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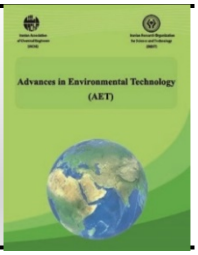

Toxicity of zero-valent iron nanoparticles and its fate in *Zea mays*

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A B S T R A C T

Application of nanotechnology has gained remarkable interest in recent years and environmental exposure to nanomaterials is becoming inevitable. Therefore, nanotoxicity problem is gaining more attention. Zero-valent iron nano particles (nZVI) are being used widely for different purposes such as environmental remediation. Excessive amounts of nanomaterials may pose inhibitory effects on growth of plants cultivated in nZVI-affected soils which has been addressed in this research. Moreover, fate of nZVI in plants was investigated in the present study. Plant seeds were exposed to different concentrations of nZVI i.e. 0, 100, 250, 500, 800 and 1000 mg/kg. *Z. mays* was selected as the model plant in this study and found to be a tolerant plant species in presence of low to moderate levels of nZVI in soil. However, addition of higher doses of nZVI reduced seedling emergence and biomass establishment. Results indicated that the total Fe concentrations in *Z. mays* treated with nZVI increased compared to the control. Considerably higher accumulation of Fe in roots of *Z. mays* compared to the shoots in all treatments was found. Results indicated that the total Fe contents in *Z. mays* treated with nZVI were higher than those in control, with the highest Fe accumulation capacity of 24666.2 µg per pot which was obtained in soil received 500 mg/kg nZVI. Overally, toxic effects of higher doses of nZVI on plants were observed in this study. Intelligent use of nZVI for environmental purposes such as applying low to moderate levels of nZVI in soil remediation activities could remarkably prevent their adverse impacts on plant species, promote plant phytoextraction capability, and reduce nZV emission in the environment.

1. Introduction

Using nanomaterials in different areas such as environmental remediation activities has attracted global attention. Recently, concerns over the adverse environmental and human health effects of nanomaterials have been raised due to the significant rise in utilization of nanomaterials. Nanotechnology has become a dynamically developing industry, with a wide variety of applications in different sectors such as energy, food and agricultural industries. Products that are derived from nanotechnology are usually known as nanomaterials [1]. For instance, nanomaterials could be used in agriculture to obtain agricultural products more rapidly and with higher yield [2]. In recent years, nanomaterials have also drawn global attention to be used for environmental remediation purposes [3]. Nanomaterials could interact with living organisms in the environment implying the serious threat for the environment and human health, particularly if nanomaterials are overused [4]. Nanomaterials may enter the environment intentionally or unintentionally. For instance, synthesized nanomaterials may enter soil resources intentionally when they are employed for soil remediation purposes. Unintentional release of nanoparticles to the environment may be attributed to solid waste streams, wastewater effluents and atmospheric depositions. Some plants are able to uptake nanomaterials from soil and accumulate them in their roots and shoots [5]. The interaction between nanomaterials and plants may result in alteration of plant gene expression and corresponding biological pathways; thereby change plant establishment and growth behavior

