Impact of copper toxicity on stone-head cabbage (*Brassica oleracea* var. *capitata*) in hydroponics

Sajid Ali[*1], Muhammad Shahbaz[1,2], Ahmad Naeem Shahzad[3], Hafiz Azhar Ali Khan[1], Moazzam Anees[1], Muhammad Saleem Haider[1] and Ammara Fatima[4]

[1]Institute of Agricultural Sciences, University of the Punjab, Lahore 54590, Pakistan.

[2]Biology Department, Colorado State University, Fort Collins, Colorado 80523, USA

[3]Department of Agronomy, Bahauddin Zakaryia University, Multan, Pakistan.

[4]Department of Environmental Science, Lahore College for Women University, Lahore, Pakistan.

Corresponding Author:

Address: Institute of Agricultural Sciences, University of the Punjab, Lahore-54590
                    Pakistan

Email address: sajid.iags@pu.edu.pk

Phone Number: **Off.:** +92 42 9923 1846
                    Cell: +92 321 955 8969

ABSTRACT

Arable soils are frequently subjected to contamination with copper as the consequence of imbalanced fertilization with manure and organic fertilizers and/or extensive use of copper-containing fungicides. In the present study, the exposure of Stone-head cabbage (*Brassica oleracea* var. *capitata*) to elevated Cu^{2+} levels resulted in leaf chlorosis and lower biomass production at ≥ 2 µM. The nitrate content of the root was not affected at all Cu^{2+} levels, whereas that was substantially decreased at ≥ 5 µM Cu^{2+} in the shoot. The decrease in nitrate contents can be related to lower nitrate uptake rates because of growth inhibition by Cu-toxicity. The sulfate content of the shoot strongly increased at ≥ 2 µM Cu^{2+} indicating an increase in demand for sulfur under Cu stress. Moreover, there was a noticeable increase in water-soluble non-protein thiol content in the root and to a lesser extent in the shoot at ≥ 2 µM. Enhanced sulfate and water-soluble non-protein thiols upon exposure at elevated Cu^{2+} concentrations need further

study to evaluate their direct relation with the synthesis of metal-chelating compounds (i.e. phytochelatins).

Keywords: Leaf chlorosis, non-protein thiol, nutrient uptake, Cu contamination, biomass, Copper; Toxicity; Brassica; Cabbage; Sulfur; Thiols; Hydroponics

Short Title: Copper toxicity in Stone-head cabbage in hydroponics

INTRODUCTION

Transition metals such as copper (Cu), zinc (Zn) and molybdenum (Mo) are essential for the growth and development of plants, but they rapidly get toxic at higher levels (Kopsell and Kopsell, 2007). Cu contamination in agricultural soils as a consequence of mining metals, dispersal of sewage sludge, arbitrary and improper application of agrochemicals, addition of organic fertilizers and frequent use of irrigation with low quality water is a well-known problem (Dach and Starmans, 2005; Yruela, 2009). Cu being redox active metal can exist in both Cu^{2+} and Cu^+ forms in living organisms. At the protein level, Cu serves as a co-factor for various enzymes such as Cu/Zn-superoxide dismutase (Cu/ZnSOD), cytochrome c oxidase, ascorbate oxidase, amino oxidase, laccase, plastocyanin (PC), and polyphenol oxidase (Yruela, 2005; 2009; Pilon et al., 2006). However, redox cycling between Cu^{2+} and Cu^+ could induce oxidative stress by producing highly toxic hydroxyl radicals (Yruela, 2005, 2009).

Plants exposed to elevated levels of Cu show unspecific toxicity symptoms. Elevated Cu levels in soils primarily result in stunted root growth and leaf chlorosis (Kopsell and Kopsell, 2007; Shahbaz et al., 2010a). Copper toxicity-induced reduction in chlorophyll contents hinder the development of chloroplast, thalakoid membrane and photosystem II (PSII), which are considered as the most sensitive Cu toxicity sites (Pätsikkä et al., 1998, 2002; Burkhead et al., 2009; Yruela, 2005, 2009; Shahbaz et al., 2010b). At cellular level, toxicity may lead to binding of sulfhydryl groups in proteins, insufficiency or excess of other essential ions, oxidative damage and reduced cell transport (De Vos et al., 1993; Yruela, 2009). Furthermore, Cu-toxicity can change the mineral composition of plants i.e. for instance, Fe contents may decrease in the shoot (Pätsikkä et al., 1998; 2002; Kopsell and Kopsell, 2007; Shahbaz et al., 2010b), Ca and Mg may decrease in the root and Zn contents may increase in both root and shoot upon Cu exposure at elevated levels (Shahbaz et al., 2010b).

