPs was poured into a Petri dish (9 × 1.5 cm) and was rapidly solidified at -20 °C.

Morphological studies

Seed germination

The seed of *Triticum aestivum*, Gaspard cultivar, was obtained from the Seed and Plant Improvement Institute (Karaj, Iran). The seeds were kept for 4 weeks in a refrigerator (4 °C) for cold treatment before being used. They were

then immersed in a 2.5% sodium hypochlorite solution for 15 min to ensure surface sterility (USEPA, 1996) and were rinsed at least 5 times with sterilized water. Ten seeds were placed on the surface of the agar in Petri dishes with about 1 cm spacing between them. The plates were then placed in a dark incubator at controlled temperature of 30 °C for 5 days. Seed germination rates were calculated after 48 hours as soon as the minimal length of the radicles was about 1 mm.

Root and shoot elongation

After an incubation period of 5 days, seedlings were separated from the medium. Their root and shoot length were then measured using a ruler. Any seedling with roots outside the agar media was omitted from the process.

Dry biomass

The 5-day seedlings were separated from their medium and were washed thoroughly using distilled water. Afterwards, they were dried at 80 °C for 24 hours in an oven before being weighed.

Bioaccumulation and bioavailability of aluminum

Bioaccumulation of aluminum in the roots and shoots was determined by Inductivity Coupled Plasma (ICP) mass spectrometry (SpectrAA 220 Varian) using a solution containing 0.1 g of dried root or shoot digested by5 mL HNO_3 (65%) for 24 hours.

Bioavailability was estimated at the end of the experiment (t = 5 days) by calculating the bioaccumulation factor, defined as the ratio of Al (mg/kg) concentration in the roots in each treatment over the nAl_2O_3 concentration in the agar media (mg/L) similar to the procedure described by Lee et al., (2008).

In order to determine the amount of Al ions released during NPs preparation, its quantity was measured in the 1000 mg/L of nanoparticles suspension after vigorous shaking and filtering by ICP. Effects of ions released during NPs preparation were investigated on the growth of wheat using $AlCl_3$ through a method similar to the one described earlier in Preparation of Culture Media Section.

Extraction and assay of antioxidant enzymes

Protein extraction was performed by homogenizing 0.3 g of fresh weight (frozen sample) in 4 mL of HEPES-KOH buffer (pH 7.8) containing 0.1 mM EDTA at 4 °C. The homogenous solution was centrifuged at 18000 rpm for 20 min at 4 °C. Superoxide dismutase (SOD) (EC 1.15.1.1) activity was assayed by monitoring the inhibition in photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Giannopolitis and Reis, (1977). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition in the rate of NBT reduction measured at 560 nm. The activity of catalase (CAT) (EC 1.11.1.6) was determined by decline in absorbance at 240 nm following the decomposition of H_2O_2 according to Cakmak and Horst (1991) method. Activity of ascorbate peroxidase (APX) (EC 1.11.1.11) was measured spectrophotometrically by recording the decrease in absorbance at 290 nm for 1 min (Nakano and Asada, 1981). The rate constant was calculated using the extinction coefficient of 2.8 $mM^{-1}cm^{-1}$ per mg protein.

Protein concentration

Protein contents were determined by the method of Bradford (1976), with bovine serum albumin (BSA) as a standard.

Statistical analysis

Experiments were conducted in completely random designs in triplicate for each treatment. All experiments were repeated at least three times. The results are expressed as mean values ± standard deviation (SD). Duncan's multiple range tests were used to compare mean of the treatments at $P<0.05$. Significance of the difference between mean values was determined by one way analysis of variance.

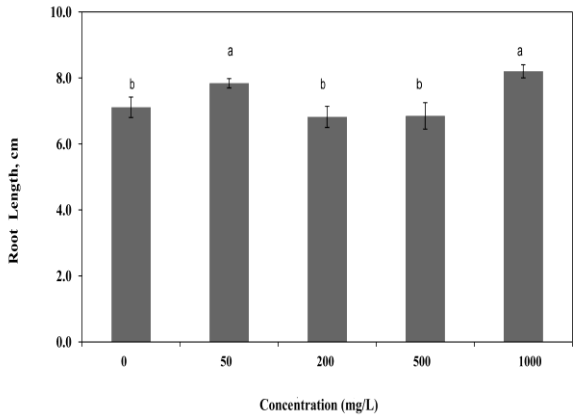

Fig. I. Effect of different concentrations of nano-scale alumina on root elongation of *T. aestivum* after 5-days. Different letters indicate significant differences at $p < 0.05$ according to Duncan's Multiple Range Tests. Bars represent one standard deviation of the mean (n = 3 replicates).