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in soils containing nanomaterials. Plant behavior in presence of nanomaterials in soil depends on different factors e.g. plant species, nanomaterial types, nanomaterial characteristics, concentration of nanomaterial and exposure duration [6]. Adverse effects of excessive amounts of metallic nanoparticles on plant growth have been reported in the literature. For instance, applying Ag nanoparticles exhibited inhibitory effects on *Lemna minor L. clone St growth, even when low doses of silver nanoparticles were applied (5 mg/L in aquatic environment)* [7]. In a previous study, negative impacts of TiO_2 nanoparticles on germination of *O. sativa* was found [8].Nanoscale zero-valent iron (nZVI) is among the most frequent nanomaterials used worldwide. Zero-valent iron nanoparticles has been utilized in soil remediation and water treatment works mainly due to its high specific surface area, strong reducibility, low cost and less toxicity compared to many other nanomaterials such as Ag nanoparticles [9; 10]. Application of nZVI as a feasible and prospective strategy to remediate heavy metal-polluted soils has been suggested by several researchers [11,12]. Based on the literature, immobilization of heavy metals in soil is the main mechanism by which nanoparticles of zero-valent iron are supporting remediation of metal-contaminated soils. It should be noticed that, the effectiveness of nZVI to immobilize metals in soils depends on different factors, such as soil properties, dose of applied nZVI, and presence of non-target pollutants. In a remediation study, immobilization of Pb, As and Cr increased more than 82% through application of 10% of nZVI; however, application of nZVI could not considerably enhance immobilization of Cd [11]. Using nanomaterials to facilitate phytoremediation of polluted soil is also gently drawing global attention; however, addition of nZVI to the soils may result in plant stresses, especially when excessive amounts of nZVI are used [9,13,14]. Enhanced growth of some plants was observed when lower levels of nZVI were applied soil, though higher rates of nZVI caused nanotoxicity [15]. Application of high levels of some nanomaterials for remediation purposes has raised environmental concerns, due to their ambiguous fate in the environment and probable toxic effects on living organisms such as plant species; however, contradictory results have been reported in the literature. For instance, adverse effects of 500 mg/kg and higher doses of nZVI on rice seedlings was reported in the literature [16], while application of similar quantities of nZVI promoted root growth for *A. thaliana* [17]. To date, only a limited number of studies have been conducted to evaluate the toxicity of nanomaterials to plant and their fate in planted soil. Considering the increasing worldwide production and utilization of nanoparticles such as nZVI for environmental remediation works, it is necessary to gain profound insight into the fate of zero-valent iron nanoparticles in planted soil and their potential toxicity for plant growth. That could help pursuing soil cleanup activities using nZVI, while understanding nZVI probable toxic effects on plants and keeping their applied doses as minimum as possible. To date, toxicity of nZVI to *Z. mays* and its growth behavior in soil treated with different doses of nZVI have rarely been investigated. In addition, accumulation capacity of *Z. mays* roots and shoots for different levels of nZVI was addressed for the first time in this study. The main objectives of this study were to investigate the effect of zero-valent iron nanoparticles on *Z. mays* growth behavior in soil and fate of the nanoparticles in the plant. In this study (i) the effect of nZVI treatments on the seedling emergence of *Z. mays*; (ii) *Z. mays* growth behavior in different nZVI treatments; (iii) uptake and translocation of iron by Z. mays and (iv) accumulation capacity of *Z. mays* for iron in different treatments were investigated.

2. Materials and methods

Soil provided from non-contaminated lands was sieved through a 2-mm mesh to exclude the gravel and large debris, and then air-dried for one week. The sieved soil was thoroughly mixed manually before adding nanomaterials to soil. Synthesized nZVI were applied to the soil at desired levels of nZVI per kilogram of soil by suspending 100, 250, 500, 800 and 1000 mg of the nZVI in 300 mL of distilled water separately, sonicated in water bath for 30 min at 30°C with occasional stirring. Each pot filled with 1.5 kg of soil. The control pot was also received 300 ml of distilled water initially [18]. The pots were kept in a dark room and stabilized for three weeks at 70% of field capacity using tap water before planting. Chemical analysis of the pots soil was carried out prior to sowing the seeds. Briefly, the soil pH was measured in suspension using a 1:2.5 (w/v) ratio of soil-water ratio. Phosphorus was determined by Olsen P extracting solution (0.5 M NaHCO3, pH 8.5), total nitrogen by the Kjeldahl measurement (VELP Scientifica, UDK 142, Italy), organic carbon (OC) content was measured according to the Walkley-Black method, in which organic carbon is oxidized using potassium dichromate [19]. Electrical conductivity (EC) was measured using a conductivity meter in a soil-water extract (1:2.5 soil: water ratio (w/v)). The soil texture was determined using a Bouyoucos densitometer which is classified as Clay-Loam (CL). Selected characteristics of the used soil are as follow: Clay content: 27%, Silt content: 35%, Sand content: 38%, OC: 0.98%, pH: 7.7, EC: 2.12 (dS/m), Nitrogen; 1.45%, phosphorous: 9.1 (mg/kg). The total concentrations of Fe in soil samples were determined through acid digestion with a mixture of 6 mL nitric acid and 2 mL chlorhydric acid in a microwave reaction system. Obtained extract was then analyzed for metals concentrations using an atomic absorption spectrophotometry according to the standard methods. All the analytical determinations were carried out in triplicate and the mean values were reported [11].

