

## Article

# Silicon-rich materials detoxify multiple heavy metals in wheat by regulating oxidative stress and heavy metal subcellular distribution

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**Abstract:** Silicon is an essential trace nutrient for plant growth and is frequently employed to remediate soils contaminated with heavy metals in agriculture. However, silicon's role and mechanism in reducing heavy metal toxicity have not been well understood, especially for multi-heavy metals. In this study, the effects of silicon-rich materials (silicate, rice husk biochar (RHB), and bentonite) on growth trait, antioxidant response, and heavy metal accumulation and distribution of wheat grown in two soils polluted by multiple heavy metals (Cd, Zn, Pb, and As) were investigated. The results revealed that the addition of silicon-rich materials enhanced plant growth, improved the photosynthetic attributes in leaf tissues, and decreased the contents of Cd, Zn, Pb, and As in wheat shoots and grains. The examination of the subcellular distribution of heavy metals in plants implied that silicon-rich materials transferred heavy metals as intracellular soluble fractions to the cell walls, indicating the reduction of mobility and toxicity of heavy metals in the plants. In addition, the application of the silicon-rich materials reduced oxidative damage in plants by downregulating plant antioxidant response systems and decreasing the production of malondialdehyde (MDA), ascorbic acid (AsA), and glutathione (GSH). Moreover, fractionation analysis of soil heavy metals showed that silicon-rich amendments could convert bioavailable heavy metals into immobilized forms. The results indicated that silicon-rich materials could remediate multi-heavy metal-polluted soils and promote wheat production.

**Keywords:** Silicon, Heavy metals, Oxidative stress, Wheat, Cellular

## 1. Introduction

In developing and developed regions of the world, soil heavy metal pollution mainly comes from mining, wastewater, pesticides, and fertilizers applications [1,2]. For most cases, soils are contaminated by multiple heavy metals, which shows combined ecological effects and makes soil remediation more challenging [1,3-6].

Chemical passivation remediation is a cost-effective and generally used technology. It is conducted by adding chemical passivation to contaminated soil to change the physical and chemical properties of the soil as well as to reduce the bioavailability and migration of soil heavy metals [7,8]. However, most studies focus on restoring single heavy metal pollution rather than multiple heavy metal contamination in soils [4,9].

Multi-heavy metals pollution in soils mainly includes cadmium (Cd), lead (Pb), zinc (Zn), and arsenic (As), which exceed National standard by 7%, 1.5%, 0.9%, and 2.7%, respectively, based on the report of the current status of soil contamination in China [10]. These metals are easily accumulated in the food chain and show high toxicity to plants and humans [11,12]. Toxic symptoms of heavy metals in higher plants can manifest as a dramatic decrease in plant biomass and elongation, impairing plant metabolism and physiological structure. For instance, reports found that heavy metal negatively affects plant photosynthesis, leading to leaf chlorosis, chlorophyll synthesis decrease, and nutrient uptake obstruction, ultimately inducing growth retardation [13,14]. Moreover, excessive heavy metals tend to disrupt the main protective enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) in plants, which may cause an imbalance between production and scavenging of reactive oxygen species (ROS) [15,16].

Silicon helps reduce and restrict heavy metal uptake and translocation in plants, which play a role in alleviating heavy metal toxicity and promoting plant growth [17]. Previous studies found that silicon application reduced the concentration and stress of heavy metals in different plants, including maize [18], rice [19,20], wheat [21,22], pigeon pea [23,24], coriander [25], and cabbage [23,24], and others. Besides, silicon can facilitate nutrient uptake, chlorophyll biosynthesis, and photosynthesis in plants [26,27]. Moreover, silicon forms a strong silica barrier in the plant cell wall, which can increase the thickness of the cell wall by co-precipitating with heavy metals, thus prohibiting heavy metals from crossing the cell membrane and entering the cytoplasm. The mechanisms of silicon action, such as reduction in the active heavy metals, enhancement in plant antioxidant enzymes, and conversion of heavy metals to metabolically inactive fractions, have been well studied [17,28].

Rice husk biochar (RHB) is a common Si-rich agricultural waste, and it has excellent porous properties and a large surface area. Previous studies have reported that RHB enhanced soil fertility and reduced the bioavailability of heavy metals in soils [29-31]. In recent years, environmental problems due to the improper placement of RHB have threatened ecological safety [32]. RHB is rich in amorphous silicon and is the most preferred silicon source for plants [17]. Jeer, *et al.* [33] reported that the abundant Si and K in RHB played a beneficial role in plant growth and effectively controlled the damage by yellow stem borers. More precisely, soil physical properties and plant nutrient uptake were enhanced after RHB application [32,34,35]. Besides, RHB can also alleviate metal toxic effects on plants, and increase soil pH and plant antioxidant enzyme activity [36]. Therefore, RHB has a great potential in soil conditioning with the advantages of cost-effectiveness, carbon sequestration, and waste recycling.

Bentonite is a kind of silicate clay mineral with montmorillonite as the main component, which has the characteristics of pore structure, large surface area, and high negative charge. It has been reported that bentonite is an effective adsorbent for heavy metals such as lead and cadmium. Moreover, bentonite has a unique crystal structure and contains trace elements essential for plants, making it able to enhance soil properties.

Although the numerous advantages of RHB, very few reports on the remediation of soil pollution and plant-heavy metal toxicity by RHB are available. Thus, the action mechanisms by which RHB remediates soil heavy metal pollution remain largely unknown. This study proposed that silicon-rich materials (silicate, RHB, and bentonite) regarded as soil amendments could enhance wheat growth and mitigate multiple heavy metal toxicity to different extent. The objectives of this study were i) to investigate the effects of the silicon-rich materials on the wheat growth and photosynthesis indicators, ii) to examine the content and subcellular distribution of heavy metals in wheat tissues, iii) to evaluate the oxidation stress responses and

detoxifying ability, and iv) to determine the soil heavy metal fractionation after the application of Si-rich amendments.

