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Magnetic (Fe_3O_4) Nanoparticles Reduce Heavy Metals Uptake and Mitigate Their Toxicity in Wheat Seedling

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Academic Editors: Elena Cristina Rada and Lucian-Ionel Cioca
Received: 25 March 2017; Accepted: 3 May 2017; Published: 10 May 2017

Abstract: Heavy metal pollution is not only a hazard to living organisms but also an important worldwide environmental concern. Experiments were performed to investigate the physiological mechanisms of magnetic (Fe_3O_4) nanoparticles (nano-Fe_3O_4) mitigation of the toxicity of heavy metals (Pb, Zn, Cd and Cu) in wheat seedlings. All the Petri dishes with germinating seedlings (1d) were covered, sealed with parafilm, and placed in a dark growth chamber. All parameters (seedling growth inhibition, heavy metal accumulation, enzymatic activities, and reducing effects of nano-Fe_3O_4 on heavy metal toxicity) were analyzed only after five days. The results showed that the tested heavy metals significantly affected the growth of wheat seedling by decreasing root length, shoot length and even death at 10 mM concentration in the case of Cd and Cu. Heavy metals exposure also showed that superoxide dismutase (SOD) and peroxidases (POD) activities decreased significantly when the malondialdehyde (MDA) content was significantly higher in wheat seedlings. Addition of magnetic (Fe_3O_4) nanoparticles (2000 mg/L) in each heavy metal solution (1 mM) significantly decreased the growth inhibition and activated protective mechanisms to reduce oxidative stress induced by heavy metals in the wheat seedlings. The reducing effects of nano-Fe_3O_4 against heavy metals stress could be dependent on the increase in the enzyme activity (SOD and POD). Their protective role was confirmed by the decrease in MDA content. The alleviating effect of nano-Fe_3O_4 is associated with their adsorption capacity of heavy metals.

Keywords: alleviation; heavy metals; magnetic (Fe_3O_4) nanoparticles; inhibition; oxidative stress; wheat

1. Introduction

Heavy metal pollution is not only a hazard to living organisms but also an important worldwide environmental concern [1–4]. These metals occur naturally in the biosphere as a result of volcanic eruptions and weathering of rocks. Recently, more heavy metals have been released into the environment due to various human activities such as mining, fossil fuel combustion and poor sewerage systems [2]. Agricultural soils are usually contaminated by heavy metals such as lead (Pb), cadmium (Cd), zinc (Zn) and copper (Cu) which are readily available for uptake by plants [5]. Plants support the ecological system and provide food for the human population [6–8]. Some plants can tolerate and serve as potential pathways for integrating the undesirable heavy metals into plant tissues [2]. The tolerance to heavy metals can lead to a greater risk to human health [9]. Various plants and crops have been shown to be hyperaccumulators of heavy metals [5,10,11]. Studies have shown that over 80% of heavy metals could be ingested via the food crops [12]. According to Kikuchi et al. [13] (pp. 294–299), during 1998–2001, 36–50% of the Cd consumed by Japanese was from rice. Some heavy metals (e.g., Mn and Zn) are beneficial to microorganisms, plants, and animals [14]. However, these metals at high concentrations in contaminated soils and water can be toxic to plants (primary producers) [11,15,16]. Many soil factors such as pH and cation exchange capacity as well as plant species, cultivars and age, could influence heavy metal uptake by plants. Plants are at a higher risk of lipid peroxidation if they are exposed to heavy metals [17], which is usually tested through its end product, Malondialdehyde (MDA) [18]. Under stressful conditions, MDA level in plant tissues is usually elevated in plants [19]. In previous reports, higher plants exposed to heavy metals have shown increased MDA content [18,19]. Studies with Cd, Pb, and Cu had enhanced lipid peroxidation and increased reactive oxygen species (ROS) in various plants [18,20,21]. However, plants have evolved protective enzymatic mechanisms like superoxide dismutase (SOD) and peroxidases (POD) that can scavenge the ROS and alleviate the resultant negative effects [22–25]. To maintain cell membrane stability, SOD can sequester O_2, reducing lipid membrane peroxidation, while POD can reduce H_2O_2 accumulation and eliminate MDA, hence keeping the integrity of cell membrane lipids [18,26].

China is the most populous country in the world with about 1.3 billion people. About 67% of this population is living in rural areas and more than 300 million people are directly involved in agricultural activities [27]. The arable land is only 7% of the global amount and more than 75% of the total cultivated land is used for producing food crops [27]. Currently, China produces 18% and 50%, respectively, of the cereal grains and vegetables in the world [28]. However, agricultural development in China is confronted with many challenges, among them soil contamination. Studies have shown that 13,330 ha of farmland have been contaminated by heavy metals from phosphate fertilizer application, industrial emission and municipal wastes in 11 provinces [5]. Reliable approaches are desired to prevent heavy metal accumulation in crops and consequently protect living organisms including human from related health hazards.

Nanotechnology has been growing in importance as a potential technology that could be used to clean the environment. Nanoparticles, often characterized by a significant amount of surface area, have unique properties and potential applications in reducing the negative effects of heavy metals on the natural resources [29,30]. Few studies have utilized nanotechnology to investigate plant phytotoxicity caused by heavy metals in various environmental mediums. The remediation of polluted soils and water using nanoscale materials continues to gain relevance especially in light of new environmental molecular science and engineering techniques [4]. Although nanoparticles can be cost effective in reducing heavy metal toxicity in plants [4], alleviation of heavy metal-induced root growth inhibition and oxidative stress in plant has been hardly studied [4,5]. Magnetic (Fe_3O_4) nanoparticles were successfully used to adsorb heavy metal ions [30]. In our previous study (data not published), we evaluated the alleviation of Cd-induced root growth inhibition and oxidative stress in the seedlings of cucumber and wheat using different sizes (6 nm, 50 nm, and 100 nm) of magnetic (Fe_3O_4) nanoparticles and bulk-Fe_3O_4. Four concentrations (0, 50, 500, and 2000 mg/L) of nano-Fe_3O_4 or bulk and 25 mg/L (Cd) were used in that experiment. The addition of nano-Fe_3O_4 (6 nm) significantly decreased the Cd accumulation, Cd-induced seedling growth inhibition and oxidative stress in the seedlings of both cucumber and wheat with increasing concentration. Therefore, we hypothesized that different heavy metal (Pb, Zn, Cd and Cu) treatments may cause inhibition in root growth, and induce oxidative stress in the wheat seedlings. Under this stress, the inhibitory effects of the selected heavy metals would be reduced and the antioxidant mechanisms activated with the addition of magnetic (Fe_3O_4) nanoparticles (6 nm). We also hypothesized that the reducing effects of nano-Fe_3O_4 would vary with the heavy metal types. To test these hypotheses, root elongation, accumulation of heavy metals, activities of