Zero-valent iron nanoparticles were prepared using borohydride reduction method. $FeSO_4.7H_2O$ (4 g) was dissolved in 200 mL of methanol/deionized water (30%:70% (v/v)). The pH was adjusted to 6.8 using 3.8 M NaOH. The $NaBH_4$ (1.5 g) powder was dissolved in 10 mL of deionized water and added dropwise to the prepared mixture in ultrasonic shaker at 25°C for 45 min. The mixture was then stirred thoroughly at 250 rpm for another 45 min. The obtained particles were then centrifuged at 5000 rpm for 15 min, washed at least three times with methanol, filtered, dried under vacuum condition for 6 h and pulverized. The ferrous iron was reduced to zero-valent iron based on the following equation [20]:

$$\mathrm{Fe(H_2O)_6}^{3+} + 3\mathrm{BH_4}^{-} + 3\mathrm{H_2O} \rightarrow \mathrm{Fe} \downarrow + 3\mathrm{B(OH)_3} + 10.5\mathrm{H_2} \tag{1}$$

The prepared nZVI nanoparticles were immediately suspended in deionized water into tightly dark, stoppered bottles at room temperature. Then, the volume of the suspension was made up to 100 mL with deionized water, containing the desired concentration of the nZVI per 100 ml deionized water. The size of nZVI were determined using transmission electron microscopy (TEM), manufactured by PHILIPS (EM208 S), with an acceleration voltage of 100 kV. The size of the nZVI particles covers a range between 40 and 100 nm. Selection of nZVI concentration range was based on the preliminary experiments, which showed that nZVI concentration of lower than 100 mg kg^{-1} had negligible influence on plant growth and metal uptake. Applying higher than 1000 mg kg^{-1} nZVI to soil exhibited severe inhibitory effects on plant growth. Therefore, the nZVI concentration range was selected between 100 and 1000 mg/kg. Plant seeds were sown in plastic pots containing different concentrations of nZVI. Nanoparticles of ZVI at doses of 0, 100, 250, 500, 800 and 1000 mg kg^{-1} were applied to soil. *Z. mays* plants were cultivated over a 60-day period in a greenhouse. The seeds were planted in the 1.5-2.0 cm depth of the surface soil in each pot. Pots were kept in a greenhouse under natural sunlight (10-12 h light) to imitate real-world conditions, and irrigated two or three times per week. Pot experiments were carried out in three replicates. Pots were monitored to assess seedling emergence rate in different treatments and cultivated plants were harvested after 60 days, then the biomass and length of roots and shoots were measured. Plants were dried in an oven at 70 °C for 48 h to obtain dry weight of the biomass. After harvesting, plant roots and shoots were thoroughly rinsed with distilled water, dried at room temperature (22-25°C), and then oven-dried at 60 °C for 12 h. The obtained samples were ground through a 200 mesh. Plant samples (0.5 g) digested in a digestion tube at a temperature of 100-230 °C with 4.0 ml HNO_3 and 1.0 ml $HClO_4$. Extracts were filtrated by a MILEXHA 0.45 μm diameter filter to obtain a clear solution, and then analyzed for the total concentration of Fe by flame atomic absorption spectroscopy (Perkin Elmer 700) [21,22]. The bio concentration factor (BCF) is used to calculate the metal uptake capacity from soil to plant tissues. It can be measured for each plant part, such as roots and shoots. Translocation factor (TF) is also an important tool to assess the potential of a given plant for phytoremediation purposes. It is determined from the ratio of the concentration of an element in plant's shoots compared to that in the plant's roots. BCF and TF were calculated using the following equations [23]:

$$\mathrm{BCF} = \frac{\text{Concentration of metal in roots}}{\text{Concentration of metal in test soil}} \tag{2}$$

$$\mathrm{TF} = \frac{\text{Concentration of metal in shoots}}{\text{Concentration of metal in roots}} \tag{3}$$

All statistical analyses were performed using IBM SPSS Statistics 24. All results in this paper were presented as the mean with standard errors (n=3). Significance of differences was determined using one-way analysis of variance (ANOVA), followed by least significant difference (LSD) test. Significance level was considered at P=0.05.

