Research Article

Effects of Nanoparticle Hydroxyapatite on Growth and Antioxidant System in Pakchoi (*Brassica chinensis L.*) from Cadmium-Contaminated Soil

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The effects of nanoscale particle hydroxyapatite (nHAP) on biomass, Cd uptake, the level of chlorophyll, vitamin C, malondialdehyde (MDA), and the activities of antioxidant enzymes, including SOD, CAT, and POD in pakchoi in Cd-contaminated soil, were evaluated by conducting pot experiment. Results showed that, by application of the $5\,g \cdot kg^{-1}$, $10\,g \cdot kg^{-1}$, $20\,g \cdot kg^{-1}$, and $30\,g \cdot kg^{-1}$ nHAP in $10\,mg \cdot kg^{-1}$ Cd-contaminated soil, the biomass of plant increased by 7.97%, 13.21%, 19.53%, and 20.23%, respectively. In addition, the reduction of Cd in shoots was 27.12%, 44.20%, 50.91%, and 62.36% compared to control samples. It was found that the supplement of the nHAP can increase the level of chlorophyll and vitamin C and decrease the level of MDA in plant shoots. Furthermore, the increment activities of SOD, CAT, and POD can be observed after addition of nHAP in Cd-contaminated soil. The results confirmed that nHAP can be applied to reduce the plant uptake of Cd and resist the Cd stress in the plant in Cd-contaminated soil.

1. Introduction

Cadmium (Cd) is a toxic element which causes serious pollution in both aquatic and terrestrial environments. The accumulation of Cd in soils and their subsequent transfer through the food chain is extremely hazardous to human health. Moreover, Cd taken in by the plant has been shown to interfere with a number of essential metabolic processes, such as having a major negative influence on water and nutrient uptake, resulting in wilting and necrosis of the leaves [1, 2]. Furthermore, Cd has been reported to interact with components of the photosynthetic apparatus and to inhibit the electron transport processes, thereby diminishing chlorophyll biosynthesis and photosynthetic carbon assimilation [3, 4].

Importantly, Cd has also been proven to induce oxidative stress by causing an overproduction of reactive oxygen species (ROS) and enhancing the level of lipid peroxidation in plant leaves and roots [2, 5, 6]. The overproduction of ROS induces protein, DNA, and lipid damage. Therefore, it is very important for plant cells to control the overproduction of ROS by coordinating the action of several antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) [7–9]. These enzymes protect against oxidation in the cell by quenching free radicals. For example, SOD catalyzes the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2 and plays an important role in removing ROS. Subsequently, the H_2O_2 produced by SOD is broken down to H_2O and O_2 by CAT, POD, and the ascorbate-glutathione cycle, another critical defense mechanism against oxidative stress by directly reducing H_2O_2, and then the oxidized form of ascorbate is, in turn, reduced by dehydroascorbate reductase using glutathione as a substrate. The oxidized glutathione can then be reduced by glutathione reductase in the presence of NADPH [2], allowing the cells to survive under high ROS levels. The response of antioxidant enzymes to Cd stress has been found to be different among various species and tissues [10]. In general, the activity of antioxidative enzymes is thought to be inversely proportional with Cd concentration. However, Cd has been reported to inactivate

some antioxidative enzymes, such as SOD [11], likely due to SOD's sensitivity to inhibition by Cd binding to –SH groups of the enzyme.

The application of HAP is a cost effective and environmentally friendly technique known to significantly immobilize Cd in contaminated soil [12]. Moreover, nanoscale particle HAP (nHAP) has been proven to be an extremely effective remediation material in Cd-contaminated soil [13], which has been hypothesized to help plants resist Cd-induced stress. However, few studies have been carried out on the effect of nHAPs on the activity of antioxidant enzymes in the plant following Cd contamination of the soil.

Therefore, the purpose of this study was to evaluate the effect of nHAP on Cd-induced stress in pakchoi (*Brassica Chinensis L.*), a common leafy vegetable consumed by a large majority of the Chinese population. We chose pakchoi for this experiment because of the general health concern with heavy metal accumulation in leafy vegetables in China. Moreover, we investigated the possible induction of oxidative stress by Cd and whether or not the alteration of these enzymes involved in the antioxidant defense system includes SOD, POD, and CAT. Finally, we studied content changes of chlorophyll, vitamin C, and malondialdehyde (MDA) and their possible involvement in Cd-induced toxicity.