SOD and POD, and MDA content were measured in this study. To better understand the effects of nanoparticles on reducing the phytotoxicity of heavy metals, we conducted study with magnetic (Fe_3O_4) nanoparticles on the toxicity and oxidative stress induced by four heavy metals (Pb, Zn, Cd and Cu) to early seedling growth of wheat (*Triticum aestivum* L.). Wheat is common model that has been extensively used in previous studies [16,31]. Moreover, wheat species have great economic and ecological relevance and are among the widely consumed plant crops in the world [6]. Furthermore, we provide a basis for developing approaches to reduce risks associated with the toxicity of heavy metals and maintaining sustainable plant production. The growth of seedlings, the accumulation of heavy metals and the oxidative stress were investigated at 1 mM and 10 mM of the selected heavy metals with or without nano-Fe_3O_4 (2000 mg/L). According to the US EPA [32] (pp. 96–163) guidelines, 2000 mg/L of nano-Fe_3O_4 is the highest concentration of NPs that can be regarded to have minimal phytotoxicity if no adverse effect on root growth are observed in tested plants. In addition, based on our previous study (data not published), this concentration of nano-Fe_3O_4 had greater effect in reducing toxicity of Cd (25 mg/L) in wheat and cucumber seedlings. Similarly, Wang et al. [14] (pp. 231–140) reported that copper (Cu), lead (Pb) and zinc (Zn) affected radicle elongation of wheat at 1 mM and 10 mM. Our results could be helpful in improving the protection of plant growth in polluted areas using magnetic (Fe_3O_4) nanoparticles.

2. Materials and Methods

2.1. Nano-Fe_3O_4, Heavy Metals and Plant

Magnetic (Fe_3O_4) nanoparticles with an average size of about 6.8 nm were synthesized following the procedures from Xiao et al. [33] (pp. 6315–6324). The selected heavy metals, $PbCl_2$, $ZnSO_4 \cdot H_2O$, $CdCl_2 \cdot 2.5H_2O$ and $CuCl_2 \cdot 2H_2O$, were purchased from Xilong Chemical Co., Ltd., Shantou, China. Other chemicals of analytical grade were obtained from the Beijing Chemical Works. Wheat (*Triticum aestivum* L.) seeds were bought from Chinese Academy of Agricultural Sciences. The average germination rate was greater than 95% according to our preliminary experiment. The seeds were stored at 4 °C until use.

2.2. Characterization of Nano-Fe_3O_4

The TEM pictures of nano-Fe_3O_4 (Figure 1) were obtained from Tecnai G2 20 S-Twin transmission electron microscope 119 (FEI company, Japan) operating at 200 KV for morphological observations. The agglomeration of nano-Fe_3O_4 in water was evaluated using Dynamic Light Scattering (DLS) equipment (ZETA SIZER 90, Nano series, Malvern, UK).

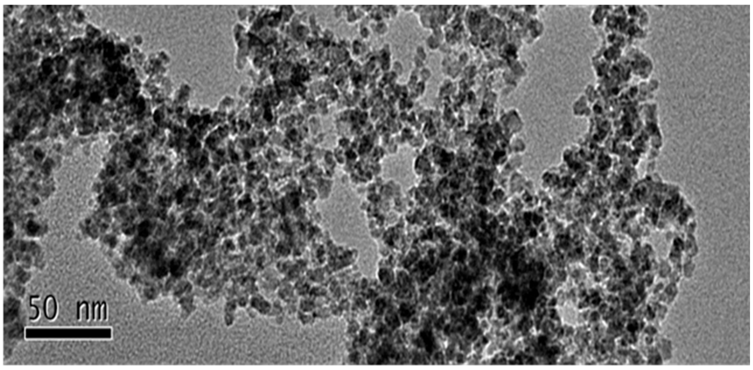

Figure 1. TEM images of nano-Fe_3O_4.

2.3. Preparation of NPs Suspensions and Heavy Metal Solutions

The effects of magnetic (Fe_3O_4) nanoparticles on wheat seedling, the toxic effects of the selected heavy metals on the seedling growth and the effects of nano-Fe_3O_4 on reducing the phytotoxicity and mainly the oxidative stress caused by heavy metals in wheat seedlings were examined using a solution culture system. The growth of seedlings was investigated at 1 mM and 10 mM, while the accumulation of heavy metals and the oxidative stress were investigated only at 1 mM of the selected heavy metals with or without nano-Fe_3O_4 (2000 mg/L). Nano-Fe_3O_4 particles were suspended directly in deionized water or the metal solutions, and then an ultrasonic vibration was applied for 40 min to obtain properly dispersed and stable nano-Fe_3O_4 suspensions of each concentration.

2.4. Seedling Exposure

Wheat seeds were sterilized by soaking in 10% sodium hypochlorite solution for 10 min before being rinsed three times with DI-water [32]. The seeds (50 per group) were then placed on a wet filter paper in 100 mm × 15 mm Petri dishes. Only distilled water was added to the Petri dishes. The Petri dishes were covered and seeds allowed producing radicals over 24 h at 25 °C and 75% relative humidity in a dark growth chamber. Once 90–95% of the wheat seeds produced a radical, further procedures were performed as described below.

2.4.1. Effects of Nano-Fe_3O_4 and Heavy Metals (Pb, Zn, Cd and Cu) on Seedling Growth

The experiment was performed to investigate the effects of nano-Fe_3O_4 or heavy metals on: (1) seedlings viability; (2) seedling growth; and (3) heavy metal-induced root growth inhibition in the seedlings of wheat. Ten seedlings were placed on a piece of wet paper in 100 mm × 15 mm Petri dishes with about 1 cm or an appreciable distance between each seedling [6], and 5 mL of a test medium (nanoparticles suspensions or metal solutions) was added. Distilled water was added to the Petri dishes for the control. All the Petri dishes were covered, sealed with parafilm, and put in a dark growth chamber at 25 °C with 75% relative humidity. After 5 days, seedling roots and shoots lengths were measured using a millimeter ruler. Seedling viability, seedling growth and the inhibitory effects of nanoparticles and heavy metals were also determined.