60   Root growth is more severely affected by elevated Cu than shoot growth and the major

61   proportion of Cu uptake retains in the root. Increased Cu contents in the plant tissues induce the

62   synthesis of metal-binding compounds (*viz*. phytochelatins), which are most likely glutathione-

63   derived compounds. (Inouhe, 2005; Ernst et al., 2008). Inductions of phytochelatins presume that

64   more sulfur is needed for synthesis of these compounds, which results into higher absorption,

65   and incorporation of sulfate. Nonetheless, the role of phytochelatins in detoxification of Cu is not

66   very clear yet (Ernst et al., 2008; Yruela, 2005; 2009; Shahbaz et al., 2010a).

67   *Brassica* and other vegetable crops are often grown in the surrounding areas of big cities and

68   industrial areas in developing countries like Pakistan, where they may be subjected to air and

69   heavy metals pollution (Yang *et al*. 2006). The direct application of sewage water to vegetables

70   is not only the source of many nutrients, but it is often contaminated with high levels of Cu and

71   other heavy metals. As a result of continues untreated sewage application, heavy metals not only

72   accumulate in the soil but also in vegetables (Younas *et al*. 1998; Butt *et al*. 2005). High Cu

73   content in crop plants might not only negatively affect plant growth and functioning, but will

74   also enter the food chain (Brun *et al*. 2001).

75   The present study used the hydroponics system which allows very close control over water

76   soluble Cu and other mineral concentrations as compared to soil-grown system. Cabbage is a

77   very important vegetable in all over the world. In Pakistan, cabbage is cultivated on almost 4.9

78   thousand hectares with 76.7 thousand tonnes annual production (FAO, 2013). The present study

79   was aimed to investigate the response of growth, pigment contents and sulfur metabolism of

80   Stone-head cabbage grown in hydroponics to the copper exposure.

81   **MATERIAL AND METHODS**

82   Stone-head cabbage (*Brassica oleracea* var. *capitata* F_1) seeds were germinated to sand in a

83   green house. The seedlings collected at ten days after germination were transferred on an aerated

84   25% modified Hoagland nutrient solution in a 11 liter container (**15.8" L x 10.3" W x 7" H; 3**

85   **plants set^{-1} and 12 sets container^{-1})** in a greenhouse for 10 days. The nutrient solution consists of

86   1.25 mM $Ca(NO_3)_2.4H_2O$, 1.25 mM KNO_3, 0.25 mM KH_2PO_4, 0 / 0.5 mM $MgSO_4.7H_2O$, 11.6

87   μM H_3BO_3, 2.4 μM $MnCl_2.4H_2O$, 0.24 μM $ZnSO_4.7H_2O$, 0.08 μM $CuSO_4.5H_2O$, 0.13 μM

88    $Na_2MoO_4.2H_2O$ and 22.5 μM Fe^{3+}-EDTA with supplemental concentrations of 0, 2, 5 and 10

89   μM $CuCl_2$ and pH 5.9-6.0. The nutrient solution was continuously aerated with Aqua-Supreme -

90 Air Pump - Model AP-4.The photoperiod was 14 h. 30 and 25°C (±5°C) temperatures were set

91 for day and night respectively, whereas the relative humidity was maintained at 60-70%.

92 **Pigment contents**

93 Whole shoot was homogenized (in 100% acetone @ 10 mL per g FW) followed by

94 centrifugation at 800 g for 20 minutes. Lichtenthaler (1987) was followed for the determination

95 of chlorophyll a, b and total carotenoid contents.