## 2. Materials and methods

### 2.1 Soils and amendments tested

Two soils (yellow brown (YB) and calcareous alluvial (CA)) used in the current trial were collected from 0-20 cm topsoil in the farm of Huazhong Agricultural University and Jiangnan Plain, respectively. The basic physicochemical properties of soil samples are presented in Table S1.

Three amendment materials,  $K_2SiO_3$  (Sinopharm, AR), rice husk biochar (RHB), and bentonite, were used in this trial at the application rate of 2 g/kg soil-based on our previously published work (Huang, *et al.* [37]). Fresh rice husk was collected from a rice mill in Wuhan, China. For RHB production, the rice husk was slowly pyrolyzed for 2 h in an electric furnace at 700 °C. RHB produced at high temperatures has better adsorption properties [38]. The bentonite was purchased from Xinyang, China. Physicochemical characteristics of RHB and bentonite are listed in Table S2.

### 2.2. Experimental design

To get multi-heavy metal-polluted soils for this experiment, the soils of YB and CA were firstly spiked with disodium hydrogen arsenate (200 mg/kg), lead acetate (1000 mg/kg), cadmium chloride (20 mg/kg), and zinc chloride (500 mg/kg). A plastic pot (10 cm × 10 cm) was filled with 5 kg of spiked soil. The soils were fertilized with 1.287 g urea (applied in three steps), 0.162 g ammonium dihydrogen phosphate, 0.555 g potassium sulfate per kg soil, and 1 mL of Arnon trace element solution [39] per kg soil. The treated soils with 80% field water capacity were then covered with a plastic membrane and aged for two months at 30-35 °C.

Five soil treatments were designed as follows: (1) Control (without amendment), (2) T1 (potassium silicate) (2 g/kg soil), (3) T2 (RHB 2 g/kg soil), (4) T3 (RHB 2 g/kg soil +Bentonite 1 g/kg soil), (5) T4 (RHB 2 g/kg soil +Bentonite 2 g/kg soil). The treatments were added to soils and mixed in the required doses. All treatments were replicated in different pots four times in each of the two soils (40 pots).

*Triticum aestivum* L. var. Zhengmai 9023, a common wheat species in the middle and lower reaches of the Yangtze River in China, was applied as the experimental subject. The disinfected seeds of wheat were evenly sown and germinated in unpolluted soils. Three weeks later, eight wheat seedlings of similar sizes were transplanted in each pot containing contaminated soil. Shoot samples were taken at seedling stages (71 days), while grain samples were taken at the maturity stage (125 days). After washing with distilled water, the harvested plants were separated. Part of the fresh samples was used for subcellular distribution and antioxidant systems analysis. The other part was dried overnight at 60 °C for plant dry weight and heavy metal concentration determination.

### 2.3. Analysis methods

#### 2.3.1. Soil analysis

After harvest, soil materials were separated and collected from roots and air-dried before analysis. The fractionation of soil heavy metals was analyzed by the improved BCR continuous extraction method [40]. Briefly, 1 g soil sample was put into a separate 50 mL centrifuge tube and equilibrated sequentially. Firstly, water-soluble and exchangeable metal, known as fraction 1 (F1), was extracted by shaking with 0.11 mol L<sup>-1</sup> acetic acid. Secondly, the remaining soil samples were extracted for reducible metal bound to Fe and Mn fraction (F2) by adding 20 mL 0.1 mol L<sup>-1</sup> hydroxylamine hydrochloride. Thirdly, oxidizable metal attached to organic matter and sulfides (F3)

was extracted by digesting soil residues with 8.8 mol L<sup>-1</sup> hydrogen peroxide and 1.0 mol L<sup>-1</sup> ammonium acetate. Finally, the final residual fraction (F4) was extracted with HCl-HNO<sub>3</sub>-HClO<sub>4</sub> acids. All the soil suspensions were centrifuged, filtered, and preserved at 4 °C for measurement, as described in 2.3.2.

### 2.3.2. Element analysis

Plant samples were digested after dried and ground using an HNO<sub>3</sub>/HClO<sub>4</sub> procedure. Cd, Pb, and Zn concentrations in the plant and soil samples were determined using an atomic absorption spectrophotometer (VarianAA240FS-VGA77) [41]. The content of As was measured by atomic fluorescence photometer (AFS-8220, Beijing Jitian Instruments Co., China), according to Qiao, *et al.* [42]).

### 2.3.3. Plant subcellular fractionation

After being harvested at the seedling stage, plants were washed and weighed for subcellular fractionation using the gradient centrifugation technique reported by Weigel and Jger [43]. The 0.5 g of fresh sample was taken with ceramic scissors and then immediately put into a pre-cooled (4 °C) glass mortar containing an extraction solution of 250 mM sucrose and 50 mM Tris-maleate (pH 7.8), 1mM magnesium chloride, and 10 mM cysteine. The homogenate was subjected to centrifugation at 6000 r/min for 30min, and the residue at the bottom was referred to as cell wall extract (E1). The supernatant was centrifuged again at 5500 r/min for 45 min, and the bottom debris was designated as the organelle fraction (E2). The resultant supernatant was named cytosol fraction (E3), including macromolecular organic matter and inorganic ions in the cytoplasm and vesicles. Heavy metal contents in E1-E3 were determined by the method in 2.3.2.