2.4.2. Effects of Nano-Fe_3O_4 on the Reducing Heavy Metal-Induced Root Growth Inhibition and Their Accumulation in Seedling

This experiment was conducted to investigate the effects of nano-Fe_3O_4 on the reducing heavy metal-induced root growth inhibition and their accumulation in the seedling. All seedlings received heavy metal solutions (1 mM and 10 mM) with or without nano-Fe_3O_4 (2000 mg/L). Each heavy metal concentration without nano-Fe_3O_4 was compared with the corresponding concentration having nano-Fe_3O_4. The seedlings growth conditions were similar with those of Section 2.4.1. After 5 days, root and shoot lengths of wheat seedlings were measured using a millimeter ruler. After that, root and shoot were separated and dried at 60 °C. The dry samples were reduced to fine particles by a high-speed pulverizer. Each sample with an amount of 15–20 mg was soaked in 5 mL HNO_3 for 24 h, and then 3 mL H_2O_2 was added. After 30 min, the mixture was digested using a heating plate at 160 °C in a laboratory fume chamber for 5 h until 1 mL of solution remained. The residues were then transferred into a 10 mL flask and diluted with deionized water to the specific volume. Total Pb, Zn, Cd and Cu contents in the tissues of wheat seedlings grown in the heavy metal solutions with or without nano-Fe_3O_4 were measured using inductively coupled plasma mass spectrometry (ICP-MS, Thermo X7, USA).

2.4.3. Effects of Nano-Fe_3O_4 on Reducing the Oxidative Stress Induced by Heavy Metals in the Wheat Seedlings

This experiment aimed to examine the oxidative stress induced by heavy metals and investigate the effects of nano-Fe_3O_4 on the reduction of heavy metal stress in the seedlings. Seedlings received heavy metal solutions (1 mM) with or without nano-Fe_3O_4 (2000 mg/L). The experiment was performed in two steps: (1) effects of each heavy metal (1 mM) and nano-Fe_3O_4 (2000 mg/L) were first compared to those of deionized water (control); and (2) effects of each heavy metal solution (1 mM) without nano-Fe_3O_4 (2000 mg/L) were compared with the corresponding concentration added nano-Fe_3O_4. The first step aimed to evaluate the stress effect of heavy metals or nanoparticles in wheat seedlings, while the second examined the alleviation effects of nano-Fe_3O_4 on the oxidative stress induced by the tested heavy metals in the wheat seedlings. The seedlings growth conditions were similar to those in Section 2.4.1.

Antioxidant Enzyme Activities and Lipid Peroxidation Assays

Wheat seedlings treated with heavy metal (1 mM) solutions or heavy metals (1 mM) + nano-Fe_3O_4 (2000 mg/L) for 5 days were used to determine the activities of antioxidant enzyme and MDA content. The fresh roots or shoots (0.2 g) from each treatment were homogenized with 1.8 mL of 0.05 M sodium phosphate buffer (pH 7.8) under ice bath to make 10% sample compound liquid. The homogenate was centrifuged at 10000× *g* and 4 °C for 15 min, and the supernatant collected for the analyses of superoxide dismutase (SOD), peroxidase (POD) activities and malondialdehyde (MDA) contents as previously described by [34].

2.5. Statistical Analysis

All treatments were performed on four replicates, and the results presented as mean ± SD (standard deviation). Statistical differences were performed using Microsoft Excel and One-way ANOVA followed by Tukey's HSD or Bonferroni test. $p < 0.05$ was considered to be a significant difference. Other relevant calculations about the selected heavy metals exposure were made based on Ahmad et al. [31] (pp. 1569–1574) and Shaikh et al. [16] (pp. 14–23). The inhibitory rate (%) of the shoot and root seedlings were determined by the following formula given by Chou and Lin. [35] (pp. 353–367):

$$\text{Inhibitory rate } (\%) \text{ of Shoot} = \frac{\text{Shoot length of control} - \text{shoot length of treatment}}{\text{Shoot length of control}} \times 100$$

$$\text{Inhibitory rate } (\%) \text{ of Root} = \frac{\text{Root length of control} - \text{Root length of treatment}}{\text{Root length of control}} \times 100$$

3. Results and Discussion

3.1. Characterization of Nano-Fe_3O_4

TEM image is shown in Figure 1. The particle sizes ranged from about 3.70 nm to 12.10 nm with average sizes about 6.85 ± 1.70 nm. When suspended in water, the hydrodynamic diameters of 50 mg/L of nano-Fe_3O_4 were larger than the results measured by their corresponding TEM image (Figure 1). These were due to their agglomeration in water and the dry state of the sample used for TEM measurement. The nano-Fe_3O_4 surfaces were negatively charged in water (–12.7 ± 0.99 mV).

3.2. Effects of Nano-Fe_3O_4 and Heavy Metals (Pb, Zn, Cd and Cu) on Seedling Growth

Table 1 shows the effects of nano-Fe_3O_4 and four heavy metals (Pb, Zn, Cd and Cu) on seedling viability and root elongation of wheat seedlings grown in Petri dishes. In general, wheat seedlings showed varying degrees of sensitivity to the four heavy metals tested. One hundred percent viability was observed in control seedlings as well as in nano-Fe_3O_4 treatment. However, viability and growth of wheat seedlings were reduced by all four heavy metals tested. As the concentration of the heavy metal increased, the rate of seedling viability decreased. The seedling viability rate was not affected by Pb and Zn at lower concentration of 1 mM, but was significantly affected by Cd and Cu at the same concentration. Inhibition rates of seedling viability were: 40%, 12.5%, 7.5% and 2.5% at 1 mM; and 100%, 100%, 82.5%, and 22.5% at 10 mM for Cd, Cu, Pb and Zn, respectively.

Table 1. Heavy metals exposure and growth of wheat seedlings.

NPs or Heavy Metals	Concentration	Seedlings Viability (%)	Inhibition (%)	Root Length (cm)	Inhibition (%)	Shoot Length (cm)	Inhibition (%)
H_20	0	100 ^{a}	0	10.93 ± 2.29 ^{a}	0	7.03 ± 1.61 ^{a}	0
Nano-Fe_3O_4	2000 mg/L	100 ^{a}	0	11.32 ± 2.02 ^{a}	−3.56816	8.03 ± 1.85 ^{a}	–14.22
Pb	1 mM	92.5 ^{a}	7.5	3.01 ± 1.01 ^{b}	72.46	4.06 ± 0.71 ^{b}	42.24
Zn		97.5^{a}	2.5	4.06 ± 1 ^{b}	62.85	5.11 ± 0.79 ^{b}	27.31
Cd		60 ^{b}	40	1.62 ± 0.40 ^{b}	85.17	2.14 ± 0.23 ^{b}	69.55
Cu		87.5 ^{b}	12.5	2.33 ± 1.39 ^{b}	78.77	3.14 ± 0.64 ^{b}	55.33
Pb	10 mM	17.5 ^{b}	82.5	1.51 ± 0.77 ^{b}	83.53	1.61 ± 0.45 ^{b}	77.09
Zn		77.5 ^{b}	22.5	1.64 ± 0.74 ^{b}	84.99	2.06 ± 0.50 ^{b}	70.69
Cd		0	100	0	100	0	100
Cu		0	100	0	100	0	100

The values were expressed as mean ± SD (standard deviation). Four replicated samples with 10 seedlings were used in each replication. In each column, values marked with the same letters (a) are not significantly different versus controls (water) at ($p < 0.05$), while the values marked with the small letters (b) are significantly different as compared with control ($p < 0.05$).

