Influence of selenium combined with rice straw on the inhibition of copper phytotoxicity to pak choi

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1. Introduction

Cu is an essential micronutrient for most biological organisms [1]. It serves as co-factor for a large array of proteins involved in diverse physiological processes in plants, such as: photosynthesis, the electron-transport chain, respiration, cell-wall metabolism, and hormone signaling at low concentrations [1]. Excess Cu is cytotoxic due to its role in the catalysis of reactions that generate reactive oxygen species, ultimately leading to increased oxidative stress in plants [1]. Cu ions enter agricultural soils through anthropogenic activities, such as metalliferous mining, pesticides, fertilizer, and sewage irrigation [2]. Cu ions are taken up by plant roots and transported to edible plant parts, induce detrimental effects on plant growth and productivity, and then pose a potential threat to human health [5]. Therefore, to reduce toxic Cu concentrations and promote agroenvironmental sustainability and food safety, feasible countermeasures for the remediation of Cu-contaminated farmlands are urgently needed.

Over the past several decades, techniques such as soil washing [6], low-temperature thermal desorption [7], and phytoremediation [8] have been applied to the treatment of heavy metals (HMs)-polluted soils. Although soil washing method can remove soluble and exchangeable HMs from polluted soils, it can also remove essential soil elements [9]. The soil disturbance associated with soil washing also needs to be considered. The effects of thermal treatment on soil properties require careful balance. Therefore, other approaches that can maintain essential soil elements while reducing Cu accumulation in plants need to be identified.

Se is an essential micronutrient for humans and animals [10]. It is predominantly obtained by the consumption of cereals, vegetables, meat, and fish [10]. Recently, the Se application in soils has been confirmed as an effective strategy for mitigating HMs accumulation and the deleterious effects on plants [11,12]. Many studies have shown that Se can exert an antagonistic effect with HMs. Therefore, it alleviates the toxic effects of HMs on plants by restoring damaged cell membranes and rebuilding chloroplasts [13], improving antioxidant ability [14], forming macromolecular complexes with HMs, and promoting the removal of free radicals produced by HMs [15]. The antagonism or detoxification between Se and Pb, Cd, As, and Hg were also reported [16]. However, little is known regarding the effect of Se application to alleviate Cu accumulation in plants. The heavy metals pollution combined with metal cations such as Cu and Cd; Cu, Pb, and Cd; and Pb and Cd were studied, which showed that Cu exist antagonism effects with Pb and Cd [17,18]. But the effects between Cu and anion were less reported. Based on the biochemical function of Se, Se application at appropriate dosages may hypothesized that it plays the important role in limiting mobility, bioavailability, and toxicity of Cu in soils.
Rice straw (RS) is the main organic matter (OM) that is available for most rice farmers and serves as an important source of K [19]. Many publications have demonstrated the role of soluble and insoluble OM in the mobility and bioavailability of various HMs [20,21]. On the one hand, OM can enhance the complexation, sorption, and precipitation of trace HMs in soils and thus reduce their mobility [22]. On the other hand, it can enrich soil solution with organic chemicals that may act as chelates and enhance the bioavailability of trace HMs [23]. Moreover, the organic chemicals in soils were affected directly and indirectly by the Se application. The transfer of Se from the soils to plants changed the root exudates or modified the microbial products in rhizosphere [24,25]. Nevertheless, these studies only evaluated the effect of Se application on naturally occurring OM in the soils. Thus, it did not provide sufficient information on the potential mechanism through which Se combined with different OM content (through adding RS) on the mobility and bioavailability of Cu in soils. We propose that Se combined with RS can inhibit Cu phytotoxicity better than that of exogenous-only Se treatments.

Total soil HMs contents do not accurately indicate the possible HMs transfer from soils to plants [26]. The uptake and accumulation of HMs by plants highly depend on their bioavailability in soils [27]. The sequential-extraction technique provides basic information on HMs fractions and the actual and potential transport among different chemical forms [28–30]. These fractions possess different abilities to either retain or release Cu, thereby influencing the mobility and bioavailability of Cu in soils [31]. Therefore, studying the transformation of fractions in soils is necessary to evaluate the bioavailability of Cu in soils.

The present study aimed to investigate the effects of the Se application combined with RS on the inhibition of Cu bioavailability in plants based on the determination of total Cu concentrations in plants and the distribution of Cu fractions in soils. The Cu fractions transformation in soils is a good indicator to assess the limitations of the absorption and bioaccumulation of Cu in plants. The objectives were as follows: 1) clarify the role of exogenous Se combined with RS application on Cu uptake and translocation in plants; 2) explore the inhibition mechanism of Cu phytotoxicity from the changes in Cu fractions in soils; and 3) determine the appropriate concentration of Se combined with RS that significantly prevented the Cu uptake by plants.

2. Materials and Methods

The detailed treatment plan of the study is as follows:

Step 1: Preparing experimental materials: collecting experimental soils; homogenizing soils; determining basic physicochemical properties of soils.