3. Results and discussion

Seedling emergence of *Z. mays* in different treatments was monitored during the experiment. Applying nZVI concentrations of up to 250 mg/kg to soil did not change germination rate compared to the control, but nZVI with concentrations of 500 to 1000 mg kg^{-1} reduced *Z. mays* germination (Figure 1). Application of 800-1000 mg/kg nZVI reduced germination rate significantly ($P<0.05$), with the lowest germination rate of 80±5% in 1000 mg/kg nZVI treatment. Exposure of peanut seed to nZVI at low concentrations stimulated the germination and development of seeds, which was attributed to the internalization of nZVI by the plants [10]. In this study, delayed germination was also observed due to addition of 800 mg/kg and 1000 mg/kg nZVI. Overally, *Z. mays* showed high tolerance to the presence of different levels of nZVI in soil. Positive effect of lower doses of other nanoparticles such as titanium dioxide nanoparticles on germination of soybean was also reported in the literature [3], which is consistent with the finding of this research.

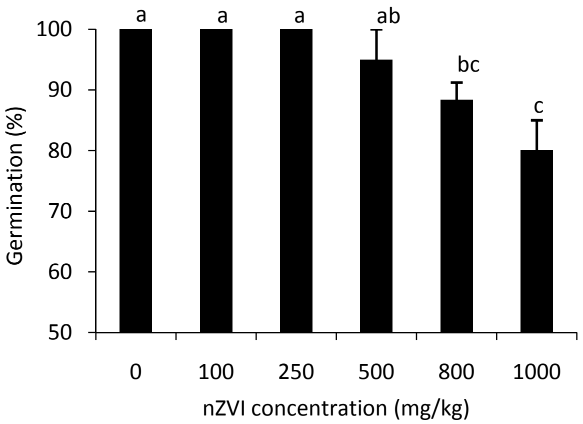

Fig. 1. Final seedling emergence of *Z. mays* in soil treated with nZVI. Error bars represent standard deviation of three replicates. Different letters indicate significant differences between the treatments (mean ± standard deviation; n = 3).

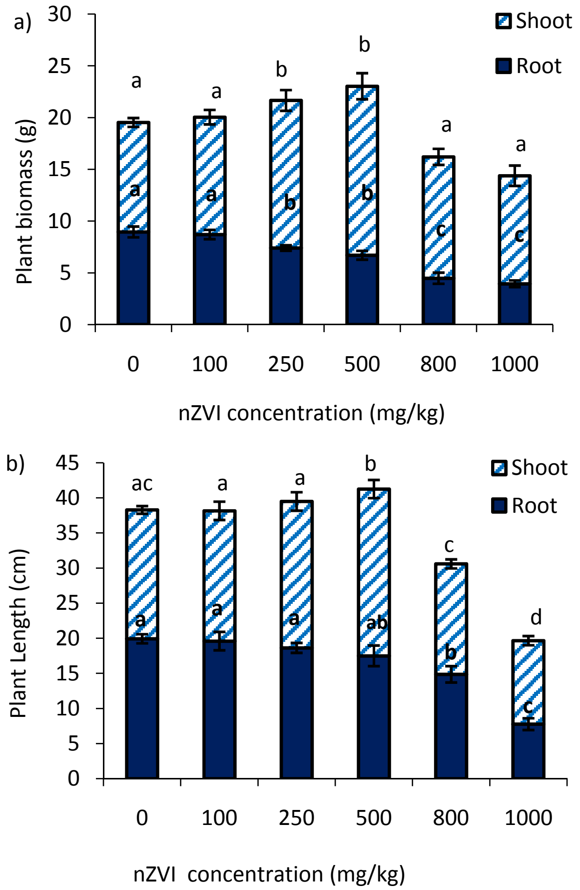

Fig. 2. The biomass (a) and length (b) of *Z. mays* grown in different nZVI treated soil samples at 60 days. Error bars represent standard deviation of three replicates. Different letters represent significant differences in roots and shoots, respectively, between the treatments (mean ± standard deviation; n = 3).