2. Materials and Methods

Experiment soil was collected from a vegetable garden. After being dried and ground to pass a 1 mm sieve, 1.0 g of homogenized soil sample was digested with HNO_3, $HClO_4$, and HF. The samples were heated until the color became clear, dissolved with several drops of 1% HNO_3, filtered, diluted to a volume of 50 mL with distilled water, and analyzed for the total content of Cd [14]. Soil pH was measured in 1 : 2.5 soil/water with a combination pH electrode. Soil organic matter was determined by wet digestion with $K_2Cr_2O_7/H_2SO_4$. Available nitrogen (N), phosphorus (P), and potassium (K) were analyzed according to standard methods recommender by Lu [15]. Some basic physiochemical properties of the soil and the concentrations of Cd in this soil were listed in Table 1.

2.1. Treatments.
Two-kilogram soil sample was packed into each pot. The Cd was supplied to soil as solutions of $CdSO_4$. The three treatments were T0 (without Cd added), T1 (5 mg·kg^{-1} Cd added), and T2 (10 mg·kg^{-1} Cd added). Soil amendment used in this experiment was nanometer particle hydroxyapatite (nHAP, bough from Emperor Company, China; particle diameter <60 nm). No Cd was detected in this material. nHAP was applied at five level for T0, T1, and T2 treatment level: 0 g·kg^{-1}, 5 g·kg^{-1}, 10 g·kg^{-1}, 20 g·kg^{-1}, and 30 g·kg^{-1}, respectively. There were four replicates in each treatment.

Soil amendments were thoroughly mixed with soil prior to potting; nitrogen (4 g·pot^{-1} soil as NH_4NO_3) and potassium (4 g·pot^{-1} soil as KCl) were added in soil as fertilizers. Pots containing 2.0 kg of soil were used in this experiment. The pots were kept moist for 2 weeks before planting

TABLE 1: Basic properties of the soil.

pH	Organic matter (g·kg^{-1})	Available (mg·kg^{-1})			Total (mg·kg^{-1})
		N	P	K	Cd
6.42	17.63	98.32	17.20	74.81	0.13

vegetable. 10 seedlings of pakchoi were transplanted into the soil and thinned to four per pot on day 10 and kept in the green house (with natural light) until harvest. Plants were harvested 2 months after emergence.

After harvest, the plants were removed from the soils. After being washed with deionised water, the plants were cut into two parts: shoots and roots. Some fresh leaves were taken for the chlorophyll, MDA, vitamin C, and enzyme assay. The other shoots and roots were put into the oven to dry at 70°C until reaching constant weight, passed through 2 mm sieve for further experiment.

Cd in plant was extracted by using acid digestion mixture (HNO_3 and $HClO_4$). The supernatant was obtained from the digested, filtered, reconstituted to the desired volume, and determined by inductively coupled plasma optical emission spectrometry (ICP-OES) for Cd content. Standard reference materials of plant (GSV-4, GBW07605, tea leaves) were used for quality control.