96 **Nitrate and sulfate contents**

97 Frozen root and shoot material was homogenized in de-mineralized water (10 mL per g fresh

98 weight) and one layer of Miracloth filter was used to filter the homogenate. The supernatant was

99 incubated in a water bath at 100°C for 10 min. The remainder was centrifuged for 15 min (0°C)

100 at 30,000 g. The anions were separated by HPLC and Maas et al. (1986) was followed for their

101 refractometric determination using a Knauer differential refractometer (model 98.00, Bad

102 Homburg, Germany).

103 **Water-soluble non-protein thiols**

104 Extraction medium containing 80 mM sulfosalicylic acid, 1 mM EDTA, and 0.15% (w/v)

105 ascorbic acid with an Ultra Turrax at 0°C (10 mL per g fresh weight) was used for the

106 homogenization of fresh plant matter. The resultant homogenous material was passed through

107 one layer of Miracloth which was then centrifuged at 30,000 g for 15 min (0°C). De Kok et al.

108 (1988) was followed for the determination of total water-soluble non-protein thiol content

109 colorimetrically at 413 nm after reaction with 5, 5'-dithiobis [2-nitrobenzoic acid].

110

111 **RESULTS**

112 **Plant biomass in response to Cu exposure**

113 Exposure of Stone-head cabbage to higher concentrations of Cu^{2+} (≥ 2 μM) in nutrient

114 solution caused chlorosis of both the shoot and young emerging leaves, that ultimately reduced

115 both root and shoot biomass production (Fig. 1). A 10 day exposure to increasing Cu^{2+}

116 concentrations in nutrient media led to a significant reduction of both root and shoot biomass

117 production at ≥ 2 μM Cu^{2+}. Shoot to root ratio improved at ≥ 5 μM Cu^{2+}, demonstrating that

118 when exposed to copper, root growth was more affected than shoot growth (Fig. 1). Root dry

119 matter content increased at 10 μM Cu^{2+}, whereas shoot dry matter content increased at ≥ 5 μM

120 Cu^{2+} (Fig. 2).

Pigment content in response to Cu exposure

121

122   The total chlorophyll (Chl. *a+b*) and carotenoids contents of Stone-head cabbage were

123 significantly decreased upon exposure at ≥ 2 µM Cu^{2+} (Fig. 3). There were significant decreases

124 in chlorophyll *a/b* and chlorophyll/carotenoid ratios when exposed to increased Cu^{2+}

125 concentrations (10 µM Cu^{2+}). Ten µM Cu^{2+} exposure resulted in the start of rapid development

126 of shoot chlorosis and significantly faster reduction in chlorophyll *a* contents of chlorophyll *b*

127 and carotenoids, ultimately leading to a significant reduction in chlorophyll *a/b* and

128 chlorophyll/carotenoid ratios (Fig. 3).

Sulfate and water-soluble non-protein thiol contents in response to Cu exposure

129

130   Elevated Cu^{2+} levels showed a significant effect on concentration of the nitrate, sulfate and

131 water-soluble non-protein thiol in stone-head cabbage. The nitrate contents of the roots showed a

132 non significant response to the Cu exposure at different levels, however in shoots it were

133 significantly decreased at ≥ 5 µM Cu^{2+} (Fig. 4). Sulfate contents in the roots were not affected;

134 however, Cu^{2+} treatments of ≥ 2 µM substantially increased the sulfate contents of the shoot

135 (Fig. 4). There was slight decrease in nitrate to sulfate ratio in the root and a strong decrease in

136 the shoot when exposed to elevated levels of Cu (Fig. 4). Furthermore, the exposure to ≥ 2 µM

137 Cu^{2+} resulted in a solid raise in water-soluble non-protein thiol contents in the roots and to a

138 smaller degree in the shoots at 10 µM Cu^{2+} (Fig. 5).

DISCUSSION

139

140   Cu exposure at elevated levels (< 2 µM Cu^{2+}) to Stone-head cabbage significantly

141 decreased the production of root and shoot biomass and raised the ratio of the shoot to the root.