Biomass and length of roots and shoots were determined after harvesting to evaluate the impacts of nZVI treatment on plant growth. Figure 2a shows that applying nZVI to the clean soil reduced root biomass of *Z. mays* particularly at higher nZVI doses, while enhanced shoot biomass of plants in all treatments compared to the control, except the 1000 mg/kg nZVI treatment. Application of 1000 mg/kg nZVI slightly reduced shoot biomass by 1.32% compared to the control treatment ($P>0.05$). Decrease in roots biomass with the rising of nZVI levels was observed. The most significant increase in shoots biomass (54.34%) was gained in 500 mg/kg nZVI treatment, compared to the control ($P<0.05$). Total plant biomass increased with the application of 100 to 500 mg/kg nZVI, while declining trend was achieved in treated soils with higher nZVI concentrations. The extent of plant growth promotion or inhibition in presence of nanoparticles in soil was reported to be dependent on plant type as well as nanoparticles types and concentrations [24]. However, contradictory results have been reported in the literature. For instance, the biomass of wheat decreased in presence of 90 mg/kg nanomaterials in soil [25], whereas addition of 100 to 300 mg/kg TiO_2 nanoparticles to soil increased soybean biomass in soil [3]. The length of roots followed the same trend as root biomass due to the application of nZVI to soil and declined with increasing nZVI concentrations (Figure 2b). The growth of *Z. mays* in the test soil treated with 1000 mg/kg nZVI with the root length of 7.8±0.8 cm and shoot length of 11.9±0.7 was suppressed in comparison with the growth yielded in the control treatment that was statistically significant ($P<0.05$). Amplified phytotoxicity of contaminants by addition of nZVI to soil has also been reported in the literature [10]. Sensibility of root extension and biomass of *Z. mays* to high concentrations of nZVI (mainly greater than 800 mg/kg) was found in this study, whereas lower concentrations of applied nZVI i.e. 250-500 mg/kg promoted plant growth in clean soil. Application of 0.01% TiO_2 NPs to soil slightly increased shoot length of leguminous crops [26]. Boosted ability of edible plants such as tomato to take up nutrients (N, P, Ca, Mg) from soil, and therefore enhanced growth of plants in soils treated with nanomaterials was also reported [27]. *Z. mays* is a reasonably fast growing species that makes it suitable for phytoremediation purposes since establishment of considerable biomass is an important factor affecting phytoremediation potential of plants [28]. Plant tolerance and establishment of considerable biomass are important factors for successful remediation of soils affected by nanomaterials and/or other contaminants. Phytoextraction of heavy metals was reported to be ten times more economical compared to conventional remediation techniques [29]. Among the various remediation approaches, phytoremediation is one of the most promising, ecologically friendly, and economical remediation approaches, which can uptake metals from soil [30]. Using plant species provides an opportunity to yield several indirect contaminant attenuation mechanisms, which promotes the elimination of contaminants from soil. However, contaminant removal mechanisms involved in phytoremediation are complex and not limited only to the direct metabolism of contaminants by plants. Indirect attenuation mechanisms are also involved in phytoremediation such as the metabolism of contaminants by plant-associated microorganisms in the rhizosphere and plant-induced modifications in the contaminated environment [3,9,15]. Selected plants for phytoextraction should have rapid growth, extended root system, and high biomass production [31]. To be brief, results indicated that low to moderate concentrations of nZVI improved plant growth, while elevated concentrations of nZVI i.e. 800 and 1000 mg/kg in soil posed inhibitory effects on growth of *Z. mays*. Phytoextraction is known to be the main mechanism by which metals are removed from soil by plants [32]. Table 1 presents the distribution of Fe in plant roots and shoots in different treatments. Iron is an essential micronutrient for plants growth and survival involved in cellular functions, such as DNA synthesis, nitrogen fixation and photosynthesis [33]; however, high doses of Fe in soil may exhibit toxic effects on plant growth, as observed in this study. Accumulation of metals in plants may induce oxidative stress due to the production and accumulation of reactive oxygen species (ROS). Low concentrations of nZVI were found to slightly reduce the oxidative stress in plants cultivated on contaminated soils, while increased oxidative stress as a result of addition of high concentrations of nZVI has been reported in the literature [9]. Therefore, the high accumulation of both nZVI may induce significant oxidative stress and damage to antioxidant enzymes of plants, which is suggested to be further studied in the future.

