CrossMark

ORIGINAL ARTICLE

Physiological and biochemical mechanisms of silicon-induced copper stress tolerance in cotton (*Gossypium hirsutum* L.)

Shafaqat Ali[1] · Muhammad Rizwan[1] · Najeeb Ullah[2] · Saima Aslam Bharwana[1] ·
Muhammad Waseem[3] · Muhammad Ahsan Farooq[1] · Ghulam Hasan Abbasi[4] ·
Mujahid Farid[5]

Abstract Accumulation of excess copper (Cu) in agricultural soils can decrease growth and quality of crops grown on these soils and a little information is available on the role of silicon (Si) in reducing Cu toxicity in plants. A hydroponic study was conducted to investigate the effects of Si (1.0 mM) on growth and physiology of cotton seedlings grown on different Cu (0, 25, and 50 μM) concentrations. Elevated levels of Cu decreased growth, biomass, photosynthetic pigments, and gas exchange characteristics, and increased the electrolyte leakage (EL), hydrogen peroxide (H_2O_2), and thiobarbituric acid reactive substances (TBARS) contents in leaf, stem, and roots of cotton seedlings. Cu stress alone decreased the activities of key antioxidant enzymes in cotton seedlings. Exogenous application of Si alleviated the toxic effects of Cu on cotton seedlings by improving growth, photosynthetic pigments, and gas exchange characteristics under Cu stress. The Si application decreased Cu concentrations in leaves, stem, and roots as compared with the control plants. Furthermore, Si decreased oxidative stress as evidenced by decreased EL, H_2O_2, and TBARS contents, and increased the antioxidant enzyme activities in cotton seedlings. This study provides evidences of Si-mediated reduction of Cu toxicity in cotton seedlings at physiological and biochemical levels.

Keywords Silicon · Oxidative stress · Antioxidant enzymes · Photosynthesis · Growth inhibition · Cu tolerance

✉ Muhammad Rizwan
mrazi1532@yahoo.com

1 Department of Environmental Sciences and Engineering, Government College University, Allama Iqbal Road, 38000 Faisalabad, Pakistan

2 Department of Plant and Food Sciences, Faculty of Agriculture and Environment, The University of Sydney, Eveleigh NSW 2015, Australia

3 Department of Microbiology, Government College University, Allama Iqbal Road, 38000 Faisalabad, Pakistan

4 Department of Soil Science University College of Agriculture and Environmental Sciences, The Islamia University of Bahawalpur, Bahawalpur, Pakistan

5 Department of Environmental Sciences, University of Gujrat, Hafiz Hayat Campus, Gujrat, Pakistan

Introduction

Contamination of agricultural soils with toxic heavy metals has become a global problem and poses a serious threat to crops cultivated on these soils (Nagajyoti et al. 2010; Ali et al. 2014; Rehman et al. 2015). Heavy metals, such as cadmium (Cd), lead (Pb), and chromium (Cr), have no known biological functions in plants, while other heavy metals, such as copper (Cu), zinc (Zn), manganese (Mn), and iron (Fe), are essential minerals and are required for normal plant growth and development. However, essential heavy metals can cause toxicity to plants at elevated concentrations (Wuana and Okieimen 2011; Keller et al. 2015; Ramzani et al. 2016). Among these essential heavy metals, Cu is mainly released into agricultural water and soil through industrial, mining, agricultural, and urban activities. Copper is required for many important growth processes, such as respiratory electron transport reactions and photosynthesis (Yruela 2009, 2013). However, excessive Cu concentrations in plant tissues can cause toxicity and plant growth inhibition (Adrees et al. 2015a; Christiansen

🖉 Springer

et al. 2015). Several studies reported that Cu toxicity decreased growth, photosynthesis, water, and mineral nutrients in plants (Michaud et al. 2008; Bravin et al. 2010; Ando et al. 2013; de Freitas et al. 2015; Keller et al. 2015).

Due to redox-active nature, higher cellular Cu concentration damages lipid membrane by producing reactive oxygen species (ROS) in different plant organs (Adrees et al. 2015a; Habiba et al. 2015). Madejon et al. (2009) and Liu et al. (2014a) reported that excess Cu enhanced malondialdehyde (MDA) content in maize leaves. Similarly, excessive Cu increased the MDA and hydrogen peroxide (H_2O_2) contents in rice (*Oryza sativa* L.), shoot, and root (Thounaojam et al. 2012; Lin et al. 2013). Plants can tolerate a certain level of Cu by different mechanisms, such as stimulation of antioxidant enzymes, sequestration in roots, and through formation of complexes with different organic molecules (Thounaojam et al. 2012; Kang et al. 2015; Keller et al. 2015). Among these tolerance mechanisms, the antioxidative defense system plays an important role in reducing the Cu toxicity in plants (Adrees et al. 2015a). Increased antioxidant enzyme activities have been reported in plant species, such as chickpea (*Cicer arietinum* L.) (Kumar et al. 2014), and *Brassica napus,* in response to Cu stress (Feigl et al. 2015; Habiba et al. 2015). Although, higher Cu levels may down-regulate antioxidant enzyme activities, e.g., lower Cu (100 μmol L^{-1}) increased and higher Cu (1000 μmol L^{-1}) decreased guaiacol peroxidase (POD) activity in maize leaves (Liu et al. 2014a).

Various materials have been used to increase heavy metal tolerance in plants (Ali et al. 2013a, b; Adrees et al. 2015b; Rizwan et al. 2016a, b). Silicon (Si) plays an essential role in inducing tolerance to abiotic stresses in plants, including uptake and toxicity of heavy metals (Rizwan et al. 2012, 2015; Adrees et al. 2015b). Exogenously applied Si has been found to ameliorate the negative effect of metal induced toxicity on plant growth, e.g., Cd toxicity in durum wheat (Rizwan et al. 2016c) and maize (Vaculik et al. 2015), Zn toxicity in rice (Gu et al. 2012) and maize (Bokor et al. 2014), and Mn toxicity in rice (Li et al. 2013a, b). The mechanisms evoked by Si include enhancement of photosynthesis restricted metal uptake by plants, immobilization in the roots, and stimulation of antioxidant enzymes (Adrees et al. 2015b). The role of Si in reducing toxicity of metals, such as Cd, Pb, Mn, and Zn, has already been explored, but limited information is available on the effects of Si on Cu-stressed plants (Collin et al. 2014; Adrees et al. 2015b). Silicon application decreased Cu toxicity in plants by limiting Cu uptake, translocation, and adsorbing Cu on the root surface (Keller et al. 2015; Mateos-Naranjo et al. 2015). Silicon may alleviate Cu toxicity by immobilization in leaf phytoliths, although this mechanism was limited in bamboos and wheat (Collin et al. 2014; Keller et al. 2015). On the other hand, Si may reduce Cu-induced toxicity be capturing production of ROS and regulating antioxidant enzymes; however, limited data are available (Adrees et al. 2015b).

Cotton is cultivated as a fiber and food crop in many parts of the world. Both biotic and abiotic stresses are mainly responsible for the reduction in growth and yield of plants (Angelova et al. 2004; Liu et al. 2014b; Mei et al. 2015). Based upon the above discussion, we hypothesized that Si may alleviate Cu toxicity in cotton seedlings by reducing Cu-induced physiological and biochemical damages in cotton. Thus, the main objective of this study was to explore the mechanisms through which Si can minimize Cu-induced damages in cotton seedlings.