Results

Effects of nAl_2O_3 on seed germination

The percentage of seed germination was the same as that in control plants, approximately 90% germination for all treatments (Table 1).

Effects of nAl_2O_3 on root and shoot elongation and dry biomass

As shown in Fig. (I) root growth was affected by NPs. Root lengths in the presence of 50 and 1000 mg/L NPs were significantly larger than in control (*p<0.05*) while they were slightly (not significantly) decreased when compared to control in both 200 and 500 mg/L treatments. As shown in Table 1, nano-scale alumina did not have any significant inhibitory or increasing effects on shoot length and dry biomass.

Bioaccumulation and bioavailability of aluminum in seedlings

To determine absorption and transportation of NPs by seedlings, bioaccumulation of Al was measured using ICP. As reported in Table 2, by increasing the concentration of NPs in media, the content of Al is elevated in the root except for cases with highest concentration (1000 mg/L). On the other hand, bioavailability which is defined by the bioaccumulation factor decreases with increasing concentration of nano-scale alumina in the medium (Table 2).

Bioaccumulation and bioavailability of Al in the shoot were measured as well and the findings were insignificant (data not shown). Al ions content in 1000 mg/L suspension were measured using ICP and found to be negligible as 1.22 ± 0.03 mg in 1000 mg/L. Interestingly, at this level of Al ion no adverse effects were found on the morphological properties of wheat (data not shown).

Effect of nAl_2O_3 on activity of antioxidant enzymes

The activity of key antioxidant enzymes including, SOD, CAT and APX in *T. aestivum* that were grown in the presence of alumina nanoparticles were analyzed (Fig. II). As shown in

Table 1
Seed germination (percentage), shoot length (cm), dry biomass (mg), of *T. aestivum* exposed to various concentrations of nano-scale alumina after 5 days

Concentration of nano-scale alumina (mg/L)	Seed germination %	Shoot length, cm	Dry biomass (seedling), mg
0	96.66 ± 5.77[a]	5.04 ± 0.15[a]	418.33 ± 35.38[a]
50	100.00 ± 0.00[a]	5.11 ± 0.23[a]	401 ± 25.51[a]
200	93.33 ± 5.77[a]	4.96 ± 0.28[a]	387.66 ± 11.59[a]
500	93.33 ± 11.54[a]	4.84 ± 0.42[a]	395.33 ± 15.63[a]
1000	96.66 ± 5.77[a]	5.28 ± 0.19[a]	436 ± 28.16[a]

Data show mean ± SD, n = 3 replicates. In each group, the same letters indicate no significant differences at *p<0.05* according to Duncan's Multiple Range Tests. For further details see Experimental procedures.

Fig. (II A), activity of SOD was increased in the 200 and 500 mg/L treatments; at 50 and 1000 mg/L treatments SOD activities were the same as the control. Activities of CAT enzyme (Fig. IIB) were increased for concentrations up to 200 mg/L and were decreased with increasing NPs concentration. As shown in Fig. (II B), CAT activity in 200 and 500 mg/L was significantly higher in comparison to the control. This is contrary to APX activity. As shown in Fig. (II C), APX activity is significantly decreased in the presence of NPs, except for 200 mg/L treatment that is similar to control. It should be noted that at the highest concentration, the activity for every antioxidant enzymes was significantly decreased.

Discussion

Due to widespread production and use of NPs, it is expected that they find their way into the environment, be taken up by living organisms (in particular plants) and consequently find their way into the food chain (Dionysiou, 2004; Leacoanet et al., 2004; Nowack and Bucheli, 2007). Objectives of the present study were to investigate, the effects of nano-scale alumina on the growth of wheat (as it is one of the most widely cultivated crops in the world) and bioaccumulation of these particles in different parts of the seedlings. Finally, response of seedlings to NPs was analyzed by assaying several antioxidant enzymes activity.

During Preparation stage of suitable culture media it was noted that all concentrations of alumina NPs had the same appearance as the control expect the 1000 mg/L for which the medium's color was changed slightly to milky.

