

Bioaccumulation and phytotoxicity of ZnO nanoparticles in soil-grown *Brassica chinensis* and potential risks

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Abstract

A pot-culture experiment was carried out to explore the phytotoxicity and accumulation of ZnO nanoparticles (ZnO NPs) in pakchoi (*Brassica chinensis*), as well as its potential risk. Zn was mainly accumulated in the shoots of pakchoi and showed the order of cell wall fraction > soluble fraction > organelles fraction. Endosomes were observed in roots cell by transmission electron microscope (TEM) and it is proving that endocytosis is a possible way for NPs to enter the cells. The results showed that higher concentration of ZnO NPs treatments resulted in an increase in the content of photosynthetic pigments and malondialdehyde (MDA), Catalase (CAT) and ascorbate peroxidase (APX) activity, while the activity of superoxide dismutase (SOD) and guaiacol peroxidase (G-POD) decreased significantly compared with the control. These changes suggest that the mechanism of ZnO NPs phytotoxicity may induce strongly oxidative stress and damage biomembrane. Generally, ZnO NPs had a similar impact on the growth and absorption of Zn in pakchoi with Zn²⁺. Simultaneously, the treatment of ZnO NPs affected the nutritional quality and food safety of pakchoi at high concentration.

1. Introduction

Zinc oxide nanoparticles (ZnO NPs) are one of the most used forms of metal-oxide nanoparticles (MONPs) and have been widely used in various products such as personal care products, textiles, paintings, industrial coatings, dye-sensitized solar cells, plastics, glass, ceramics, cement, nanomedicine, as well as optic and electronic materials cosmetics (Balážová et al. 2018, Liu et al. 2020, Wang et al. 2013, Zoufan et al. 2020). It has been estimated that the global annual production of ZnO NPs ranges from 550 to 35,000 tons (Liu et al. 2020, Piccinno et al. 2012, Rajput et al. 2018), and about 8–28% of them are released into the soil environment (Medina-Velo et al. 2017).

Soil is considered as a major sink of ZnO NPs compared to atmospheric and aqueous ecosystems (Liu et al. 2020, Rajput et al. 2018). In recent years, ZnO NPs have been employed as a novel macronutrient nanofertilizer in the Zn deficient soil (Song & Kim 2020, Sun et al. 2020, Yusefi-Tanha et al. 2020), as an antibacterial agent for plant protection (Singh et al. 2018), as well as an effective agent for reducing metal uptake by plants (Rizwan et al. 2019, Sharifan et al. 2019a, 2020). ZnO NPs may enter the soil medium by direct (agricultural products) or indirect (biosolids) pathways, and then can be absorbed and accumulated by plants, causing an effect on plant growth (Zeb et al. 2021). Large scale applications of ZnO NPs are linked to a high environmental distribution, which demands a better understanding of the interaction of these NPs with plants.

Plants are essential components of various ecosystems (Balážová et al. 2018, Yusefi-Tanha et al. 2020) and are prone to be directly influenced by ZnO NPs in soils, posing possible risks to food safety and human health (Baskar et al. 2020). From an ecological perspective, understanding the phytotoxicity and uptake of ZnO NPs in plant species is of significant importance (Zafar et al. 2016). Phytotoxicity of ZnO NPs is largely related with nanoparticle size and concentration, surface coating, plant species and growth matrix (Liu et al. 2020). A latest study showed that lower concentrations of ZnO NPs (especially 10 and

25 mg/L) enhanced seed germination and improved seedling growth of *Vicia faba*, while higher concentrations (100 and 200 mg L⁻¹) resulted in phytotoxicity (Youssef & Elamawi 2020). Length and weight of *Brassica nigra* plants were adversely affected by ZnO NPs in culture conditions, but no significant effects were observed in the soil matrix (Zafar et al. 2019). Moreover, ZnO NPs were highly toxic to the leaves of *Glycine max* (Priester et al. 2017), while they were relatively less toxic to *Medicago sativa* leaves (Bandyopadhyay et al. 2015). Therefore, further studies were needed to deeply reveal the phytotoxicity mechanisms of ZnO NPs in plants.

Both positive and negative effects of ZnO NPs on plants' physiological and biochemical parameters have been reported (Elhaj Baddar & Unrine 2018, Liu et al. 2020, Tripathi et al. 2017). ZnO NPs can be served as an effective seed treatment to enhance both Zn nutrition and plant growth (Elhaj Baddar & Unrine 2018). Furthermore, ZnO NPs (< 400 mg kg⁻¹) can improve photosynthetic pigments and decrease lipid peroxidation in *Coriandrum sativum* (Reddy Pullagurala et al. 2018). Beneficial effects of ZnO NPs in plants are also recorded in *Zea mays* and *Triticum aestivum* (Reddy Pullagurala et al. 2018, Tripathi et al. 2017). Adverse effects of ZnO NPs on seed germination, root elongation, plant biomass and chlorophyll content have been confirmed in several plants, such as *Abelmoschus esculentus* (Baskar et al. 2020), *Brassica juncea* (Rao & Shekhawat 2014), *Brassica nigra* (Zafar et al. 2016), *Chenopodium murale* (Zoufan et al. 2020), *Schoenoplectus tabernaemontani* (Zhang et al. 2015a), *Triticum aestivum* (Watson et al. 2015), *Zea mays* (Wang et al. 2016) and *Cucumis sativus* (Zhang et al. 2015c). However, phytotoxicity of ZnO NPs in pakchoi (*Brassica chinensis*) is still unclear and the potential risk of *Brassica chinensis* grown in ZnO NPs polluted soil is currently unreported.

Pakchoi (*Brassica chinensis*) is one of the most important leafy vegetables worldwide, which contains rich essential nutrients (Cu, Ca, Fe, and so on), abundant vitamins and minerals. Due to the strong dissolution of ZnO NPs, their phytotoxicity on pakchoi, as well as a dose-response relationship relating ZnO NPs and Zn ions, need to be investigated. Thus, the paper aims to (1) realize the absorption and accumulation of ZnO NPs in pakchoi, (2) determine the biochemical parameters of pakchoi treated with ZnO NPs, (3) compare the effects of ZnO NPs to Zn²⁺ on pakchoi, determine the phytotoxicity of NPs to plant and (4) assess the potential food risks of pakchoi on safety and quality.

2. Materials And Methods

2.1 ZnO NPs characterization

Zinc oxide nanoparticles (ZnO NPs, 30 ± 10 nm, 99% purity) were obtained from Shanghai Macklin Biochemical Co., China. The morphology and particle size distribution of the nanomaterials used in the experiment were observed by transmission electron microscopy (TEM, JEM-2800), and the crystal form and purity were confirmed via X-ray powder diffraction (XRD, Ulitma IV). The TEM image and diffraction pattern are shown in

[Fig. S1](#). From [Fig. S1A](#), it can be clearly seen that the NPs were predominantly spherical, even though NPs with tetragonal or other irregular shapes can also be seen. The average particles size was about 30 nm. XRD diffraction peak showed that the ZnO NPs were hexagonal, with good crystallinity and high purity ([Fig. S1B](#)). ZnO NPs formed aggregation in aqueous solution definitely. The hydrodynamic size distribution of ZnO NPs suspensions with different concentrations was measured with dynamic light scattering (DLS) in this study. The relative particle size of ZnO NPs in the suspension was mainly distributed within the range of 348 - 435 nm for 50 - 1000 mg L⁻¹ ([Fig. S1C](#)). The zeta potential peaked at 19.53 ± 0.55 mV when the ZnO NPs suspensions concentration was 1000 mg L⁻¹ ([Fig. S1D](#)).

2.2 Soil treatments

Fresh and clean soil samples were collected, dried naturally, and passed through a 20-mesh sieve for later use. 1 kg of soil samples were weighed and added into the pots, which is 11 cm high with an outer diameter of 18 cm. A total of nine treatment groups were prepared including a negative control (CK), with three replicates in each treatment group. The pakchoi seedlings were treated with 50, 100, 500 and 1000 mg kg⁻¹ of ZnO NPs, respectively. Each group was correspondingly labeled as N50, N100, N500, N1000 separately. (These concentrations were selected because they clearly affected the growth and physiological metabolism of pakchoi plants, previously). While the Zn²⁺ treatment groups were labeled as I50, I100, I500 and I1000 respectively. (Zn²⁺ was obtained by the guaranteed reagent ZnSO₄, which provided an equal amount of Zn compared to ZnO NPs.) Each group of accurately weighed ZnO NPs was added to the weighed clean soil to make a contaminated soil of the aforementioned concentration through a stepwise stirring manner. Stock solution of ZnSO₄ were prepared, and a corresponding volume of stock solution was added into the weighed soil and stirred well. The pots were placed in the dark and balanced for a month, watered to equivalent of 80% field capacity weekly (Ebbs et al. 2016).