2.2. Enzyme Assay.
Enzyme extractions were carried out at 4°C. The plant leaves were homogenized to powder in liquid nitrogen and extracted in 100 mmol·L^{-1} potassium phosphate buffer (pH 7.5), which contained 1% (w/v) insoluble polyvinylpolypyrrolidone (pvpp) and 1 mmol·L^{-1} EDTA. The homogenate was centrifuged at 25000 ×g for 20 min and the supernatant was applied for enzyme assay. SOD, POD, and CAT activities were determined by the method of Gajewska et al. [16]. Total SOD activity was determined spectrophotometrically based on pyrogallol autoxidation. The reaction contained 0.1 mM EDTA, 0.055 μM NBT, 1.4% Triton X-100, 50 mM Tris-cacodylic buffer, pH 8.2, enzyme extract (about 20 μg protein), and 16 μM pyrogallol. The reaction system was incubated in 37°C for 5 min. The reaction was stopped by addition of 3.5 mL of mixture including 0.35 M formic buffer, pH 3.5, 0.6% Triton X-100, and 3.5% formaldehyde and absorbance was assayed at 540 nm. One unit of SOD activity was expressed as the amount of enzyme that caused inhibition of NBT reduction by 50%. POD activity determinate reaction mixture contained 5.4 mM guaiacol, 15 mM H_2O_2, 50 mM sodium acetate buffer pH 5.6, and enzyme extract (about 5 μg protein). The increase in absorbance due to the oxidation of guaiacol to tetraguaiacol was assayed at 470 nm. CAT activity determinate reaction mixture consisted of 15 mM H_2O_2, 50 mM sodium phosphate buffer pH 7.0 and enzyme extract (about 5 μg protein). Decomposition of H_2O_2 was assayed at 240 nm.

2.3. Determination of Chlorophyll, Vitamin C, and MDA Content.
Before extraction, fresh leaf samples were cleaned with deionized water to remove any surface contaminants. The chlorophyll photosynthetic pigments were extracted in 80%

TABLE 2: Biomass of pakchoi (*Brassica chinensis L.*) grown in the uncontaminated soil and contaminated soil with nHAP (total weigh, g/pot, fresh weigh).

nHAP treatment level (mg·kg^{-1})	Cd treatment level (T0) 0 mg·kg^{-1}	Cd treatment level (T1) 5 mg·kg^{-1}	Cd treatment level (T2) 10 mg·kg^{-1}
0.0	71.22 ± 4.08[ab]	59.55 ± 4.49[b]	50.80 ± 5.30[b]
5.0	72.36 ± 2.47[a]	62.19 ± 3.91[ab]	54.85 ± 1.06[ab]
10.0	67.36 ± 5.71[ab]	65.05 ± 2.77[a]	57.51 ± 3.83[ab]
20.0	66.54 ± 1.63[ab]	65.27 ± 5.87[a]	60.73 ± 4.48[a]
30.0	64.33 ± 6.53[b]	62.49 ± 2.28[ab]	61.08 ± 5.79[a]

Mean values denoted by the same letter in a column do not differ significantly according to the Duncan test.

TABLE 3: Concentration of Cd in pakchoi (*Brassica chinensis L.*) grown in Cd contaminated soil in pot culture receiving nHAP amendments (mg·kg^{-1}, dry weigh).

nHAP treatment level (mg·kg^{-1})	Cd treatment level (T0) 0 mg·kg^{-1}		Cd treatment level (T1) 5 mg·kg^{-1}		Cd treatment level (T2) 10 mg·kg^{-1}	
	Shoot	Root	Shoot	Root	Shoot	Root
0.0	0.33 ± 0.02[a]	0.58 ± 0.03[a]	18.35 ± 0.58[a]	24.87 ± 1.06[a]	30.45 ± 1.71[a]	41.96 ± 2.85[a]
5.0	0.21 ± 0.01[b]	0.45 ± 0.01[b]	17.08 ± 0.73[b]	23.80 ± 2.44[a]	22.19 ± 1.38[b]	31.26 ± 2.46[b]
10.0	0.24 ± 0.02[b]	0.42 ± 0.02[b]	14.97 ± 0.44[c]	19.44 ± 1.63[b]	16.99 ± 0.89[c]	19.58 ± 0.97[c]
20.0	0.25 ± 0.04[b]	0.41 ± 0.02[b]	7.55 ± 0.36[d]	14.39 ± 0.48[c]	14.93 ± 0.63[d]	17.35 ± 1.22[cd]
30.0	0.19 ± 0.03[c]	0.44 ± 0.03[b]	6.12 ± 0.51[e]	10.86 ± 0.57[d]	11.46 ± 0.69[e]	14.52 ± 0.73[d]

Mean values denoted by the same letter in a column do not differ significantly according to the Duncan test.