In the present study, we observed the toxicological effects of four heavy metals (Pb, Zn, Cd and Cu) on the growth of wheat seedlings. The inhibitory effects of the selected heavy metals on the root and shoot lengths of wheat seedlings were also investigated (Table 1). Nano-Fe_3O_4 at 2000 mg/L did not show significant differences ($p < 0.05$) from the control (DI-water) after five days. Wang et al. [4] (pp. 48–54) obtained similar results in a study using nano-Fe_3O_4 on the root growth of carrot, cucumber, lettuce and tomato.

However, their results indicated that root and shoot lengths were negatively affected by the present of heavy metals in the solutions. Wheat seedlings grew very slowly in Pb and Zn treatments and were more retarded with Cd and Cu when they were exposed to 1 mM concentration. Alfalfa [36], *Agropyron elongatum* and *Bromus inermis* [37] seedlings have also shown decreased root and shoot lengths. A possible explanation could be ascribed to the reduction in mitotic cells of the root meristematic zone. A similar phenomenon was observed in a previous study on the effect of lead and cadmium on the growth of *Leucaena leucocephala* seedlings [38]. At 10 mM, seedling growth was severely retarded and short browning roots were observed in Pb and Zn treatments. The results indicated that Cd had the highest negative effect on seedling length followed by Cu. The treatments of Cd and Cu inhibited growth and even led to the death of wheat seedling at 10 mM concentration. Cd and Cu have been known to be very toxic to many plants [14], and this was confirmed in our present study with the wheat seedlings. The highest concentration of heavy metals caused negative effects on many parameters of plant growth such as the decrease in nutritive elements and water absorbance, disturbance in water balance, inhibition of enzyme activity, decrease in metabolism and photosynthesis and respiration, and even death of plants [37]. The heavy metal-toxic ranks of root and shoot elongations were not different, although the inhibitory effects in roots were much higher than those of shoot growth. These observations could be attributed to the higher accumulation of the selected heavy metals in roots of wheat seedlings than those in the shoots. In general, selected heavy metal solutions demonstrated a concentration-dependent inhibition on seedling growth. A similar phenomenon has been reported in a previous study on phytotoxicity of heavy metals on plants [16,39–42]. Mahmood et al. [43] (p. 451) found that the effects of Cu on root length of rice and wheat seedlings were more pronounced than that of Pb and Zn, while the root length of all crop seedlings was significantly affected by increasing Cu, Pb and Zn. The treatment of Zn showed the lowest inhibitory effect on the root and shoot elongation of wheat seedlings in our experiments. Wang et al. [4] (pp. 48–54) confirmed a similar result in wheat seedlings. Based on the mentioned observations from our study, the order of heavy metal toxicity to seedling growth was: Cd > Cu > Pb > Zn. A similar order of toxicity was observed by Wang et al. [14] (pp. 231–240) when the same metals, excluding Cd, were used to treat wheat seedlings. Munzuroglu et al. [2] (pp. 203–213) and Jadia and Fulekar [39] (pp. 547–558) made similar observations and confirmed this order when they studied the effects of Cd, Cu, Pb, and Zn and other heavy metal (Hg and Co) inhibition of germination of wheat.

3.3. Effects of Nano-Fe_3O_4 on Reducing Root Growth Inhibition Induced by Heavy Metals (Pb, Zn, Cd and Cu)

Seedlings were exposed to heavy metals (1 mM and 10 mM) or heavy metals (1 mM and 10 mM) + nano-Fe_3O_4 (2000 mg/L). Each heavy metal concentration without nano-Fe_3O_4 was compared to one containing nano-Fe_3O_4. Wheat seedlings showed varying degrees of sensitivity to the four heavy metals tested in the presence of nano-Fe_3O_4. The shoot and root length of seedlings are illustrated in Figure 2.

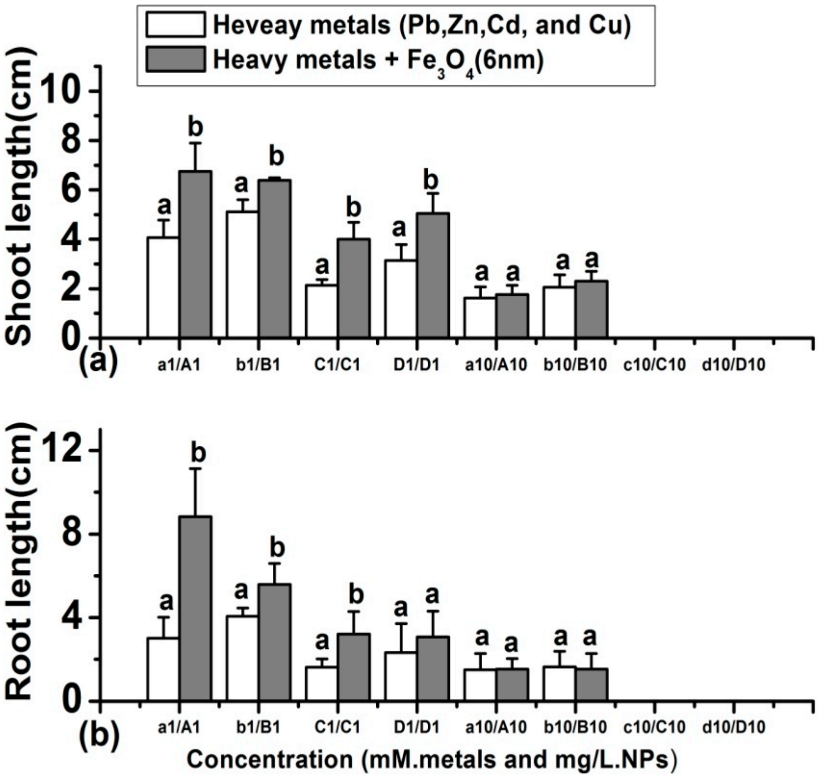

Figure 2. Wheat shoot length (**a**); and root length (**b**) treated with 1 mM and 10 mM of four heavy metals (Pb, Zn, Cd and Cu) with or without nano-Fe_3O_4 (2000 mg/L). The values are expressed as mean ± SD (standard deviation) of the four replicated samples with 10 seedlings for each replication. Values marked with the same letters are not significantly different versus controls (corresponding heavy metal solution without nano-Fe_3O_4) at $p < 0.05$. A, B, C and D = addition of nano-Fe_3O_4 (2000 mg/L) and Pb, Zn, Cd and Cu at 1 mM and 10 mM, respectively.