Materials and methods

Plant materials and growth conditions

The glasshouse experiment was conducted using a cotton cultivar BR001, a gluphosinate-resistant transgenic cotton cultivar containing the Bar gene (Daud et al. 2009). Healthy and uniform seeds were soaked in distilled water for 4 h at 35 °C and then were sown in trays containing layers of sterilized sand (about 5 cm) in the growth chamber under 28–30 °C temperature, 60 % relative humidity, and 16 h photoperiod at 450–500 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). Sand used for seed germination was thoroughly washed with distilled water, then oven dried at 40 °C till constant weight. Two weeks after sowing, the uniform seedlings were transplanted into thermo pole sheets floating in plastic jars containing modified Hoagland's solution (20 L). The basic nutrient solution was comprised of: $(Ca(NO_3)_2$ 2.5 mM, $MgSO_4$ 1 mM, KCl 0.5 mM, KH_2PO_4 0.5 mM, $FeCl_3$ 0.1 μM, $CuSO_4$ 0.2 μM, $ZnSO_4$ 1 μM, H_3BO_3 20 μM, H_2MoO_4 0.005 μM, and $MnSO_4$ 2 μM). The solution was continuously aerated by bubbling air through the nutrient solution. The pH was maintained 6.2 ± 0.1 throughout the experiment by adding with 1 M H_2SO_4 and/or NaOH when required. The design of the experiment was complete randomized design (CRD).

Treatments

Two weeks after transplanting, three Cu levels (0, 25, and 50 μM) and two levels of Si (0 and 1 mM) were introduced using $CuSO_4 \cdot 5H_2O$ and $Na_2SiO_3 \cdot 9H_2O$, respectively, with six treatment combinations and six replications. The Cu and Si treatments were based on our previous experiments indicating Cu stress on brassica seedlings (Habiba et al. 2015) and Si effect on reducing Cu toxicity in wheat seedlings (Keller et al. 2015) within these Cu and Si

concentrations. The nutrient solutions of each jar were renewed every 3 days during the first 2 weeks of treatments, and then every 2nd day during the additional two weeks of the Cu/Si treatments to maintain the nutrient and treatment levels in the growth medium as constant as possible. In total, the plants were grown for 8 weeks, such as 2 weeks in sand, then 2 weeks in the solution without Cu and Si treatments and for 4 weeks with Si/Cu treatments when required.

Plant sampling and analysis

After 4 weeks of treatments, the plants were harvested and washed with distilled water and different growth parameters, such as plant height, root length, number of leaves per plant, and leaf area were recorded. Fresh weights of leaves, stem, root, and flower were separately calculated. The plant material was first air-dried for about 4 days under the shade and then oven dried at 70 °C for at least 72 h and then dry weights were measured.

Photosynthetic pigments and gas exchange parameters determination

Gas exchange characteristics, such as transpiration rate (Tr), stomatal conductance (gs), water use efficiency (WUE), and net photosynthetic rate (Ps), were determined from the youngest fully expanded healthy leaves using an Infra-Red Gas Analyzer (IRGA) (Analytical Development Company, Hoddesdon, England). Four weeks after treatment, gas exchange measurements were taken between 10:00 am and 11:00 am during the sunny day with the growth conditions as described above.

The photosynthetic pigments were extracted from the same leaves used for gas exchange measurements in 85 % (v/v) aqueous acetone in dark by continuous shaking until the color was completely disappeared from the leaves. The supernatant was then collected from the assay mixture after centrifuging at $4000 \times g$ for 10 min at 4 °C. Chlorophylls (chl a and chl b) and carotenoid contents were measured by light absorbance at 663, 644, and 452.5 nm by a spectrophotometer (Halo DB-20/DB 20S, Dynamica Company, London, UK) (Metzner et al. 1965). The concentrations of pigments were calculated using the adjusted extinction coefficients (Lichtenthaler 1987).

Determination of EL, TBARS, and H_2O_2 contents

Electrolyte leakage (EL) was measured according to the method described by Dionisio-Sese and Tobita (1998). After 4 weeks of treatments, the youngest fully expanded leaves were cut into small pieces (5 mm length) and positioned in test tubes containing 8 mL deionized water.

The tubes were placed in an incubator in a water bath at 32 °C for 2 h and then the electrical conductivity of initial medium (EC_1) was assessed. After this, all samples were placed in an autoclave at 121 °C for 20 min, cooled to 25 °C and again electrical conductivity (EC_2) was measured. EL was calculated using the following formula:

$$EL = (EC_1/EC_2) \times 100. \tag{1}$$

Thiobarbituric acid reactive substances (TBARS) were determined by the method of Heath and Packer (1968), with some modifications (Dhindsa et al. 1981; Zhang and Kirkham 1994). The reaction was completed using the thiobarbituric acid (TBA). 0.25 g fresh weight was taken and mixed in 5 mL 0.1 % TCA. The mixture was centrifuged at 12,000 rpm for 15 min. For 1 mL of aliquot of the supernatant, 4 mL of 20 % TCA comprising of 0.5 % TBA was added. The sample mixture was heated at 95 °C for 30 min and then rapidly cooled in ice. After centrifugation at $10,000 \times g$ for 10 min, the absorbance of the supernatant mixture was measured at 532 nm, for nonspecific absorbance at 600 nm, values were subtracted. The MDA content was calculated by means of an extinction coefficient of 155 mM^{-1} cm^{-1}.

Hydrogen peroxide (H_2O_2) was extracted by homogenizing 50 mg root/leaf soft tissue with 3 mL of phosphate buffer (50 mM, pH 6.5). The homogeneous mixture was centrifuged at $6000 \times g$ for 30 min. For the estimation of H_2O_2 content, extracted sample solution (3 mL) was mixed in 1 mL of 0.1 % titanium sulfate in 20 % (v/v) H_2SO_4. The mixture was centrifuged at $6000 \times g$ for 20 min. The strength of yellow color of the supernatant mixture was evaluated at 410 nm. H_2O_2 contents were calculated using the extinction coefficient of 0.28 μmol^{-1} cm^{-1}.

Determination of antioxidant enzyme activities

For antioxidant enzyme activities, fully expanded leaves and roots were taken after 8 weeks of treatments. Antioxidant enzymes, including ascorbate peroxidase (APX), superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), in roots and leaves were evaluated spectrophotometrically. The leaves and roots were first chopped with a mortar and pestle under chilled conditions with liquid nitrogen. This pattern was standardized in 0.05 M phosphate buffer (maintaining pH at 7.8) and filtered through four layers of muslin cloth and centrifuged at $12,000 \times g$ for 10 min at 4 °C. Finally, this enzyme extract was used for quantification of SOD and POD activities following Zhang (1992).

Catalase activity was determined according to Aebi (1984). The assay mixture (3.0 mL) consisted of 100 μL enzyme extract, 100 μL H_2O_2 (300 mM), and 2.8 mL 50 mM phosphate buffer with 2 mM CA (pH 7.0). The

CAT activity was assayed by measuring the decrease in absorbance at 240 nm because of the H_2O_2 disappearance ($\varepsilon = 39.4$ mM^{-1} cm^{-1}). Ascorbate peroxidase activity was assayed by adopting the method of Nakano and Asada (1981). The reaction mixture consisted of enzyme extract (100 μL), ascorbate 100 μL (prepared from 7.5 mM), 2.7 mL of 25 mM potassium phosphate buffer with 2 mM EDTA (pH 7.0), and 100 μL H_2O_2 (300 mM). The oxidation activity of ascorbate was determined by the variation in wavelength at 290 nm ($\varepsilon = 2.8$ mM^{-1} cm^{-1}).

Metal content analysis

For determining Cu contents in plant tissues, roots, stem, and leaf samples (0.5 g) were collected in a 100 mL flask and then 15 mL of concentrated HNO_3 was added. After mixing, the flasks were placed on a hot plate and temperature was gradually increased up to 275 °C. Dense yellow fumes appeared from the flask and the hydrogen peroxide was continuously added until dense yellow fumes disappeared. When the samples became colorless, the flasks were removed from a hot plate, and volume was made up to 25 ml using distilled water. Copper contents in root, stem, and leaf tissues were determined using flame atomic absorption spectrometry (AAS) (novA A400, Analytik Jena, Germany).