acetone at 4°C. The resulting suspension was centrifuged for 5 min at 3000 ×g; then the absorbance of the supernatant was assayed at 460, 645, and 665 nm with an UV spectrometer [17]. The MDA of plant leaves was determined according to Lin et al. [18]. MDA was assayed by measuring the concentration of thiobarbituric acid-reacting substances. The leaves were homogenized in 5% (w/v) trichloroacetic acid (TCA). After centrifugation, a sample of the supernatant was addition of 20% TCA consisting of 0.5% (w/v) thiobarbituric acid. The mixture was incubated at 95°C for 30 min. Then the absorbance of the supernatant was assayed at 532,600, and 450 nm with an UV spectrometer. Vitamin C was determined according to the method of Lu [15]. The leaves were homogenized in 2% oxalic acid. After centrifugation, a sample of the supernatant was addition of 1% oxalic acid, a drop of thiourea, and 2,4-dinitrophenylhydrazine. The mixture was incubated at 37°C for 3 hours. After addition of 85% H_2SO_4 2 mL, the absorbance of the supernatant was assayed at 540 nm with an UV spectrometer.

2.4. Statistical Analysis. The means and standard deviations (SD) were calculated by Excel 2003. Statistical analysis including the analysis of variance was conducted using SPSS version 17.0 software (SPSS Inc., USA) and differences ($P < 0.05$) between means were determined using the Duncan test. The figures were plotted by origin 7.5.

3. Results and Discussions

3.1. Effect of nHAP on the Growth and Uptake of Cd by Pakchoi. The biomass of the pakchoi was significantly decreased by Cd application at T1 and T2 treatment level compared to the T0 treatment level (Table 2). As Table 2 showed, the reduction of biomass of pakchoi was 16.4% and 28.7% at T1 and T2 treatment level without adding of nHAP which compared to that of the control. However, it was reported [19] that excessive Cd contents in soil can restrain the development of root system of plant, inhibit photosynthesis, and even reduce the biomass of plant. This result corresponded with the opinion. The biomass of the pakchoi increased with the increasing of the nHAP addition level except applying for 30 g·kg^{-1} in T1 treatment level. As the result showed, by supplement of 5, 10, 20, and 30 g·kg^{-1} nHAP, the increment of pakchoi biomass was 4.4%, 9.2%, 9.6%, and 4.9% in T1 treatment level and 8.0%, 13.2%, 19.5, and 20.2% in T2 treatment level as compared to the unamendment treatment in T1 and T2 level. It can be observed that, after adding the level of 20 g·kg^{-1} nHAP to T1 and 30 g·kg^{-1} nHAP to T2 Cd treatment level, the biomass of plant reached the top value in each Cd-contaminated level. This result may be due to the fact that the nHAP can release the toxic effect on pakchoi caused by Cd treatment, resulting in the increment of the biomass of pakchoi. Another reason may lie in the fact that more phosphate nutrition supplement after phosphate amendments addition can promote the growth of the pakchoi [12].

The application of nHAP significantly reduced the concentration of Cd in the shoots and roots of the pakchoi grown in the Cd-contaminated soil (Table 3). It was shown that, after the addition of the 5, 10, 20, and 30 g·kg^{-1} nHAP, the reduction of concentrations of Cd in the pakchoi shoots was 6.9%, 18.4%, 58.9%, and 66.6% in T1 treatment level

and 27.1%, 44.2%, 51.0%, and 62.4% in T2 treatment level as compared to the unamendment treatment in T1 and T2 level. The reduction of concentrations of Cd in the pakchoi roots was 4.3%, 21.8%, 42.1%, and 56.3% in T1 treatment level and 25.5%, 53.3%, 58.7%, and 65.4% in T2 treatment level as compared to the unamendment treatment in T1 and T2 level. It can be found that the application of 30 g·kg^{-1} nHAP has the best effect on reducing Pb content in pakchoi shoots and roots in both T1 and T2 treatment level.

In this study, it can be found that, while plant exposure to high concentration of Cd treatment level, some toxicity symptoms like chlorosis and growth reduction were observed. This toxic effect is attributed to the over accumulation of Cd in leaves and roots. Studies conducted on various plant species have confirmed that Cd can interfere with many metabolic processes. It blocked down the water and nutrient uptake, resulting in visible symptoms of injury in plants such as chlorosis and necrosis of leaves, and inhibited the growth of roots [2]. However, plant growth was promoted by the supplement of nHAP to the Cd-contaminated soil. This result may be due to the fact that the nHAP can alleviate the toxic effect on plant caused by Cd treatment, resulting in the increment of the biomass of plant.