The nano-Fe_3O_4 suspension had no statistically obvious effect on all heavy metal toxicities in the seedlings of wheat at high concentration (10 mM). Cd and Cu toxicity did not change with the addition of nano-Fe_3O_4 at high concentration (10 mM). They both caused the death of seedlings at 10 mM concentration in the presence of nano-Fe_3O_4 after 5 days. Interestingly, the growth of root and shoot of seedlings were promoted in the presence of nano-Fe_3O_4 compared with heavy metal solutions (1 mM) without nano-Fe_3O_4. In detail, the addition of nano-Fe_3O_4 (2000 mg/L) significantly

($p < 0.05$) decreased root growth inhibition induced by the selected heavy metals (Pb, Zn, Cd and Cu) by 193.91%, 37.56%, 97.72%, and 31.89% in the root; and 65.75%, 25.06% 87.35% and 60.96% in the shoot, respectively, at 1 mM concentration (Figure 2). The resulting rank order of decreased seedling growth inhibition induced by the selected heavy metals was: Pb > Cd >Zn > Cu in the roots, and Cd > Pb > Cu > Zn in the shoots. According to Wang et al. [4] (pp. 48–54), the root growth of cucumber, tomato, lettuce, and carrot increased in the presence of nano-Fe_3O_4 and other nanoparticles compared with control (Cd, EC_{50}). These results suggested that nano-Fe_3O_4 can alleviate the toxicity of the selected heavy metals in the growth of plants. However, it should be noted that the effect of nano-Fe_3O_4 on heavy metal-induced seedling growth inhibition in wheat seedlings could depend on the concentrations of the heavy metals tested.

3.4. Effect of Nano-Fe_3O_4 on Heavy Metals Accumulation in Wheat Seedlings

Table 2 shows the effects of nano-Fe_3O_4 on heavy metals accumulation in wheat seedlings. In this study, effects of heavy metal solutions with nano-Fe_3O_4 were compared to those of heavy metals without nano-Fe_3O_4 to determine the effects of nanoparticles on heavy metals accumulation in wheat seedlings. Seedlings received heavy metal solutions (1 mM) with or without nano-Fe_3O_4 (2000 mg/L). The addition of nano-Fe_3O_4 showed significantly positive effects on the heavy metals accumulation in wheat seedlings (Table 2). In plants that are not hyper accumulators of heavy metals (e.g., Cd and Cu), roots have been shown to be the primary site of phytochelatin synthesis and hence heavy metal accumulation [44]. As seen, the analysis of heavy metals uptake indicated that most of the tested heavy metals accumulated in roots, but only lower levels of heavy metal contents were translocated and accumulated in shoots after five days. Similar results have been found in *Allium sativum* [44], wheat seedlings [4] and water hyacinths [45]. The higher concentration in the shoot was found with Zn treatment. Analyzing the accumulation of Pb, Cu, and Zn in native plants, Yoon et al. [11] (pp. 456–464) observed that the maximum values of tested heavy metals in plants were in the roots. The accumulation of heavy metals was in the order of root > shoot for all of the tested heavy metals. Among the heavy metals, the accumulations were in the order Pb > Zn > Cd > Cu and Zn > Pb > Cd > Cu for root and shoot samples, respectively. Research conducted by Yoon et al. [11] (pp. 456–464) showed that accumulation of heavy metals in 36 plants of 17 species were in the order: Pb> Zn > Cu. This rank order, except for Cd, which was not present in their study, corresponds with the results obtained for seedling roots in the study presented here. Lapalikar et al. [46] (pp. 49–53) found that the heavy metals accumulated in *Jatropha curcas* leaf samples were in the following order: Zn > Cd > Cu. This rank order, except for Pb, which was not present in their study, corresponds with results obtained for shoots in our study. Plant uptake of heavy metals can be influenced by many factors including the plant species, transport across the plasma membrane of root epidermal cells, mass flow of water into the roots, and cation exchange capacity [47]. The higher concentration in the roots and shoots were found with Pb (808.5 ± 41.72 mg/kg) and Zn (39.2 ± 9.04 mg/kg) treatments, respectively. Similar results were reported by Yilmaz et al. [48] (pp. 189–199) who observed that the Pb levels in eggplant seedlings were found to be 551–995 mg/kg in roots. A previous study showed that the higher level of metal in sunflower plants was from Zn treatment following by Cu and Cd [39].

Table 2. Heavy metal (Pb, Zn, Cd and Cu) concentrations in seedlings.

Heavy Metals	Heavy Metal Concentrations (mg.kg^{-1}) in Seedlings			
	Shoot		Root	
	Heavy Metals	Heavy Metals + NPs	Heavy Metals	Heavy Metals + NPs
Pb	13.1 ± 3.66 ^{a}	10.7 ± 1.37 ^{a}	808.5 ± 41.72 ^{a}	371.5 ± 72.29 ^{b}
Zn	39.2 ± 9.04 ^{a}	34.7 ± 3.35 ^{a}	238.8 ± 43.99 ^{a}	181.6 ± 18.42 ^{b}
Cd	7.3 ± 0.92 ^{a}	0.030 ± 0.003 ^{b}	139.8 ± 23.40 ^{a}	48.2 ± 5.91 ^{b}
Cu	6.0 ± 0.66 ^{a}	2.8 ± 0.5 ^{b}	109.7 ± 17.39 ^{a}	34.4 ± 5.90 ^{b}

The values are expressed as the mean ± standard deviation (SD). In each line, values marked with the same letters (a) are not significantly different versus controls (corresponding heavy metal solution without nano-Fe_3O_4) at ($p < 0.05$), while the values marked with the small letters (b) are significantly different versus controls ($p < 0.05$).

In this study, more than 50% of all heavy metal concentrations except for Zn were significantly reduced in the root, while only the concentrations of Cd and Cu significantly reduced in the shoots with the addition of nano-Fe_3O_4. Interestingly, 99% Cd concentration was reduced by nano-Fe_3O_4 in the shoots of wheat seedlings. In detail, addition of nano-Fe_3O_4 significantly ($p < 0.05$) decreased the concentrations of Pb, Zn, Cd and Cu by 54.04%, 23.95%, 65.51%, and 68.64% in the roots; and by 17.75%, 11.39%, 99.59% and 52.94% in the shoots, respectively. The order of heavy metal accumulation did not change with the addition of nano-Fe_3O_4 in the roots but changed in the shoots between Cd and Cu. The accumulations were in the order Zn > Pb > Cu > Cd in the shoots with the presence of nano-Fe_3O_4 in the heavy metal solutions (Table 2).

3.5. Antioxidant Enzyme Activities and Lipid Peroxidation Assays

3.5.1. Effects of Heavy Metals on Antioxidative Enzyme Activity and MDA Content

In this experiment, seedlings received nano-Fe_3O_4 suspension (2000 mg/L) and heavy metal solutions (1 mM). Effects of nanoparticles or heavy metals were compared to those of water to determine the oxidative stress induced by nano-Fe_3O_4 or heavy metals.