2.3 Plant culture and harvest

Pakchoi seeds were provided by the Beijing Green gold blue Seedlings Co., China. The seeds were sterilized using 10% H₂O₂ for about 30 min and then rinsed repeatedly with

ultrapure water before germination. The germination experiment was carried out in a seedling tray at room temperature and moist soil. When the seedlings grew to 3 - 4 true leaves, the seedlings with relatively consistent growth were selected and transplanted to a balanced contaminated soil, with 3 plants per pot. Regular watering was done to keep the soil in the pot at 60% - 80% of the field water capacity, and the plants were harvested one month after transplantation. The pot-culture experiment was carried out under a glasshouse, and the irrigation water was DI water during the whole test period.

After a month of transplanting, the plants were harvested and rinsed thoroughly with tap water to remove the surface sludge and dust attached to the plants, immersed in 20 mM Na₂-EDTA solution for 20 min and then washed with DI water for several times. The water on the surface of the plants was dried with filter papers. Pakchoi plants were separated into roots and shoots and the fresh weight was weighed.

2.4 Transmission electron microscope (TEM) observation

About 1 mm fresh root tip of pakchoi was selected from 500 mg kg⁻¹ treatment group using double blades, and put into a fixative composed of 2.5% glutaraldehyde solution at 4 °C for 5 h, and for 2 h in 1% osmic acid again. After rinsing in buffer solution, root tip was dehydrated in an ethanol series, and embedded by epoxy resin steps. Finally, uranium dyed samples were sliced by an ultrathin slicer (Leica, Germany). The detailed operation referenced to Ma et al. (2013) (Ma et al. 2013). The nickel net that was placed with the processed samples was observed using TEM (JEM-2800).

2.5 Fe, Cu, Ca, P and Zn quantitative analysis in plant

Zn, Ca, Cu, P, and Fe content in pakchoi were determined by ICP-MS (Perkin Elmer ELAN DRC-e). The fresh samples were dried in the oven at 105 °C for 5 min, and at 70 °C until constant weight as described by Liu et al. (2010) (Liu et al. 2010). Briefly, 0.10 g of dried plant tissues were transferred into a digestion tubes with a mixture of 1:1 HNO₃ and H₂O₂ on hot block digester (Xiong et al. 2017). The experiment used a standard substance (GB/T 35871-2018, from the Standardization administration in China) to detect the

recovery of heavy metal in plants. The recovery rate of standard materials was 90 - 105 %. Each sample was analyzed as triplicate.

2.6 Determination of Zn subcellular distribution of pakchoi

Zinc determination in pakchoi plants was done according to the procedure by Zhou et al (Zhou et al. 2017). Briefly, 0.5 g fresh samples of shoots and roots were weighed and added into a pre-chilled mortar, 10 mL extraction buffer was added to fully grind. The buffer was composed of 1.0 mM DTT, 500 mM sucrose, 5.0 mM ascorbic acid, 50 mM HEPES and 1% Polyclar AT PVPP (w:v, pH 7.5). The homogenate was sieved through 100 mesh nylon sieve, the residue was cell wall fraction. The filtrate was centrifuged at 10000 × g for 30 min, the deposition and supernatant were organelles fraction and soluble fraction (including vacuole, cytoplasm and cytomembrane) respectively. All operations were performed at 4 °C. The fractions were dried to a constant weight, and then digested with HNO₃-HClO₄ mixture (4:1, v:v). Zn content was determined by ICP-MS, according to the method used by Liu et al. (2010) (Liu et al. 2010).

2.7 Determination of biochemical parameters

2.7.1 Determination of photosynthetic pigments contents

0.2 g fresh leave samples were cut to pieces and extracted in the mixture of 10 mL ethanol and acetone (1:1, v/v). Shook until the leaves were completely discolored in the dark, and volume was adjusted to 25 mL. The absorbance of supernatant was determined at 662 nm, 645nm and 470 nm in sequence by the UV-422G spectrophotometer.

Chlorophyll-a, chlorophyll-b, and carotenoid were calculated using the following formulas:

$$\text{Chlorophyll a (mg L}^{-1}\text{)} = (11.24 \times A_{662} - 2.04 \times A_{645}) \times 8 \quad (1)$$

$$\text{Chlorophyll b (mg L}^{-1}\text{)} = (20.13 \times A_{645} - 4.19 \times A_{662}) \times 8 \quad (2)$$

$$\text{carotenoid (mg L}^{-1}\text{)} = ((1000 \times A_{470} - 1.9 \times A_{662}) - 63.14 \times A_{645} / 214) \times 8 \quad (3)$$

2.7.2 Determination of malondialdehyde (MDA) and antioxidant enzyme

0.5 g fresh plant samples were used for testing the MDA, SOD, APX, G-POD, and CAT. Then, MDA content and antioxidant enzyme activity were determined according to the assay kits (Nanjing Jiancheng Bioengineering Institute, China).

2.8 Safety standards (Health risk assessment)

2.8.1 Translocation factor (TF)

To evaluate the transfer capacity of Zn from roots to shoots, the metal TF was calculated following the method of Liu et al. (2011) (Liu et al. 2011), mentioned as follows:

$$TF = \frac{C_{shoot}}{C_{root}} \quad (4)$$

Where C_{shoot} is the average shoot Zn concentration (DW) of shoot tissues and C_{root} is the average shoot Zn concentration (DW) of root tissues.

2.8.2 Estimated daily intake (EDI)

To assess the health risks of Zn consumed leaves of pakchoi, the EDI of Zn was calculated by the following equation (Natasha et al. 2020):

$$EDI = \frac{C_{FW} \times IR \times EF \times ED}{BW \times AT} \quad (5)$$

Here, C_{FW} is the concentration of Zn in the fresh tissues of pakchoi edible part, IR represents the day ingestion rate, the average value is 0.345 kg d⁻¹ (Hu et al. 2014). EF, ED, BW, AT present the exposure frequency (365 days per year), exposure duration (30 years for an adult) (Fan et al. 2017), the body weight (70 kg for an adult) (Datta & Young 2005), and the average lifetime (30×365), respectively (Natasha et al. 2020).

2.8.3 Hazard quotient (HQ)

The HQ equation described as follows:

$$HQ = \frac{EDI}{RfD} = \frac{C \times IR \times EF \times ED \times Cf}{BW \times AT \times RfD} \quad (6)$$

The oral reference dose (RfD, mg kg⁻¹ d⁻¹) obtained from the EPA's Integrated Risk Information System online database (IRIS) is intended for use in risk assessments for health effects known or assumed to be produced through a nonlinear (possibly threshold) mode of action. The value of IRIS used was 0.3 mg kg⁻¹ d⁻¹ (Agency. 2005).

2.9 Data analysis

All the data were analyzed with SPSS 13.0, Excel 2010 for one-way ANOVA and Tukey's HSD test, and graphed by Origin 9.0. All treatments were replicated three times in this experiment. And the data were reported as mean ± standard error (SE). Metal concentrations of plants are expressed in terms of DW.

3. Results And Discussion

3.1 Zn accumulation and distribution

According to Fig. 1, Zn was mainly accumulated in the shoot tissues of pakchoi, both ZnO NPs and Zn²⁺ appreciably enhanced the Zn content in pakchoi tissues. However, the zinc concentration of edible tissues with NPs treatments were not affected compared to control (CK). With the increase of the ZnO NPs dosage, the concentrations of Zn in pakchoi gradually increased. When the concentration of ZnO NPs was 1000 mg kg⁻¹, the Zn concentration of shoot was 315.17 mg kg⁻¹ that was significant difference compared with CK. In addition, there was inapparent difference after Zn²⁺ treatment.

There were no significant differences between Zn²⁺ and ZnO NPs treatments, but the concentration and content of Zn in each component of pakchoi treated with ZnO NPs was higher than Zn²⁺ under the same conditions. The same result was found in soybean (*Glycine max*), Chinese cabbage (*Brassica pekinensis L.*), lettuce (*Lactuca sativa L.*), cowpea (*Vigna unguiculata*) and so on (López-Moreno et al. 2010, Wang et al. 2013, Xiang et al. 2015, Xu et al. 2018a). In fact, a series of experiments suggested that changes in plant physiological characteristics exposed to ENPs or heavy metals may be due to dynamic changes in essential nutrients in plant tissues (Sharifan et al. 2019b, 2020). Therefore, the toxicity mechanism of the two treatments on plants is similar, and further experiments are needed to investigate the action pathway of ZnO NPs in plants.

The TF values were significantly higher than 1, Table 1. When the Zn concentration was increased to 500 mg kg⁻¹, the TF values decreased as compared to CK. It has been reported that ZnO NPs can affect the performance of transporters, influence the uptake of heavy metals (Sharifan et al. 2020). Therefore, at higher concentrations, the phytotoxicity of Zn affects the absorption and transport of heavy metals. In addition, the concentration and content of Zn in each component of pakchoi treated with ZnO NPs was higher than Zn²⁺ under the same conditions.