Statistical analysis

All values described in this study are mean of six replicates. Analysis of variance (ANOVA) was performed using a statistical package, SPSS version 16.0 (SPSS, Chicago, IL, USA), followed by Tukey's post hoc test between the means of treatments to determine the significant difference.

Results

Plant growth and biomass

Elevated levels of Cu in the growth media significantly inhibited plant height, root length, number of leaves per plant, and leaf area (Fig. 1). Maximum reduction was observed in response to 50 μM of Cu, which caused 64, 49, 64, and 41 % reduction in plant height, root length, number of leaves per plant, and leaf area, respectively, as compared with control. Silicon enhanced the growth of Cu-stressed cotton seedlings by increasing plant height as compared with control. The height of Cu-stressed cotton plants was significantly increased when treated with Si. For example, Si caused 56 and 78 % increase in height of plants treated with 25 and 50 μM of Cu, respectively, as compared with their respective Cu-treated plants without Si. Similarly,

increasing levels of Cu in the growth media significantly reduced the root, stem, and leaves fresh and dry weights of cotton seedlings (Table 1). Silicon addition improved the fresh and dry weights of different plant parts as compared to the respective Cu-only treatments. Root and leaf dry biomass were increased by 98.7 and 66 %, respectively, under Cu 50 μM + Si treatment as compared with 50 μM Cu treatment alone, respectively.

Photosynthetic pigments gas exchange characteristics

Copper toxicity decreased the chlorophyll a, b, total chlorophyll, and carotenoid concentrations in leaves of cotton seedlings as compared with the control (Fig. 2). At 50 μM Cu stress, chlorophyll *a*, *b*, and carotenoid concentrations decreased by about 47.7, 47 and 42 % as compared with the control, respectively. Silicon application increased the photosynthetic pigments under Cu stress as compared with the respective Cu-only treatments. At 50 μM Cu + Si treatments, the increase in chlorophyll a, b, total chlorophyll, and carotenoid concentrations was about 30, 7, 22 and 22 % as compared with 50 μM Cu-only treatment, respectively.

Gas exchange characteristics, Tr, gs, Ps, and WUE significantly decreased under Cu treatments as a dose-dependent manner (Fig. 3). The maximum reduction in Tr, gs, Ps, and WUE was about 55, 58, 65, and 22 % in 50 μM Cu stress as compared to the control, respectively. Exogenously applied Si significantly increased the gas exchange parameters of cotton seedlings under Cu stress as compared with the respective Cu-only treatments. At 50 μM Cu + Si treatment, the increase in Tr, gs, Ps, and WUE was about 55, 58, 65, and 22 % as compared with the same Cu-only treatment respectively.

Oxidative stress and antioxidant enzyme activities

Copper-induced toxicity in cotton seedlings was calculated by estimating the TBARS and H_2O_2 contents and EL in roots and leaf tissues (Fig. 4). A significant increase in TBARS, H_2O_2, and EL was observed with increasing levels of Cu in the growth medium. The highest increase in TBARS, H_2O_2, and EL was observed in plants treated with 50 μM Cu. Application of Si significantly reduced oxidative stress in cotton seedlings as indicated by decreased levels of TBARS, H_2O_2, and EL in root and shoot tissues.

There was a significant increase in the activities of antioxidant enzymes, e.g., SOD, POD, CAT, and APX in root and leaves in response to both Cu levels as compared with the control (Fig. 5). The maximum increase in antioxidant enzymes in leaves and roots was observed in 25 μM Cu treatment irrespective of Si application. In roots,

Fig. 1 Plant height, maximum root length of single root, leaf area, and number of leaves of cotton seedlings grown in hydroponics with 0, 25, and 50 μM Cu and 0 or 1 mM Si. *Bars* represent ± SD of six replicates. *Different letters* indicate significant differences among the treatments at $p < 0.05$
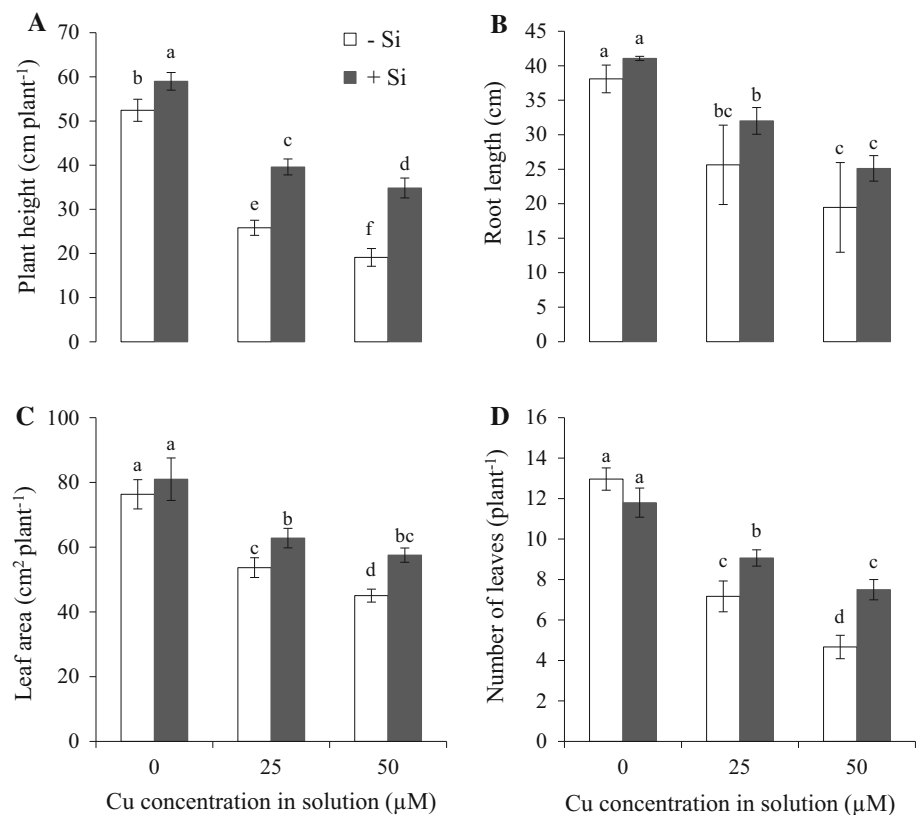

Table 1 Leaf, stem, and root fresh and dry weights of cotton seedlings grown in hydroponics with 0, 25, and 50 μM Cu and 0 or 1 mM Si

Treatments	Leaf weight (g plant^{-1})		Stem weight (g plant^{-1})		Root weight (g plant^{-1})	
	Fresh	Dry	Fresh	Dry	Fresh	Dry
Control	9.67 ± 0.55ab	2.36 ± 0.06a	11.50 ± 0.78a	4.33 ± 0.42a	6.40 ± 0.62a	2.03 ± 0.06a
Si	10.20 ± 0.36a	2.37 ± 0.09a	12.51 ± 0.85a	4.50 ± 0.46a	6.10 ± 0.30a	2.07 ± 0.13a
Cu (25 μM)	6.77 ± 0.51c	1.38 ± 0.15c	6.22 ± 0.37c	3.00 ± 0.20c	4.37 ± 0.25b	0.76 ± 0.05d
Cu25 + Si	8.77 ± 0.49b	1.99 ± 0.13b	7.77 ± 0.50b	3.53 ± 0.13b	4.70 ± 0.21b	1.34 ± 0.07b
Cu (50 μM)	3.13 ± 0.16e	1.10 ± 0.12d	4.13 ± 0.16d	1.67 ± 0.31d	3.06 ± 0.16c	0.52 ± 0.05e
Cu50 + Si	4.47 ± 0.45d	1.82 ± 0.07b	5.47 ± 0.45c	1.90 ± 0.10d	3.19 ± 0.07c	1.03 ± 0.06c

Bars represent sd of six replicates. Different letters indicate significant differences among the treatments at a $p < 0.05$

the increase in SOD, POD, CAT, and APX activities was about 128, 119, 171, and 133 % in 25 μM Cu treatment as compared with the control, respectively. The application of Si further increased the antioxidant enzyme activities as compared with the respective Cu-only treatments.

