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**Combined application of biochar and nano-zeolite enhanced
cadmium immobilization and promote the growth of Pak
Choi in cadmium contaminated soil**

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30

31 **Abstract**

32 Biochar and zeolite have been demonstrated effective to remove heavy metals in
33 soil; however, the effect of combined application of the both materials on the fraction
34 of cadmium (Cd) and soil-plant system are largely unknown. Cd fractions in soil,
35 growth and Cd uptake of Pak Choi were measured after the combined application of
36 biochar (0, 5, 10 and 20 g·kg⁻¹) and nano-zeolite (0, 5, 10, 20 g·kg⁻¹) by pot
37 experiment. Results showed that both single and combined application reduced the
38 exchangeable Cd in soil and improved the plant growth. However, combined
39 application of 20 g·kg⁻¹ biochar with 10 g·kg⁻¹ nano-zeolite showed the strongest
40 effect, with the residual Cd in soil increased by 214% as compared with control. 20
41 g·kg⁻¹ biochar with 10 g·kg⁻¹ nano-zeolite Mechanic studies showed that this
42 combination enhanced the antioxidant system, with the SOD, CAT and POD activities
43 enhanced by 56.1%, 133.3% and 235.3%, respectively. The oxidative stress was
44 reduced correspondingly, as shown by the reduced MDA contents (by 46.7%). This
45 combination also showed the best efficiency in regulating soil pH, soil organic matter
46 (SOM) and soil enzymes thus improving the plant growth. This study suggests that
47 combined application various materials such as biochar and nano-zeolite may provide
48 new strategies for reducing the bioavailability of Cd in soil and thus the accumulation
49 in edible plants.

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53 **Key words: Biochar, Nano-zeolite, Cadmium, Pak Choi, Enzymatic**
54 **activities**

1. Introduction

Heavy metals pollution in soil is one of the great challenges faced by global society [1-5]. Compared to organic contaminants, heavy metals are not degradable in soil thus are persistent in terrestrial ecosystems [6]. In 2014, the National Survey Bulletin on Soil Pollution showed that Cd pollution ranked first among inorganic pollutants, exceeding the standard rate by up to 7% [7]. Ren et al. found that under the same growth environment, there were significant differences in the absorption and accumulation of heavy metals (Cd, Cr, Cu, Ni, Pb, Zn) of 14 vegetables, among which Cd was the most accumulated heavy metal in vegetables [8]. Cd in soil can be easily absorbed by plant roots and transferred through the food chain and accumulated in the human body, resulting in teratogenicity, cancer, mutation and other risks [9]. Cd over accumulation in plant induces excessive accumulation of reactive oxygen species (ROS), which can cause plasma membrane rupture, DNA damage or other metabolic disorders in plants and consequent inhibition of plant growth and development [10-11]. The form of Cd in the environment is an important factor affecting its mobility, bioavailability and toxicity in the ecosystem. In the sequential extraction, the order of lability of the five fractions is: exchangeable > carbonate > Fe-Mn oxide > organic > residual [12]. The exchangeable fraction is the most active form with high bioavailability and thus toxicity [13]. Therefore, reducing the bioavailable fraction of Cd in soil and crops is of great significance to ensure the safe production of staple food.

Adding amendments into the soil is an efficient method to remedy soil heavy metal pollution due to its low disturbance to soil and economical cost [14]. The amendments reduce the mobility, bioavailability and bioaccessibility of the heavy metals in soil by complexation, precipitation and adsorption. At present, amendments are generally divided into organic, inorganic, organic-inorganic mixed and new material amendments [15]. Organic amendments mainly include biochar, peat, compost and straw. The main inorganic amendments were lime, zeolite, industrial waste residue, clay minerals [16-17]. The organic-inorganic amendment was the mixture of

organic and inorganic amendments in a certain proportion ^[18]. New material amendments mainly included nanomaterials, polyphenols, functional membrane materials or mesoporous materials ^[19]. For example, Guo et al. found that spraying of 10 mg·L⁻¹ fullerene nanomaterials on cucumber leaves can effectively reduce the accumulation and toxicity of As and Cd in plant ^[20]. Foliar spray of 100 mg·L⁻¹ zinc oxide nanoparticles significantly diminished the Cd content in rice shoot (30%) and rice roots (31%), respectively ^[21] Previous studies have shown that the nanoparticles obtained through polyphenolic condensation, they can adsorb the Pb²⁺ from aqueous solutions and exhibit a high removal efficiency (90%) and maximum adsorption capacity up to 584.8 mg·g⁻¹ ^[22].

Biochar and zeolite have shown great promise for water and soil remediation ^[23]. The capacity of biochar to immobilize metals in soil are largely attributed to its high surface area, porosity, cation exchange capacity and abundant surface functional groups ^[24-25]. Biochar is also capable of elevating the soil pH thus enhancing the adsorption of metals on the surface. The chemical structure is very stable thus it can stay in soil and function for a long duration. ^[26]. Moreover, biochar has shown capacity to improve plant growth whilst reducing the residual Cd in soils ^[27]. Zeolite also show equivalent ability for Cd remediation. It was reported that addition of 5% of zeolites can reduce 22% of Cd in soils ^[28]. When the size goes down to nanoscale, the adsorption capacity of zeolite toward Cu and Pb can increased by up to 20 folds as has been reported ^[29-30].

In actual agricultural practices, combined application of different amendments has been demonstrated effective which is sometimes better than single application. For example, it has been reported that combined application of hydroxyapatite and zeolite, or different organic materials and lime, can obtained significantly better effects than single application to remedy Cd and Pb contaminated soil ^[31-33]. In the long run, based on the comprehensive effects of improving soil fertility and soil structure and reducing the bioavailability of soil heavy metals, combined application of organic and inorganic amendments is expected to become the most ideal agronomic measures in the soil pollution remediation ^[34-35]. However, as a new material, nano-

zeolite has shown positive effect in soil heavy metal pollution, but the research of nano-zeolite combined with other amendments to remediate heavy metal contaminated soil is rarely reported.