The results show that nano-Fe_3O_4 at 2000 mg/L was not different ($p < 0.05$) from the controls (DI-water) after five days (Figure 3). However, when wheat seedlings were exposed to the selected heavy metals, SOD and POD activities decreased significantly in the root compared to control ($p < 0.05$). On the other hand, besides Zn, the selected heavy metals significantly decreased the SOD activity in the shoots while only Cd treatment significantly decreased the POD activities in the shoot (Figure 3). In general, SOD and POD activities were less in heavy metal treatments compared to control. The decrease in antioxidant enzymes activities might be due to the inhibition of enzymatic mechanism by excess H_2O_2 content that is a product in various cellular compartments [45], which suggested the impairment of the SOD role of scavenging oxygen. Reports on Alyssum species [49] and *Allium sativum* [44] have shown similar trends. Dey et al. [50] (pp. 53–60) observed a significant decrease in SOD activity and its loss in shoot and root of wheat under the stress of Cd and Cu. The effects of heavy metals such as Cd and Cu on antioxidant enzymatic activities such as SOD and POD have been previously observed in cucumber [51], rice, [52], pea plants [53], *Kandelia candel* and *Bruguiera gymnorrhiza* [18]. At higher concentration of Pb, the SOD decreased significantly in water hyacinth [45]. In other plants, varying changes in SOD and POD activities were observed in response to heavy metal stress [18,41,42,45,50,54,55]. An increase in the activities of the antioxidative enzyme (SOD and POD) under heavy metal stress could be attributed to the tolerance of heavy metals in plants. Previous study found that Cd stress caused reduction of POD in stems and no effect on roots [10]. Furthermore, no effect on SOD activities in stem and root were noted, while the SOD activity increased in leaf under Cd stress. Plant growth inhibition induced by the stress of heavy metals has been described by many researchers [10,44]. POD can convert H_2O_2 to H_2O, while SOD catalyzes the decomposition of O_2^- radicals to H_2O_2 and O_2 [10]. Higher plants can experience oxidative stress induced by heavy metals resulting in of superoxide radical, collectively termed as ROS such as singlet oxygen (O_2), hydroxyl radical (HO) and hydrogen peroxide (H_2O_2) [18]. The MDA concentration is usually used as the general indicator of the extent of lipid peroxidation resulting from oxidative stress [41,56]. In the present study, the selected heavy metals induced oxidative stress, characterized by a significant increase in MDA content ($p < 0.05$). In detail, MDA concentration increased significantly ($p < 0.05$) in roots of wheat seedlings treated with heavy metal solutions except for Zn, which did not show a significant difference as compared with control (Figure 3). However, in the shoots, only the MDA of wheat seedlings exposed to Cd and Cu increased significantly as compared with control ($p < 0.05$). The present study demonstrates that among the tested heavy metals, Cd and Cu might cause severe oxidative stress by stimulating the generation of the reactive oxygen species (ROS) which may result in damage plant tissues. Similar observations have been found in cucumber plants [41], wheat seedlings [50], strawberry [57], *Festuca arundinacea* [42], and Lactuca [58]. The accumulation of MDA at a higher level could explain the presence of the poisonous ROS [10,59,60]. Damages may occur when the production of ROS exceeds the ability of the antioxidant mechanism to scavenge them and protect plant cells [58,59]. Zn and nano-Fe_3O_4 had similar influence on the MDA content in root and shoot, but also the addition Pb and nano-Fe_3O_4 on root compared to the controls, which suggested the tolerant nature of wheat seedlings under Pb and Zn stress [11].

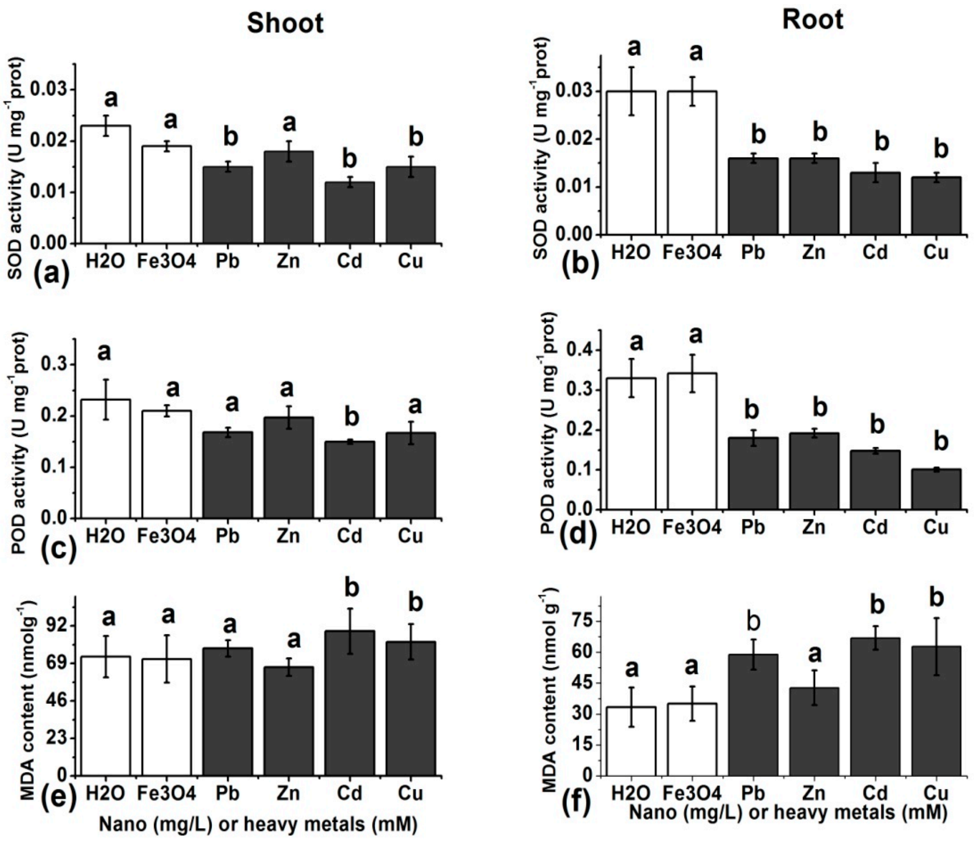

Figure 3. SOD and POD activities and the content of MDA in the root and shoot of wheat seedlings treated with H2O, nano-Fe_3O_4 (2000 mg/L) and four heavy metals (Pb, Zn, Cd and Cu) at 1 mM concentration: SOD in shoot (**a**); and root (**b**); POD in shoot (**c**); and root (**d**); and MDA content in shoots (**e**); and roots (**f**). The values were expressed as mean ± SD (standard deviation) of four replications. Values marked with the same letters are not significantly different versus controls (water) at ($p < 0.05$).