Step 2: Pot experiments: preparing 81 plastic pots containing 2.5 kg of soil; adding chemicals (Se and Cu) with different concentrations and RS with different contents; mixing basal fertilizer; keeping soil moisture at 70%; harvest.

Step 3: Preparing sample: collecting soil samples (before planting and after harvest); collecting pak choi samples (separate shoots and roots).

Step 4: Chemical analysis: Determining Cu concentrations in soils and Cu fractions in soils; determining Cu concentrations in plants.

2.1 Experimental Materials

Agricultural soils were collected at a depth of 0 cm to 20 cm at the Chom Sao area, Hung Dinh commune, Thuan An town, Binh Duong Province, Vietnam, according to Environmental Quality Standard of Vietnam (TCVN 5297:1995). Soil samples were completely air dried at room temperature, homogenized, and passed through a 5 mm sieve. The experimental soil is clay loam soil that basic physicochemical properties were as follows: proportion 3.09 g/cm³; density 1.15 g/cm³; humidity 21.1%; pH₄Cl 3.8; pH₂H₂O 3.7; total N 0.132%; total P₂O₅ 0.032%; total organic carbon 4.07%; total Se 0.31 mg/kg; and total Cu 5.1 mg/kg. These soil properties were determined according to the procedures described by Bao [32]. All chemicals were purchased from a reagent factory in Tianjin, China.

2.2 Pot experiments

The dosages of chemistry exposure to soil samples in this study were set as 0, 1.0, and 2.5 mg/kg soil for Se (added as Na₂SeO₃) and 0, 50, and 200 mg/kg soil for Cu (added as CuSO₄·5H₂O) [33]. RS was also added to each pot at 0, 10, and 20 g/kg soil. One treatment without Se, Cu and RS was prepared and set as the control treatment. This experiment had a completely randomized design with three replicates, including a total of 81 pots for 27 treatments (Table 1).

Different concentrations of Se or Cu-spiked solutions were aspirated on dry soils by using a plastic nebulizer. After the soils were homogenized and equilibrated for 30 days, basal fertilizer comprising 0.15 g/kg N (urea, AR) and 0.033 g/kg P (monopotassium phosphate, AR) were thoroughly mixed in a plastic pot (diameter: 18 cm; height: 15 cm) containing 2.5 kg of the equilibrated soil. Soils moisture content was kept at approximately 70% water-holding capacity within the equilibrating period. Pak choi seeds were sowed in each pot, and the seedlings were thinned to five in each pot after 10 days of germination. The plants were grown in pots in a greenhouse and watered periodically to keep soil moisture at 70% of
the field capacity. The plants grow at temperatures between 24°C and 29°C during the day and 16°C to 24°C at night. Plants were harvested after 38 days.

### 2.3 Sample preparation

Soil samples were collected from each pot before planting and after harvest. Then, the soil samples were placed in sealed polyethylene bags to prevent cross-pollination, completely air dried at room temperature, homogenized, and passed through a 100-mesh sieve (0.15 mm) for chemical analyses of total Cu concentrations and Cu fractions.

Pak choi samples were thoroughly washed with deionized water, and their shoots and roots were separated. The samples were oven-dried at 90°C for 30 min and kept at constant weight at 50°C. The dried samples were ground into fine powder and then stored in a dark room at room temperature.

### 2.4 Chemical analysis

#### 2.4.1 Determination of Cu concentration in plants and soils

Plant samples were digested using 4:1 (v/v) HNO$_3$–HClO$_4$, whereas soil samples for determining Cu concentrations were digested with an oxidative acid mixture of 3:1 (v/v) HNO$_3$–HClO$_4$ at 160°C. In a typical procedure, 0.5 g of each sample was precisely weighed in a 100 mL glass tube. HNO$_3$ and HClO$_4$ with a combined volume of 10 mL were added and kept overnight at room temperature. Acid digestion was conducted in an automatic temperature-controlled furnace until the digestion solution became clear. After acid digestion, the sample solutions were cooled and diluted with deionized water in a glass tube. Cu concentration in the digestion solution was determined with an atomic absorption spectroscopy (AAS AA-7000, Shimadzu Corporation, Kyoto, Japan) system according to the Standard Method TCVN 6496:2009 developed by the Ministry of Natural Resources and Environment of Vietnam. The specs of AAS used for chemical analysis: lamp intensity 6 mA; wavelength 324.8 nm; slit width 0.5 nm; compressed air and acetylene; lamp type BGC – D2.

#### 2.4.2 Determination of Cu fractions in soils

Cu fractions analysis applied the five-step sequential extraction method described by Hu et al. [33]. According to this method, Cu fractions were divided into exchangeable, bound to carbonates, bound to Fe–Mn oxidates, bound to OM, and residual fractions.