In the present study, a life cycle study of Pak Choi was performed, and the concentration dependent effects of the amendments (biochar combined with nano-zeolite) were evaluated by measuring the soil pH, SOM, enzyme activities and the antioxidative responses in the plant Pak Choi. The objectives of this study were to provide a preliminary study on the efficacy of combined application of nano-zeolite and biochar for remediation of Cd contaminated soil and reduction of Cd absorption by Pak Choi.

2 Materials and methods

2.1 Sample preparation and characterization

Meadow soils (0–20 cm depth) were sampled at Zhangyi Town, Shenyang City. The soil pH and total organic matter content were 5.85 and 13.07 g·kg⁻¹, respectively; the total Cd content was 0.47 mg·kg⁻¹.

Biochar was produced from rice husk by thermal decomposition at 600 °C under anaerobic conditions for 6–8 h. The pH of biochar was 8.80. Total Cd content was 0.17 mg·kg⁻¹. Total specific surface area was 286.3 m²·g⁻¹. Nano-zeolite with a size of 70 ± 10 nm was purchased from Zhengzhou environmental protection company. The pH of nano-zeolite was 9.1. Total Cd content was 0.09 mg·kg⁻¹. Total specific surface area was 569.6 m²·g⁻¹. Scanning electron microscope (SEM, HITACHI-Regulus8100) and Fourier transform infrared spectroscopy (FTIR, General Purpose Mid-IR Bruker Alpha) were used to characterize the morphology and structure of biochar and nano-zeolite. The SEM images confirmed that the pore size of biochar was 3 ~ 5 μm and the diameter of nano-zeolite ranged from 70 to 80 nm (Fig. S1). Organic functional groups including C-H, C-O, C=C on the biochar and Si-O, Al-O and Si-Al-OH on the nano-zeolite were observed by FTIR (Fig. S1).

2.2 Experimental design

Pot experiment was conducted in the Experimental Station of Shenyang Agricultural University. The soil sample was air-dried and sieved to remove the impurities such as stones, grasses and branches for pot experiment. 1.5 kg soil was placed in each pot. Soil was mixed thoroughly with amendments and basal fertilizer containing urea, phosphate and potash fertilizers. The doses of fertilizers were 0.26 g·kg⁻¹ (N), 0.2 g·kg⁻¹ (P₂O₅), 0.3 g·kg⁻¹ (K₂O), respectively. Biochar and nano-zeolite were used as amendments. Biochar was set at four levels: B0 (0 g·kg⁻¹), B5 (5 g·kg⁻¹), B10 (10 g·kg⁻¹), and B20 (20 g·kg⁻¹). Nano-zeolite (N) was set at four levels: N0 (0 g·kg⁻¹), N5 (5 g·kg⁻¹), N10 (10 g·kg⁻¹), and N20 (20 g·kg⁻¹). Experiments were planned in completely randomized design with sixteen treatments and three replicates for each treatment. Contaminated soil with 5 mg·kg⁻¹ Cd with no amendments was set as control (CK). The experimental design was shown in **Table 1**. Adding the configured Cd (NO₃)₂·4H₂O solution ensured the final concentration of 5 mg·kg⁻¹ Cd in each pot. Soil was homogeneously moistened to 70% of the field capacity with deionized water. The pots are placed randomly. Ten seeds were sown in each pot. Fifteen days after sowing, the seedlings were thinned to three per pot. The Pak Choi was harvested after 70 days of treatments.

Table 1 Experimental design and does of treatments

Biochar	Nano-zeolite			
Dose (g·kg ⁻¹)	0	5	10	20
0	B0N0 (CK)	N5	N10	N20
5	B5	B5N5	B5N10	B5N20
10	B10	B10N5	B10N10	B10N20
20	B20	B20N5	B20N10	B20N20

2.3 Determination of pH, SOM and Cd fractionations in soil

After harvest of plants, all soil samples of each treatment were collected from each pot. Soil samples were air-dried, crushed, and sieved through 20 and 100 meshes. Soil pH was determined by measuring the soil/water mixture (w/v: 1/2.5) by a pH meter

(ORION STAR A211, Thermo Scientific) ^[36]. SOM was determined by K₂Cr₂O₇-external heating method ^[36]. The distribution of Cd fractions in soil was determined using the sequential extraction technique described by Tessier et al. ^[37]. Consequently, five sequential extractions, including exchangeable form (EX-Cd), carbonate bound form (CAB-Cd), organic bound form (OM-Cd), iron manganese oxide bound form (FMO-Cd) and residual form (RES-Cd) were extracted. The fraction of Cd was measured by flame atomic absorption spectrometer (FAAS, Z-2000, HITACHI) ^[38]. Cd standard solution in a concentration range from 0 to 2 mg·L⁻¹ was used as external reference. The spike recovery of Cd was 93.7% ± 7.1%. The limit of detection for Cd was 0.005 mg·L⁻¹.

2.4 Soil enzyme activities

The catalase activity was determined using the potassium permanganate titrimetric method and expressed as the volume of 0.1 mol·L⁻¹ KMnO₄ (ml) consumed per gram dry soil in 1 h ^[39]. The urease activity was measured by the sodium phenate-sodium hypochlorite colorimetric method and expressed as the milligram of NH₃-N in per gram dry soil after 24 h ^[40]. The invertase activity was determined by 3, 5 - dinitrosalicylate acid colorimetry and expressed as the amount of glucose (mg) produced per gram dry soil in 24 h ^[41].

2.5 Pak choi growth and Cd contents

Before harvest, the portable Chlorophyll Analyzer (SPAD-502PLUS) was used to measure the chlorophyll contents in pak choi leaves. Three homogeneous leaves of each Pak Choi were selected for determination. Pak choi was harvested and washed three times with tap water and then three times with deionised water. The water on the surface of plants were absorbed by filter paper. The fresh weights were measured by electronic balance (BSA224S-CW, Sartorius Group). Roots and aerial parts were separated and the height, leaf length and leaf width were measured with a ruler. The measurement standard of Pak Choi hight was determined to be from the rhizome division to the longest leaf tip. Three uniform leaves were selected from each Pak Choi to determine the leaf length and width.