3.5.2. Alleviation of Oxidative Stress Induced by Heavy Metals in Wheat Seedlings by Nano-Fe_3O_4

Nano-Fe_3O_4 (2000 mg/L) was added to the selected heavy metal solutions to examine their effects on heavy metal-induced oxidative stress in the seedlings of wheat. The addition of nano-Fe_3O_4 to each heavy metal solution was compared to its corresponding heavy metal solution without nano-Fe_3O_4. In general, the addition of nano-Fe_3O_4 showed varying degrees of reduction to the oxidative stress induced by the tested heavy metals (Figure 4). In detail, the SOD activity significantly increased in the shoots by 26.35%, 38.13% and 31.57% with the addition of nano-Fe_3O_4 to the solution (1 mM) of Pb, Cd and Cu, respectively (Figure 4). No significant effects of the addition of nano-Fe_3O_4 and Zn were noted on SOD activity in the shoots and roots of wheat seedlings. However, the addition of nano-Fe_3O_4 to the Pb solution significantly ($p < 0.05$) increased the SOD activity in roots by 71.61% compared to control (corresponding metal solution without nano-Fe_3O_4) at ($p < 0.05$). In the wheat seedlings, there were positive effects on the POD activity in the shoot with the addition of nano-Fe_3O_4 to all selected metals.

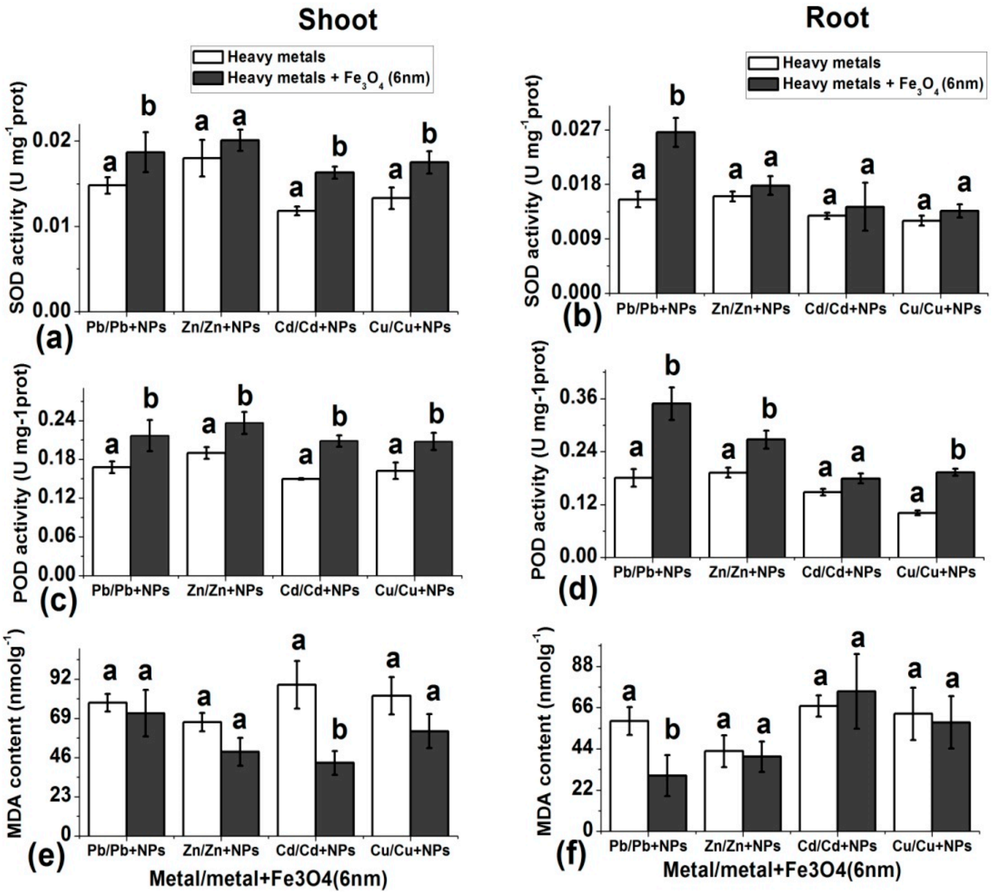

Figure 4. SOD and POD activities and MDA content in the root and shoot of wheat seedlings treated with 1 mM of four heavy metals (Pb, Zn, Cd and Cu) with or without nano-Fe_3O_4 (2000 mg/L): SOD in shoot (**a**); and root (**b**); POD in shoot (**c**); and root (**d**); and MDA content in shoots (**e**); and roots (**f**). The values were presented as mean ± standard deviation (SD) of four replications. The values marked with the same letters are not significantly different versus controls (corresponding heavy metal solution without nano-Fe_3O_4) at ($p < 0.05$).

A significant increase in POD activities in the shoot of wheat by 28.98%, 24.39%, 39.05%, 27.97% and 32.86%, was observed with the addition of nanoparticles and Pb, Zn, Cd and Cu, respectively. In the root, POD activity significantly increased by 93.28%, 39.11% and 91.66% with the addition of nano-Fe_3O_4 to the solutions of Pb, Zn and Cu, respectively (Figure 4). No significant effects of the additions of nano-Fe_3O_4 and Cd on POD activity were noted in the roots of wheat seedlings. Accumulation of reactive oxygen species (ROS) and oxidative stress might due to the disruption of the balance between their production and the antioxidative system activity such as POD and SOD [61]. In higher plants, heavy metals induce oxidative stress and the main response to their stress is an increase in SOD and POD activities. These enzymatic antioxidants provide the necessary protective mechanism that can mitigate the oxidative damage in plants [18]. The tested heavy metal treatment alone (1 mM) caused a decrease in the SOD and POD activities in shoot and root of wheat seedlings except for the Zn in the shoot (Figure 3). The presence of nano-Fe_3O_4 significantly up-regulated this decrease. Plants develop enzymatic mechanisms such as SOD and POD to scavenge ROS, and prevent oxidative stress [58,62]. The increase in SOD and POD activities in the shoots and roots of wheat seedlings suggests that their activities were rapidly induced by the addition of nano-Fe_3O_4 to the heavy metal treatments. The addition of nano-Fe_3O_4 did not change the effects of Zn on SOD activity in shoot and of Zn, Cd and Cu on SOD in root (Figure 4), suggesting that the oxygen scavenging function of SOD was impaired due to the metal stress, which had not been affected by the presence of nano-Fe_3O_4. However, the increase in SOD activity observed in shoot and root could be the result of a direct effect of nano-Fe_3O_4 on the stress of Pb, Cd and Cu in shoot and Pb in the root, respectively. We suggest that this increase in SOD activity might lead to an increased ability of the shoots and roots of wheat seedlings to scavenge O_2^- radical and prevent the oxidative stress to cells [18,61]. There were positive effects on the POD activities in the shoot and root with the addition of nano-Fe_3O_4 to all selected heavy metals except for Cd (roots). Hence, the presence of nano-Fe_3O_4 could increase the POD activity, which can decrease H_2O_2 accumulation and maintain cell membrane integrity [18,26]. The result also showed that the MDA concentration in the wheat seedlings did not change significantly with the addition of nano-Fe_3O_4, except for the addition nano-Fe_3O_4 and Pb in root and with Cd in the shoot, where the MDA concentration significantly decreased by 49.46% and 51.46%, respectively (Figure 4). The current study strongly shows that nano-Fe_3O_4 can play a protective role against oxidative stress caused by heavy metals mainly Pb and Cd. A similar observation has been reported by Lin et al. [5] (pp. 343–351), who found that the addition of Se could reduce Cd toxicity in rice.