1. Exchangeable fraction (EXC-Cu): 0.1 mol L$^{-1}$ NH$_4$HAC 25°C, shaken for 2 h, liquid/soil = 10:1;
2. Bound to carbonates fraction (CAB-Cu): 1 mol L$^{-1}$ NaAc 25°C, shaken for 2 h, liquid/soil = 10:1;
3. Bound to Fe–Mn oxidates fraction (FEM-Cu): 0.1 mol L$^{-1}$ NH$_2$OH + 0.01 mol L$^{-1}$ HCl 25°C, shaken for 0.5 h, liquid/soil = 10:1;
4. Bound to OM fraction (OM-Cu): 0.01 mol L$^{-1}$ HNO$_3$, 30% H$_2$O$_2$ heated for 2 h in 85°C, shaken intermittently, liquid/soil = 10:1;
5. Residual fraction (RES-Cu): 15 mL of HNO$_3$, 5 mL of HF, 5 mL of HClO$_4$ heated to 300°C for 2 h, until the solutions became clear.

The extracted supernatant was analyzed by AAS.

### 2.5 Quality control

Different quality-assurance and quality-control measures were included in the sample preparation and chemical analyses, including the use of certified reference materials for instrumental calibration, determination of the method detection limit, and analyses of reagent blanks, sample duplicates, and spiked samples. The certified reference materials analyzed along with each batch of samples in this study were QCVN 03-MT:2015/BTNMT (agricultural land, Vietnam) from the Vietnamese national standard reference material. The measured Cu concentration for QCVN 03-MT:2015/BTNMT was 100 mg/kg.

### 2.6 Statistical data analysis

Data were subjected to statistical analysis using SPSS 20.0 software. All results are presented as the mean ± standard deviation of three replicates. This study used Dunnnett's multiple comparison test of one-way ANOVA. For all tests, $P < 0.05$ was considered as a significant difference.
The bioconcentration factors (BCFs) and the translocation factors (TFs) are two indicators, which were used to describe the uptake capacity of Cu from soil to shoot and the translocation of Cu from root to shoot [34]. BCF and TF are defined as:

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BCFs = \frac{\text{total Cu concentration in pak choi shoot}}{\text{total Cu concentration in soil}}
\]

\[
TFs = \frac{\text{total Cu concentration in shoot}}{\text{total Cu concentration in root}}
\]

3. Results

3.1 Effect of Se combined with RS on pak choi growth under Cu stress

For single Cu treatments, at low Cu concentration 50 mg/kg, the shoot and root dry weight of pak choi slightly increased by 16.8% and 16.7%, compared with the control treatment (Figure 1). Conversely, the shoot and root dry weight of pak choi decreased by 28.6% and 27.1% at high Cu concentration 200 mg/kg, compared with Cu treatment at 50 mg/kg. These results suggested that at high concentrations (exceeding the standard) Cu inhibited the growth of pak choi.

For Cu and Se co-exposure treatments, at high Cu concentration (200 mg/kg), the shoot and root dry weight of pak choi dramatically increased by 20.7%–40.3% and 21.0%–41.0% (\(P < 0.05\)), compared with single Cu treatments (Figure 1). At low Cu concentration (50 mg/kg), no significant differences (\(P > 0.05\)) was found for the shoot and root dry weight of pak choi, compared with single Cu treatments. These results indicated that the supplementation of appropriate Se concentration can alleviate Cu toxicity with concentration exceeding the standard and thus enhancing the growth of pak choi.

For Cu and RS co-exposure treatments, with both RS contents at 10 and 20 g/kg, the shoot dry weight of pak choi no significantly increased by 2.4%–6.7% and 4.8%–13.3% (\(P > 0.05\)), compared with single Cu treatments (Figure 1). The root dry weight significantly increased to 16.7%–30.0% with high RS content (20 g/kg) (\(P < 0.05\)), compared with single Cu treatments.

![Figure 1](#). The dry weight of pak choi tissues: shoot (A), root (B). Data are presented as means ± SD (\(n = 3\)). Small stars indicate significant difference from no rice straw treatments (for all treatments), big stars indicate significant difference from no Se treatments (for only Cu-Se co-exposure treatments) (One-way ANOVA, followed by Dunnett’s test, *\(P < 0.05\)).
For Cu-Se and RS co-exposure treatments, the dramatic increase of the shoot and root dry weight of pak choi (21.1%–25.0%, \( P < 0.05 \)) at 50 mg/kg Cu and 2.5 mg/kg Se were observed, especially with high RS content (20 g/kg), compared with co-exposed Cu–Se treatments (Figure 1). At high Cu concentration (200 mg/kg), no significant increase (\( P > 0.05 \)) was found for the shoot and root dry weight of pak choi, compared with co-exposed Cu–Se treatments. These results indicated that the supplementation of RS in Cu–Se co-exposure treatments enhanced the growth of pak choi compared with only Cu–Se co-exposure treatments.

### 3.2 Effects of Se combined with RS on Cu bioavailability in soils

For single Cu treatments, the Cu concentrations in shoot and root reached up to 42.2 and 284.3 mg/kg at high Cu concentration (200 mg/kg), which increased to 5.9- and 11.1-fold compared with the control treatment (Figure 2).

![Figure 2](image_url)

**Figure 2.** The concentrations of Cu in pak choi tissues: in shoot (A), in root (B). Data are presented as means ± SD (\( n = 3 \)). Small stars indicate significant difference from no rice straw treatments (for all treatments), big stars indicate significant difference from no Se treatments (for only Cu-Se co-exposure treatments) (One-way ANOVA, followed by Dunnett’s test, * \( P < 0.05 \)).