Figure 2 showed the distribution of Zn in the subcellular fraction, and the subcellular distribution of Zn in pakchoi treated showed the order of cell wall fraction > soluble fraction > organelles fraction. Zn was stored mainly in the cell wall of leaves (79.1%) and roots (85.3%) treated by ZnO NPs, which is consistent with most studies (Cheng et al. 2018, Wang et al. 2017, Xin et al. 2017, Xu et al. 2018b, Zhou et al. 2017). The proportion of Zn showed an increase in the soluble fraction but decreased in the organelle of pakchoi tissues treated by ZnO NPs. Rather, exposure to Zn²⁺ resulted in a decrease in the proportion of organelles and soluble fractions. The cell walls are the first barrier for heavy metals uptake and can restrict their transport across the cytomembrane (Wang et al. 2017), the organelles are the main place for cell metabolic activities (Xin et al. 2017). Studies have shown that soluble components are mainly composed by vacuoles (Dou et al. 2009), and vacuolar regionalization is identified as one of the most important heavy metal detoxification mechanisms (Zhang et al. 2015b). Therefore, the subcellular result might be related to the defense mechanism of pakchoi cells, and indicated that Zn²⁺ caused lower damage to normal metabolism than ZnO NPs.

3.2 TEM analysis

In order to understand the possible pathway of NPs into plant cells, the root cells were observed by TEM in this study. Figure 3 illustrated the possibility of the presence of ZnO NPs in pakchoi root cells. Compared with the TEM image of control (Fig. 3E) ZnO NPs were found in the entire cell (Fig. 3A). We could see NPs not only in the cytoplasm (Fig. 3D) but also in the intercellular spaces (Fig. 3B) of pakchoi root cells. Many of round vesicles (range from about several hundred nanometers to a few microns) were observed in Fig. 3A, these vesicles were similar in size and shape to the endosomes observed in tobacco BY-2 cells by Lam, et al (Lam et al. 2007).

As confirmation that ZnO NPs can enter cells, it is important to understand the main transmembrane pathway for uptake of ZnO NPs. We conjectured that Fig. 3C may be the early stage of the formation of vesicles. Early endosome with tubular vesicles structure morphology appearance is not observed in our study. Therefore, the vesicles observed in the TEM image may be late endosomes. The presence of endosomes suggests that ZnO NPs may enter the cells through endocytosis if our conjecture was correct, thus proving that endocytosis is a possible way for NPs to enter the cells. It was reported that NPs can also enter the plant cell through ion channels, aquaporin or carrier protein except endocytosis (Jasmina et al. 2010).

3.3 Pakchoi growth and photosynthesis

As shown in Fig. 4, the biomass of pakchoi reached the maximum value of 7.0 g at 500 mg kg⁻¹ of ZnO NPs. But, the biomass of pakchoi increased with the increase of Zn²⁺ concentration. Under the same conditions, Zn²⁺ promoted the biomass of pakchoi more than ZnO NPs, especially at 1000 mg kg⁻¹. It may be related to the fact that ZnO NPs could enhance the accumulation of Zn more than Zn²⁺ (Fig. 1), and caused more effect to plant growth than Zn²⁺.

The content of chlorophyll directly affects the photosynthesis efficiency of plants, which in turn affects the growth of plants. Figure 5 depicted that with increasing the concentration of ZnO NPs, the contents of chlorophyll (Fig. 5a) and carotenoid (Fig. 5b) without exception were increased. In Fig. 5, it is shown that chlorophyll and carotenoid are increased significantly under N1000 and I100 treatments, compared with control. There is no obvious difference in the effect of Zn²⁺ and ZnO NPs treatments on the pigment content of pakchoi leaves.

ZnO NPs can significantly promote the growth of edible plants by increasing chlorophyll content and total biomass. It was found that certain concentration of ZnO NPs increased the growth of mung (*Vigna radiata*) and cotton (*Gossypium spp*) (Pramod et al. 2011, Venkatachalam et al. 2017). The phytotoxicity of metal NPs is considered to be due to the toxicity of NPs themselves and the ions dissolved from NPs (Rao & Shekhawat 2014). TEM analysis of pakchoi revealed that there were suspected NPs in root cells. Some studies have found that metal NPs may form small masses or break into ions inside the plant (Večeřová et al. 2016). According to the results of Zn content, biomass and photosynthetic pigments of pakchoi, ZnO NPs and Zn²⁺ promoted the growth of pakchoi by increasing chlorophyll content and total biomass, but there were not statistical differences between the phytotoxicity of ZnO NPs and Zn²⁺ under the same conditions.

3.4 Antioxidant enzyme activities and lipid peroxidation content

To understand the oxidative stress produced by pollutant entering plants. This study measured the activities of antioxidant enzymes (SOD, CAT, APX and G-POD) and MDA content of pakchoi leaves. SOD catalyzes the disproportionation reaction of O₂^{•-} to H₂O₂, which is then reduced to H₂O and O₂ by CAT and peroxidase. Figure 6a showed that the SOD activity increased by 6% at the 50 mg kg⁻¹ of ZnO NPs. As the concentration of pollutions increased, SOD activity decreased. The activity of SOD in shoots decreased by 33%, 52% in 1000 mg kg⁻¹ of ZnO NPs and Zn²⁺ respectively. On the other hand, there was more effect of Zn²⁺ on SOD activity than ZnO NPs, compared with control.

Similar to the role of CAT, APX can remove H₂O₂ in plants, and G-POD is related to plant anti-aging. Within a certain range (less than 500 mg kg⁻¹), there was a dose-response relationship between ZnO NPs and CAT activity (Fig. 6b). But no obvious change was detected in CAT activity of pakchoi shoots when treated with Zn²⁺. CAT activity in the plants grown at 500 mg kg⁻¹ of ZnO NPs and 1000 mg kg⁻¹ of Zn²⁺ increased approximately 186% and 177% separately. The difference of APX (Fig. 6c) and G-POD (Fig. 6d)

activity between ZnO NPs treatments were similar to that of CAT activity. The maximum of APX activity increase to 101% at 500 mg kg⁻¹ and the maximum of G-POD activity was found at 100 mg kg⁻¹. Compared with ZnO NPs, Zn²⁺ treatment has less effect on the activity of APX and G-POD.

The content of MDA is usually used to characterize the degree of cell membrane lipid peroxidation. To evaluate the intensity of oxidative stress under ZnO NPs treatments, the MDA content was measured. As the concentration of ZnO NPs increased to 1000 mg kg⁻¹, MDA content showed an increase of approximately 187% compared with the control (Fig. 6e). MDA content was not significantly different from that of the control ($p < 0.05$) between the pakchoi treated with Zn²⁺. Similar to APX and G-POD activity, Zn²⁺ treatment has less effect on MDA content compared with ZnO NPs.

The changes of antioxidant enzyme activities and lipid peroxidation content suggested that ZnO NPs within a range of concentration strongly induce oxidative stress. ZnO NPs might affect functionality of transporter. Low concentration of ZnO NPs promoted SOD removal of O₂[·]. As the concentration increased, the phytotoxicity of ZnO NPs affected the performance of SOD. The activity of other antioxidant enzymes was similar to SOD. The results suggested that low concentration MONPs could alleviate the phytotoxicity by increasing the activity of enzymatic antioxidants, while higher concentration inhibited the activity of the antioxidant enzyme system. Some studies had proved this conclusion (Ajey et al. 2016, Qian et al. 2013, Zoufan et al. 2020). In the present study, Zn²⁺ treatment had less effect on the activity of APX, G-POD, CAT, and the content of MDA compared with ZnO NPs.

3.5 Nutrient elements in shoots

Pakchoi is not only rich in minerals and vitamins, but also has a high content of essential nutrients (Ca, P, Fe, et al.) The concentration of several nutrients of edible tissues accumulation in pakchoi treated by ZnO NPs is shown in Fig. 7. The increase of ZnO NPs concentration, especially at 100 mg kg⁻¹, promoted almost 74% of the absorption of Ca by pakchoi. The content of P is much higher than other nutrient elements. Similar to Ca, the concentration of P increased by 31.90% when treated with ZnO NPs (100 mg kg⁻¹). Fe and Cu content increased by approximately 88.3%, and 31.66% at 50 mg kg⁻¹, respectively. The impact of ZnO NPs in accumulation of few essential nutrients in shoots follows the order of Fe > Ca > P > Cu. Similar results were also found in cilantro and spinach for absorbing Fe and Cu (Sharifan et al. 2020).