Table 1. Distribution of Fe in roots and shoots of *Z. mays* grown in clean soil in different nZVI treatments, bio concentration factors (BCF) and translocation factors (TF) of Fe in *Z. mays* grown in different soil treatments. Standard deviations for three replicates are presented.

nZVI concentration (mg/kg)	**Fe concentration (mg/kg)**		**Bio concentration and translocation factors**	
	Roots	**Shoots**	**BCF**	**TF**
0	589.63±67.47	347.88±43.33	1.64±0.12	0.59±0.13
100	854.4±46.55	538.27±45.31	1.74±0.08	0.63±0.04
250	966.85±61.06	705.80±144.90	2.14±0.06	0.73±0.13
500	1217.84±157.70	1010.81±109.89	2.46±0.05	0.83±0.05
800	1504.86±112.15	857.77±107.96	1.94±0.12	0.57±0.03
1000	1704.7±156.02	903.49±102.75	1.85±0.09	0.53±0.04

In terrestrial species, transport of contaminants towards the plant is dominated by the uptake of water by roots, and distribution within the plant mainly relies on xylem or phloem transport [34,35]. Plants can uptake contaminants like heavy metals from soil and transfer them to the harvestable parts through a sub-process called phytoextraction. This process often occurs with metals, radionuclides and certain organic compounds that are resistant to plant metabolism. Phytoextraction of heavy metals was reported to be ten times more economical compared to conventional remediation techniques [29].

Table 1 indicates that the total Fe levels in roots were significantly higher than those in shoots, which may be attributed to the direct exposure of the *Z. mays* roots to the nZVI in soil. Concentrations of Fe in roots increased with nZVI concentration to hit a plateau of 1704.70 mg kg^{-1} in treated soil with 1000 mg/kg nZVI, which was 2.89 times greater than the corresponding value in control treatment. In 100-500 mg/kg nZVI treatments, the concentrations of Fe in shoots as well as TF values increased with the rising of nZVI; which suggested that low to moderate contents of nZVI could promote translocation of Fe in *Z. mays*. BCF and TF values of Fe in nZVI treatments ranged from 1.74 to 2.46 and 0.53 to 0.83, respectively, with the highest values obtained in 500 mg/kg nZVI treatment. Despite the increase in concentrations of Fe in the roots of *Z. mays*, BCF values were alleviated by addition of 800-1000 mg/kg nZVI. In addition, translocation factors of Fe declined in 800-1000 mg/kg nZVI treatments implying that application of high concentrations of nZVI had inhibitory effects on Fe uptake by plant and translocation of Fe within the plant, which might be caused by the plugging of the pathway of Fe from the root to the shoot by nZVI as also suggested by Wang et al. (2016). The limited ability of plants to transfer some metals to the aerial parts of plants has also been reported in the literature as a result of blocking the root apex in plants as a result of exposure to metals [36]. Apoplastic barriers could be develop near the root apex in plants grown under the stress of metals, which reduce the translocation of sorbed metals from root to aerial parts [37].

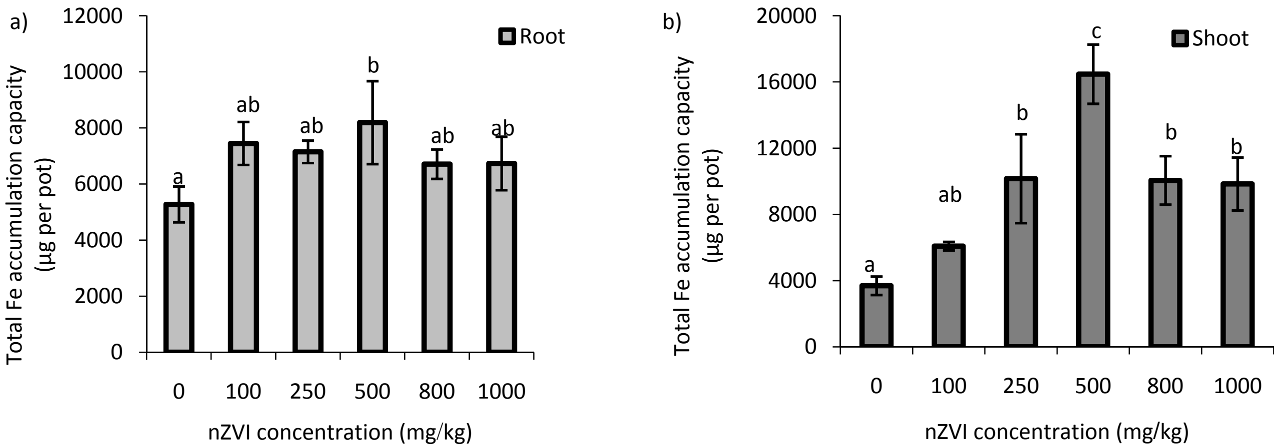

Fig. 3. The Fe accumulation capacity in roots (a) and shoots (b) of *Z. mays* grown in different nZVI-containing soils at 60 days. Error bars represent standard deviation of three replicates. Different letters represent significant differences in roots and shoots, respectively, between the treatments (mean ± standard deviation; n = 3).