For Cu and Se co-exposure treatments, at low Cu concentration (50 mg/kg), Se significantly increased Cu bioavailability in soils (\( P < 0.05 \)) that the Cu concentrations in shoot and root of pak choi dramatically increased by 22.8%–40.2% and 33.5%–37.2%, compared with single Cu treatments (Figure 2). At high Cu concentration (200 mg/kg), Se can inhibit the Cu phytotoxicity of pak choi. The dramatic decrease of the Cu concentrations (\( P < 0.05 \)) in the pak choi root (31.7%–56.2%) was higher than that in the pak choi shoot (18.2%–30.3%), compared with single Cu treatments. And a sharp decline in the Cu concentration was found at the highest Se concentration (2.5 mg/kg) treatments. All these results revealed that the Se application of appropriate concentration (2.5 mg/kg) was an easy way to inhibit Cu excessive accumulation in pak choi.

For Cu and RS co-exposure treatments, at low Cu treatment (50 mg/kg), the Cu concentrations in shoot and root of pak choi dramatically increased (\( P < 0.05 \)) by
39.7% and 64.3% with low RS contents (10 g/kg); the Cu concentrations in shoot and root of pak choi no significantly increased ($P > 0.05$) by 29.2% and 32.8% with high RS contents (20 g/kg); compared with single Cu treatments (Figure 2). At high Cu treatment (200 mg/kg), the significant decrease ($P < 0.05$) in the Cu concentrations in shoot and root of pak choi were found; indicating that added RS decreased the bioavailability Cu at concentrations exceeding the standard.

For Cu–Se and RS co-exposure treatments, at low Cu concentration (50 mg/kg), the Cu concentrations in shoot and root of pak choi significantly increased ($P < 0.05$) by 21.7%–29.8% and 39.9%–43.1%, with low RS content at 10 g/kg; conversely, the Cu concentrations in shoot and root of pak choi no significantly decreased ($P > 0.05$) with high RS content at 20 g/kg, compared with co-exposed Cu–Se treatments (Figure 2). In particular, in Cu-treated soils at 200 mg/kg, the Cu concentrations in shoot and root of pak choi dramatically decreased ($P < 0.05$) from 12.5% to 27.6% and 19.4% to 35.3%, compared with co-exposed Cu–Se treatments, and a significantly higher value was observed at RS 20 g/kg. These findings suggested that RS and Se co-exposure can significantly inhibit Cu bioavailability in soils.

### 3.3 Effects of Se combined with RS on the Cu bioconcentration and translocation factors

For single Cu treatments, the BCFs of Cu significantly increased by 70.8%–262.5% upon increasing the concentration of Cu in soils, while the TFs of Cu significantly decreased by 20.1%–47.2%, compared with control treatment (Figure 3).

For Cu and Se co-exposure treatments, at low Cu concentration (50 mg/kg), the BCFs of Cu significantly increased by 24.4%–48.8%, but the TFs of Cu did not significantly change, compared with single Cu treatments (Figure 3). At high Cu concentration (200 mg/kg), the BCFs of Cu decreased by 8.0%–14.9%; on contrary, the TFs of Cu significantly increased by 19.7%–59.1%, compared with single Cu treatments. These results suggested that Se promotes the Cu accumulation in pak choi shoot at low concentration or Se greatly inhibited Cu to enter pak choi shoot at high concentration, but Se did not change the transfer efficiency from root to shoot.

For Cu–Se and RS co-exposure treatments, at low Cu concentration (50 mg/kg), the TFs of Cu slightly decreased by 7.2%–15.0%; the BCFs of Cu increased by 15.7%–26.8% with low RS content (10 g/kg), conversely, BCFs of Cu showed no significant change with high RS content (20 g/kg); compared with co-exposed Cu–Se treatments (Figure 3). At high Cu concentration (200 mg/kg), the TFs of Cu showed no drastic change; whereas the BCFs of Cu significantly decreased by 10.3%–41.9% compared with co-exposed Cu–Se treatments. These results indicated that with the addition of RS in Cu–Se co-exposure treatments can inhibit Cu accumulation better than that of exogenous only Cu–Se co-exposure treatments.

### 3.4 Effects of Se combined with RS on changes in Cu fractions in soils

The distributions of Cu fractions in soils with different concentrations of Se, Cu, and RS are presented in Figure 4. Cu fractions were arranged following the sequential mobile levels, namely, EXC-Cu, CAB-Cu, FEM-Cu, OM-Cu, and RES-Cu.

Before planting, Cu primarily existed in FEM-Cu (36.1%) and RES-Cu (37.1%) fractions in native soil (control). The other stable fraction (OM-Cu) also accounted for 12.7% of the total soils Cu, whereas the mobile fractions were rather low (5.3% for EXC-Cu and 8.8% for CAB-Cu).