3.2. Effect of nHAP on the Content of Chlorophyll, MDA, and Vitamin C in Pakchoi Leaves.

Chlorophyll content in the vegetable shoots is an important index indicating the effectiveness of the heavy metal in the plant. In this study, the content of chlorophyll in pakchoi leave decreased with increasing concentrations of Cd in soil (Figure 1). As compared to the control, the content of chlorophyll decreased by 27.19% in T1 treatment and 32.28% in the T2 treatment. Alternatively, the supplementation of nHAP caused the content of chlorophyll to increase. Chlorophyll levels reached their maximum value both in the T1 and T2 treatment after application of 20 g·kg^{-1} nHAP, increasing by 29.99% and 31.30% which compared to the T1 and T2 treatment without adding nHAP. Results conclusively showed that the application of nHAP increased the content of chlorophyll in pakchoi under Cd stress.

Figure 1 showed that Cd toxicity increased the total amount of malonaldehyde (MDA) in pakchoi leaves. There was an increase in MDA by 54.82% and 144.23% in the T1 and T2 levels, respectively, as compared to the control. Supplementation of a high concentration of nHAP (30 g·kg^{-1}) caused a subsequent decrease in MDA levels, reducing the content of MDA in pakchoi leave by 33.23% and 53.00% in T1 and T2 treatment, respectively, as compared to that of the control. This result confirmed that nHAP reduced Cd-induced plant and improved the physiological function in plant.

Increased production of ROS results in the damage biomembrane proteins and phospholipids, leading to membrane destabilization and destructurization [5, 6]. In this experiment, we hypothesized that Cd may be involved in lipid peroxidation and membrane damage, based on an observed increase in the MDA content in the plant leaves. The addition of nHAP to Cd-treated soil caused a significant decrease in the MDA content, indicating that nHAP supplementation inhibits ROS.

Furthermore, we found that Cd caused a marked decrease in the total content of chlorophyll. It has been suggested that a reduction in chlorophyll was responsible for the inhibition of photosynthesis and growth via Cd contamination [20]. On one hand, Cd may be interfering with enzymes involved in chlorophyll biosynthesis, such as aminolevulinic acid dehydratase and protochlorophyllide reductase, thus leading to reduction of chlorophyll content. On the other hand, decreases in chlorophyll may be due to oxidation stress caused by Cd [2, 4, 21]. One way or the other, nHAP protected and restored chlorophyll levels, indicating that the supplementation of nHAP probably maintains chlorophyll synthesis by blocking the Cd toxicity effect on photosynthetic pigments [2], resulting in less damage to the chlorophyll and relieving oxidation stress.

We hypothesize that the high concentration of MDA following Cd-induced stress in the plant was likely the result of an increase in the levels of oxygen free radical. As such, we wanted to look at the role of vitamin C in Cd-induced stress due to its critical action in clearing free radicals from the plant tissue. As shown in Figure 1, Cd treatment led to a significant decrease in vitamin C content within the pakchoi leaves. However, when being under T1 and T2 treatment levels, the content of vitamin C was significantly decreased (21.88% and 38.86%), as compared to the control. Moreover, the amount of vitamin C increased with increasing nHAP concentration. The content of vitamin C in pakchoi reached its maximum value of 13.95 mg/100 g following 20 g·kg^{-1} of nHAP in T1 treatment level, increasing by 48.26%, as compared with plant material under T1-contaminated conditions without any amendment application. Furthermore, nHAP added to T2 polluted soil also increased vitamin C content, reaching its maximum value of 13.60 mg/kg following the supplementation of 30 g·kg^{-1} nHAP to the soil, a value of 40.69% greater T2 treatment controls. These results suggested that the addition of nHAP protected against Cd-induced stress by inhibiting free radicals through a vitamin-C mediated effect.