Copper contents

Copper concentrations significantly increased in root, stem, and leaves with increasing Cu levels in the growth medium (Table 2). The highest Cu contents were found in root followed by stem and leaf tissues, respectively. Exogenous application of Si significantly decreased the Cu concentrations in all plant parts as compared with the respective

Cu treatments alone. Copper concentration decreased in leaf, stem, and roots by 45, 24, and 6 % in 25 μM Cu + Si treatment as compared with the same Cu-only treatment, respectively. Similarly, at 50 μM Cu + Si treatment, the reduction in leaf, stem, and roots Cu concentration was about 37, 21, and 9 % as compared with the 50 μM Cu treatments, respectively.

Discussion

In this study, increasing supply of Cu in the growth medium has increased Cu concentrations in leaves, stem, and roots with a maximum accumulation in root tissues

Fig. 2 Chlorophyll *a*, Chlorophyll *b*, total chlorophyll, and carotenoids concentrations in leaves of cotton seedlings grown in hydroponics with 0, 25, and 50 μM Cu and 0 or 1 mM Si. *Bars* represent ± SD of six replicates. *Different letters* indicate significant differences among the treatments at a $p < 0.05$
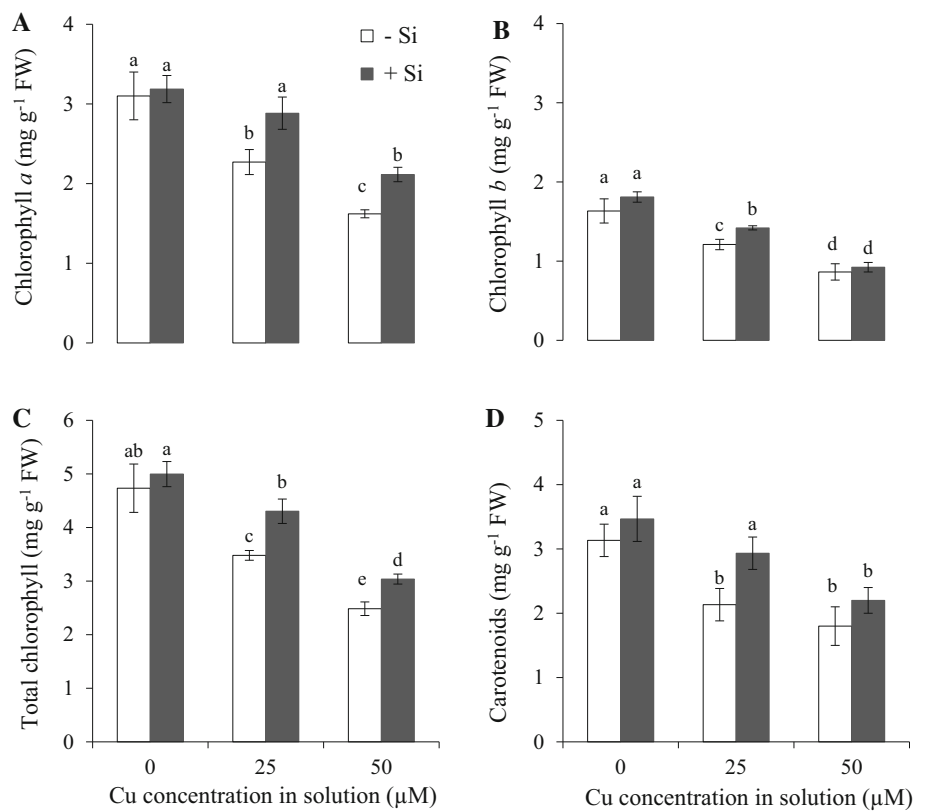

Fig. 3 Transpiration rate (E), stomatal conductance (gs), net photosynthetic rate (Pn), and water use efficiency in leaves of cotton seedlings grown in hydroponics with 0, 25, and 50 μM Cu and 0 or 1 mM Si. *Bars* represent ± SD of six replicates. *Different letters* indicate significant differences among the treatments at a $p < 0.05$
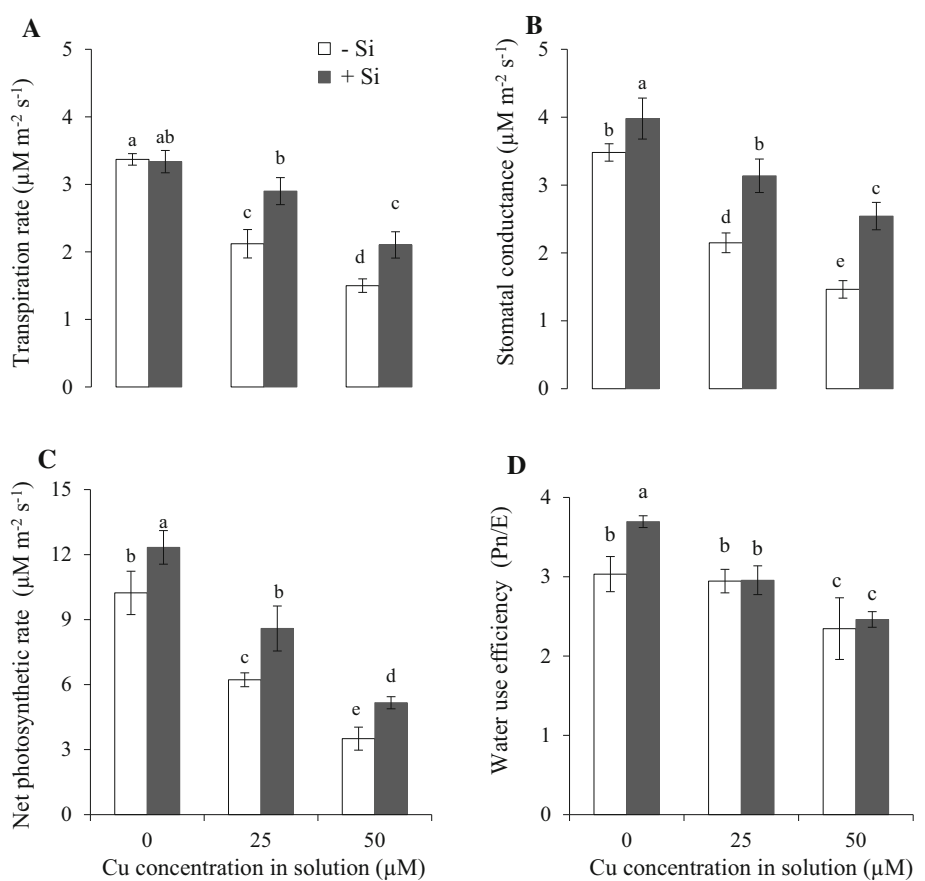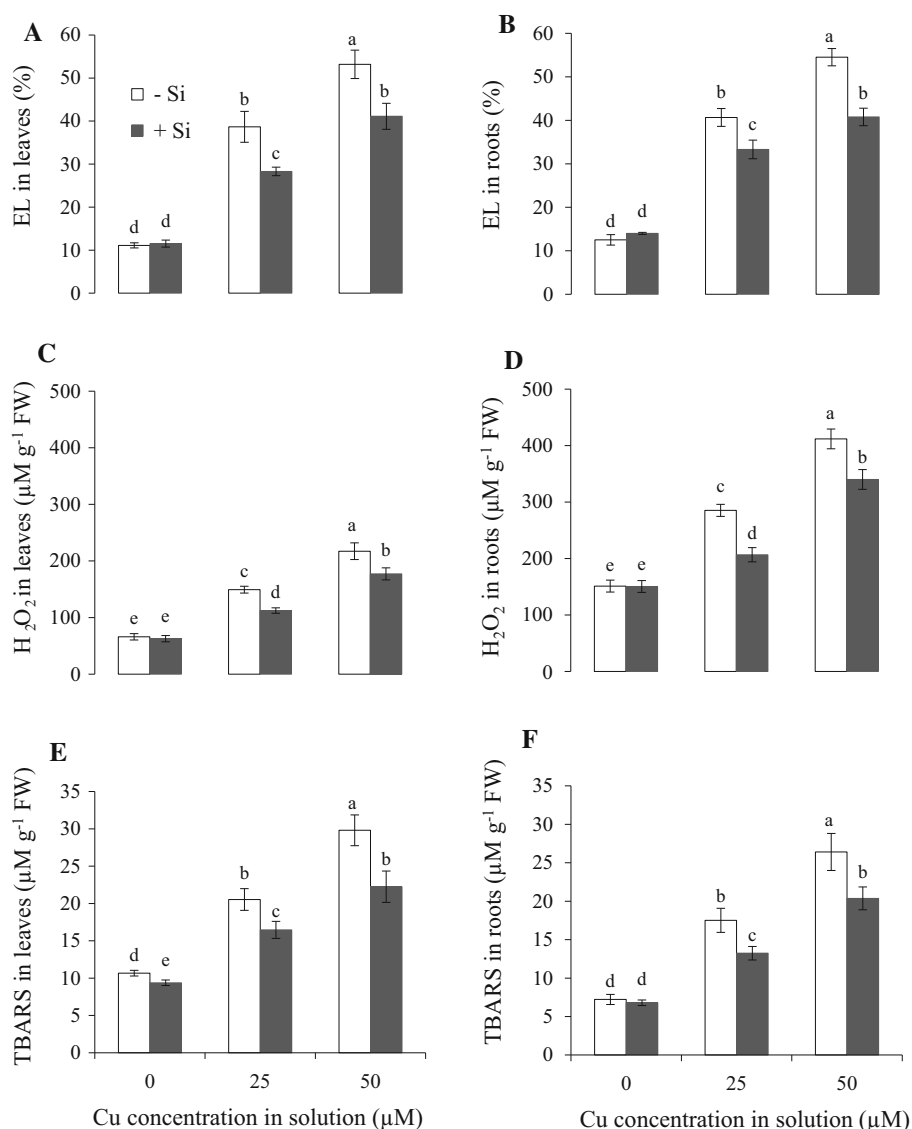
Fig. 4 Electrolyte leakage (EL), hydrogen peroxide (H_2O_2), and thiobarbituric acid reactive substances (TBARS) in leaves and roots of cotton seedlings grown in hydroponics with 0, 25, and 50 μM Cu and 0 or 1 mM Si. *Bars* represent ± SD of six replicates. *Different letters* indicate significant differences among the treatments at a $p < 0.05$