For single Cu treatments, the proportions of EXC-Cu and CAB-Cu fractions increased by 32.1% and 17.0% at low Cu concentration (50 mg/kg); whereas significantly increased by up to 2.5-fold and approximately 2.3-fold at high Cu concentration (200 mg/kg), compared with the control treatment. Whereby, a significant decrease in RES-Cu fraction (19.6%) was observed; meanwhile, the proportions of FEM-Cu fraction still accounted for dominance in soil (38.3%) at high Cu concentration (200 mg/kg).

For Cu and Se co-exposure treatments, at low Cu concentration (50 mg/kg), the proportions of OM-Cu and RES-Cu fractions decreased from 3.4% to 7.7% and 5.3% to 28.1%; whereas those of EXC-Cu, CAB-Cu, and FEM-Cu fractions increased by 7.1%–21.4%, 4.9%–19.4%, and 3.2%–18.5%; compared with single Cu treatments. At high Cu concentration (200 mg/kg), the distributions of Cu fractions changed following an opposite trend. The proportions of FEM-Cu, OM-Cu, and RES-Cu fractions markedly increased to 13.1%–27.9%, 43.2%–62.5%, and 24.0%–35.7%; by contrast, the proportions of EXC-Cu and CAB-Cu fractions dramatically decreased to 38.5%–66.9% and 41.9%–71.4%, compared with single Cu treatments. The maximum increasing proportion was at Se 2.5 mg/kg. These results suggested that Se can enhance the compact Cu binding in soils with Cu at high concentrations (200 mg/kg).

For Cu and RS co-exposure treatments, with low RS content (10 g/kg), the proportions of OM-Cu and RES-Cu fractions increased to 16.5%–97.7% and 3.5%–28.6%, compared with single Cu treatments. With high RS content (20 g/kg), the proportions of OM-Cu and RES-Cu fractions markedly increased to 50.4%–233.0% and 17.5%–55.6%; by contrast, the proportions of EXC-Cu, CAB-Cu, and FEM-Cu fractions decreased to 5.7%–57.7%, 12.6%–56.7%, and 3.6%–42.2%, compared with single Cu treatments. These results suggested that RS can enhance Cu binding in soils as well.
For Cu–Se and RS co-exposure treatments, the proportions of OM-Cu and RES-Cu fractions markedly increased to 24.5%–187.0% and 4.5%–59.0%; but those of EXC-Cu, CAB-Cu, and FEM-Cu fractions decreased to 20.0%–55.3%, 18.5%–52.8%, and 3.9%–50.0%, compared with co-exposed Cu–Se treatments. These results further indicated that exogenous Cu–Se and RS co-exposure treatments can inhibit Cu accumulation better than that of exogenous Cu–Se co-exposure treatments.

After harvest, Cu tended to transfer into FEM-Cu, OM-Cu and RES-Cu fractions for all treatments. For Cu and Se co-exposure treatments, the proportions of FEM-Cu, OM-Cu, and RES-Cu fractions markedly increased by 7.1%–20.4%, 1.7%–21.6%, and 12.8%–69.4%; whereas the proportions of EXC-Cu and CAB-Cu fractions considerably decreased to 67.2%–70.0% and 68.2%–70.7%; compared with those in soils before planting. For Cu–Se and RS treatments, a similar trend was observed. Specifically, the proportions of FEM-Cu, OM-Cu, and RES-Cu fractions increased by 4.3%–20.7%, 1.2%–23.2%, and 9.9%–29.0%; whereas the proportions of EXC-Cu and CAB-Cu fractions considerably decreased to 68.9%–71.7% and 68.6%–73.3%; compared with those in soils before planting. These results suggested that Cu became less mobile with time and its bioavailability in soils significantly decreased.

### 3.5 Correlation between different Cu fractions in soils with uptake of Cu and pak choi growth

To consider the effect of changes in Cu fractions on Cu bioavailability in soils and pak choi growth, we analyzed the correlations between them (Table 2). Results showed that the uptake of Cu by shoot and root was significantly positively correlated with EXC-Cu and CAB-Cu fractions ($R^2 > 0.5$, $P < 0.01$ for Cu concentrations in root; $R^2 > 0.4$, $P < 0.05$ for Cu concentrations in shoot), whereas significantly negatively correlated with
Figure 4. The Cu fractions proportions in soils before planting (A) and after harvest (B).

Table 2. Correlation between copper fractions in soil with the uptake of copper by pak choi and pak choi growth.

| Correlation coefficient | Before planting | After harvest |
|-------------------------|-----------------|---------------|
|                         | EXC-Cu | CAB-Cu | FEM-Cu | OM-Cu | RES-Cu | EXC-Cu | CAB-Cu | FEM-Cu | OM-Cu | RES-Cu |
| Cu (shoot)              | 0.418*  | 0.411*  | 0.273  | 0.017 | −0.707** | 0.389* | 0.396* | 0.286  | 0.107 | −0.689** |
| Cu (root)               | 0.507** | 0.511** | 0.172  | 0.011 | −0.657** | 0.476* | 0.498** | 0.200  | 0.116 | −0.591** |
| DW (shoot)              | −0.568** | −0.563** | −0.358 | 0.452* | 0.443*  | −0.570** | −0.564** | −0.399* | 0.355 | 0.360   |
| DW (root)               | −0.709** | −0.713** | −0.510** | 0.707** | 0.484*  | −0.729** | −0.709** | −0.578** | 0.659** | 0.319   |

Notes: *P < 0.05, **P < 0.01, n = 27. Cu (shoot): Cu concentration in shoot; Cu (root): Cu concentration in root; DW (shoot): shoot dry weight; DW (root): root dry weight.