### 2.3.4. Determination of plant photosynthetic parameters

The photosynthetic parameters were determined by LI-6400XT photosynthetic analysis instrument (U.S.A. LI-COR) with effective light radiation of 1200 µmol/m<sup>2</sup>/s, the CO<sub>2</sub> concentration of 400 µmol/mol and leaf chamber temperature of 25°C. The leaves were rinsed one day before the measurements. Photosynthetic rate (Pn), Stomatal conductance (Gs), Intercellular CO<sub>2</sub> concentration (Ci), and transpiration rate (Tr) parameters were recorded at the exact position of the fully expanded upper leaves between 9:00 to 11:00 am [44]. The parameter recording was conducted for two consecutive days, and the average value was calculated.

### 2.3.5. Plant antioxidant system

To obtain enzyme extract, 0.5 g fresh leaf tissues were ground in a pre-cooled mortar containing 5 mL of phosphate buffer (pH 7.8) and some quartz. The enzyme extract was centrifuged at 11000 r/m for 15 min under 4 °C [45]. After the addition of nitro-blue tetrazolium (NBT), SOD activity was measured at 560 nm using a spectrophotometer, according to Beauchamp and Fridovich [46]. POD activity was assessed by monitoring guaiacol's oxidation, resulting in a decrease in absorbance (470 nm) according to the protocol of Zhou and Leul [47]. The disappearance of hydrogen peroxide was observed at 240 nm absorbance, and the chemical reaction of the enzyme extract with hydrogen peroxide was calculated to estimate the catalase (CAT) activity [48].

For pretreatments, 0.5 g leaf tissues were ground with 5 mL 10% (w/v) of trichloroacetic acid and centrifuged at 11000 r/m for 15 min under 4 °C. Ascorbic acid AsA content was assessed by the method of Mukherjee and Choudhuri [45]. Dinitrophenylhydrazine and thiourea were mixed with trichloroacetic acid extract, boiled, and added to sulfuric acid, and the absorbance was read at 530 nm out to estimate the AsA content. The content of glutathione GSH was determined according

to the method of Griffith [49]. Leaf tissue was extracted with trichloroacetic acid and mixed with dinitrothiocyanobenzene, and the change in absorbance was measured at 412 nm out. The extracts were heated by mixing the extracts with thiobarbituric acid, and the spectral luminosity measured at 532 nm was used to assess the malondialdehyde MDA content [50].

#### 2.4. Statistical analysis

The results were subjected to one-way ANOVA and underwent the Duncan test ( $P < 0.05$ ) by employing SPSS software (version 21). The data were expressed as the means  $\pm$  SE (standard error) with the average of four replicates adopted. Tables and figures were drawn by using Excel 2018 and Origin pro-2019.

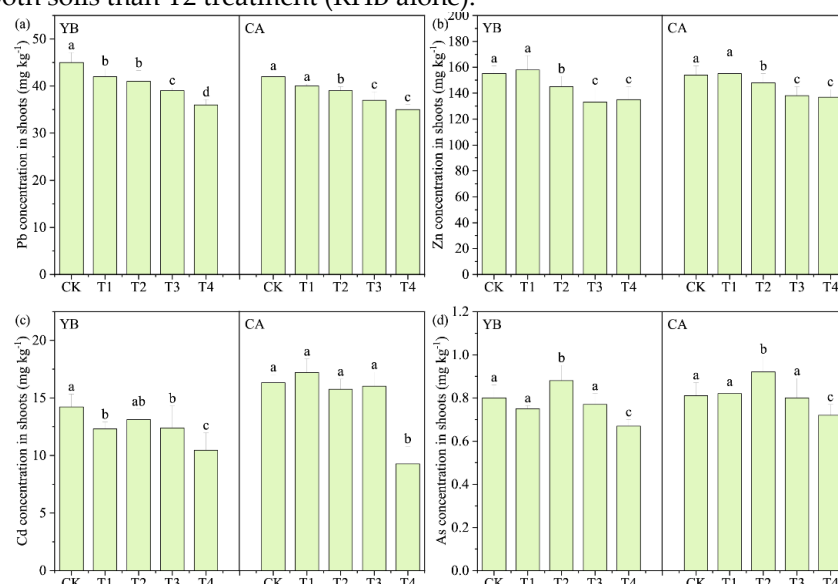
### 3. Results

#### 3.1. Growth and grain yield

As shown in Table S3, applying four Si-rich materials (T1-T4) at a 2 g/kg level resulted in a notable increase in wheat growth traits compared to the control. For example, T2 treatment (RHB 2 g/kg) improved plant biomass in the seedling and maturity stage by 19.2%, 10.8% for YB soil, and 8.9%, 3.1% for CA soil, compared to the control. The best growth results were obtained in T4 treatment. This optimal Si treatment (T4) exhibited significantly higher wheat growth efficiency in both soils with an increment of 26.8% (YB) and 10.3% (CA) in the seedling stage and 26.9% (YB) and 15.5% (CA) in the maturity stage.