(Table 2). Similar Cu deposition in roots has been reported in other plant species, such as bamboo, wheat, and cotton (Collin et al. 2014; Keller et al. 2015; Mei et al. 2015). This restricted Cu translocation to shoots might be responsible for Cu tolerance in plants (Adrees et al. 2015a; Rizwan et al. 2016c). It was also supported by Si-induced inhibited Cu transport to aerial plant part, enabling plants to defy Cu-induced injury. Contrasting effects of Si on Cu concentrations have previously been reported in the literature (Adrees et al. 2015b). Keller et al. (2015) reported that 1.0 mM Si application decreased Cu concentrations in roots and shoots of 21-day-old wheat seedlings. Similarly, 1.0 mM Si decreased Cu concentration in shoot tissues of *Erica andevalensis* plants exposed to 500 μM Cu. Contrarily, Li et al. (2008) and Collin et al. (2012) observed that Si application has no effect on Cu uptake by *A. thaliana* and bamboo exposed to 30 and 1.5 μM Cu,

respectively. This variation in Si effect on Cu uptake by plants might be due to difference in growth conditions, exposure time, and the species studied. In this study, the Si-mediated decrease in Cu uptake by cotton plants might be associated with an increased uptake of other nutrients, deposition of Si in root endodermis, and/or adsorption of Cu on the root surface, as was observed in wheat with Si under Cu stress (Keller et al. 2015). It has been reported that Si application decreased the symplasmic and increased apoplasmic Cd concentrations in maize roots (Vaculik et al. 2012). In addition, Si application decreased Cu translocation from root to shoot in *Spartina densiflora* (Mateos-Naranjo et al. 2015).

Elevated Cu concentrations in cotton tissues induced phytotoxic effects, causing growth inhibition (Fig. 1, Table 1). This growth and biomass reduction might be the result of Cu-induced impairment (rigidity) of root cell wall,

Fig. 5 Effect of Cu concentrations (0, 25, and 50 μM) with and without 1 mM Si on superoxide dismutase (**a**, **b**), guaiacol peroxidase (**c**, **d**), catalase (CAT) (**e**, **f**), and ascorbate peroxidase (G, H) in leaves and roots of cotton. *Bars* represent ± SD of six replicates. *Different letters* indicate significant differences among the treatments at a $P < 0.05$
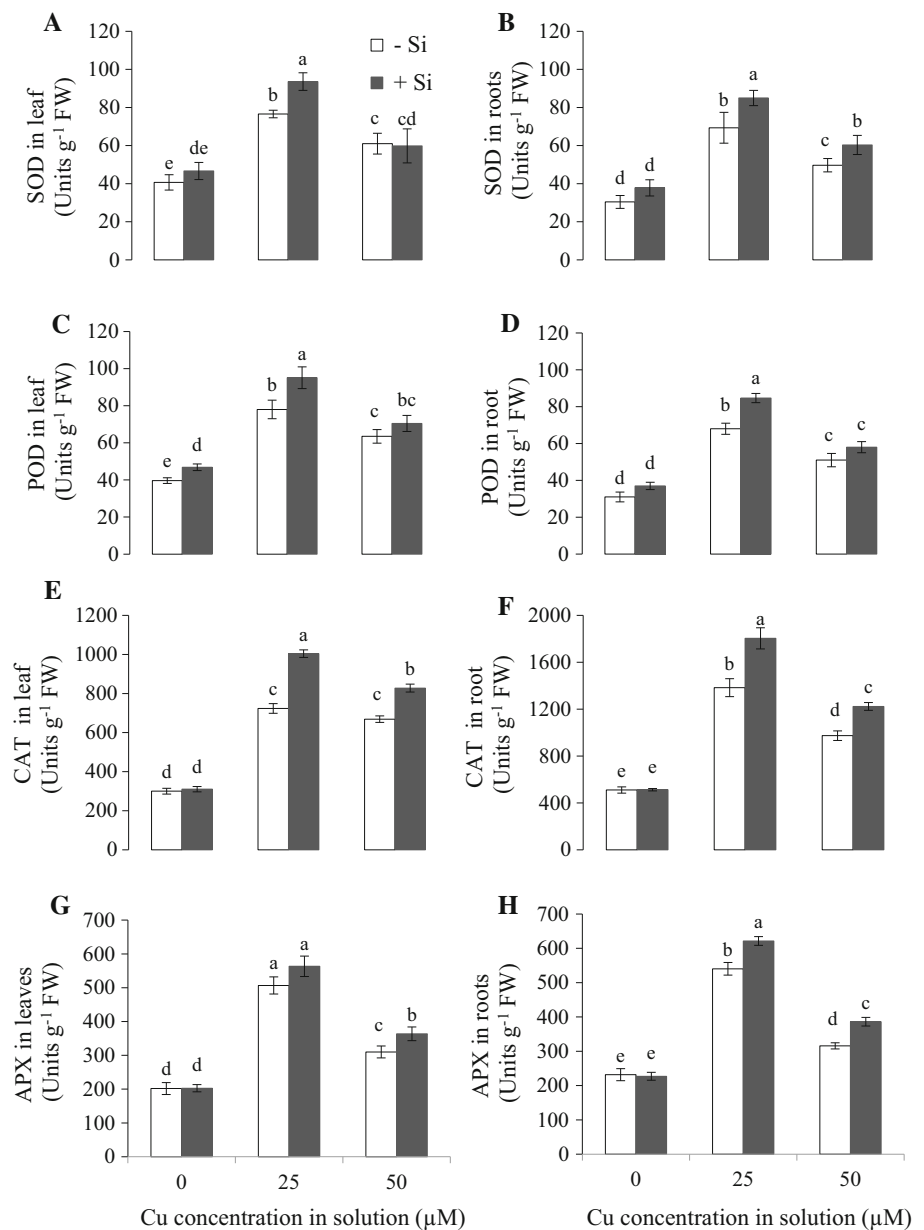