RES-Cu fraction ($R^2 > 0.65, P < 0.01$), and no correlated with OM-Cu and FEM-Cu fractions ($R^2 < 0.3, P > 0.05$) for before planting treatments. A similar trend was observed for after harvest treatments, these correlations decreased less ($R^2_{after} < R^2_{before}$). This analysis only occurs the correlation for the mobile fractions, such as EXC-Cu and CAB-Cu fractions, because their high availability and easy absorption by plants. Whereas the semi-mobile fractions had lower availability, such as FEM-Cu and OM-Cu fractions, showed that no correlation, because they almost only exist in soils. Moreover, the proportions change of
FEM-Cu and OM-Cu fractions also is affected by the OM content, pH, clay minerals in soils. For pak choi growth, results showed that shoot and root dry weight of pak choi was significantly positively correlated with OM-Cu and RES-Cu fractions ($R^2 > 0.4$, $P < 0.05$), whereas significantly negatively correlated with EXC-Cu, CAB-Cu, and FEM-Cu fractions ($R^2 > 0.5$, $P < 0.01$; except FEM-Cu with dry weight shoot) for before planting treatments. After harvest, this correlation decreased less. The result of the correlation analysis suggested that the Cu fractions between before planting and after harvest can predict uptake Cu capability by pak choi and pak choi growth.

4. Discussion

The bioavailability and mobility of Cu is affected by the OM content, pH, clay minerals, metal oxides, and other factors [35–37].

4.1 Effect of exogenous single Cu on Cu bioavailability in soils

In the present study, the experimental soil was clay loam soil comprising phyllosilicate minerals rich in silicon and aluminum oxides and hydroxides (sec. Materials and methods). Cu does not exist as ion in soils, and most Cu is adsorbed by iron and aluminum oxides in Cu$^{2+}$–O–Fe$^{3+}$ or Cu–O–Al forms [38], thereby decreasing its mobility. According to the principle of hard and soft acids and bases, the ‘classic’ trace metals (Cu, Co, Ni, Al, Cd, and Zn) have strong binding abilities with hard ligands (O-containing functional groups). Therefore, the fractions of Cu in native soil are primarily FEM-Cu (36.1%) and RES-Cu (37.1%) fractions.

For single Cu treatments, Cu tends to increase in mobile fractions. At low Cu concentrations (50 mg/kg), the proportions of EXC-Cu and CAB-Cu fractions increased by 32.1% and 17.0%, compared with the control treatment (Figure 4). Thus, pak choi may actively uptake Cu as nutrients to support growth; which increased by 16.8% and 16.7% in the shoot and root dry weight of pak choi, compared with the control treatment (Figure 1). This conclusion was completely consistent with that of Feng et al. for hydroponic solution at low concentrations (50–100 μM), which plant may actively uptake Cu as nutrients to support plant growth [39]. At high Cu concentrations (200 mg/kg), the Cu concentrations in shoot and root increased reached up to 5.9- and 11.1-fold compared with the control treatment (Figure 2). This result posed to the shoot and root dry weight of pak choi to decrease by 28.6% and 27.1%, compared with single Cu treatments at 50 mg/kg (Figure 1). This phenomenon can be explained by the significant increase of the proportions of EXC-Cu and CAB-Cu fractions (up to 2.5-fold and approximately 2.3-fold compared with the control treatment) (Figure 4). Meanwhile, the RES-Cu fraction significantly decreased (19.6%) (Figure 4). The proportions of FEM-Cu fraction still accounted for dominance in soil (38.3%) at high Cu concentrations (200 mg/kg) (Figure 4). The result was in agreement with many previous studies [33,40]. For example, Luo et al. found that Fe-Mn oxide Cu was the dominant fraction in Cu-polluted soil, whereas residual Cu was dominant in unpolluted soils [40].

4.2 Effect of Cu–Se co-exposure on Cu bioavailability in soils

For Cu–Se co-exposure treatments, at low Cu concentrations (50 mg/kg), Se significantly increased Cu bioavailability in soils. Cu primarily presented in EXC-Cu, CAB-Cu, and FEM-Cu fractions, which increased by 7.1%–21.4%, 4.9%–19.4%, and 3.2%–18.5%, compared with single Cu treatments (Figure 4). These percentages increased with increased Se concentration in soils. The Cu concentrations in shoot and root of pak choi dramatically increased ($P < 0.05$) (Figure 2). Hu et al. showed that Cu in the stems of Danshen (Salvia miltiorrhiza) were higher when Se was added to the soils [41].