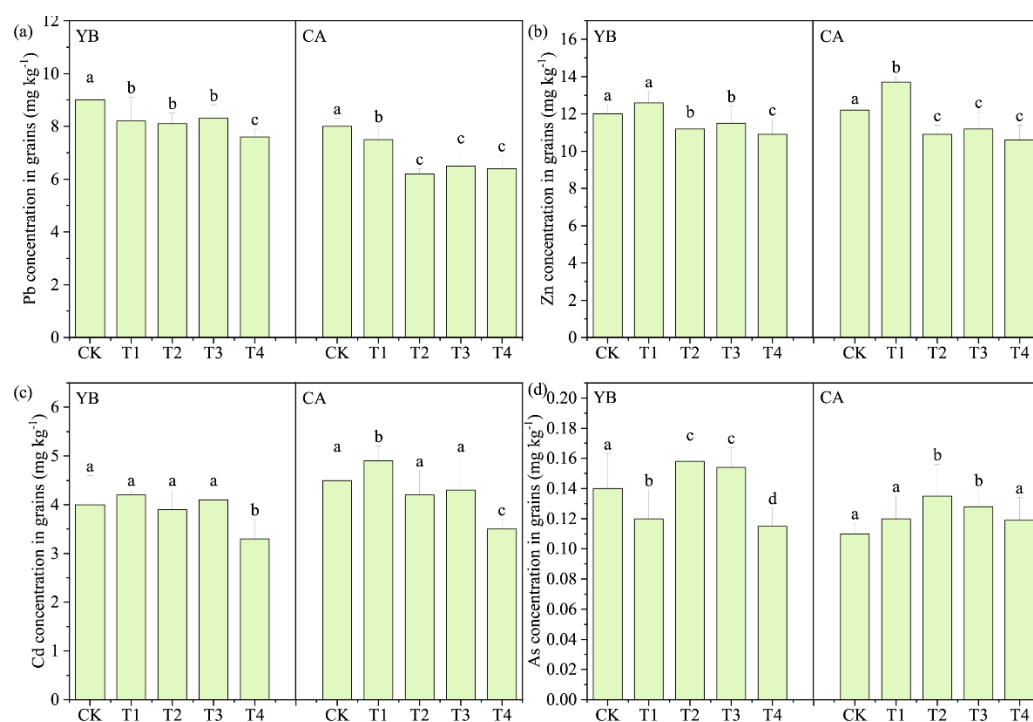
#### 3.2. Heavy metal accumulation in shoots and grains

With Si-rich amendments in both soils, heavy metal concentrations in wheat shoots and grains were significantly reduced, indicating that Si treatments could decrease the heavy metal accumulation (Figure 1, 2). All four treatments effectively reduced Pb concentrations in wheat shoots and grains (Figure 1a, 2a). Among these, T4 treatment (RHB 2 g/kg soil + Bentonite 2 g/kg soil) displayed the best effect as reducing by 20.1%, 16.7% (YB), and 15.6%, 19.8% (CA) in shoots and grains for two soils, respectively. Furthermore, T4 treatment led to 6.17% to 12.2% lower Pb concentration in both soils than T2 treatment (RHB alone).



**Figure 1.** Effects of Si-rich amendments (T1-T4) on heavy metal accumulation in wheat shoots in YB and CA soils. (a) Pb. (b) Zn. (c) Cd. (d) As. Different lower-case letters above the columns in the graph indicate a significant difference ( $p < 0.05$ ) between treatment groups.





**Figure 2** Effects of Si-rich amendments (T1-T4) on heavy metals accumulation in wheat grains in GB and CA soils. (a) Pb. (b) Zn. (c) Cd. (d) As. Different lower-case letters above the columns in the graph indicate a significant difference ( $p < 0.05$ ) between treatment groups.

In this experiment, all the treatments except T1 effectively reduced Zn content by 6.4% to 14.2% (YB) and 3.9% to 13.1% (CA) in wheat shoots and grains, respectively (Figure 1b, 2b). The addition of bentonite (T3 and T4) led to better results than RHB (T2) application alone, reducing wheat grain Zn content by 4.2% (T3), 9.1% (T4) for YB soil, and 8.1% (T3), 13.1% (T4) for CA soil, compared to that in control.

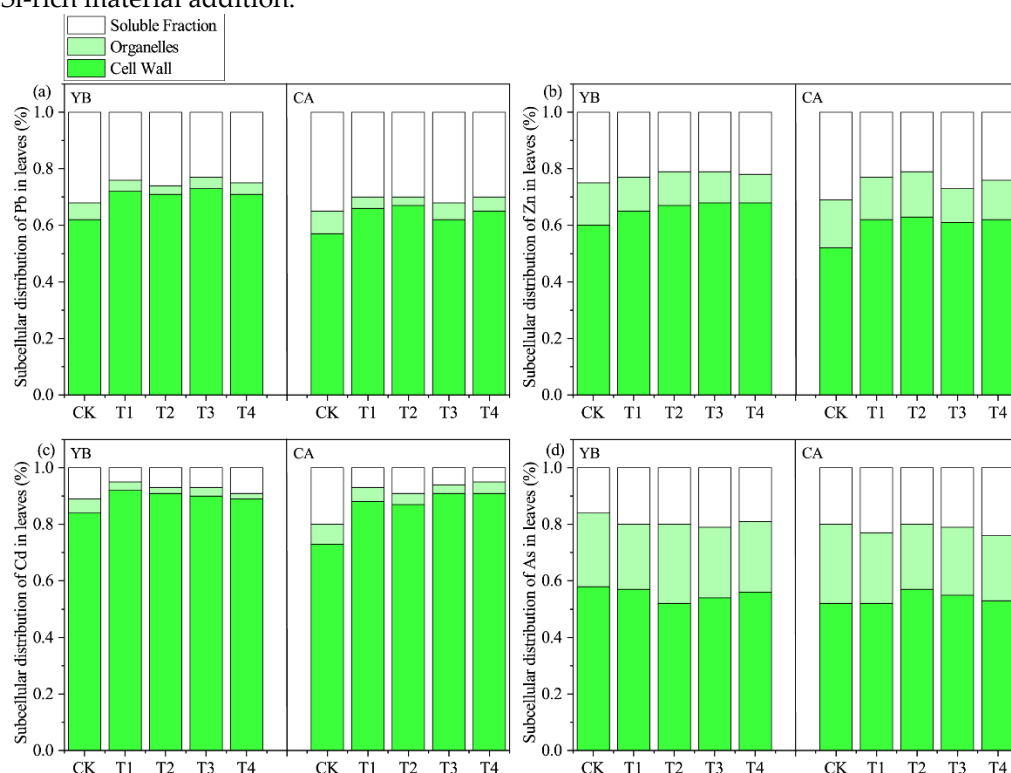
Cd content in wheat shoots was reduced by 13.4%, 12.9%, and 26.5% for yellow-brown soil (YB) after T1, T3, and T4, respectively (Figure 1c, 2c). In contrast, no significant reduction in Cd concentration was observed in wheat shoots for CA soil, except for T4, in which Cd concentration was reduced by 22.1%. Compared to control, the Cd content in wheat grains was decreased by 2.5% to 22.1%. However, there was no significant difference between the control and T1, T2, or T3.