3.6 Health risk assessment

The hazard quotient (HQ) is widely used to assess the non-carcinogenic risk level due to heavy metal consumption (Hu et al. 2013, Xu et al. 2015). The potential health risk of Zn in the edible part of pakchoi are presented in Table 1. The HQ exceeded threshold limits (1.0) for N1000 and I100, and the HQ value was close to the limit when the concentration of ZnO NPs is higher than 100 mg kg⁻¹. When the concentration of Zn²⁺ is 100 mg kg⁻¹, HQ reaches the highest value of 1.03 and then gradually decreases, strangely. The EDI and HQ were more than one times higher at N1000 compared with control. There was a dose-dependent relation between health risk parameters and ZnO NPs applied, but the health risk of Zn²⁺ at high concentration need to more research.

Recently, some studies (Ji et al. 2018, Natasha et al. 2020, Zhang et al. 2018, Zhong et al. 2018) have focused on the risk assessment of crops triggered by heavy metals. For the human body, zinc is an essential nutrient, but excessive zinc can cause considerable effects on protein synthesis, induce a copper deficiency in the body, and even cause cancer (Zhou et al. 2016). Xiong et al. (Xiong et al. 2017) indicates that only a part of micro-sized metal particles is bio-accessible for the plant. But the current risk assessment of NPs focuses on the effects of metal species on plants, rather than NPs on human health. Thus, the toxicity of NPs in plants and human health need further investigations. In our experiment the distribution of Zn in root cells and Zn content in various tissues of pakchoi were measured, the intake risk of ZnO NPs into human body could not be assessed. Therefore, the existence and distribution of ZnO NPs in usable tissues needs to be further explored to determine the possibility of human intake risk.

4. Conclusions

The effects of ZnO NPs and Zn²⁺ on pakchoi were similar, but pakchoi had a greater ability to absorb and transfer Zn under the NPs treatment. ZnO NPs can promote the growth of pakchoi by increasing chlorophyll content and total biomass, yet nonetheless Zn²⁺ promoted the biomass of pakchoi more than ZnO NPs. Based on the discovery of vesicles on roots cell, ZnO NPs may enter the cell through endocytosis. ZnO NPs could enhance the accumulation of Zn more than Zn²⁺, which will cause more serious impact on homeostasis of pakchoi. ZnO NPs changed the activities of antioxidant biomarkers and MDA content, suggesting oxidative stress induction in pakchoi. In particular, ZnO NPs can increase levels of lipid peroxidation and disrupt the permeability of biomembrane. Moreover, ZnO NPs affect the nutritional quality of pakchoi especially the content of trace elements (Ca, Fe, P, Cu), simultaneously, HQ values indicate that NPs would cause food safety risk.

Declarations

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Authors' contributions Meimei Shen: Data Curation, Formal analysis, Writing - Original Draft, Writing - Review & Editing; Weitao Liu: Conceptualization, Methodology, Resources, Supervision, Writing - Review & Editing, Funding acquisition; Aurang Zeb: Investigation, Formal analysis, Data Curation; Jiapan Lian: Investigation, Visualization; Xiji Hu: Data Curation; Jiani Wu: Investigation. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflicts of interests The authors declare that they have no competing interests.

Ethical approval and consent to participate Not applicable.

Consent to publish All the authors have agreed for authorship, read and approved the manuscript, and given consent for submission and subsequent publication of the manuscript.

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References

Agency. USEP (2005) Intergrated Risk Information System (IRIS). Environmental Protection Agency, United States

Ajey, Singh, N.B., Singh, Imtiyaz, Hussain, Himani, Singh, Vijaya, Biotechnology YJJo (2016) Green synthesis of nano zinc oxide and evaluation of its impact on germination and metabolic activity of *Solanum lycopersicum*. J. Biotech. 233:84-94

Balážová Ľ, Babula P, Baláž M, Bačkorová M, Bujňáková Z, Briančin J, Kurmanbayeva A, Sagi M (2018) Zinc oxide nanoparticles phytotoxicity on halophyte from genus *Salicornia*. Plant Physiol. Biochem. 130:30-42

Bandyopadhyay S, Plascencia-Villa G, Mukherjee A, Rico CM, José-Yacamán M, Peralta-Videa JR, Gardea-Torresdey JL (2015) Comparative phytotoxicity of ZnO NPs, bulk ZnO, and ionic zinc onto the alfalfa plants symbiotically associated with *Sinorhizobium meliloti* in soil. Sci. Total Environ. 515–516:60-69

Baskar V, Safia N, Sree Preethy K, Dhivya S, Thiruvengadam M, Sathishkumar R (2020) A comparative study of phytotoxic effects of metal oxide (CuO, ZnO and NiO) nanoparticles on *in-vitro* grown *Abelmoschus esculentus*. Plant Biosyst. 155:374-383.

Cheng Y, Wang C, Chai S, Shuai W, Sha L, Zhang H, Kang H, Fan X, Zeng J, Zhou Y, Wang Y (2018) Ammonium N influences the uptakes, translocations, subcellular distributions and chemical forms of Cd and Zn to mediate the Cd/Zn interactions in dwarf polish wheat (*Triticum polonicum* L.) seedlings. Chemosphere 193:1164-1171

Datta SP, Young SD (2005) Predicting metal uptake and risk to the human food chain from leaf vegetables grown on soils amended by long-term application of sewage sludge. Water, Air, Soil Pollut. 163:119-136

Dou CM, Fu XP, Chen XC, Shi JY, Chen YX (2009) Accumulation and detoxification of manganese in hyperaccumulator *Phytolacca americana*. Plant Biology 11:664-670

Ebbs SD, Bradfield SJ, Kumar P, White JC, Musante C, Ma XM (2016) Accumulation of zinc, copper, or cerium in carrot (*Daucus carota*) exposed to metal oxide nanoparticles and metal ions. Environ. Sci.-Nano

- Elhaj Baddar Z, Unrine JM (2018) Functionalized-ZnO-Nanoparticle Seed Treatments to Enhance Growth and Zn Content of Wheat (*Triticum aestivum*) Seedlings. *J. Agric. Food Chem.* 66:12166-12178
- Fan Y, Li H, Xue Z, Zhang Q, Cheng F (2017) Accumulation characteristics and potential risk of heavy metals in soil-vegetable system under greenhouse cultivation condition in Northern China. *Ecol. Eng.* 102:367-373
- Hu W, Huang B, Shi X, Chen W, Zhao Y, Jiao WJE, Safety E (2013) Accumulation and health risk of heavy metals in a plot-scale vegetable production system in a peri-urban vegetable farm near Nanjing, China. *Ecotoxicol. Environ. Saf.* 98:303-309
- Hu W, Yong C, Huang B, Human SNJ, Journal ERAAI (2014) Health Risk Assessment of Heavy Metals in Soils and Vegetables from a Typical Greenhouse Vegetable Production System in China. *Hum. Ecol. Risk Assess.* 20:1264-1280
- Jasmina, Kurepa, Tatjana, Paunesku, Stefan, Vogt, Hans, Arora, Bryan, Letters MJN (2010) Uptake and Distribution of Ultrasmall Anatase TiO₂ Alizarin Red S Nanoconjugates in *Arabidopsis thaliana*. *Nano Lett.* 10:2296-2302.
- Ji Y, Wu PJ, Zhang J, Zhang J, Zhou YF, Peng YW, Zhang SF, Cai GT, Gao GQ (2018) Heavy metal accumulation, risk assessment and integrated biomarker responses of local vegetables: A case study along the Le'an river. *Chemosphere* 199:361-371
- Lam SK, Siu CL, Hillmer S, Jang S, An G, Robinson DG, Jiang L (2007) Rice SCAMP₁ Clathrin-Coated, trans-Golgi-located Tubular-Vesicular Structures as an Early Endosome in Tobacco BY-2 Cells. *Plant Cell* 19:296-319
- Liu W, Zhou Q, Zhang Y, Wei S (2010) Lead accumulation in different Chinese cabbage cultivars and screening for pollution-safe cultivars. *J. Environ. Manage.* 91:781-8
- Liu W, Zhou Q, Zhang Z, Hua T, Cai Z (2011) Evaluation of cadmium phytoremediation potential in Chinese cabbage cultivars. *J. Agric. Food Chem.* 59:8324-30
- Liu W, Zeb A, Lian J, Wu J, Xiong H, Tang J, Zheng S (2020) Interactions of metal-based nanoparticles (MBNPs) and metal-oxide nanoparticles (MONPs) with crop plants: a critical review of research progress and prospects. *Environ. Rev.* 28:294-310.
- López-Moreno ML, de la Rosa G, Hernández-Viezcás JÁ, Castillo-Michel H, Botez CE, Peralta-Videa JR, Gardea-Torresdey JL (2010) Evidence of the Differential Biotransformation and Genotoxicity of ZnO and CeO₂ Nanoparticles on Soybean (*Glycine max*) Plants. *Environ. Sci. Technol.* 44:7315-7320