In order to measure the Cd contents, the plant samples were dried at 105°C for 30 min and further dried under 60°C until a constant weight achieved. The dry samples were then ground into fine powders with a mortar. The powdered samples (0.5 g) were digested with 8 ml HNO₃ for 80 minutes by microwave digester (Microwave Reaction System Mars6, PYNM). After digestion, the samples were then heated under 180 °C in a digestion heating block (EHD-12, PYNM) until the residue was close to 1ml. The residual solutions were then diluted to 25ml with deionized water. The Cd concentrations were then measured by graphite furnace atomic absorption spectrometry (GFAAS, Z-2000, HITACHI).

2.6 Determination of antioxidant enzyme activities and MDA content in Pak Choi

Fresh samples were excised and homogenized with cold phosphate-buffered saline (PBS) (50 mM, pH 7.8), and centrifuged at 10 000g and 4 °C for 10 min. The supernatants were collected for further analyses. The activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) activities and malondialdehyde (MDA) contents were measured using assay kits purchased from Beyotime (Shanghai, China) according to the manufacture's instruction. To ensure accuracy and linearity, standard SOD, CAT, POD and MDA with known concentrations (6 concentrations) were prepared and analyzed following the same procedure described in the kits for sample analysis. Six replicate samples were tested for each data point and the experiments were repeated three times ^[42].

2.7 Data analysis

The statistical analysis was performed on IBM SPSS 19.0. One way ANOVA were used to determine the differences between groups. Principal component analysis (PCA) ^[43] was used to analyze the reasonable application dose of biochar and nano-zeolite. Origin 2019 was used for plotting. **In order to compare the effect of combined application of biochar and nano-zeolite on passivating the absorption of Cd by Pak Choi under Cd stress, PCA was used to conduct a unified and comprehensive analysis on the data of each index, determine the principal component and the contribution rate**

of each index, and use the results of principal component analysis to explain the overall effects of the combined application strategy ^[44].

3 Results and Discussion

3.1 Effects of biochar and nano-zeolite on soil pH and SOMs

The effect of biochar and nano-zeolite on soil pH and SOMs were shown in **Fig.1**. Both single and combined application of amendments significantly increased the soil pH (**Fig.1a**) and SOM contents (**Fig.1b** $P < 0.05$). Compared with the control, the single application of nano-zeolite increased the pH by 17.3%, 22.8%, 29.7%, and SOM by 17.0%, 25.5%, 12.4%, respectively. Single treatment with biochar also increased the pH by 16.0%, 17.8%, 27.4%, and the SOM content by 18.2%, 25.5%, 36.3%, respectively. The effect of combined application seems stronger than the single application. The combined application increased the by 19% ~ 37.5%. B20N20 treatment caused the highest increase of the soil pH (by 37.5%). Overall, the combined application of biochar and nano-zeolite cause higher increase of SOM than the single application when the doses of nano-zeolite were 5 g·kg⁻¹ or 10 g·kg⁻¹. B20N10 treatment caused the highest increase of the SOM (by 49.8%). However, when the dose of nano-zeolite reached 20 g·kg⁻¹, the effects are equal or even less than the single application of biochar. These results indicate that combined application has better performance in enhancing soil SOMs while high dose of nano-zeolite may compromise such effect.

Mobility of heavy metal is mainly governed by soil pH. Heavy metal complexation is often favored by high pH which reduces the desorption of heavy metals from soils. The increase of soil pH due to the application of biochar and nano zeolite thus facilitated the immobilization of the Cd. There are various exchangeable cations (K⁺、Na⁺、Ca²⁺、Mg²⁺) in the porous structure of nano-zeolite, which can replace H⁺ and Al³⁺ in soil, resulting in the decrease of hydrolytic acid and the increase of pH. Biochar can increase soil pH because the ash of biochar contained alkaline oxides or carbonates of Na, K, Ca and Mg. These substances are alkaline in aqueous solution, so they also increase soil pH ^[45]. The results of Wu et al. ^[46] were

similar to ours, on the 60th day of soil incubation experiment, they found that the pH of 10 mg · kg⁻¹ Cd contaminated soil increased by 20.1% and the exchangeable Cd decreased by 33.0% when 10% biochar and 10% zeolite were applied combinedly. Because biochar itself contained rich organic matter the application of biochar can increase the content of organic matter in the soil [47]. Kim et al. [48] applied biochar at the ratio of 1%, 2% and 5%, and the content of SOMs increased from 14.6 g·kg⁻¹ to 20.5 g·kg⁻¹. Ge et al. [49] found that the combined application of biochar and zeolite improved the stability of biochar and made the soil structure more stable. Therefore, the combined application of biochar and zeolite can more effectively improve the content of SOMs than biochar alone.

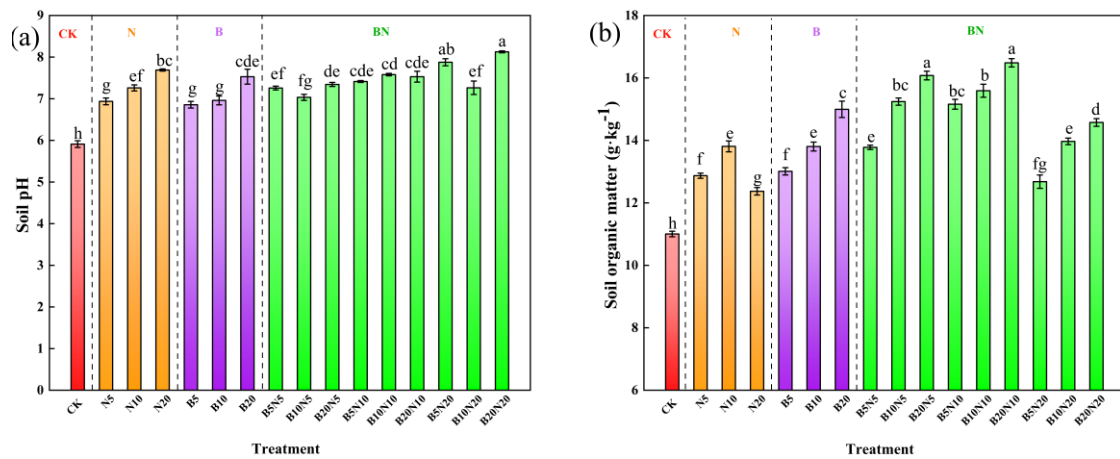


Fig. 1 Effects of single and combined application of biochar and nano-zeolite on the soil pH (a) and SOM contents (b). Different Lowercase letters indicates significant difference at $P < 0.05$.