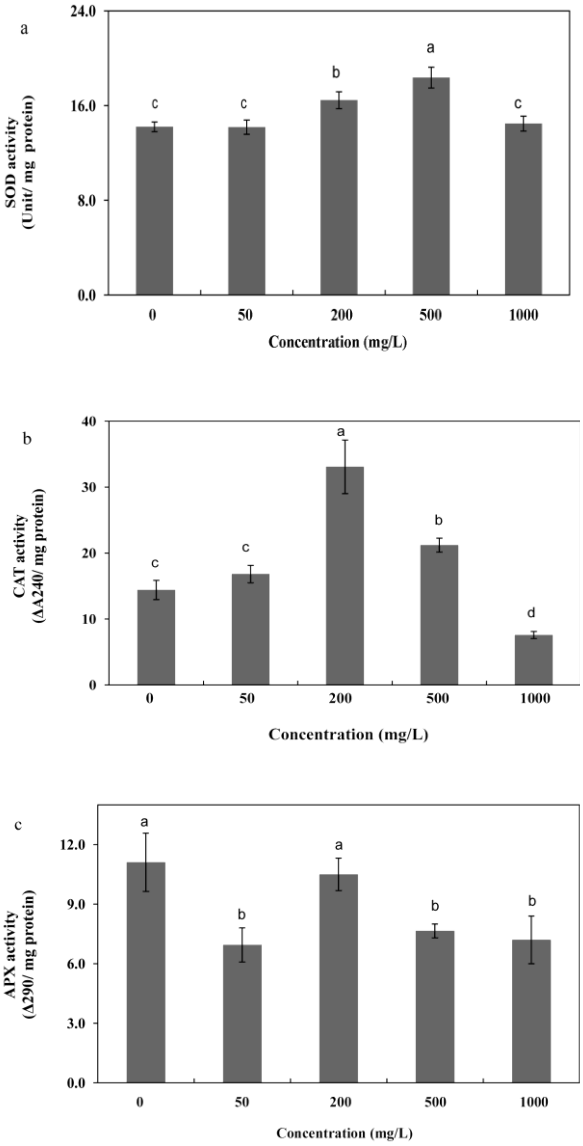

Fig. II. Activity of antioxidant enzymes; Letters a, b and c represent SOD, CAT and APX activity, respectively. Different letters indicate significant differences at $p < 0.05$ according to Duncan's Multiple Range Test. Bars represent one standard deviation of the mean (n = 3 replicates).

Table 2
Bioaccumulation and bioavailability of nano-scale alumina after 5 days in root of *T. aestivum*. Data show mean ± SD, n = 3 replicates.

Concentration of nano-scale alumina (mg/L)	Bioaccumulation in root (mg/ kg)	Bioavailability in root (L/kg)
0	nd	-
50	1633.3 ± 404^{b}	32.6 ± 8^{b}
200	7633.3 ± 1514^{a}	38.1 ± 7.5^{a}
500	10300 ± 858^{a}	20.6 ± 3.7^{a}
1000	3800 ± 2150^{b}	3.8 ± 2.1^{b}

In each group, different letters indicate significant differences at $p < 0.05$ according to Duncan's Multiple Range Tests. For further details see Experimental procedures. nd, not detected.

This change can be attributed to the high concentration and agglomeration of NPs; as been reported previously, aggregation of nanoparticles is a common phenomenon (Borm et al., 2006).

The effects of nAl_2O_3 on morphological properties of wheat seedlings

Simplicity and sensitivity in seed germination and root elongation makes them a useful test subject for investigating the phytotoxicity of pollution on plants (Wang et al. 2001; Munzuroglu and Geckil 2002). As reported in Table 1, seed germination was not affected by alumina NPs. This may be the result of selective permeability of seed coats that does not allow this material to pass through it (Wierzbicka and Obidzinska 1998; Lin and Xing 2007). These findings agree with recent reports stating that seed germination for different plant species was not affected by nAl_2O_3 (Yang and Watts, 2005; Lin and Xing, 2007; Lee et al., 2010). It has also been reported that the process of seed germination in wheat is not affected by the presence of aluminum ion under experimental settings (Jamal et al., 2006).

As shown in Fig. (I) root growth was significantly promoted at 50 and 1000 mg/L concentration of these particles. Note that the mechanism for this effect has not been clearly understood. Furthermore, the positive effect of nAl_2O_3 on root elongation in *Arabidopsis thaliana* has been reported by Lee et al. (2010). Improvement in wheat root growth has also been reported at low doses of Al (Aniol, 1989). The increasing root length in cases with 1000 mg/L treatment can be explained by the presence of NPs in aggregate form causing changes in color of the medium and a significant decrease in bioaccumulation and bioavailability of Al (Table 2). On the other hand, the root length in presence of 200 and 500 mg/L NPs was reduced slightly (not significantly) as compared to control root length. This observation can be attributed to high Al content in the roots, as reported in Table 2. The decrease in root elongation in the presence of nAl_2O_3 for various plant species has been reported (Yang and Watts, 2005; Lin and Xing, 2007).