- Ma X, Gurung A, Deng Y (2013) Phytotoxicity and uptake of nanoscale zero-valent iron (nZVI) by two plant species. *Sci. Total Environ* 443:844-849
- Medina-Velo IA, Peralta-Videa JR, Gardea-Torresdey JL (2017) Assessing plant uptake and transport mechanisms of engineered nanomaterials from soil. *MRS Bull.* 42:379-383
- Natasha, Shahid M, Farooq ABU, Rabbani F, Khalid S, Dumat C (2020) Risk assessment and biophysiochemical responses of spinach to foliar application of lead oxide nanoparticles: A multivariate analysis. *Chemosphere* 245:125605
- Piccinno F, Gottschalk F, Seeger S, Nowack B (2012) Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. *J Nanopart Res.* 14:1109
- Pramod M, Dhoke SK, Khanna AS (2011) Effect of Nano-ZnO Particle Suspension on Growth of Mung (*Vigna radiata*) and Gram (*Cicer arietinum*) Seedlings Using Plant Agar Method. *J. Nanotech.* 696535
- Priester JH, Moritz SC, Espinosa K, Ge Y, Wang Y, Nisbet RM, Schimel JP, Susana Goggi A, Gardea-Torresdey JL, Holden PA (2017) Damage assessment for soybean cultivated in soil with either CeO₂ or ZnO manufactured nanomaterials. *Sci. Total Environ.* 579:1756-1768
- Qian H, Peng X, Han X, Ren J, Sun L, Fu ZJ (2013) Comparison of the toxicity of silver nanoparticles and silver ions on the growth of terrestrial plant model *Arabidopsis thaliana*. *J Environ Sci*, 25:1947-1956
- Rajput VD, Minkina TM, Behal A, Sushkova SN, Mandzhieva S, Singh R, Gorovtsov A, Tsitsuashvili VS, Purvis WO, Ghazaryan KA, Movsesyan HS (2018) Effects of zinc-oxide nanoparticles on soil, plants, animals and soil organisms: A review. *Environ. Nanotech. Monitor. & Manage.* 9:76-84
- Rao S, Shekhawat GS (2014) Toxicity of ZnO engineered nanoparticles and evaluation of their effect on growth, metabolism and tissue specific accumulation in *Brassica juncea*. *J. Environ. Chem. Eng.* 2:105-114
- Reddy Pullagurala VL, Adisa IO, Rawat S, Kalagara S, Hernandez-Viezcás JA, Peralta-Videa JR, Gardea-Torresdey JL (2018) ZnO nanoparticles increase photosynthetic pigments and decrease lipid peroxidation in soil grown cilantro (*Coriandrum sativum*). *Plant Physiol. Biochem.* 132:120-127
- Rizwan M, Ali S, Zia ur Rehman M, Adrees M, Arshad M, Qayyum MF, Ali L, Hussain A, Chatha SAS, Imran M (2019) Alleviation of cadmium accumulation in maize (*Zea mays* L.) by foliar spray of zinc oxide nanoparticles and biochar to contaminated soil. *Environ. Pollut.* 248:358-367
- Sharifan H, Ma X, Moore JM, Habib MR, Evans C (2019a) Zinc Oxide Nanoparticles Alleviated the Bioavailability of Cadmium and Lead and Changed the Uptake of Iron in Hydroponically Grown Lettuce (*Lactuca sativa* L. var. *Longifolia*). *ACS Sustain. Chem. Eng.* 7:16401-16409

- Sharifan H, Wang X, Ma XM (2019b): Impact of nanoparticle surface charge and phosphate on the uptake of coexisting cerium oxide nanoparticles and cadmium by soybean (*Glycine max.* (L.) merr.). *Int. J. Phytorem.* 22:305-312
- Sharifan H, Moore J, Ma X (2020): Zinc oxide (ZnO) nanoparticles elevated iron and copper contents and mitigated the bioavailability of lead and cadmium in different leafy greens. *Ecotoxicol. Environ. Saf.* 191:110177
- Singh A, Singh NB, Afzal S, Singh T, Hussain I (2018) Zinc oxide nanoparticles: a review of their biological synthesis, antimicrobial activity, uptake, translocation and biotransformation in plants. *J. Mater. Sci.* 53:85-201
- Song U, Kim J (2020) Zinc oxide nanoparticles: a potential micronutrient fertilizer for horticultural crops with little toxicity. *Hortic. Environ. Biote.* 61:625-631
- Sun H, Du W, Peng Q, Lv Z, Mao H, Kopittke PM (2020): Development of ZnO Nanoparticles as an Efficient Zn Fertilizer: Using Synchrotron-Based Techniques and Laser Ablation to Examine Elemental Distribution in Wheat Grain. *J. Agric. Food Chem.* 68:5068-5075
- Tripathi DK, Shweta, Singh S, Singh S, Pandey R, Singh VP, Sharma NC, Prasad SM, Dubey NK, Chauhan DK (2017) An overview on manufactured nanoparticles in plants: Uptake, translocation, accumulation and phytotoxicity. *Plant Physiol. Bioch.* 110:2-12
- Večeřová K, Večeřa Z, Dočekal B, Oravec M, Pompeiano A, Tříška J, Urban O (2016) Changes of primary and secondary metabolites in barley plants exposed to CdO nanoparticles. *Environ. Pollut.* 218:207-218
- Venkatachalam P, Jayaraj M, Manikandan R, Geetha N, Sahi SVJPPB (2017) Zinc oxide nanoparticles (ZnONPs) alleviate heavy metal-induced toxicity in *Leucaena leucocephala* seedlings: A physiochemical analysis. *Plant Physiol. Bioch.* 110:59-69
- Wang F, Liu X, Shi Z, Tong R, Adams CA, Shi X (2016) Arbuscular mycorrhizae alleviate negative effects of zinc oxide nanoparticle and zinc accumulation in maize plants – A soil microcosm experiment. *Chemosphere* 147:88-97
- Wang P, Menzies NW, Lombi E, McKenna BA, Johannessen B, Glover CJ, Kappen P, Kopittke PM (2013) Fate of ZnO Nanoparticles in Soils and Cowpea (*Vigna unguiculata*). *Environ. Sci. Technol.* 47:13822-13830
- Wang Y, Chai L, Yang Z, Mubarak H, Xiao R, Tang C (2017) Subcellular distribution and chemical forms of antimony in *Ficus tikoua*. *Int. J. Phytoremediat.* 19:97-103
- Watson JL, Fang T, Dimkpa CO, Britt DW, McLean JE, Jacobson A, Anderson AJ (2015) The phytotoxicity of ZnO nanoparticles on wheat varies with soil properties. *Biometals* 28:101-112

- Xiang L, Zhao H-M, Li Y-W, Huang X-P, Wu X-L, Zhai T, Yuan Y, Cai Q-Y, Mo C-H (2015) Effects of the size and morphology of zinc oxide nanoparticles on the germination of Chinese cabbage seeds. *Environ. Sci. Pollut. Res.* 22:10452-10462
- Xin J, Zhao X, Tan Q, Sun X, Hu C (2017) Comparison of cadmium absorption, translocation, subcellular distribution and chemical forms between two radish cultivars (*Raphanus sativus* L.). *Ecotoxicol. Environ. Saf.* 145:258-265
- Xiong T, Dumat C, Dappe V, Vezin H, Schreck E, Shahid M, Pierart A, Sobanska S (2017) Copper Oxide Nanoparticle Foliar Uptake, Phytotoxicity, and Consequences for Sustainable Urban Agriculture. *Environ. Sci. Technol.* 51:5242-5251
- Xu J, Luo X, Wang Y, Feng Y (2018a) Evaluation of zinc oxide nanoparticles on lettuce (*Lactuca sativa* L.) growth and soil bacterial community. *Environ. Sci. Pollut. Res.* 25:6026-6035
- Xu L, Lu A, Wang J, Ma Z, Pan L, Feng X, Luan YJE, Safety E (2015) Accumulation status, sources and phytoavailability of metals in greenhouse vegetable production systems in Beijing, China. *Ecotoxicol. Environ. Saf.* 122:214-220
- Xu S, Hu C, Tan Q, Qin S, Sun X (2018b) Subcellular distribution of molybdenum, ultrastructural and antioxidative responses in soybean seedlings under excess molybdenum stress. *Plant Physiol. Biochem.* 123:75-80
- Youssef MS, Elamawi RM (2020) Evaluation of phytotoxicity, cytotoxicity, and genotoxicity of ZnO nanoparticles in *Vicia faba*. *Environ. Sci. Pollut. Res.* 27:18972-18984
- Yusefi-Tanha E, Fallah S, Rostamnejadi A, Pokhrel LR (2020) Zinc oxide nanoparticles (ZnONPs) as a novel nanofertilizer: Influence on seed yield and antioxidant defense system in soil grown soybean (*Glycine max* cv. Kowsar). *Sci. Total Environ.* 738:140240
- Zafar H, Ali A, Ali JS, Haq IU, Zia M (2016) Effect of ZnO Nanoparticles on *Brassica nigra* Seedlings and Stem Explants: Growth Dynamics and Antioxidative Response. *Front. Plant Sci.* 7:535
- Zafar H, Abbasi BH, Zia M (2019) Physiological and antioxidative response of *Brassica nigra* (L.) to ZnO nanoparticles grown in culture media and soil. *Toxicol. Environ. Chem.* 101:281-299
- Zeb A, Liu W, Wu J, Lian J, Lian Y (2021) Knowledge domain and emerging trends in nanoparticles and plants interaction research: A scientometric analysis. *NanoImpact* 21:100278
- Zhang D, Hua T, Xiao F, Chen C, Gersberg RM, Liu Y, Stuckey D, Ng WJ, Tan SK (2015a) Phytotoxicity and bioaccumulation of ZnO nanoparticles in *Schoenoplectus tabernaemontani*. *Chemosphere* 120:211-219
- Zhang H, Guo Q, Yang J, Shen J, Shao CJE, Safety E (2015b) Subcellular cadmium distribution and antioxidant enzymatic activities in the leaves of two castor (*Ricinus communis* L.) cultivars exhibit