3.2 Soil enzyme activity

Soil enzymes were sensitive to the changes of their living environment and play an important role in the function of soil system [50]. They are used as indicators to evaluate the soil health and activity. The effects of biochar and nano-zeolite on soil enzyme activities are shown in **Fig. 2**. Soil enzyme activities were affected in varying degrees after application of amendments. Compared to the control, the treatment of 10 g·kg⁻¹ biochar combined with 20 g·kg⁻¹ nano-zeolite (B10N20) treatment showed the largest increase (46.1%) in soil catalase activity. B20N5, B20N10 and B20N20 also increased the catalase activity by 39.9%, 37.1% and 42.5% respectively ($P < 0.05$),

and N20, B10, B20, B10N5, B5N10, B10N10 and B5N20 significantly increased the catalase activity by 27.3%, 17.3%, 35.7%, 18.7%, 11.3%, 33.1% and 19.2%, respectively (Fig. 2a). The effects of amendment on soil urease activity is largely dependent on the amount of applied nano-zeolite. Among them, 20 g·kg⁻¹ biochar combined with 10 g·kg⁻¹ nano-zeolite (B20N10) had the largest increase (by 69.7%) in urease activity (Fig. 2b). Compared with the control, the soil invertase activity increased by 31.2%, 74.0% and 32.2% under the single treatment of B10, B20 and N10, respectively. Combined treatments showed stronger promotive effects, with the activity increased up to 99.4% by the B20N20 treatments (Fig. 2c).

Hydrogen peroxide can oxidize sulfhydryl groups in protein molecules and damage cells. Catalase is an oxidoreductase, which can decompose hydrogen peroxide in soil and reduce its toxicity to soil microorganisms. An increase in the catalase activity suggests an enhanced decomposition of H₂O₂ and antioxidative capacity in soil to cope with Cd stress. Urease is a peptide and amino hydrolase, which is closely related to the nitrogen cycle in soil and can catalyze the hydrolysis of urea to carbon dioxide and water [51]. Previous studies showed that the mixed application of biochar and phosphate solubilizing bacteria can increase soil urease activity, sucrase activity and catalase activity by up to 189.33%, 117.81% and 125%, respectively [52]. Our results also showed that combined application of biochar and nano-zeolite increased soil urease activity. This phenomenon might be due to multiple reasons. Biochar can provide carbon source and stimulate the activities of microorganisms in soil by increasing energy supply, whilst nano-zeolite has large porosity which makes the soil fluffy and provides habitat for the growth of soil microorganisms. Moreover, biochar and nano-zeolite have strong adsorption capacity, which can adsorb the reaction substrate and promote the enzymatic reaction. Xu et al. found that biochar was applied to the paddy soil, the urease activity increased significantly, which may be due to that the application of biochar increased the soil nutrients and fertility, promoted the hydrolysis of carbon and hydrogen bonds in urea and organic molecules, and then accelerated the consumption of NH₄⁺, thus promoting the urease hydrolysis process [53].

Soil sucrase, as a hydrolase, plays an important role in the carbon cycle in the soil system, which is closely related to the utilization rate of soil nutrients. It can hydrolyze sucrose in the soil, provide energy for organisms, and play an important role in increasing soluble nutrients in the soil [54]. The enhanced activity of sucrase by biochar and nano-zeolite treatments might be due to the increase of SOM contents, which not only increased the nutrients for soil microbial activities, but also provided rich substrates for enzymatic reaction, resulting in the increase of soil invertase activity [55-56].

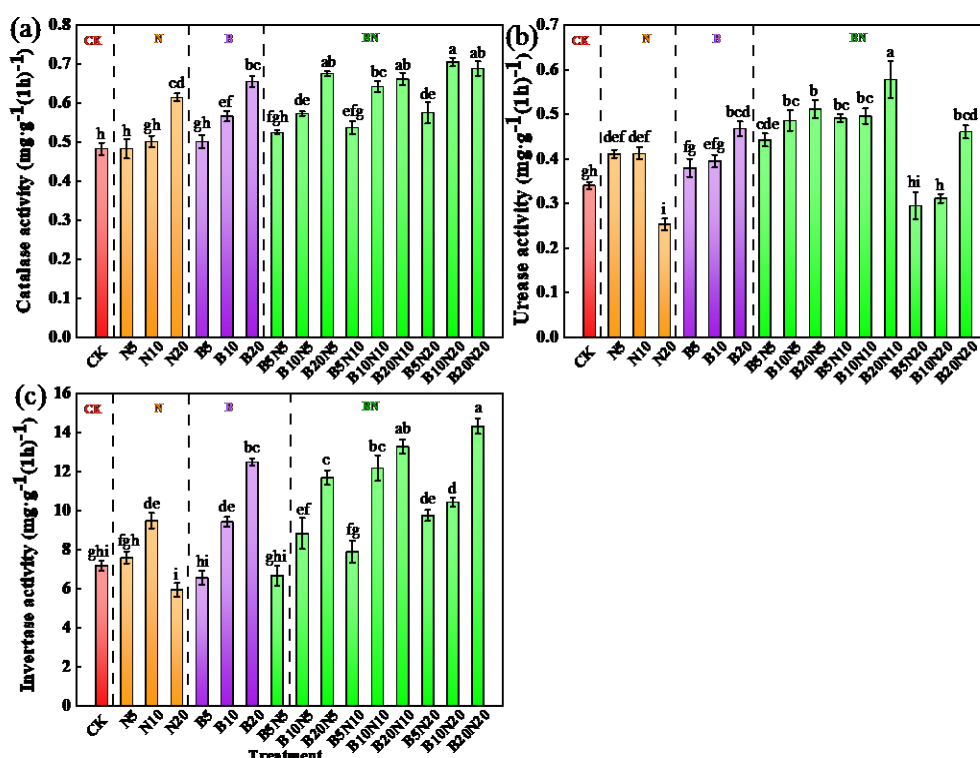


Fig.2 Effect of biochar and nano-zeolite combined application on the activities of catalase (a), urease(b), invertase(c). Values with different letters are significantly different ($P < 0.05$)