At high Cu concentrations (200 mg/kg) treatments, the presence of Se played an important inhibition role against Cu uptake. Similarly, the antagonistic effects between Se and Cu have been demonstrated in the fern Pteris vittata L [42]. The Cu concentrations in shoot and root of pak choi to be significantly reduced ($P < 0.05$) by 18.2%–30.3%, and 31.7%–56.2%, compared with single Cu treatments (Figure 2). Thereby, causing the shoot and root dry weight of pak choi also dramatically increased ($P < 0.05$) by 20.7%–40.3% and 21.0%–41.0%, compared with single Cu treatments (Figure 1). This phenomenon can be caused by the significant increase of the proportions of FEM-Cu, OM-Cu, and RES-Cu fractions (13.1%–27.9%, 43.2%–62.5%, and 24.0%–35.7%), compared with single Cu treatments (Figure 4). These results are completely consistent with previous studies, which also found that the added Cu and Se transformed from soluble and exchangeable to stable fractions with time (e.g. Fe–Mn oxide-bound Cu and Se) [33,43]. These findings may be explained by chemical reactions, i.e. Cu can lead to changes in fraction transformation and bioavailability [44] by the following mechanisms: 1. The addition of Se under low pH conditions (3.7; sec. Materials and methods), Se$^{2-}$ exhibited an enhanced reducing ability and increased Fe$^{2+}$ concentrations in the soil solution by reducing high-valence Fe in the soils, posing dramatically enhanced the Fe content of Fe plaque on root surfaces [45]. This can cause the large part of the adsorbed fraction of Cu on iron oxides in soils to have a high retention capacity for Cu [46,47], lead to
the proportions of FEM-Cu fraction increased. 2. With low-pH soil (3.7; sec. Materials and methods), the release of carbon-rich root exudates that can facilitate the formation of sulfides (S\(^2\)-) was promoted [48]. Sulfate reduction in contaminated soils may mobilize Cu through the formation of Cu-rich sulphide colloids. Free Cu\(^{2+}\) ions tightly combined with S\(^2\)- in soils to form CuS complex [49-51]. Se often exists as an isomorphous substituent of sulfur (S) in sulfide crystal lattices. S and Se also have the same atomic structure, the same charge (S\(^2\)- and Se\(^2\)-), and similar atomic and ionic radii (S: 0.184 nm, Se: 0.191 nm), indicating that Se can easily be incorporated into the crystalline lattices of S [52]. Therefore, this study predicted that S\(^2\)- can be replaced by Se\(^2\)- to form inert CuSe precipitates; posing to significantly increased the proportions of RES-Cu fraction (Figure 4). 3. Se reduced the PCs accumulation in the presence of Cu, due to by the transformation of SeO\(_2\)\(^2\)- into organic Se (SeCys or SeMet), lead to the decrease of the Cu concentration in plants [53].

4.3 Effect of Cu–Se and RS co-exposure on Cu bioavailability in soils

Soil OM content plays a very important role in the mobility and availability of Cu in soils by strong affinity with Cu [54,55].

For Cu and RS co-exposure treatments at low Cu concentrations (50 mg/kg), with low RS contents at 10 g/kg, the proportion of OM-Cu fraction increased to 16.5%–97.7%, compared with single Cu treatments (Figure 4). The addition of RS modulated Cu (as nutrients) uptake by secreting root exudate, which increases small organic ligands in the rhizosphere [56,57], thereby facilitating trace-Cu mobilization from OM-Cu fraction. RS was decomposed by microbes into some micro-organic materials through gut digestion, these micro-organic materials could chelate soils Cu ion and thus increased the quantity of available Cu in soils [58]. Moreover, the addition of RS increased soil pH by about 0.4 pH unit on average, and significantly increased dissolved organic carbon in soils [59]. This finding was consistent with the results of [60], who found that in soil solutions 99.99% of the Cu is bound to the dissolved OM. Whereby, Cu concentrations in pak choi tissue dramatically increased (P < 0.05) (Figure 2). Cui et al. also demonstrated that with the addition of RS, total soluble Cu concentration increased from 0.82 µmol l\(^{-1}\) to 1.22 µmol l\(^{-1}\) [59].

With high RS contents (20 g/kg), Cu mobility and availability deceased in the presence of high OM content [61]. The proportions of OM-Cu fractions markedly increased to 50.4%–233.0%, compared with single Cu treatments (Figure 4). This finding was due to the high affinity of OM for Cu in soils [62], which forms very strong complexes with Cu [63,64]. The OM-Cu complex was generally the most predominant form. Some studies have reported that about >90% of the total soil Cu was found as OM-Cu form depending on Cu concentrations in Cu-contaminated soil solutions [65]. The root dry weight significantly increased to 16.7%–30.0% (P < 0.05), however, the shoot dry weight of pak choi no significantly increased (P > 0.05), compared with single Cu treatments (Figure 1). These results were consistent with the study of Wang et al., which strain significantly enhanced root biomass instead of shoot biomass [66]. This was explained that: first, the C/N ratio of RS was high, and soil available N decreased rapidly during RS decomposition; second, the population of soil microbes increased after RS addition, and more plant-available nutrients were fixed by microbes, which inhibited plants growth [66].