Table 2 Copper concentrations in roots, stem, and leaves of cotton seedlings grown in hydroponics with 0, 25, and 50 μM Cu and 0 or 1 mM Si

Treatment	Cu in roots (mg kg^{-1})	Cu in stem (mg kg^{-1})	Cu in leaves (mg kg^{-1})
Control	21.83 ± 0.76f	9.64 ± 0.56f	7.03 ± 0.20f
Si	26.50 ± 0.50e	15.67 ± 0.45e	9.35 ± 0.40e
Cu (25 μM)	1826.00 ± 18.77c	152.33 ± 7.07c	39.32 ± 1.64c
Cu25 + Si	1707.67 ± 14.74d	114.67 ± 6.61d	21.43 ± 1.93d
Cu 50 μM)	2208.67 ± 24.23a	239.33 ± 9.50a	55.09 ± 3.14a
Cu50 + Si	2015.10 ± 19.03b	189.00 ± 7.55b	34.27 ± 1.45b

Bars represent sd of six replicates. Different letters indicate significant differences among the treatments at a $p < 0.05$

which decreased nutrient uptake by plants (Kopittke and Menzies 2006; Feigl et al. 2015). Cu toxicity also impaired various physiological processes of plants, as was evidenced

by inhibiting gas exchange parameters, such as Tr, gs, WUE, and Ps in this study (Figs. 2, 3). Reduction in photosynthesis and gas exchange parameters is the early

response of plants to Cu toxicity (Adrees et al. 2015a) and might be the result of structural damage to photosynthetic apparatus, as has been reported by Feigl et al. (2015) in *Brassica juncea*. Exposure to excess Cu decreased photosynthetic pigments in many plant species, such as wheat (Keller et al. 2015), rapeseed, and Indian mustard (Feigl et al. 2015; Habiba et al. 2015).

Exogenously applied Si ameliorated the negative effects of Cu toxicity on growth, biomass, and photosynthesis of cotton seedlings (Figs. 1, 2, 3; Table 1). The Si-mediated growth improvement has been observed in metal (Li et al. 2013, b; Mateos-Naranjo et al. 2015; Vaculik et al. 2015; Khaliq et al. 2016; Rizwan et al. 2016c), drought, and salt stressed plants (Rizwan et al. 2015). Photosynthesis recovery in metal stressed (Mn, Cd and Cr) plants has linked with Si-induced protection of chloroplast machinery (Feng et al. 2010; Vaculik et al. 2015). In addition, Si might have ameliorated the nutrient imbalance of Cu-stressed cotton by repairing the cellular membrane, as has been evidenced by a relatively lower EL under Si + Cu than Cu application alone (Fig. 4). The Si-induced increased mineral nutrient uptake in wheat (Keller et al. 2015) and did not affect the mineral nutrients in *S. densiflora* (Mateos-Naranjo et al. 2015) which might be due to the variation in plant species and growth conditions. In addition, increase in plant growth may be due to the sequestration of Cu ions in leaf phytoliths, reducing cellular Cu mobility, but this phenomenon varied with plant species (Oliva et al. 2011; Keller et al. 2015) and needs further in depth investigations.

A direct effect of Cu toxicity in plants is the oxidative stress caused by overproduction of ROS (Adrees et al. 2015a). In plants, excess Cu could enhance the ROS production due to the Fenton– or Haber–Weiss reactions (Mittler 2002). In this study, elevated levels of Cu in cotton tissues increased ROS production in both leaves and roots of cotton seedlings (Fig. 4). Several studies have reported the Cu-mediated ROS production in many plant species, such as maize (Madejon et al. 2009; Dresler et al. 2014), wheat (Gajewska and SkLodowska 2010), rice (Thounaojam et al. 2012), *Brassica* species (Feigl et al. 2015), bamboo (Chen et al. 2015), soybean (Sanchez-Pardo et al. 2014), and cotton (Mei et al. 2015). Overproduction of ROS has also been observed in cotton under Ni (Khaliq et al. 2016) or Cd stress (Farooq et al. 2016). Our results showed that lower Cu (25 μM) enhanced while higher Cu (50 μM) decreased the activities of antioxidant enzymes (Fig. 5). Similar changes in antioxidant enzyme activities have been observed in cotton and other plant species exposed to either Cd or Cu stress (Farooq et al. 2013; Feigl et al. 2015). Up-regulation of antioxidant enzyme activities in response to lower Cu concentrations might be due to stimulation of plant defense system against Cu stress,

although higher Cu concentrations reduced activities of antioxidant enzymes by elevating ROS accumulation in plant tissues. Increase in ROS contents along with a decrease in enzyme activities in 50 μM Cu treatment indicated that plant suffered from Cu toxicity.

By contrast, Si application decreased oxidative stress by enhancing the activities of antioxidant enzymes (Figs. 4, 5). Similar effects of Si have been reported in several other plant species exposed to different heavy metals (Adrees et al. 2015b). The Si-mediated increase in antioxidant enzyme activities may accelerate the elimination of H_2O_2 under Cu stress. The APX is involved in H_2O_2 detoxification and the increase in APX activity might be involved in scavenging intracellular H_2O_2 level (Mittler 2002). The Si-mediated enhancement in antioxidant enzyme activities is considered as a defense mechanism in cotton seedlings against Cu stress. On the other hand, Si application decreased SOD, POD, and CAT activities in peanut and maize roots under Al and Zn stress as compared with respective metal treatments alone (Bokor et al. 2014; Shen et al. 2014). This showed that the Si effect on the activities of antioxidant enzymes varied with plant species and metal stress applied. Furthermore, the variation in the response of antioxidant enzyme activities in plant species indicated that Si-mediated modulation in antioxidant defense system might be a secondary response. However, further studies are still needed to clarify this phenomenon.

Conclusion

Results obtained from this study showed that Cu is toxic to cotton seedlings and decreased cotton growth, biomass, photosynthetic pigments, and gas exchange characteristics while increased TBARS, EL and H_2O_2 contents and antioxidant enzyme activities in both roots and shoots. Silicon application has the potential to reduce Cu accumulation in cotton seedlings and increased plant growth and photosynthesis through enhancing the activities of antioxidant enzymes under Cu stress. Silicon application may provide a useful option for safe cultivation in Cu-contaminated soils. Further field scale studies are needed to determine the long-term effects of Si application for improving cotton growth in Cu-contaminated soils.

Author contribution statement SA, MR, and SAB conceived the idea and designed research. MAF and MF conducted the experiment. SAB and MF did the analysis. SA, MR, MW, and UN analysed the data and developed the first full draft of the manuscript. GHA critically reviewed the manuscript. All authors contributed to the subsequent development and approved the final manuscript.