This phenomenon could also adversely affect phytoremediation of contaminants in presence of high levels of nanomaterials in soil. Recently, some studies conducted on nanotechnology-assisted phytoremediation to promote removal of contaminants such as heavy metals from soil by using association of plants and nanomaterials [38,39]. When the pathway of contaminants from the roots to the shoots are blocked by excessive amounts of nZVI, target contaminants could not transfer effectively from the roots to the shoots, therefore accumulation of heavy metals in shoots could be decreased, which is not favourable in the context of phytoextraction. Reduced absorption of Cr by edible rape in soil treated with high concentrations of nZVI was reported in the literature [40]. In addition, application of high doses of nZVI inhibited the growth of hybrid poplars [15], suggesting the importance of using low concentrations of nZVI for environmental remediation purposes. The exposure to nZVI may also change physiological characteristics of plant species. Addition of high levels of nZVI to soil could reduce plant's chlorophyll content due to the adverse impact of excessive amounts of nZVI on the biochemical factors such as lipid peroxidation in photosynthesis membranes [41,42]. Zero-valent iron nanoparticles can move along the plant pipeline upon internalization of nZVI by plants and the plant metabolism may be disturbed by nZVI [10]. Fe accumulation capacity of *Z. mays* does not only depend on Fe concentration in plant organs, but also depends on plant dry biomass, which was calculated and illustrated in Figure 3. Fe accumulation capacity of plant species in soils affected by nZVI has rarely been determined. The greatest total Fe accumulation capacity in *Z. mays* was reached 24666.2 µg per pot, which was achieved by addition of 500 mg/kg nZVI to soil, followed by the 250 mg/kg nZVI treatment. In this study, root biomass of *Z. mays* was lower than that of shoots in all treatments. Therefore, it seems that higher accumulation capacity of the roots compared to the shoots of *Z. mays* at 250-1000 mg/kg nZVI treatments could be attributed to the greater concentrations of Fe in roots rather than shoots. Figure 3a and Figure 3b illustrate that accumulation capacity of Fe in the roots and shoots of *Z. mays* enhanced in all nZVI treatments compared to control treatment; however, accumulation capacity of the roots and shoots declined at higher doses of nZVI. Accumulation capacity of a given plant species is suggested to be a beneficial indicator of the ability of plants to uptake and accumulate contaminants in soil that could be more highlighted in phytoremediation studies.

4. Conclusions

This study aimed to assess the effect of nZVI on *Z. mays* growth behavior in soil and fate of the nanomaterials in the plant. *Z. mays* was found to be a tolerant plant species in presence of nZVI in soil; however, germination of *Z. mays* reduced significantly up on addition of 800-100 mg/kg nZVI to soil. Application of 100 to 500 mg/kg nZVI promoted biomass of *Z. mays*, while addition of higher nZVI levels to soil showed inhibitory effects on plant biomass production. The total Fe contents in *Z. mays* treated with nZVI were greater than those in non-nZVI treatment. Fe concentrations in the roots and shoots of *Z. mays* grown in 500 mg/kg nZVI treatment increased by, respectively, 106.54% and 190.56% compared to the control. Considerably higher accumulation of Fe in roots of *Z. mays* compared to the shoots in all treatments suggesting that *Z. mays* roots were the preferential Fe storage organ. Low to moderate concentrations of nZVI effectively increased accumulation capacity of *Z. mays* for Fe in this study. In summary, the addition of low to moderate concentrations of nZVI did not show toxic effects on the plant, while application of 800-1000 mg/kg nZVI exhibited inhibitory effects on *Z. mays* growth. Using nanomaterials require significant cautions to be taken into account to avoid spreading their excessive levels in the environment.

Acknowledgments

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