3.3. Effect of nHAP on the Antioxidant Enzymes Activity in Pakchoi Leaves.

SOD activity shown significantly decreased in T1 and T2 treatment plant compared to the control (Figure 2). The application of nHAP (5 to 30 g·kg^{-1}) to the test soil with Cd led to an increment of SOD activity, indicating that the supplement of nHAP increased the activity of SOD in pakchoi leaves. T1 and T2 treatment caused an increase in POD activity up to 26.26% and 34.25% compared to the control. The application of nHAP to Cd treatment greatly enhanced the POD activity. When nHAP was supplied to a high level of 20 g·kg^{-1} in T1 treatment and 30 g·kg^{-1} in T2 treatment, there was an enhancement of the POD activity by 13.74% and 17.53% compared to pakchoi treated with Cd alone. Similar to POD, the CAT activity induced an increment by 10.13% and 37.16% in Cd treated vegetable compared to control. Supplementation of nHAP at the concentration of 30 g·kg^{-1} in both T1 and T2 led to a maximum enhancement in CAT activity, which was increased by about 50.80% and 38.83% compared to Cd treatment alone, indicating that applying with nHAP can improve the functioning of CAT.
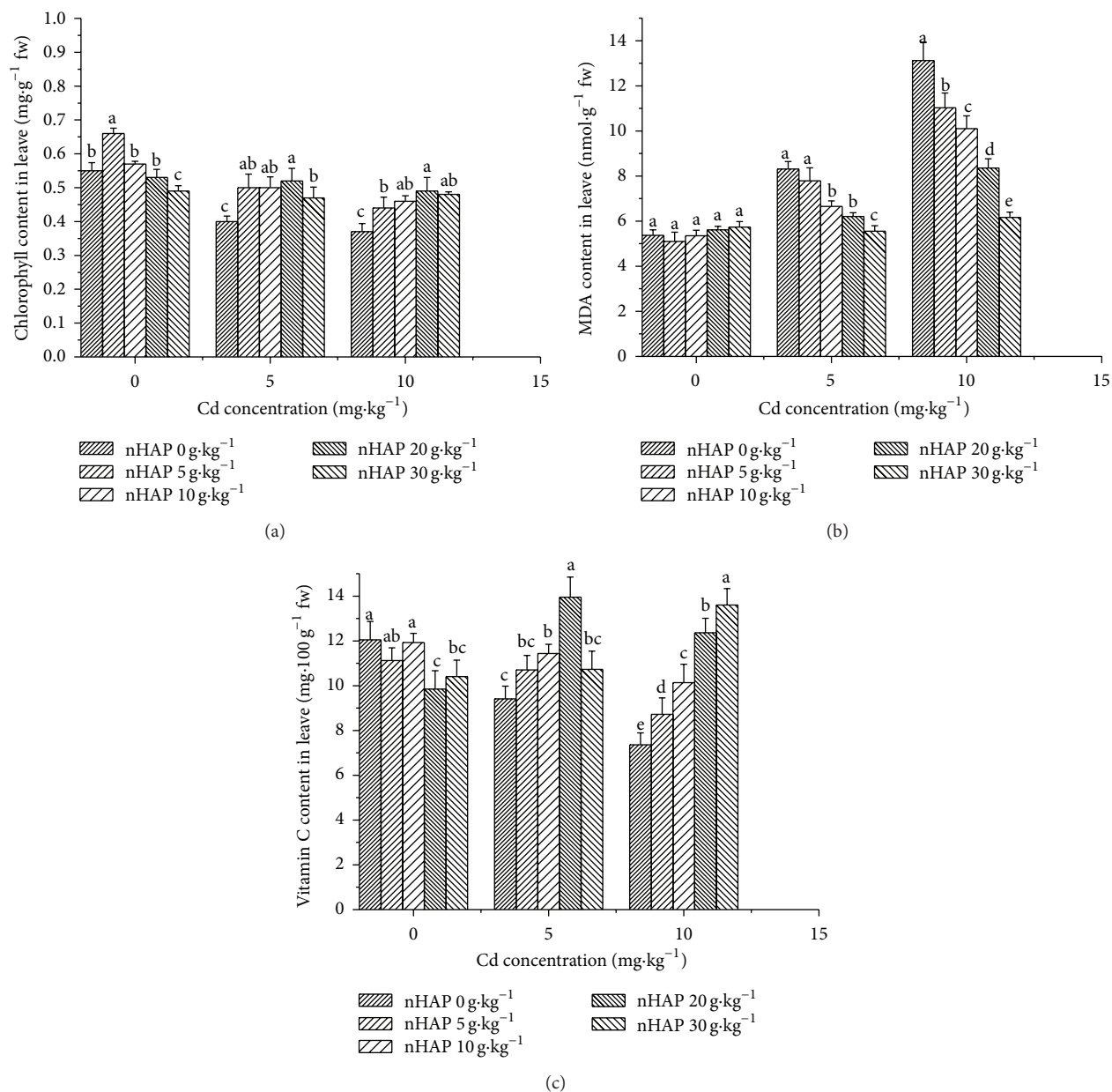
(a)