References

Adrees M, Ali S, Rizwan M, Ibrahim M, Abbas F, Farid M, Rehman MZ, Irshad MK, Bharwana SA (2015a) The effect of excess copper on growth and physiology of important food crops: a review. Environ Sci Pollut Res 22:8148–8162

Adrees M, Ali S, Rizwan M, Rehman MZ, Ibrahim M, Abbas F, Farid M, Qayyum MK, Irshad MK (2015b) Mechanisms of silicon-mediated alleviation of heavy metal toxicity in plants: a review. Ecotoxicol Environ Saf 119:186–197

Aebi H (1984) Catalase in vitro. Methods Enzymol 105:121–126

Ali B, Tao Q, Zhou Y, Gill RA, Ali S, Rafiq MT, Xu L, Zhou W (2013a) 5-Aminolevolinic acid mitigates the cadmium-induced changes in Brassica napus as revealed by the biochemical and ultra-structural evaluation of roots. Ecotoxicol Environ Saf 92:271–280

Ali B, Wang B, Ali S, Ghani MA, Hayat MT, Yang C, Xu L, Zhou W (2013b) 5-Aminolevulinic acid ameliorates the growth, photo-synthetic gas exchange capacity, and ultrastructural changes under cadmium stress in Brassica napus L. J Plant Growth Regul 32:604–614

Ali B, Qian P, Jin R, Ali S, Khan M, Aziz R, Tian T, Zhou W (2014) Physiological and ultra-structural changes in Brassica napus seedlings induced by cadmium stress. Biol Plant 58:131–138

Ando Y, Nagata S, Yanagisawa S, Yoneyama T (2013) Copper in xylem and phloem saps from rice (Oryza sativa): the effect of moderate copper concentrations in the growth medium on the accumulation of five essential metals and a speciation analysis of copper-containing compounds. Funct Plant Biol 40:89–100

Angelova V, Ivanova R, Delibaltova V, Ivanov K (2004) Bio-accumulation and distribution of heavy metals in fibre crops (flax, cotton and hemp). Indus Crops Prod 19:197–205

Bokor B, Vaculik M, Slováková L, Masarovič D, Lux A (2014) Silicon does not always mitigate zinc toxicity in maize. Acta Physiol Plant 36:733–743

Bravin MN, Le Merrer B, Denaix L, Schneider A, Hinsinger P (2010) Copper uptake kinetics in hydroponically-grown durum wheat (Triticum turgidum durum L.) as compared with soil's ability to supply copper. Plant Soil 331:91–104

Chen J, Shafi M, Li S, Wang Y, Wu J, Ye Z, Peng D, Yan W, Liu D (2015) Copper induced oxidative stresses, antioxidant responses and phytoremediation potential of Moso bamboo (Phyllostachys pubescens). Sci Rep. doi:10.1038/srep13554

Christiansen KS, Borggaard OK, Holm PE, Vijver MG, Hauschild MZ, Peijnenburg WJ (2015) Experimental determinations of soil copper toxicity to lettuce (Lactuca sativa) growth in highly different copper spiked and aged soils. Environ Sci Pollut Res 22:5283–5292

Collin B, Doelsch E, Keller C, Panfili F, Meunier JD (2012) Distribution and variability of silicon, copper and zinc in different bamboo species. Plant Soil 351:377–387

Collin B, Doelsch E, Keller C, Cazevieille P, Tella M, Chaurand P, Panfili F, Hazemann JL, Meunier JD (2014) Copper distribution and speciation in bamboo exposed to a high Cu concentration and Si supplementation. First evidence on the presence of reduced copper bound to sulfur compounds in Poaceae. Environ Pollut 187:22–30

Daud MK, Variath MT, Ali S, Jamil M, Khan MT, Shafi M, Shuijin Z (2009) Genetic transformation of bar gene and its inheritance and segregation behavior in the resultant transgenic cotton germplasm (br001). Pak J Bot 41:2167–2178

de Freitas TA, França MGC, de Almeida AAF, de Oliveira SJR, de Jesus RM, Souza VL, Mangabeira PA (2015) Morphology, ultrastructure and mineral uptake is affected by copper toxicity in young plants of Inga subnuda subs. luschnathiana (Benth.) TD Penn. Environ Sci Pollut Res 22:15479–15494

Dhindsa RS, Plumb-Dhindsa P, Thorne TA (1981) Leaf senescence correlated with increased levels of membrane permeability and lipid peroxidation and decreased levels of superoxide dismutase and catalase. J Exp Bot 32:93–101

Dionisio-Sese ML, Tobita S (1998) Antioxidant responses of rice seedlings to salinity stress. Plant Sci 135:1–9

Dresler S, Hanaka A, Bednarek W, Maksymiec W (2014) Accumulation of low-molecular-weight organic acids in roots and leaf segments of Zea mays plants treated with cadmium and copper. Acta Physiol Plant 36:1565–1575

Farooq MA, Ali S, Hameed A, Ishaque W, Mahmood K, Iqbal Z (2013) Alleviation of cadmium toxicity by silicon is related to elevated photosynthesis, antioxidant enzymes; suppressed cadmium uptake and oxidative stress in cotton. Ecotoxicol Environ Saf 96:242–249

Farooq MA, Ali S, Hameed A, Bharwana SA, Rizwan M, Ishaque W, Farid M, Mahmood K, Iqbal Z (2016) Cadmium stress in cotton seedlings: physiological, photosynthesis and oxidative damages alleviated by glycinebetaine. South Afr J Bot 104:61–68

Feigl G, Kumar D, Lehotai N, Pető A, Molnár Á, Rácz É, Ördög A, Erdei L, Kolbert ZS, Laskay G (2015) Comparing the effects of excess copper in the leaves of Brassica juncea (L. Czern) and Brassica napus (L.) seedlings: growth inhibition, oxidative stress and photosynthetic damage. Acta Biol Hung 66:205–221

Feng J, Shi Q, Wang X, Wei M, Yang F, Xu H (2010) Silicon supplementation ameliorated the inhibition of photosynthesis and nitrate metabolism by cadmium (Cd) toxicity in Cucumis sativus L. Sci Hort 123:521–530

Gajewska E, SkŁodowska M (2010) Differential effect of equal copper, cadmium and nickel concentration on biochemical reactions in wheat seedlings. Ecotoxicol Environ Saf 73:996–1003

Gu HH, Zhan S, Wang SZ, Tang YT, Chaney RL, Fang XH, Cai XD, Qiu RL (2012) Silicon-mediated amelioration of zinc toxicity in rice (Oryza sativa L.) seedlings. Plant Soil 350:193–204

Habiba U, Shafaqat Ali S, Farid M, Shakoor MB, Rizwan M, Ibrahim M, Abbasi GH, Hayat T, Ali B (2015) EDTA enhanced plant growth, antioxidant defense system, and phytoextraction of copper by Brassica napus L. Environ Sci Pollut Res 22:1534–1544

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189–198

Kang W, Bao J, Zheng J, Hu H, Du J (2015) Distribution and chemical forms of copper in the root cells of castor seedlings and their tolerance to copper phytotoxicity in hydroponic culture. Environ Sci Pollut Res 22:7726–7734

Keller C, Rizwan M, Davidian JC, Pokrovsky OS, Bovet N, Chaurand P, Meunier JD (2015) Effect of silicon on wheat seedlings (Triticum turgidum L.) grown in hydroponics and exposed to 0 to 30 μM Cu. Planta 241:847–860

Khaliq A, Ali S, Hameed A, Farooq MA, Farid M, Shakoor MB, Mahmood K, Ishaque W, Rizwan M (2016) Silicon alleviates nickel toxicity in cotton seedlings through enhancing growth, photosynthesis and suppressing Ni uptake and oxidative stress. Arch Agron Soil Sci 62:633–647

Khandekar S, Leisner S (2011) Soluble silicon modulates expression of Arabidopsis thaliana genes involved in copper stress. J Plant Physiol 168:699–705

Kopittke PM, Menzies NW (2006) Effect of Cu toxicity on growth of cowpea (Vigna unguiculata). Plant Soil 279:287–296