As shown in Figure 1d and 2d, the content of As in wheat shoots and grains decreased with the addition of Si-rich materials to YB soil. For T4 treatment in YB soil, As content decreased by 16.2% and 17.8% in shoots and grains, respectively. However, there was very little change in wheat As accumulation in CA soil, and even a slight increase after RHB treatment (T2).

### 3.3. Subcellular distribution

In Figure 3, the heavy metal contents in subcellular fractions of wheat shoots showed a similar trend in YB and CA soils, with the highest heavy metal content observed in the soluble fraction, followed by the cell wall, and the lowest in the organelle. The addition of Si-rich soil amendments resulted in an increase in Cd, Zn, and Pb contents in the cell wall, and a reduction in the soluble fraction and the organelle. Compared to the control group, heavy metal contents in soluble fractions were decreased by 8.6%~28.1% (Pb), 8.2%~15.8% (Zn), and 18.1%~72.8% (Cd), respectively. Similarly, Pb, Zn, and Cd contents in organelle were decreased by 24.5%~61.8%, 5.8%~29.4%, and 28.5%~57.1%, respectively, while those in cell wall were elevated by 8.7%~17.3%, 8.5%~21.2%, and 6.0%~19.9%, respectively. However, the As

content in subcellular fractions of wheat shoots displayed no significant change after the Si-rich material addition.



**Figure 3** Contents of heavy metal in subcellular fractions of wheat leaves in YB and CA soils added with four amendments (T1-T4). (a) Pb. (b) Zn. (c) Cd. (d) As.

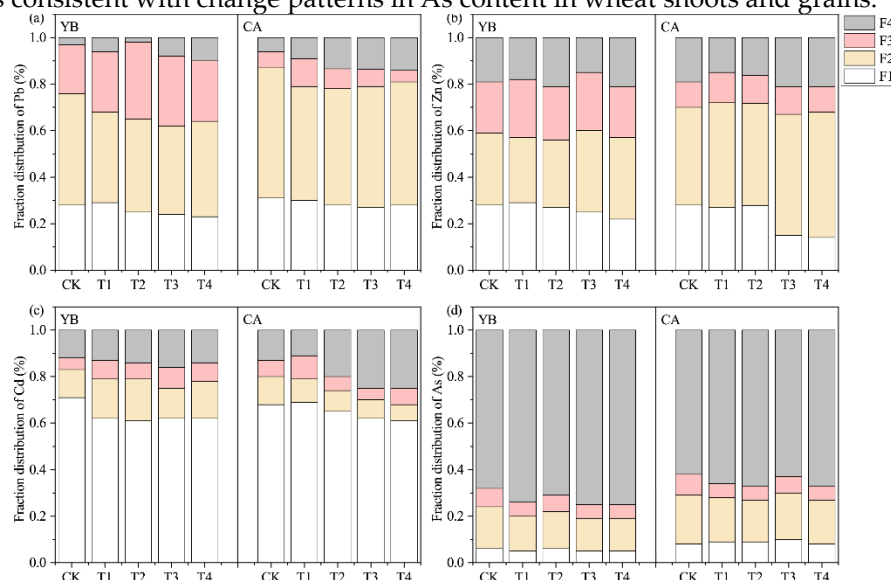
### 3.4. Photosynthetic parameters

The Si-rich soil amendments mitigated the adverse effect of multiple heavy metals on the photosynthetic parameters of wheat. Specifically, the Si-addition treatments increased Pn, Tr, and Gs values to different degrees, but the Ci value was not affected by any treatment (Figure S1, S2). In YB soil, the maximum increase in Pn, Tr, and Gs values among the four treatments was 42.24%, 31.4%, and 34.5%, respectively. In CA soil, the maximum increase in Pn, Tr, and Gs values in the four treatments was 5.5%, 38.1%, and 74.4%, respectively. Under heavy metal stress, the Si-rich soil amendment contributed to the improvement of photosynthetic parameters of wheat, which might be beneficial to wheat growth.

### 3.5. Soil heavy metal fractionation

In multiple heavy metal-contaminated soils, the application of soil amendments resulted in a significant reduction in the acid fraction of heavy metals in the soil, and an increase in the reduced, oxidized, and residual states to various degrees (Figure 4). In YB soil, all four heavy metal distributions displayed a gradual shift from the acid fraction (F1) to the reduced (F2), oxidized (F3), and residual states (F4) after applying soil conditioners. Under different treatments (T1-T4), the reducible fraction exhibited a higher Pb and Zn content than the other fractions. Particularly, more Pb and Zn in the reducible fraction were found in T3 and T4 than in T1 and T2. Meanwhile, the acid fraction was reduced by 10.7%-16.8% and 12.7%-21.4% in four treatments, respectively. Moreover, T4 showed a greater shift from an acid fraction (F1) to other fractions (F2, F3, and F4) than other treatments. The addition of T4 increased the residual Pb, Zn, Cd, and As (F4) by 231%, 10.5%, 17.1%, and 10.3%, respectively, compared with the control group. CA soil showed a consistent change in heavy metal fractions (except As) with YB soil. The four treatments decreased heavy metal acid fraction by 3.2% to 12.9% (Pb),

0.7% to 52.0% (Zn), and 4.4% to 10.3% (Cd), respectively. The changes in As fraction in the CA soil after four treatments were not significant compared to the control, which was consistent with change patterns in As content in wheat shoots and grains.