3.3 Effects of biochar and nano-zeolite on Cd fractions in soil and accumulation in Pak Choi

As shown in **Fig. 3a**, compared with the control, the exchangeable Cd content of single application or combined application of biochar and nano-zeolite decreased significantly ($P < 0.05$), with 44.8% and 43.1% of reduction observed for B20N10

and B20N20 treatments, respectively. Notably, the combined application of biochar and nano-zeolite increased the content of carbonate bound Cd ($P < 0.05$) by 34% and 44%, respectively upon B20N10 and B20N20 treatments, and by 33% and 38% upon B5N20 and B10N20 treatments. Except for B5 and N5 treatment, the single application and combined application of biochar and nano-zeolite significantly increased the content of iron manganese oxide bound Cd, with the highest increase (41%) for B20N20 treatment. The combined application also significantly increased the content of organic bound Cd. Specifically, B10N5, B20N5, B20N10, B20N20 increased the content of organic bound Cd by 26.7%, 28.1%, 28.6% and 34.6%, respectively. **The residual Cd contents in soil decreased significantly by 79.5% ~ 214.2% after combined treatment.** B20N10 immobilized the residual Cd content to the largest extent (214%).

Fig. 3b showed the Cd concentration in Pak Choi. Compared with the control, both the single and combined application of amendments significantly reduced the content of Cd in Pak Choi; however, combined application of amendments showed stronger effects ($P < 0.05$). The content of Cd in Pak Choi decreased by 17.1%, 25.6% and 35.4% with single application of biochar, and by 13.3%, 20.4% and 36.4% with single application of nano-zeolite. **The content of Cd in Pak Choi significantly decreased by 24.9%, 34.4%, 35.6%, 33.9% after combined application with of B5N5, B10N5, B20N5, B5N10.** However, the Cd content was decreased by 46.8%, 44.5%, 51.7%, 52.7% and 53.9% upon treatment with B10N10, B20N10, B5N20, B10N20, B20N20, respectively.

Soil pH was regarded as a key factor controlling the bioavailability of Cd in contaminated soil. With the increase of soil pH, the negative charge on the soil surface increased, and the CdCO_3 precipitates may form which led to the decrease of the migration and solubility of Cd^{2+} [57]. SOM is also a key factor affecting the activity of Cd in soil. Our results showed that the combined application of biochar and nano-zeolite increased the content of SOM, which may contribute to the reduced exchangeable Cd. Shan et al. showed that the exchangeable Cd content in soil decreased significantly after application of straw and pig manure; and the ratio of

humic acid to fulvic acid, an important component of SOM, increased significantly thus reduced the exchangeable Cd content [58]. Previous studies suggested that after applying biochar to the soil, the increase of SOM and other nutrients in the soil can improve the ion exchanges in the soil, so as to improve the adsorption capacity of the soil for metals such as Cd, thus can promote the complexation reaction, and reduce the mobility of Cd in the soil, and subsequent absorption by plants [59].

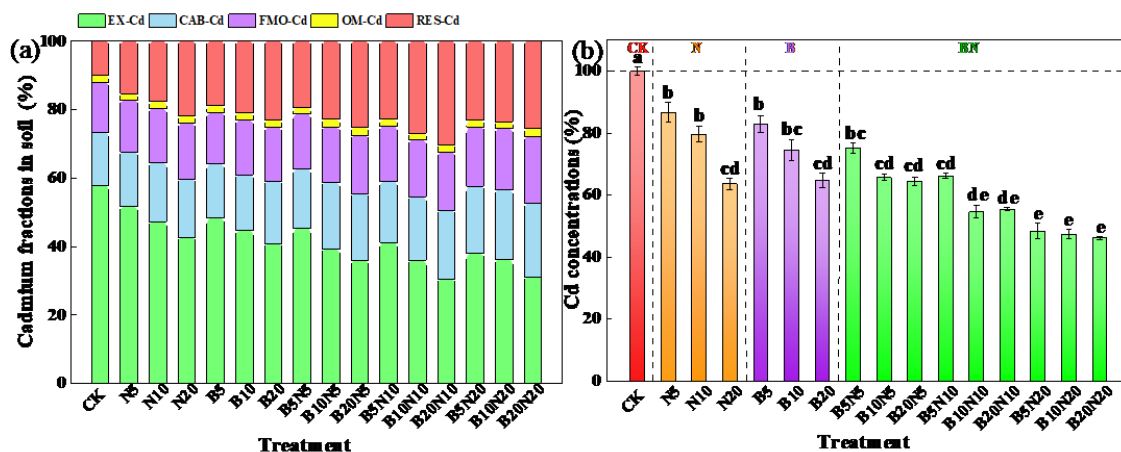


Fig.3. Effect of biochar and nano-zeolite combined application on Cd fractions in soil (a) and Cd concentrations in Pak Choi (b). Values with different letters are significantly different ($P < 0.05$). Data were expressed as mean \pm SD (n= 6).