(b)

(c)

FIGURE 1: The content of chlorophyll, MDA, and vitamin C in plant leaves after different level of nHAP treatment (fresh weigh). Among the same Cd concentration treatments, bars with same letter are not significantly different.

Cd has been proven to induce the overproduction of ROS, leading to increased lipid peroxidation levels and the broken down chlorophyll. In order to eliminate the overproduction of ROS, plants self-activate antioxidant enzymes play a key role in directly quenching free radicals. SOD is one of these antioxidant enzymes, diminishing ROS by catalyzing the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2 [7]. We found that Cd inhibits the activity of SOD, likely due to the fact that Cd competes with Zn for binding to –SH groups of SOD [2], thus resulting in a decrease in the activity of the enzyme. The application of nHAP to the Cd-contaminated soil enhanced SOD activity in the leaves of the plants. We hypothesize that the supplementation of nHAP diminishes the transfer of Cd

into plant cell by combining with it. As a result, there is less binding of Cd to SOD, therefore leading to an enhancement of the enzyme activity.

Moreover, H_2O_2 produced by SOD is broken down to H_2O and O_2 by CAT. In the current study the activity of both CAT and POD in the leaves was increased by the supplementation of Cd, an effect further enhanced by the application of nHAP to the soil. This result is likely due to the fact that when SOD activity is induced more H_2O_2 is produced and accumulated. In order to prevent the H_2O_2 from damaging lipids and chlorophyll the other antioxidant enzymes, CAT and POD, need to be overexpressed to break down this accumulation of H_2O_2 [2, 21, 22]. Thus, the
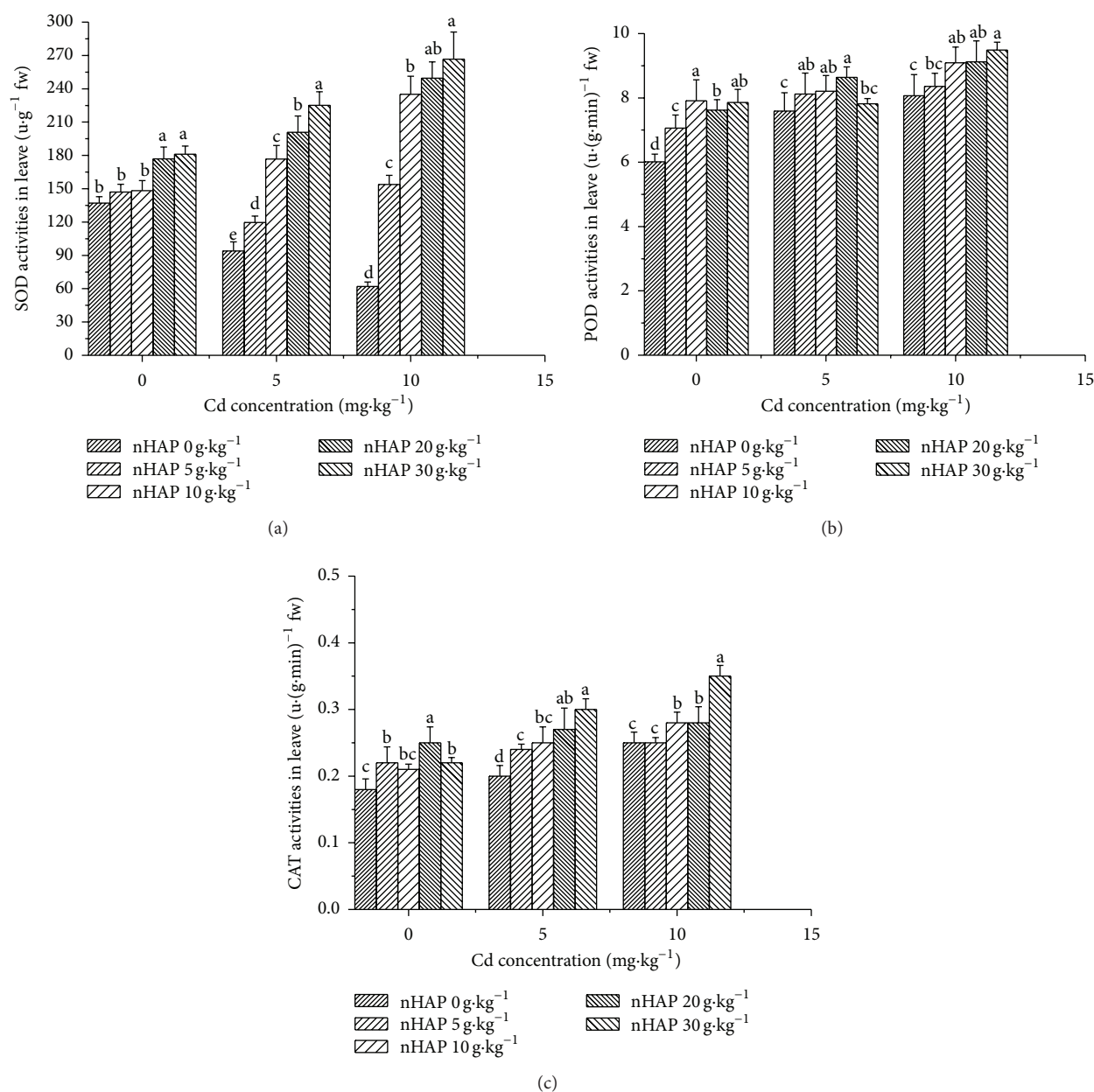
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FIGURE 2: The content of SOD, POD, and CAT in plant leaves after different level of nHAP treatment (fresh weigh). Among the same Cd concentration treatments, bars with same letter are not significantly different.