For the Cu-Se and RS co-exposure treatments, Cu–Se compounds may combine more with the dissolved OM in the rhizosphere and then form much larger Cu–Se complexes to further reduce Cu bioavailability [67], lead to the proportions of OM-Cu and RES-Cu fractions markedly increased to 24.5%–187.0% and 4.5%–59.0%, compared with co-exposed Cu–Se treatments (Figure 4). The Cu concentrations in the pak choi shoot and root dramatically decreased (P < 0.05) from 12.5% to 27.6% and 19.4% to 35.3%, in Cu-treated soils at 200 mg/kg at RS 20 g/kg (Figure 2). Cui et al. also found that the addition of RS decreased the concentrations of free Cu\(^{2+}\) [59]. This can be attributed to the adsorption capacity of RS added and the pH increase in treatments with the RS [59]. The inhibition may only significantly occur during Se exposure at an appropriate concentration (2.5 mg/kg) with significantly decreased Cu concentrations in pak choi (30.3% for shoots and 56.2% for roots), consistent with the result of our previous study [68]. These phenomena showed that Cu–Se co-exposure at a suitable concentration can significantly restrict Cu uptake by plants in case the Cu concentration exceeds the standard limit.

4.4 Effect of Se combined with RS on transfer factors and bioaccumulation factors of Cu

The BCFs of Cu change similar trend with the Cu concentrations in pak choi shoot (Figures 2 and 3) suggested that Se and RS may have a protective effect against the Cu bioaccumulation at concentrations exceeding the standard (200 mg/kg). Se combined with RS can inhibit Cu accumulation better than that of exogenous only Cu–Se co-exposure. This addition did not change the transfer efficiency from root to shoot (TFs of Cu) (Figure 3). Therefore, RS dramatically increased Cu concentrations in root than those in shoot at low Cu concentration (50 mg/kg); or Se can inhibit the Cu concentration in pak choi root was higher than that in shoot at high Cu concentration
(200 mg/kg) (Figure 2). This finding revealed that Se and RS amendment benefited the absorption of more Cu by the root but had no effect on transporting Cu in the plants because the bioavailable part of Cu can be mostly affected by pH and OM in soils. In particular, the plant rhizosphere zone can affect Cu uptake by plant [69]. Moreover, this phenomenon is due to the translocation of Cu from root to shoot following the physiological mechanism of plants; thus, this result needs further study in the future.

4.5 Effect of time on Cu bioavailability in soils with Cu–Se and RS co-exposure

After harvest, the distributions of Cu fractions changed according to the influence of Se and RS similar to the case before planting. Cu tended to transfer more into FEM-Cu, OM-Cu, and RES-Cu fractions with increased proportions of FEM-Cu, OM-Cu, and RES-Cu fractions to 4.3%–20.7%, 1.2%–23.2%, and 9.9%–69.4%, compared with those in soils before planting, in Cu–Se and RS co-exposure treatments (Figure 4). This finding agreed with a previous conclusion asserting that Cu can transform from exchangeable to stable fractions within the amendment time [43]. Specifically, the exogenous HMs that adsorbed onto the surface of solid soil as the exchangeable form rapidly entered the soil environment and then slowly transformed into other stable forms with time [70]. Soil pH was very low, reaching only 3.7 (sec. Materials and methods); thus, the mobile and semi-mobile fractions can be dissolved with time, releasing Cu$^{2+}$ ions and increasing free Cu$^{2+}$ ions [71]. These free ions tend to combine with stable fractions by strong binding affinity with S$^{2-}$, resulting in increased RES-Cu fractions (Figure 4). The presence of RS also increased the OM content of rhizospheric soil by the time, which can form Cu complexes and inhibit Cu mobility and bioavailability, leading to increased proportion of OM-Cu [72,73] (Figure 4). Moreover, with low-pH soil, plants developed various strategies such as secreting organic extrudates by the time [47,74]. With the addition of RS in soil, Fe plaque on the epidermis was sequestered, the proportion of FEM-Cu increased [34] (Figure 4), and a biomineralization rim was generated on root epidermis [75] to reduce Cu mobility. Therefore, Cu mobility in soils decreased after harvest and its uptake was restricted in plants.

5. Conclusion

Se may play an important role in prevent Cu uptake by pak choi tissue at excessive concentrations (200 mg/kg) through transformations into immobile Cu fractions. The supplementation of RS in Cu–Se co-exposure treatments can limit the absorption Cu in pak choi and enhance pak choi growth than only Cu–Se co-exposure treatments. This inhibition may significantly occur only when Se and RS were at appropriate concentration (2.5 mg/kg for Se and 20 g/kg for RS). Conversely, Se and RS increased the Cu concentrations in plants and promoted pak choi growth at low Cu concentration (50 mg/kg). Moreover, Se combined with RS only affected to the uptake of Cu by pak choi tissue but did not change the translocation efficiency from root to shoot.

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Author contributions

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