differences in Cd accumulation. *Ecotoxicol. Environ. Saf.* 120:184-192

Zhang RC, Zhang HB, Tu C, Hu XF, Li LZ, Luo YM, Christie P (2015c) Phytotoxicity of ZnO nanoparticles and the released Zn(II) ion to corn (*Zea mays* L.) and cucumber (*Cucumis sativus* L.) during germination. *Environ. Sci. Pollut. Res.* 22:11109-11117

Zhang Y, Yin C, Cao S, Cheng L, Wu G, Guo J (2018) Heavy metal accumulation and health risk assessment in soil-wheat system under different nitrogen levels. *Sci. Total Environ.* 622-623:1499-1508

Zhong T, Xue D, Zhao L, Zhang X (2018) Concentration of heavy metals in vegetables and potential health risk assessment in China. *Environ. Geochem. Health* 40:313-322

Zhou J, Wan H, He J, Lyu D, Li H (2017) Integration of Cadmium Accumulation, Subcellular Distribution, and Physiological Responses to Understand Cadmium Tolerance in Apple Rootstocks. *Front. Plant Sci.* 8:966

Zhou Q, Guo J-J, He C-T, Shen C, Huang Y-Y, Chen J-X, Guo J-h, Yuan J-G, Yang Z-Y (2016) Comparative Transcriptome Analysis between Low- and High-Cadmium-Accumulating Genotypes of Pakchoi (*Brassica chinensis* L.) in Response to Cadmium Stress. *Environ. Sci. Technol.* 50:6485-6494

Zoufan P, Baroonian M, Zargar B (2020): ZnO nanoparticles-induced oxidative stress in *Chenopodium murale* L, Zn uptake, and accumulation under hydroponic culture. *Environ. Sci. Pollut. Res.* 27, 11066-11078

Tables

Table 1

The values of TF, EDI (mg kg⁻¹ day⁻¹), HQ of Zn in pakchoi.

Treatments	CK		ZnO NPs				ZnO SO ₄			
	N50	N100	N500	N1000	I50	I100	I500	I1000		
TF	1.99	2.46	2.1	1.7	1.31	2.09	3.75	1.52	1.93	
EDI	0.15	0.25	0.29	0.28	0.31	0.16	0.31	0.26	0.21	
HQ	0.5	0.83	0.96	0.93	1.04	0.53	1.03	0.86	0.71	

Figures

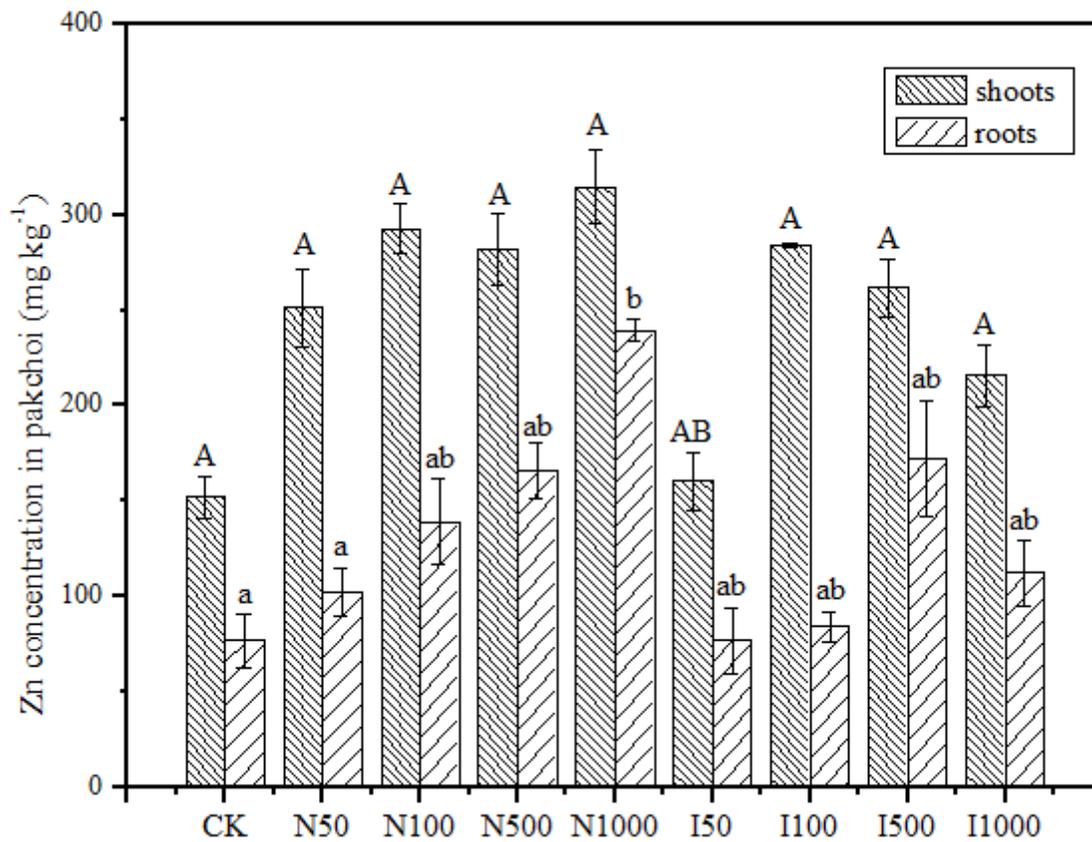


Figure 1

Zn concentrations in shoots and roots of pakchoi (DW). Different lettering indicated the significant difference at $p < 0.05$, and the error bars are standard errors.