addition of nHAP decreased the uptake of Cd, leading to an enhanced level of antioxidant enzymes and more efficient ROS scavenging activity within the cells of the plant.

Nanotechnology is an advanced modern approach widely used throughout the world. As it can offer new types of materials which provide the unique and important solutions to the limitations of other conventional materials, many researchers were focus on it [23–25]. Nanomaterials which have nanoscale dimensions range from 1 to 100 nm and often exhibit significantly changed physical, chemical, and biological properties because of their structure and larger

specific surface area [23–25]. Nanoscale particle HAP has proven to have significant effect on immobilization of heavy metal in water and soil [13]. However, few studies were carried out on the application of nHAP to the plant when facing the oxidative stress induced by the presence of Cd in contaminated soil. In this study, we confirmed that nHAP directly activities antioxidant enzymes, such as SOD, CAT, and POD in the presence of Cd contamination. Moreover, nHAP inhibited the uptake of Cd and blocked Cd-induced increases in MDA. These results suggest that applying nHAP in Cd-contaminated soil is an available approach not only

for increasing vegetable production, but also for reducing Cd uptake and improving the quality of the plant for human consumption.

4. Conclusions

The results verified the inference that the application of nHAP can increase the level of chlorophyll and vitamin C and decrease the content of MDA in plant shoots. In addition, the activities of antioxidant enzymes like SOD, CAT, and POD can be enhanced by the input of nHAP on Cd-contaminated soil. These results confirmed that the Cd bioavailability can be reduced and the resistance to Cd stress can be enhanced in Cd-contaminated soil by nHAP.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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