3.4 Effects of biochar and nano-zeolite amendments on the growth of Pak Choi

The effects of amendments on the growth of Pak Choi under Cd stress were shown in Fig. 4. Compared with the control treatment, the single application of biochar and nano-zeolite had a positive effect on the growth state of Pak Choi. High concentration of nano-zeolite (N20) led to some negative effects, but the combined application of biochar and nano-zeolite showed its advantages that alleviated the negative effects. In specific, the fresh weight increased by 18.1% ~ 69.8% after combined application. the fresh weight of Pak Choi under B20N10 treatment had the largest increase (by 69.8%). The height and leaf length of Pak Choi under the combined application of biochar and nano-zeolite increased by 7.4% ~ 90.9% and 12.8% ~ 57.2%, with the highest increase observed for B20 (90.9% and 57.2%). Except for N10, N20, B5N5,

B5N20 and B10N20, the leaf width of Pak Choi increased significantly (10.7%~65.6%), with the highest increase for B20 treatment. Compared with the control treatment, the chlorophyll content of Pak Choi under B20 treatment increased by 92.8%. In the combined application, B5N5, B10N5, B20N5, B10N10, B20N10 and B20N10 all significantly increased the chlorophyll content (by 57.3%, 109.5%, 119.4%, 36.7%, 99.2%, 31.7%), with the largest increase observed for B20N5 (119.4%).

The increased soil pH and SOM contents, reduced bioavailability of Cd in soil, increased soil enzyme activity, all contributed to the reduced toxic effects of Cd on Pak Choi ^[60-62]. The biochar has been proved to improve the growth of plants under Cd stresses. The biochar may improve the growth of plant by supplying essential plant nutrients and decreasing toxic metal in plant. It may be due to the increase in metal and nutrient competition at the surface of roots, or immobilization of metals in the soil ^[63]. In addition, previous studies have shown that nano-zeolite can effectively reduce the content of exchangeable Cd in soil, inhibit the absorption of Cd by Chinese cabbage, and thus reduce the toxicity of Cd to Chinese cabbage. On the other hand, nano-zeolite applied to soil can improve soil physical and chemical properties, such as soil cation exchange capacity, and improve the absorption of nutrient elements by plants, so as to promote plant growth ^[64].

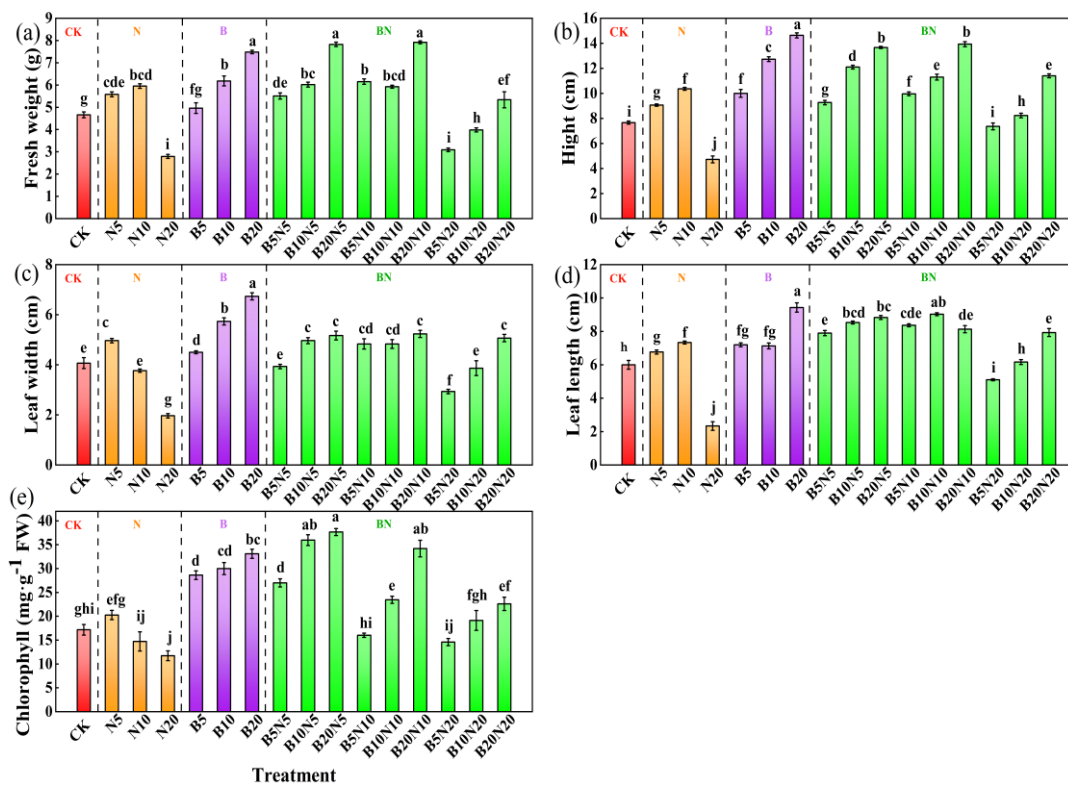


Fig. 4 Effects of biochar and nano-zeolite combined application on growth characteristics of Pak Choi. Values with different letters are significantly different ($P < 0.05$)

3.5 Effects of combined application of biochar and nano-zeolite on antioxidant enzyme system and MDA content of Pak Choi

The effect of amendments on antioxidant system in Pak Choi was shown in **Fig 5a**. Except for N20 treatment, the SOD activity was significantly increased ($P < 0.05$). The treatment with single biochar increased SOD activity by 14.6% ~ 45.4%. Nano zeolite increased the SOD activity and by 17.0% and 23.9%, respectively in N5 and N10 treatments. Combined application showed stronger effects (increased by 20.9% ~ 56.1%). Among them, 20 g·kg⁻¹ biochar combined with 5 g·kg⁻¹ nano-zeolite (B20N5) treatment showed the largest increase (56.1%) in SOD activity.

It can be seen from **Fig. 5b** that compared with the control treatment without amendments, the CAT activity increased by 51.9%~106.1% after single application of biochar. Single application of nano zeolite (N5, N10) increased the CAT activity by 34.1% and 73.0% respectively. Combined treatments showed stronger enhancing

effects, with the CAT activity increased by 64.9% ~ 133.3%. Similar trend was observed for POD (Fig. 5c).C Except for the N20 treatment, the POD activity in other treatments was significantly increased. Single application increased the POD activity by 18.9%~100.0%. Combined application enhanced the POD activities by 73.7% ~ 253.3%, suggesting enhanced positive effects compared with single application.