Kumar P, Tewari RK, Sharma PN (2008) Modulation of copper toxicity induced oxidative damage by excess supply of iron in maize plants. Plant Cell Rep 27:399–409

Kumar S, Kumar S, Prakash P, Singh M (2014) Antioxidant defense mechanisms in chickpea (Cicer arietinum L.) under copper and arsenic toxicity. Int J Plant Physiol Biochem 6:40–43

Li J, Leisner SM, Frantz J (2008) Alleviation of copper toxicity in Arabidopsis thaliana by silicon addition to hydroponic solutions. J Am Soc Hort Sci 133:670–677

Li P, Song A, Li Z, Fan F, Liang Y (2013a) Silicon ameliorates manganese toxicity by regulating manganese transport and antioxidant reactions in rice (Oryza sativa L.). Plant Soil 354:407–419

Li P, Song A, Li Z, Fan F, Liang Y (2013b) Silicon ameliorates manganese toxicity by regulating manganese transport and antioxidant reactions in rice (Oryza sativa L.). Plant Soil 354:407–419

Lichtenthaler HK (1987) Chlorophylls and carotenoids—pigments of photosynthetic biomembranes. In: Colowick SP, Kaplan NO (eds) Methods in enzymology, vol 148. Academic Press, San Diego, pp 350–382

Lin CY, Trinh NN, Fu SF, Hsiung YC, Chia LC, Lin CW, Huang HJ (2013) Comparison of early transcriptome responses to copper and cadmium in rice roots. Plant Mol Biol 81:507–522

Liu JJ, Wei Z, Li JH (2014a) Effects of copper on leaf membrane structure and root activity of maize seedling. Bot Stud 55:1–6

Liu L, Sun H, Chen J, Zhang Y, Li D, Li C (2014b) Effects of cadmium (Cd) on seedling growth traits and photosynthesis parameters in cotton (Gossypium hirsutum L.). Plant Omics J 7:284–290

Lukatkin A, Egorova I, Michailova I, Malec P, Strzałka K (2014) Effect of copper on pro-and antioxidative reactions in radish (Raphanus sativus L.) in vitro and in vivo. J Trace Elem Med Biol 28:80–86

Madejon P, Ramírez-Benítez JE, Corrales I, Barceló J, Poschenrieder C (2009) Copper-induced oxidative damage and enhanced antioxidant defenses in the root apex of maize cultivars differing in Cu tolerance. Environ Exp Bot 67:415–420

Mateos-Naranjo E, Gallé A, Florez-Sarasa I, Perdomo JA, Galmés J, Ribas-Carbó M, Flexas J (2015) Assessment of the role of silicon in the Cu-tolerance of the C 4 grass Spartina densiflora. J Plant Physiol 178:74–83

Mei L, Daud MK, Ullah N, Ali S, Khan M, Malik Z, Zhu SJ (2015) Pretreatment with salicylic acid and ascorbic acid significantly mitigate oxidative stress induced by copper in cotton genotypes. Environ Sci Pollut Res 22:9922–9931

Metzner H, Rau H, Senger H (1965) Untersuchungenzur Synchronisierbakeiteinzelner Pigmentmangel-Mutation von Chlorella. Planta 65:186–194 **(in German)**

Michaud AM, Chappellaz C, Hinsinger P (2008) Copper phytotoxicity affects root elongation and iron nutrition in durum wheat (Triticum turgidum durum L.). Plant Soil 310:151–165

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405–410

Nagajyoti PC, Lee KD, Sreekanth TVM (2010) Heavy metals, occurrence and toxicity for plants: a review. Environ Chem Lett 8:199–216

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867–880

Oliva SR, Mingorance MD, Leidi EO (2011) Effects of silicon on copper toxicity in Erica andevalensis Cabezudo and Rivera: a potential species to remediate contaminated soils. J Environ Monit 13:591–596

Ramzani PMA, Khan WD, Iqbal M, Kausar S, Ali S, Rizwan M, Virk ZA (2016) Effect of different amendments on rice (Oryza sativa L.) growth, yield, nutrient uptake and grain quality in Ni-contaminated soil. Environ Sci Pollut Res doi:10.1007/s11356-016-7038-x

Rehman MZ, Rizwan M, Ghafoor A, Naeem A, Ali S, Sabir M, Qayyum MF (2015) Effect of inorganic amendments for in situ stabilization of cadmium in contaminated soil and its phyto-availability to wheat and rice under rotation. Environ Sci Pollut Res 22:16897–16906

Rizwan M, Meunier JD, Hélène M, Keller C (2012) Effect of silicon on reducing cadmium toxicity in durum wheat (Triticum turgidum L. cv. Claudio W.) grown in a soil with aged contamination. J Hazard Mater 209–210:326–334

Rizwan M, Ali S, Ibrahim M, Farid M, Adrees M, Bharwana SA, Rehman MZ, Qayyum MF, Abbas F (2015) Mechanisms of silicon-mediated alleviation of drought and salt stress in plants: a review. Environ Sci Pollut Res 22:15416–15431

Rizwan M, Meunier JD, Davidian JC, Pokrovsky OS, Bovet N, Keller C (2016a) Silicon alleviates Cd stress of wheat seedlings (Triticum turgidum L. cv. Claudio) grown in hydroponics. Environ Sci Pollut Res 23:1414–1427

Rizwan M, Ali S, Qayyum MF, Ibrahim M, Rehman MZ, Abbas T, Ok YS (2016b) Mechanisms of biochar-mediated alleviation of toxicity of trace elements in plants: a critical review. Environ Sci Pollut Res 23:2230–2248

Rizwan M, Ali S, Adrees M, Rizvi H, Rehman MZ, Hannan F, Qayyum MF, Hafeez F, Ok YS (2016c) Cadmium stress in rice: toxic effects, tolerance mechanisms and management: a critical review. Environ Sci Pollut Res. doi:10.1007/s11356-016-6436-4

Sanchez-Pardo B, Fernandez-Pascual M, Zornoza P (2014) Copper microlocalisation and changes in leaf morphology, chloroplast ultrastructure and antioxidative response in white lupin and soybean grown in copper excess. J Plant Res 127:119–129

Shen X, Xiao X, Dong Z, Chen Y (2014) Silicon effects on antioxidative enzymes and lipid peroxidation in leaves and roots of peanut under aluminum stress. Acta Physiol Plant 36:3063–3069

Thounaojam TC, Panda P, Mazumdar P, Kumar D, Sharma G, Sahoo L, Panda S (2012) Excess copper induced oxidative stress and response of antioxidants in rice. Plant Physiol Biochem 53:33–39

Vaculik M, Landberg T, Greger M, Luxova M, Stolarikova M, Lux A (2012) Silicon modifies root anatomy, and uptake and subcellular distribution of cadmium in young maize. Ann Bot 110:433–443

Vaculik M, Pavlovič A, Lux A (2015) Silicon alleviates cadmium toxicity by enhanced photosynthetic rate and modified bundle sheath's cell chloroplasts ultrastructure in maize. Ecotoxicol Environ Saf 120:66–73

Wuana RA, Okieimen FE (2011) Heavy metals in contaminated soils: a review of sources, chemistry, risks and best available strategies for remediation. ISRN Ecol. doi:10.5402/2011/402647

Yruela I (2009) Copper in plants: acquisition, transport and interactions. Funct Plant Biol 36:409–430

Yruela I (2013) Transition metals in plant photosynthesis. Metallomics 5:1090–1109

Zhang XZ (1992) Themeasurement and mechanism of lipid peroxidation and SOD POD and CAT activities in biological system. In: Zhang XZ (ed) Research Methodology of Crop Physiology, Beijing Agriculture Press, pp 208–211

Zhang J, Kirkham MB (1994) Drought-stress induced changes in activities of superoxide dismutase, catalase and peroxidases in wheat leaves. Plant Cell Physiol 35:785–791