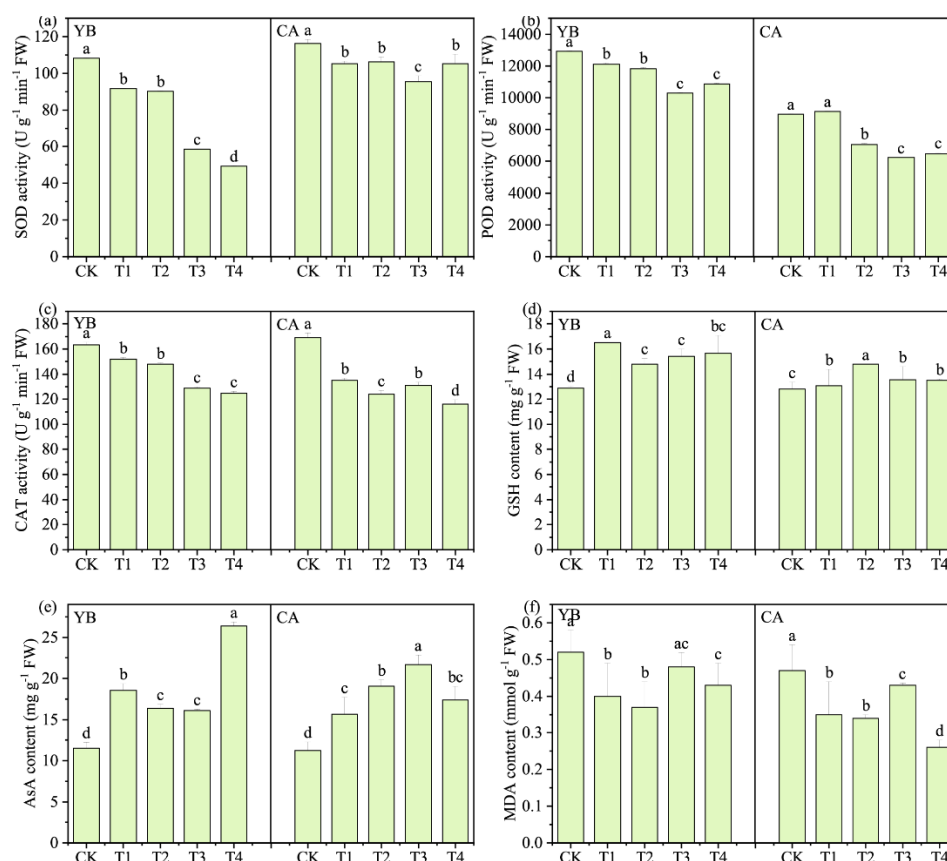


**Figure 4** Relative distribution of heavy metal soil-phase fractions after the applications of Si-rich amendments during wheat growth in YB and CA soils. F1 represents acid fraction metals; F2 represents reducible metals; F3 represents oxidizable metals; F4, residual fractions. (a) Pb. (b) Zn. (c) Cd. (d) As.

### 3.6. Antioxidant system

Figure 5 represents the effect of the treatments on the antioxidant system indicators of wheat shoots. In this antioxidant system, the antioxidant enzymes SOD, POD, and CAT activities were reduced to different degrees in both soils (Figure 5 a, b, c). For YB soil, the most significant effect was observed in T4, where SOD, POD, and CAT activities were reduced by 54.3%, 15.8%, and 23.4%, respectively, compared to the control, indicating that T4 could relieve effectively heavy metal stress. In CA soil, T3 and T4 showed better metal alleviation effects in POD and CAT activities than T1 and T2.





**Figure 5** Activities of (a) SOD, (b) POD, (c) CAT and contents of (d) GSH, (e) AsA, (f) MDA in wheat leaves in YB and CA soil with Si-rich amendments. Different lower-case letters above the columns in the graph indicate a significant difference ( $p < 0.05$ ) between treatment groups.

Our results of non-enzymatic antioxidants showed that in wheat shoots in the amended soils, the levels of GSH and AsA were increased (Figure 5d, e). In contrast, the MDA level was decreased (Figure 5f) compared to the control. T4 had the most significant effect in YB soil, with a 129.3% and 21.4% increase in AsA and GSH, respectively, and a 17.3% decrease in MDA content. In CA soil, T2 and T3 had a more significant effect on AsA, GSH and MDA. Compared to the control, the level of AsA and GSH increase of 70.0% and 15.5% (T2), and 93.0% and 5.9% (T3), and MDA content decrease of 28.8% (T2) and 8.5% (T3).

#### 4. Discussion

Cd, Zn, Pb, and As are widely distributed persistent pollutants, and their accumulation in plants affects morphological and physiological performances and reduces their production [17,51]. In this study, the addition of Si-rich materials to soils contaminated with multiple heavy metals positively affected wheat growth. Recently, soil amendments made of biomass-derived waste materials have gained broad interest due to their several abilities, such as improving soil fertility [52], up-regulating antioxidant defense system [33], and immobilizing soil pollutants [53,54]. Hence, these bio-amendments can be used to limit the mobility and toxicity of contaminants [8,13] to promote plant growth [32].