3.6. Adsorbent Studies of Magnetic (Fe_3O_4) Nanoparticles

This experiment aimed to understand the removal efficiency of heavy metals absorbed by magnetic (Fe_3O_4) nanoparticles and to confirm their reducing effects on growth inhibition and oxidative stress induced by heavy metals in wheat seedlings. The concentration of each heavy metal (Pb, Zn, Cd and Cu) was determined at three different times (1 d, 2 d and 5 d) by ICP-MS in the heavy metal solution (1 mM) or heavy metal (1 mM) + nano-Fe_3O_4 (2000 mg/L). Each concentration from the addition of nanoparticles to each heavy metal solution was compared to its corresponding heavy metal solution without nano-Fe_3O_4.

The results show that the concentrations of all tested heavy metals have been significantly reduced in the solutions with the addition of the nano-Fe_3O_4 compared to each corresponding heavy metal solution without nano-Fe_3O_4 after five days (Table 3). A previous study has shown that cadmium concentration was significantly reduced by the increase the amount of iron oxide nanoparticles in the soil [63]. The present study shows that the removal efficiency of the tested heavy metals was as follows: Pb > Cu > Cd > Zn (Table 3). According to Giraldo et al. [64] (pp. 465–474), the adsorption capacity of magnetic (Fe_3O_4) nanoparticles could be due to different electrostatic attractions between heavy metal cations and negatively charged adsorption sites. This observation was confirmed in our study through the correlation between the zeta potential (–12.7 ± 0.99 mV) and heavy metal removal efficiency by the nano-Fe_3O_4 used in this experiment. Adsorption is considered among environmental detoxification methods as a conventional but efficient approach to remove toxic ions of heavy metals and bacterial pathogens. The application of nano-Fe_3O_4 particles as adsorbents in environmental treatments could provide a convenient solution for separating and removing the contaminants by applying external magnetic fields. The decrease in the toxicity and accumulation of the tested heavy metals in wheat seedlings could not only be as a result of the adsorption capacity of the heavy metals by nano-Fe_3O_4 and the increase in the activities of enzymatic antioxidants (SOD and POD) related to the addition of magnetic (Fe_3O_4) nanoparticles into the heavy metal solutions, but also the type of heavy metal.

Table 3. Removal efficiency of four different heavy metals (Pb, Zn, Cd and Cu) absorbed by nano-Fe_3O_4.

Heavy Metals	Time	Cd, Zn, Pb and Cu Contents (mg/L)	
		Heavy Metals	Metals + Nano-Fe_3O_4
Cd	1 d	123.43 ± 4.22	1.77 ± 0.53
	2 d	121.6 ± 1.08	1.18 ± 0.2
	5 d	118.27 ± 1.05 ^{a}	0.44 ± 0.14 ^{b}
Zn	1 d	68 ± 0.07	5.74 ± 1.61
	2 d	70.42 ± 0.21	1.46 ± 0.26
	5 d	70.61 ± 1.38 ^{a}	0.98 ± 0.14 ^{b}
Pb	1 d	126.9 ± 0.62	0.1 ± 0.02
	2 d	130.87 ± 0.47	0.1 ± 0.07
	5 d	129.37 ± 0.81 ^{a}	0.05 ± 0.01 ^{b}
Cu	1 d	68.92 ± 0.32	1.51 ± 0.4
	2 d	70.63	0.34 ± 0.08
	5 d	71.01 ^{a}	0.22 ± 001 ^{b}

The values are presented as the mean ± standard deviation (SD). In each line (5 d), values marked with the same letters (a) are not significantly different versus controls (corresponding heavy metal solution without nano-Fe_3O_4) at ($p < 0.05$), while the values marked with the small letters (b) are significantly different versus controls ($p < 0.05$). Treatments: 1 mM (metal) or 1 mM (metal) + 2000 mg/L (nano-Fe_3O_4).

4. Conclusions

Our findings suggest that the tested heavy metal toxicity induced growth inhibition and oxidative stress in wheat seedlings. The addition of nano-Fe_3O_4 significantly decreased root growth inhibition, and reduced and alleviated oxidative stress induced by the heavy metals tested in the wheat seedlings. The effects of nano-Fe_3O_4 on heavy metal toxicity could be dependent on different parameters such as plant species, particle sizes and the tested concentrations in the growth medium as well as the types of heavy metals. However, the protective role of nano-Fe_3O_4 against heavy metal toxicity and oxidative stress in wheat seedlings is complex. The mechanism of prevention against toxicity and stress of the tested heavy metals is linked mainly to the decrease in their concentration in shoots and roots. Our results suggest that nano-Fe_3O_4 activate protective mechanisms that can reduce inhibition of root growth and alleviate oxidative stress induced by heavy metals in the wheat seedlings. Furthermore, the alleviating effect of nano-Fe_3O_4 is associated with their adsorption capacity of heavy metals, which could be due to a different electrostatic attraction between heavy metal cations and negatively charged adsorption sites. Magnetic (Fe_3O_4) nanoparticles have great potential for reducing the toxicity of heavy metals without negatively impacting the growth of the seedlings. Future research needs to focus on the effects of magnetic (Fe_3O_4) nanoparticles on heavy metal toxicity in plants under field conditions.

Acknowledgments: The work was supported by National Natural Science Foundation of China (No. 41371471); NSFC-Guangdong Joint Fund (U1401234); The Ministry of Science and Technology of China (No. 2011CB933400, 2012CB932504 and 2013CB932703); and National Natural Science Foundation of China (No. 11275215, 11275218, and 11375009).

Author Contributions: Yukui Rui and Zhiyong Zhang conceived the project; Alexandre Konate and Xiao He designed the experiment; Alexandre Konate performed experiments, collected, analyzed, interpreted the data, and drafted the manuscript; Yukui Rui and Zhiyong Zhang supervised the overall study from design to final manuscript; all authors have read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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