The effect of amendments on MDA content in Pak Choi was shown in Fig 5d. Compared with the control treatment without amendments, except for N20 and B5, the MDA contents decreased significantly by 13.8% ~ 46.7%. Among them, the treatment of 20 g·kg⁻¹ biochar combined with 20 g·kg⁻¹ nano-zeolite (B20N20) showed the largest reduction in MDA content, which was significantly reduced by 46.7%.

Cd stress can induce the production of O²⁻, OH⁻, H₂O₂ and other reactive oxygen species, free radicals and peroxides in plants, leading to oxidative damage. Plants can start their own defense mechanisms to remove these products, and detoxify themselves by increasing the activities of SOD, CAT and POD and decomposing excessive hydrogen dioxide [65-66]. The content of MDA can reflect the degree of damage of plant cells by reactive oxygen species, free radicals and other products [67-68]. In this study, the combined application of biochar and nano-zeolite reduced the content of Cd in Pak Choi, alleviated the toxic effects of free radicals and reactive oxygen species on Pak Choi cells and tissues, promoted the growth of Pak Choi, and improved its SOD, CAT and POD activities, so as to eliminate the products under Cd Stress (Fig. 6). The reduced amount of MDA contents suggests reduced oxidative damage caused by Cd [69]. Nano-zeolite contains Ca²⁺, which can promote the synthesis of amino acids and polyamines in Pak Choi, thus directly scavenging reactive oxygen species or indirectly scavenging reactive oxygen species by regulating antioxidant enzyme activity, thus alleviating the membrane lipid peroxidation [70]. Wu et al. [71] found that compared with the control treatment, biochar application could promote the growth of vetiver grass, reduce the content of MDA by increasing the activity of antioxidant enzymes in Vetiver grass, and alleviate the oxidative stress caused by heavy metals.

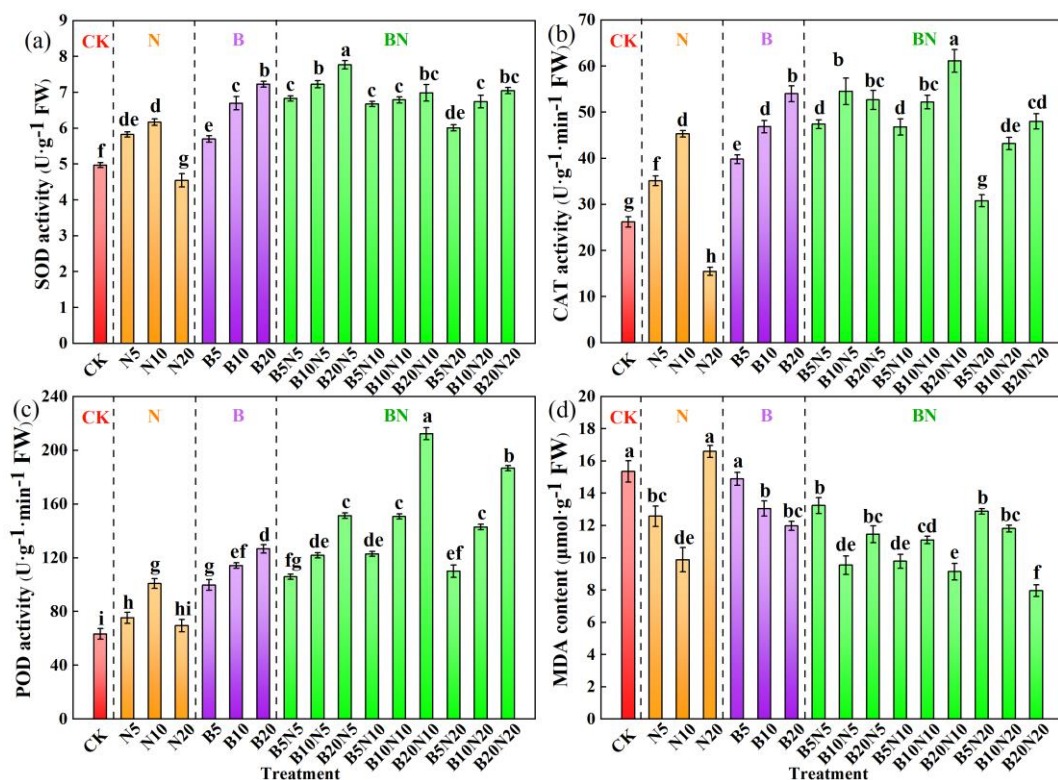


Fig. 5 Effect of biochar and nano-zeolite combined application on SOD (a), CAT (b), POD (c) and MDA (d) of Pak Choi. Values with different letters are significantly different ($P < 0.05$)

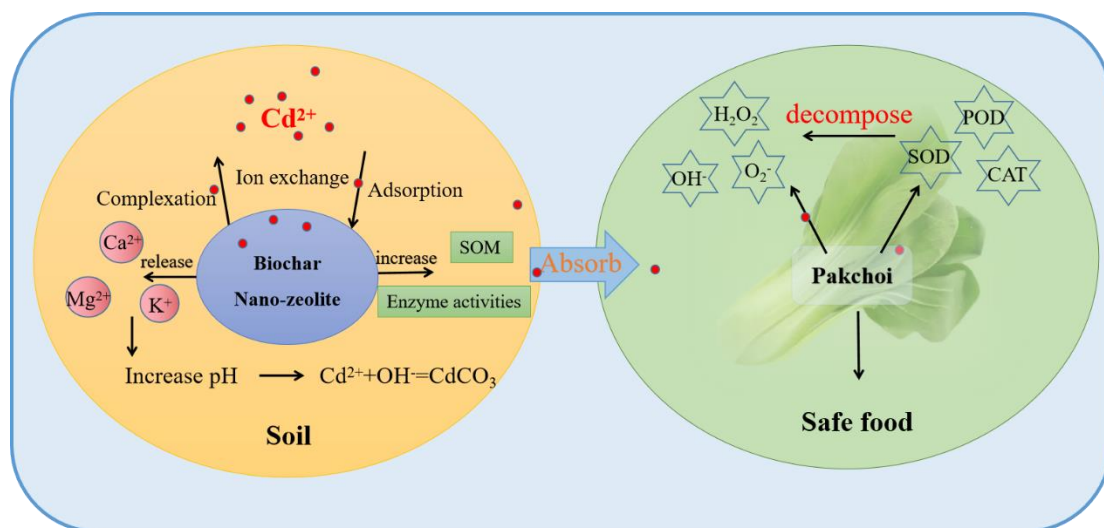


Fig. 6 Illustration of key processes of biochar and nano-zeolite in remediation of heavy metal Cd contaminated soil.