##### 4.1. Heavy metal accumulation

In the current study, all four treatments of Si-rich amendments effectively reduced heavy metal contents in wheat shoots and grains (Figure 1, 2). Our results are aligned with the previous findings in wheat [26], rice [8,20], pigeon pea [55], and palm [56]. The presence of multiple heavy metals in the soil aggravates soil acidification

[5] stimulates the uptake of heavy metals [57], resulting in serious oxidative damage [58]. The toxicity between metal elements often has additive and synergistic effects, making the management of soil combined heavy metal pollution difficult [59]. All the Si-rich amendments in this experiment were able to reduce the concentrations of all the heavy metals except As in wheat in both soils, indicating that the Si-rich materials in this experiment were able to restrain both the transfer and uptake of complex heavy metals in wheat. The treatment of RHB in combination with bentonite simultaneously lowers the contents of Cd, Zn, Pb, and As in wheat shoots and grains in YB soil. Saffari [60]) has reported that rice husk biochar can effectively reduce the bio effectiveness of heavy metals except for Cr in composite heavy metal-contaminated soil by the mechanism that rice husk biochar has high pH, large specific surface area, and cation exchange capacity, which further enhances the adsorption effect of surface complexation and ion exchange.

The cell wall is considered the first and most effective shield for heavy metal-affected plants to prevent heavy metal from flowing into intracellular sites [61]. The polysaccharide fractions in the cell wall contain many functional groups (including -COOH and -SH), which can effectively bind metal cations and restrain their transfer [17]. The experimental results showed that all four treatments decreased Pb, Zn, Cd, and As contents in cell soluble fraction (including cytoplasm and cytosol) in YB soil (Figure 3), which is consistent with the reduction of heavy metals in whole plants (Figure 1, 2). Luyckx, *et al.* [62]) have revealed that Si treatment could alter cell wall metabolism to increase plant tissue extensibility and promote cell wall sequestration of heavy metals. A similar finding was also illustrated in the study of Howladar, Al-Robai, Al-Zahrani, Howladar and Aldhebiani [26]). Our results also indicated that the concentration of Cd and As in the cell wall exhibited slight variation in CA soil, which might explain the little concentration change of Cd and As in wheat shoots and grains.

Our data showed that the supplementation of Si-rich amendments changed the soil morphology with acid fraction reduction, and reduction, oxidation, and residue fractions increased to various degrees (Figure 4). Si has been reported to reduce the exchangeable state of heavy metals in soil, alleviating heavy metal toxicity [17]. Meanwhile, RHB has special carbonaceous properties and unique functional groups (organic hydroxyl, phenolic, and carboxyl groups), increasing heavy metal adsorption on the RHB surface and reducing heavy metal bio effectiveness [36]. Besides, RHB application reduces the contents of organic bound As and ferromanganese oxidized As in the soil, suggesting that these As forms were well associated with soil extractable As concentrations [31]. On the other hand, bentonite is also commonly used as a soil heavy metal passivator with high porosity and small particle size, and it has been confirmed to be competent in reducing the exchangeable state of heavy metals in soil [63-65]. Rizwan, Ali, Qayyum, Ibrahim, Zia-ur-Rehman, Abbas and Ok [11]) have suggested that soil type influences the absorption of heavy metals in plants; however, in our results, the combination of RHB and bentonite as remediation agents in both different soils effectively increased heavy metal accumulation in cell walls and reduced the available fraction of soil heavy metals. After that, our remediation agent has great application potential for decreasing the heavy metal content in wheat shoot and grain.

#### 4.2. Plant growth

Leaf chlorosis and plant stunting are the most common toxic symptoms induced by heavy metals [66,67]. Our experiments indicated that the Si-rich amendments enhanced wheat plant biomass and yield (Table S1), mirroring previous observations [21,27,34,44]. These enhancements may be attributed to several factors, e.g., the decrease in the heavy metal availability in plants and the porous structure of RHB, which retains nutrients, increases water holding capacity, total nitrogen, and other cation concentrations [34,52,68]. Moreover, RHB contains many plant nutrients such as Si and K, enhancing soil biophysical properties and promoting plant growth [32,33].

#### 4.3. Plant photosynthesis

One of the significant consequences of heavy metal toxicity is to affect plant photosynthesis, which provides an interaction between the plant's internal metabolism and the external environment, and changes in photosynthesis can reflect environmental stress [69,70]. In this experiment, the addition of Si-rich amendments significantly enhanced photosynthesis parameters in wheat and recovered the reduced photosynthetic rate ( $P_n$ ) and stomatal activity ( $G_s$ ) caused by heavy metal stress (Figure S1, 2), which contributed to maintaining plant growth. The previous studies of Si-rich amendment application in other crops under heavy metal stress have been reported similar results [71,72]. This may be since Si increases the synthesis of photosynthetic products and eventually sustains plant growth by reconstructing the damaged chloroplast ultrastructure, reorganizing cysts and stroma, increasing chloroplast size and cell membrane fluidity, and preventing chlorophyll degradation, thus counteracting the damaging effects of heavy metals to some extent [73]. Furthermore, Si can promote protein biosynthesis in chloroplasts and induce the accumulation of photosynthesis-related proteins that can mediate electron transfer. In addition, Si can act as photoreceptors to enhance photosynthesis under heavy metal stress [71,74]. As a notable fact, the effect of RHB is similar to that of Si, indicating that Si from RHB can also be effectively utilized by plants [33].