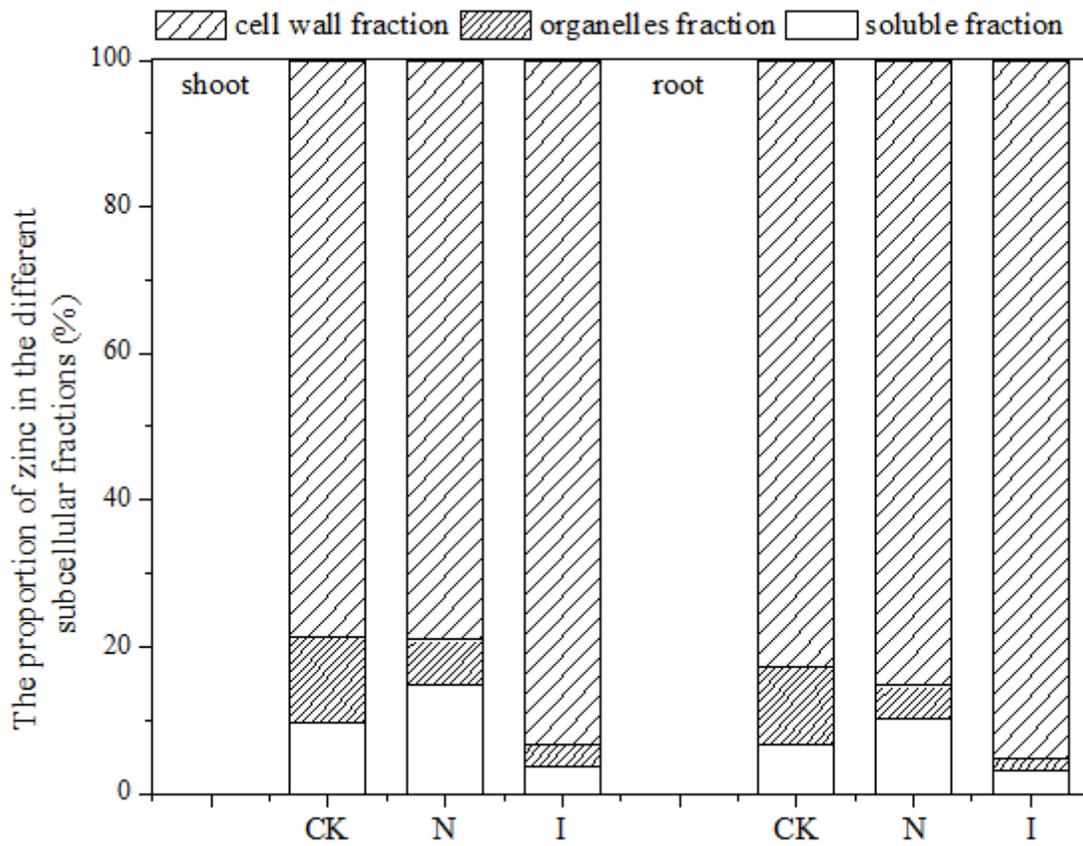


Figure 2

Zn subcellular distribution in shoots and roots of pakchoi treated with control (CK), ZnO NPs (N) and Zn²⁺ (I).

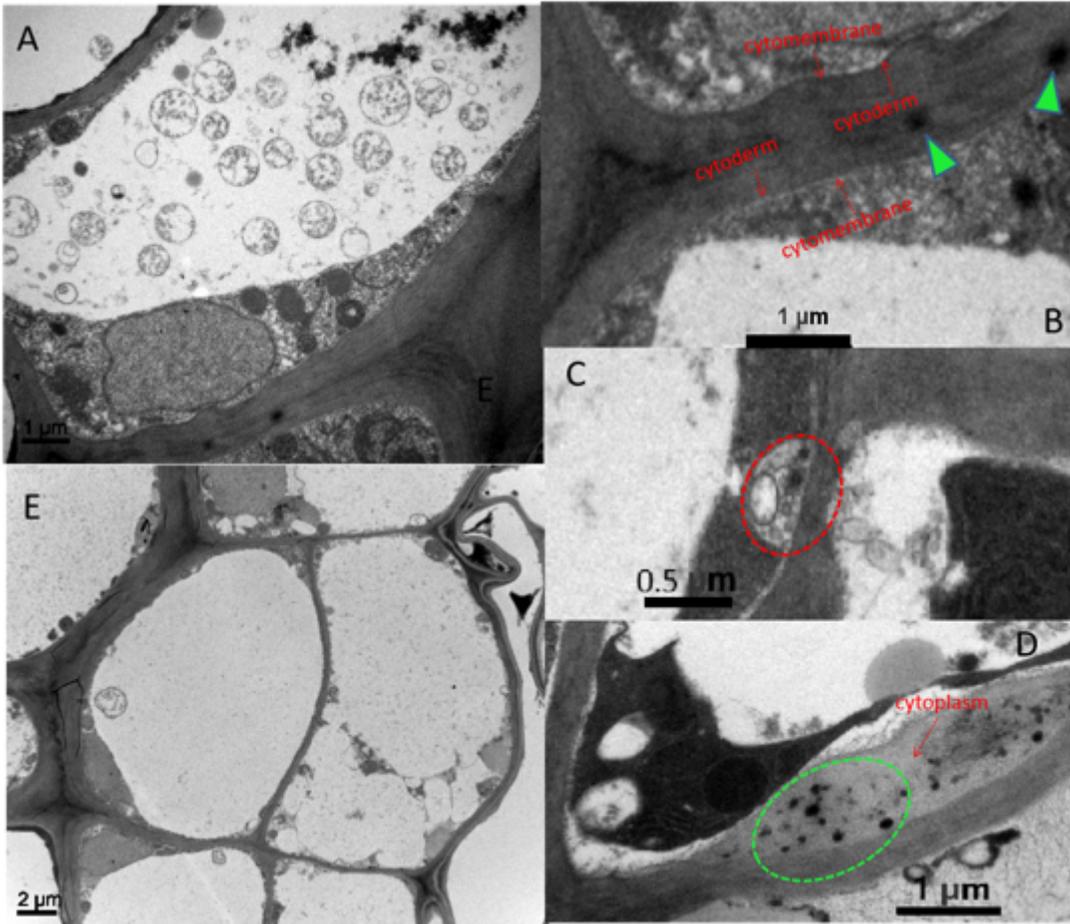


Figure 3

Transmission electron microscope (TEM) images of pakchoi root tissue. A is the TEM image of pakchoi cell treated by ZnO NPs. B, C and D are the images of the part of the cell, and green marks indicate the presence of NPs. E is the TEM image of the control.

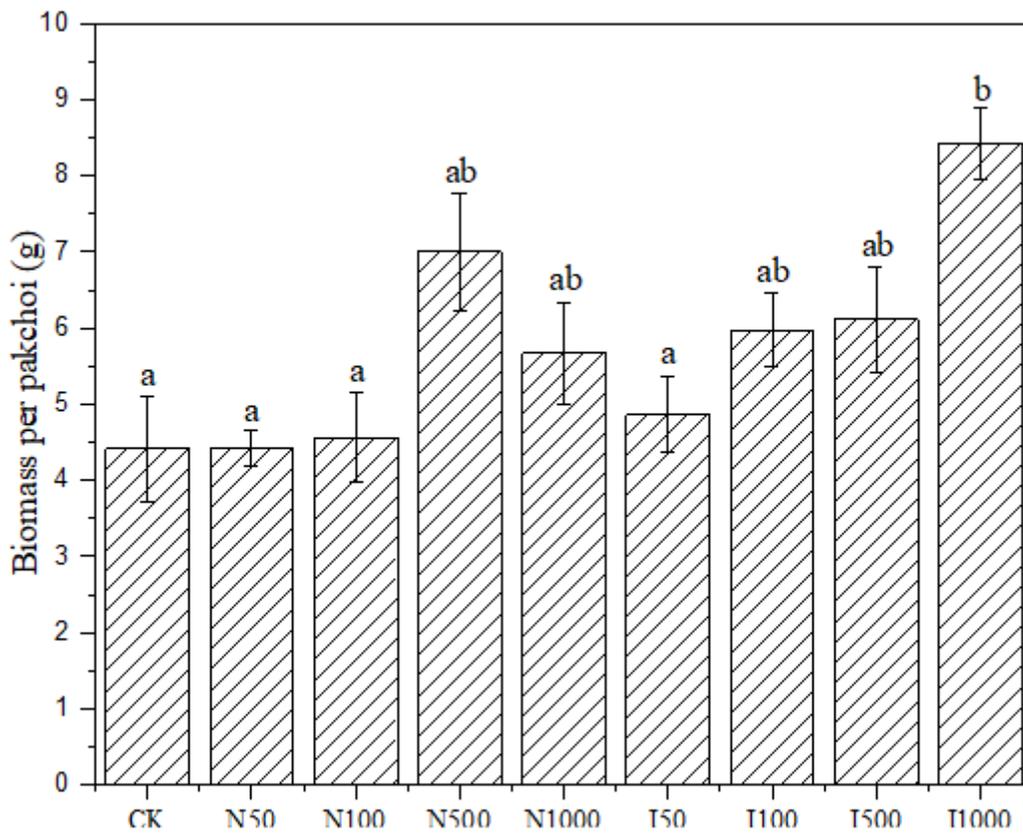


Figure 4

Average biomass of per pakchoi (FW) at different treatments. Different lettering indicated the significant difference at $p < 0.05$, and the error bars are standard errors.