3.6 Principal component analysis of amendments on soil properties,

Cd fractions and growth of Pak Choi

The effects of biochar and nano-zeolite on the main physiological indexes of Pak Choi and soil indexes were analyzed by PCA, and the results were shown in **Fig. 7**. As can be seen from **Fig. 7**, the contribution rate of PC1 is 60.0%, and that of PC2 is 22.9%. The two principal components explained 82.9% of the total variance. Therefore, the above 20 main indexes can be expressed by the two principal components. PC1 was negatively correlated with exchangeable Cd in soil, Cd content in Pak Choi and MDA content, and positively correlated with other indexes. PC2 was negatively correlated with soil pH, catalase, sucrase, carbonate bound form Cd, iron manganese oxide bound form Cd, organic bound form Cd, residual form Cd, POD and MDA, and positively correlated with other indexes. Take PC1 as the horizontal axis and PC2 as the vertical axis to establish the coordinate system and calculate the score. The color dots in the figure represent different treatments, and the corresponding values projected vertically on the coordinate axis represent the scores of each treatment. Overall, the abscissa value of B20N10 treatment in PC1 is 5.16, and the vertical axis is 0.31, with the highest total score, Therefore, B20N10 treatment (20 g·kg⁻¹ biochar combined with 10 g · kg⁻¹ nano-zeolite) can effectively reduce the exchangeable Cd content in soil and reduce the absorption of Cd by Pak Choi.

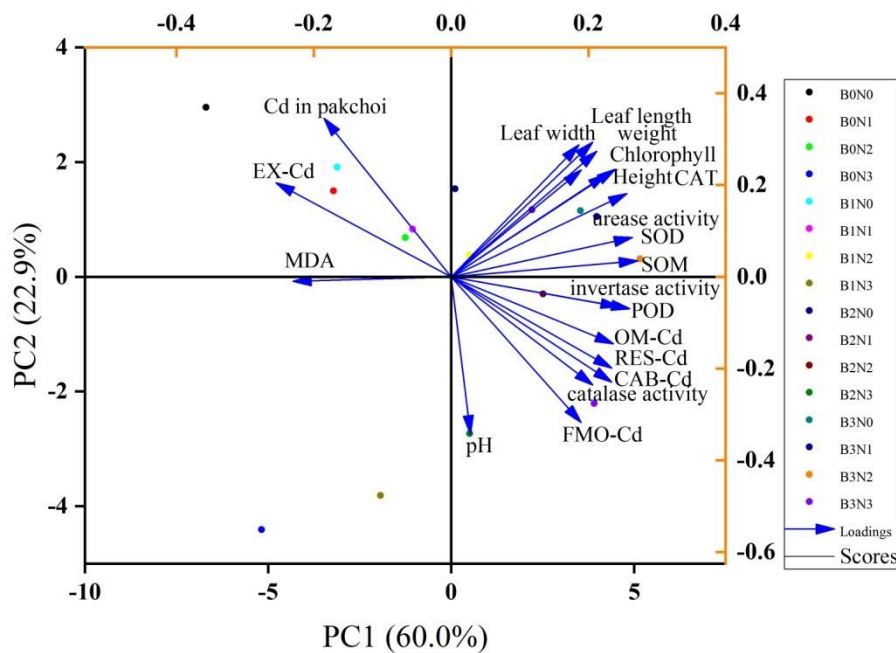


Fig. 7 Principal component analysis of soil properties, Cd fractions and physiological indexes of Pak Choi with biochar and nano-zeolite combined application.

4 Conclusions

The Cd pollution level in this study is $5\text{mg}\cdot\text{kg}^{-1}$, which is 2.5 times higher as the risk control value of Cd in the soil environmental quality. This study indicated that in such highly Cd contaminated soil, the application amount and mixing ratio of biochar and nano-zeolite have a significant impact on soil characteristics, which determine the fractions of the Cd species in soil. Combined application of biochar and nano-zeolite reduced the percentage of exchangeable Cd due to the increase of pH and SOM contents in Cd contaminated soil. This reduction consequently suppressed Cd uptake by Pak Choi. Thus, the harmful effects of Cd on the growth of Pak Choi were reduced. The combined application of both amendments significantly increased soil enzyme activities, plant antioxidant enzyme activities and growth after the application of amendments. According to the PCA, the B20N10 treatment, which contained $20\text{g}\cdot\text{kg}^{-1}$ of biochar and $10\text{g}\cdot\text{kg}^{-1}$ of nano-zeolite, showed the best improvement effect. This study suggests that combined application of biochar and nano-zeolite offered an innovative method to remediate contaminated soil.

ASSOCIATED CONTENT

Credit author statement

X.L. Dang guided experimental design and article writing ideas. S.S. Feng conducted the pot experiment, and completed most of the test work independently in laboratory, and was a major contributor in writing the manuscript. P. Zhang, T. Nadezhda, Z.L. Guo and I. Lynch put forward important guidance on article writing and language expression. Y.M. Hu, F. Jin, Y.Q. Liu, S.X. Cai, Z.J. Song, and X. Zhang assisted on the laboratory work. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Characterization of biochar and nano-zeolite including SEM and FTIR is provided in the Figure. S1.

Author Contributions

[†] The authors contributed equally to this paper.

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