#### 4.4. Antioxidant indicators

The increase in wheat yield and photosynthesis rate may also be related to mitigating heavy metal antioxidant damage to wheat by soil amendments. MDA is a lipid peroxidation product, representing the degree of heavy metal toxicity on wheat [75]. In this study, MDA content in wheat leaves treated with Si-rich amendments was lower than in control, which might be attributed to the alleviated heavy metal stress (Figure 5). Our results agreed with the previous report that the application of rice straw biochar reduced MDA and  $H_2O_2$  concentrations in wheat [76]. SOD, POD, and CAT are antioxidant enzymes and non-enzymes that scavenge reactive oxygen species (ROS) from plants; thus, they are also important indicators of whether plants are subjected to external stress [22,70]. In the present study, the application of soil remediation agents reduced the activities of SOD, CAT, and POD in wheat (Figure 5a, b, c), which coincides with the findings of Kiran and Prasad [36]. Rizwan, Ali, Abbas, Adrees, Zia-ur-Rehman, Ibrahim, Abbas, Qayyum and Nawaz [8] have also demonstrated that biochar down-regulates the enzymatic activities of antioxidant systems. Likewise, Ascorbic acid (AsA) and glutathione (GSH) can scavenge ROS from plants, and heavy metal stress leads to a decrease in AsA and GSH levels in plants [75]. In the present study, AsA and GSH contents increased to different degrees after applying soil remediation agents (Figure 5). This is consistent with the investigations of Mehmood, Saeed, Rizwan, Khan, Aziz, Bashir, Ibrahim, Ditta, Akmal, Mumtaz, Ahmed, Irshad, Imtiaz, Tu and Shaheen [15]) and Howladar, Al-Robai, Al-Zahrani, Howladar and Aldhebiani [26]). The above results suggest that the Si-rich soil amendments in this study can effectively diminish heavy metal toxicity in plants and regulate plant antioxidant enzymes and non-enzymatic systems.

#### 4.5. Detoxification mechanism

The Si-rich materials used in this experiment reduced the toxicity of four heavy metals to wheat through several mechanisms (Figure 6). Firstly, RHB treatment reduced Pb, Zn and Cd levels in wheat grains to different degrees, but there was a promotion effect on As accumulation in wheat. This may be due to the fact that RHB increased the doses of iron-reducing bacteria in the soil, which enhanced the mobility of As in the soil through reductive dissolution [77]. After adding a sufficient amount of bentonite (T4), the As content in wheat grains decreased. This is probably caused by

the adsorption of negatively charged arsenite/arsenate on the positive charge surface of bentonite [78]. Secondly, Si in the treatments contributed to the formation of Si-heavy metals co-precipitation and helped in the thickness of the cell wall to impede the transport of heavy metals [79]. Thirdly, with a strong adsorption capacity for metal cations, Si-rich materials reduced the mobile form of Pb, Zn, and Cd in the soil. Fourthly, silicon activated the antioxidant enzyme system in wheat and reduced the oxidative damage caused by heavy metals. Finally, RHB is rich in Si, N, P, K, and other nutrients, improving crop photosynthesis and promoting wheat growth compared to silicate.

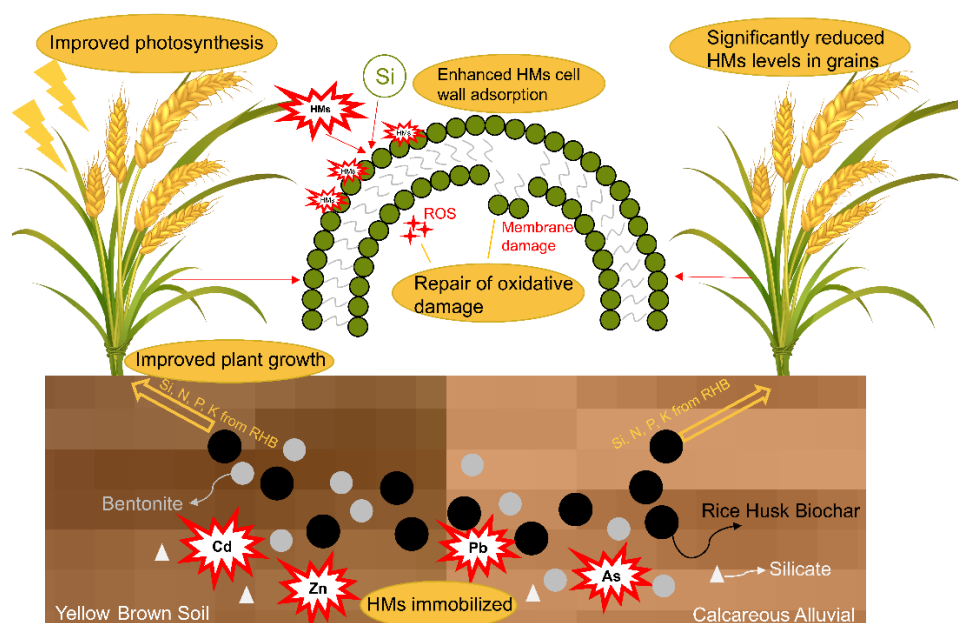


Figure 6 Detoxification mechanism of wheat in heavy metal contaminated soil by Si-rich materials

## 5. Conclusion

In this study, three silicon-rich materials, silicate, RHB, and bentonite, were applied to two soils contaminated with Cd, Zn, Pb, and As to investigate their potential effects on wheat growth and heavy metal accumulation. The results showed that applying Si-rich materials into the soil could be a practical method to reduce the uptake of combined heavy metals by wheat, thus mitigating the toxicity of heavy metals on wheat. The combined application of RHB and bentonite significantly improved plant growth, grain yield, photosynthetic parameters and reduce heavy metal concentration in plants and cell soluble fractions. Therefore, from an environmental perspective, the recycling of RHB is a promising approach for improving plant productivity. Moreover, the combined application of RHB and bentonite can effectively alleviate the multiple heavy metal-induced stress on wheat growth, which will be a good choice for heavy metal remediation in agricultural fields.

**Conflict of Interests:** The authors declare no conflict of interest.

**Acknowledgments:** This work was sponsored by the Hubei Major Project of Technique Innovation (2017ABA154) the National Key Technology Research & Development Program (2015BAD05B02).

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