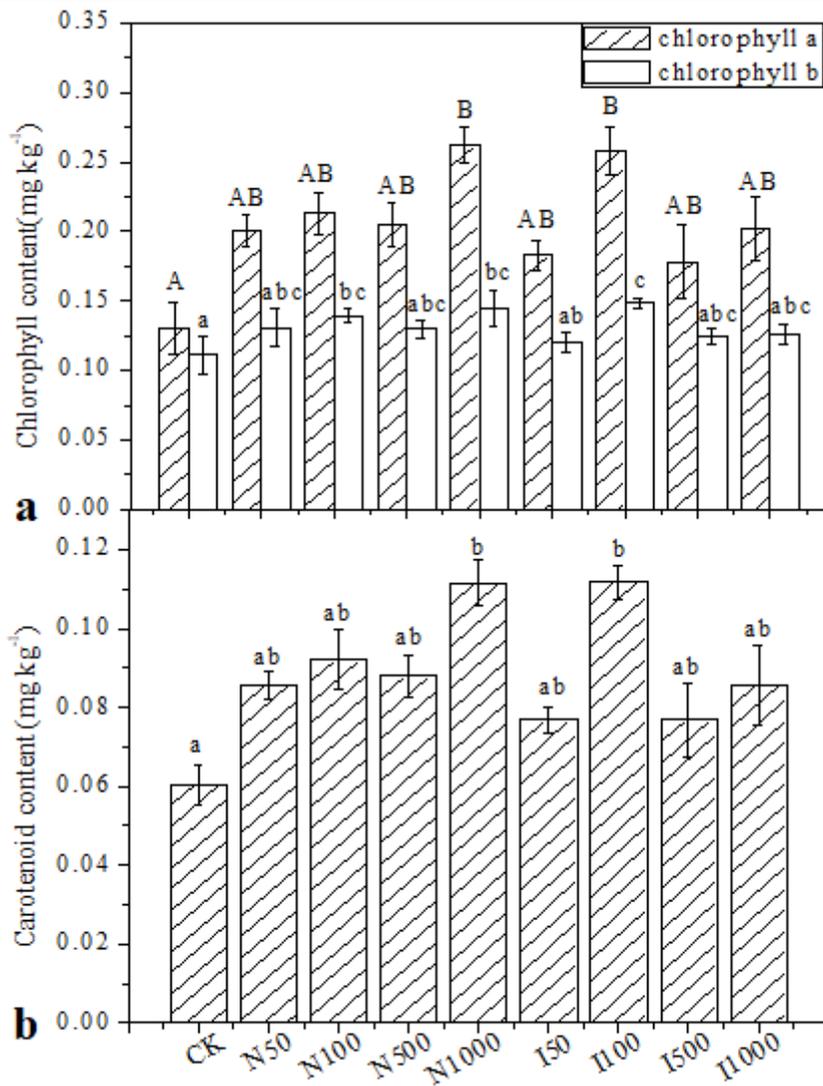


Figure 5

Effect of different treatments on (a) chlorophyll content and (b) carotenoid content in pakchoi leaves (FW). Different lettering indicated the significant difference at $p < 0.05$.

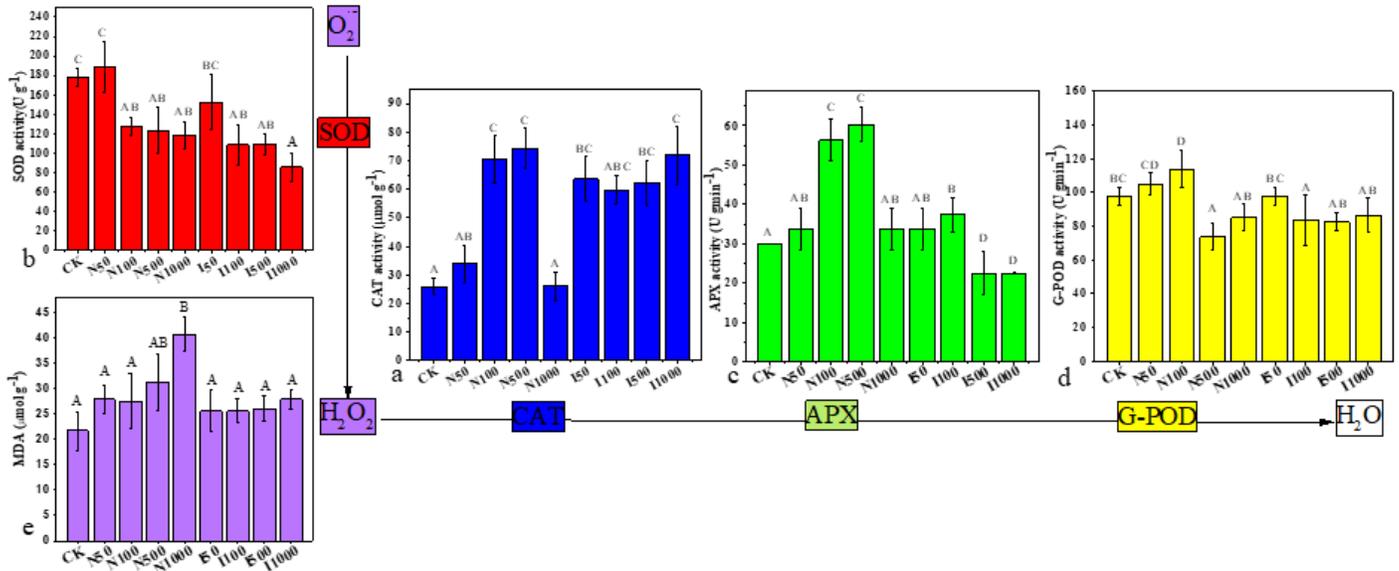


Figure 6

Effects of different treatments on the activity of several antioxidant enzymes as (a) SOD, (b) CAT, (c) APX, (d) G-POD and (e) MDA in pakchoi leaves (FW). The “asterisk” and different lettering are used to detect possible differences among the treatments at $p < 0.05$.

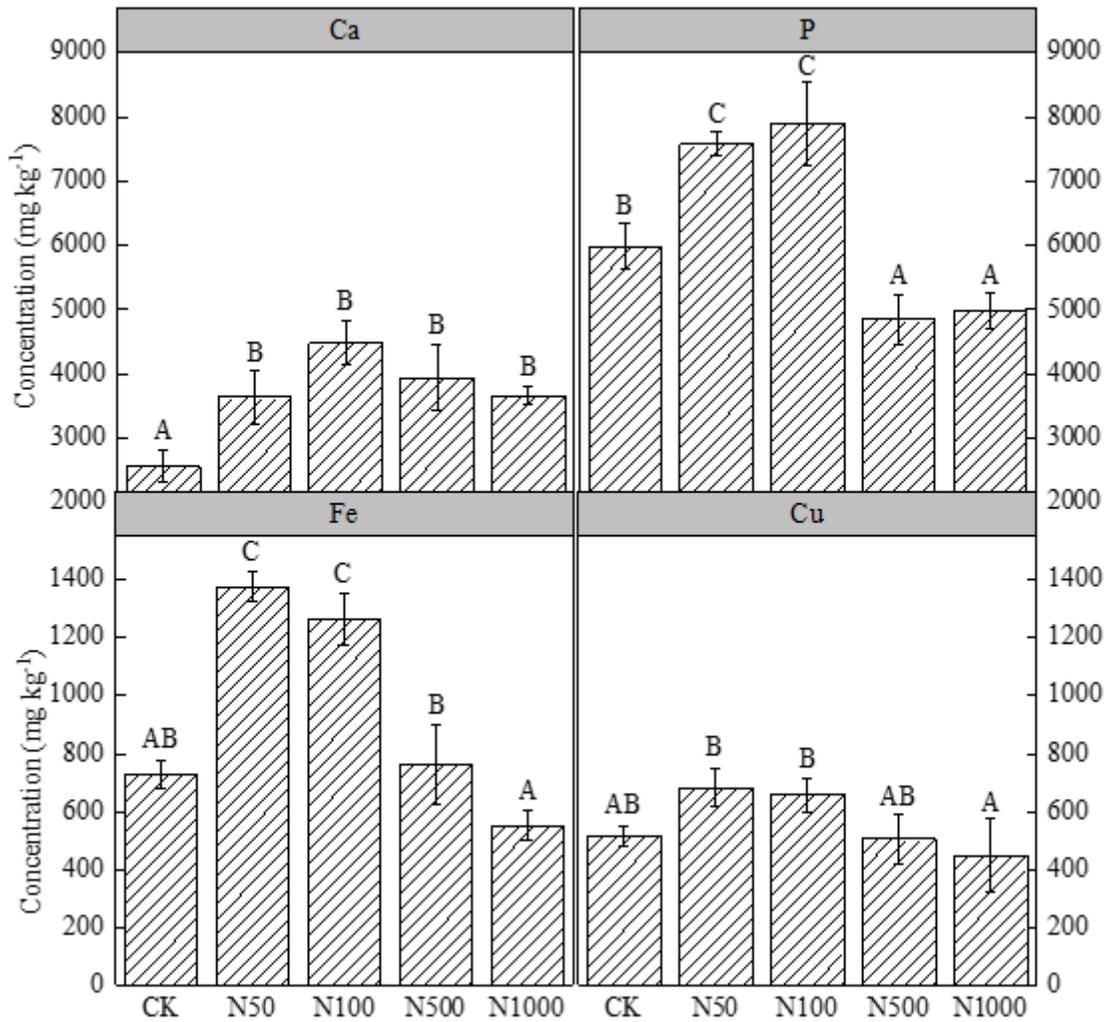


Figure 7

Trace metals concentration of Ca, P, Fe, Cu pakchoi inedible tissue (DW) treated with ZnO NPs. The CK represents the control. The error bars are standard errors.

Supplementary Files

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