In contrast, shoot length was not affected by NPs (Table 1) which may be a consequence of confronting roots with excess of NPs and low transportability of this material to the shoot (data not shown). However, as described previously, toxic symptoms seem to appear more in the roots rather than shoots (Sresty and Rao, 1999). It is worthwhile to mention that in cases of 200 and 500 mg/L treatments, shoot length and dry biomass weight were similar to those of the control (Table 1). Therefore, it may be concluded that the more uptake of these particles, the more adverse effects on the growth of seedlings (Table 2).

Bioaccumulation and bioavailability of aluminum in seedlings

Absorption of aluminum by roots was confirmed by measuring Al concentration using ICP. As reported in Table 2, aluminum content in root was elevated by the increase in NPs concentration in the growth media except for the 1000 mg/L treatment. The decrease in uptake of Al by roots in the presence of 1000 mg/L, and also the decrease of bioavailability due to high concentration of nAl_2O_3 in culture media can be attributed to agglomeration of nanoparticles at high concentration.

Negative effects of aluminum ions (aluminum nitrate), e.g., growth inhibition, on two varieties of *T. aestivum* have been reported (Jamal et al., 2006). To ensure that the effects of nano-scale alumina on root length (in 200 and 500 mg/L) are the result of NPs properties or aluminum ions that were released during media preparation, the content of aluminum ions in 1000 mg/L was measured using ICP and was found to be negligible. No morphological changes were seen in wheat when $AlCl_3$ was used as a source of Al ion in this concentration (data not shown). The present results showed that in studying the effects of alumina nanoparticles on wheat under experimental conditions, the concentration of solubilized Al ion was not enough to induce any significant effect on morphological properties of the seedlings.

Antioxidant enzymes activity

Although reactive oxygen species (ROS) are proposed to be responsible for negative effects of inhaled nanoparticles (Nel et al., 2006),

toxicity mechanism of the NPs (as well as nAL_2O_3) in plant has not yet been clearly understood. However, it is plausible that the production of ROS is responsible for inducing the nanotoxicity. Antioxidant enzymes are involved in scavenging of the ROS and are more active in the presence of biotic or abiotic stresses; hence, in order to determine the probability mechanism of nanotoxicity, activity of several antioxidant enzymes were assessed. Effects of alumina nanoparticles on the activity of antioxidant enzymes are shown in Fig. (II). As can be seen from Fig. (II A), activity of SOD increases as concentration of nano-sacle alumina is increased, up to 500 mg/L. SOD plays an important role in catalyzing $O^{\cdot -}_2$ (the fundamental free radical) to H_2O_2 (Dong et al., 2002). The SOD activity was stimulated especially for exposure concentrations of 200 and 500 mg/L, while significant differences, as compared to control, were observed at 50 and 1000 mg/L. It can be speculated that the increase in activity of SOD is an indicator of damage to the root growth (Dong et al., 2002), which is also the reason for negligible root growth inhibition at 200 and 500 mg/L concentrations.

Activities of enzymes that are responsible for elimination of H_2O_2, CAT and APX (Fridovich, 1983; Bowler et al., 1992) are shown in Fig. (II B) and (II C), respectively. High activity of CAT in treatments of 200 and 500 can be attributed to the higher activity of SOD and generation of more H_2O_2. Moreover, low activity of APX in the presence of nano-scale alumina, can be attributed to the remarkable activity of CAT as a key enzyme for eliminating H_2O_2, thereby regulating the activity of APX as reported by Ghanati et al. (2005), for low activity of APX in the presence of Al in tea cells (*Camellia sinensis*).

Conclusions

Based on the results obtained from the study, it can be speculated that the toxic effects of nano-scale alumina on plants is due to formation of ROS, following the NPs uptake. Here, it seems that the activity of antioxidant enzymes which scavenge ROS is responsible for decreasing symptoms of these particles on wheat. This suggestion can be supported by a previous study which showed that phytotoxicity effects of alumina nanoparticles on *Zea mays* are reduced in the presence of DMSO, a free hydroxyl radical scavenger that can react with free hydroxyl groups on the surface of NPs (Yang and Watts, 2005).

However, due to potentially retarding effect of alumina NPs on root growth for certain plants (Yang and Watts, 2005), it is possible that the NPs size (used in the experiment) was too large for the uptake; also with long time exposure, the toxicity effects may be observed more clearly on the morphological properties of wheat. Therefore, more investigations are needed to determine the positive or negative effects of nAl_2O_3 on wheat. Based on the results presented, the uptake and accumulation of this material can occur following the growth of wheat in the presence of the particles. Since wheat is the most staple food, NPs can find their way into the human body and interact with the cells with unexpected consequences.

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