142 Copper contamination in the root environment generally results in retarded production of root

143 and shoot biomass and a reduced photosynthetic activity. Moreover, it causes chlorosis, necrosis

144 and bleaching of pigments (Yruela, 2005, 2009; Sheldon and Menzies, 2005; Shahbaz et al.

145 2010a, b). In cabbage, the reduced production of biomass when exposed to elevated Cu levels

146 coincided with decreased pigment contents (chl a, b, carotenoids; Fig. 3) which may have

147 resulted in reduced activity of photosynthesis and the dark respiration rate (Shahbaz et al.,

148 2010a). It has been shown that Cu-toxicity damages chloroplasts either by inducing iron

149 deficiency or by replacing Mg in the chlorophyll by Cu (Pätsikkä et al., 2002; Küpper et al.,

150 2003). Cu exposure at elevated levels not only decreased the pigment content but there was also

151 a change in pigment composition. Chlorophyll a content decreased significantly faster than that

152 of chlorophyll b and carotenoids, which resulted in a decreased chlorophyll a/b and
153 chlorophyll/carotenoid ratio. Similar results were reported by Chu et al. (2006) in *Trifolium*
154 *repens* L.

155 It is shown that the production of root biomass was more influenced than that of the shoot
156 biomass production. The relatively higher reduction in the root biomass upon exposure to metal
157 contamination could be due the fact that roots come in direct contact with toxic metals (Cd, Cu).
158 Toxic metal-induced hindered root growth also reduces the uptake of essential nutrients (Sheldon
159 and Menzies, 2005).

160 Plants have evolved a tightly-controlled mechanism for the absorption, allocation and
161 assimilation of sulfate under normal conditions. (Hawkesford and De Kok, 2006). Enhanced
162 exposure of cabbage to Cu concentrations considerably affects the contents and allocation of
163 sulfur compounds in the root and shoot of cabbage (Shahbaz et al., 2010a). The raised shoot
164 sulfate contents might be attributed to Cu-toxicity induced upregulation of the sulfate suppliers
165 in roots (Shahbaz et al., 2010a). To maintain rapid growth rates under stress conditions, *Brassica*
166 species increase their demand for sulfur supply (Koralewska et al., 2008; 2009). Exposure of
167 Stone-head cabbage to elevated levels of Cu did not affect the nitrate contents in roots, however
168 at ≥ 5 µM Cu^{2+} there was a significant decrease in the shoot nitrate contents. The reduced
169 production of plant biomass at ≥ 5 µM Cu^{2+} could be attributed to reduced supply of nitrate in the
170 shoot. The decrease in nitrate: sulfate ratio in the shoot of Stone-head cabbage under elevated Cu
171 levels may be attributed to enhanced sulfate contents, however the link between uptake rates of
172 nitrate and sulfate is not evident yet (Stulen and De Kok, 2012) . Since both nitrate and sulfate
173 are involved in amino acid and protein synthesis, their uptake rates are related with growth rates
174 (Stulen and De Kok, 2012).

175 Roots accumulated a slightly raised level of water-soluble non-protein thiols when
176 compared with that of the shoot. Only a small proportion of the rise in thiol content might be
177 attributed to a Cu-induced synthesis of phytochelatins in Chinese cabbage (Shahbaz et al.,
178 2010a). Previous reports suggest that the formation of sulfur-rich metal-chelating compounds
179 (i.e. water-soluble non-protein thiols) when disclosed to prospective toxic metals may perhaps
180 require an enhanced demand for sulfur, viz. the absorption rate and incorporation of sulfate
181 (Sirko and Gotor, 2007; Ernst et al., 2008). The possible significance of sulfur nutrition upon
182 elevated copper exposure needs further investigation.

183 **CONCLUSIONS**

184   This investigation has shown that the elevated copper levels in the root surroundings

185 proved toxic for Stone-head cabbage. Copper exposure at ≥ 2 µM Cu^{2+} negatively affected the

186 plant biomass production and pigment contents. Furthermore, elevated Cu content considerably

187 affected the concentration of sulfate and water-soluble non-protein thiol of Stone-head cabbage

188 which might be due to the induction of phytochelatins to detoxify excess copper.

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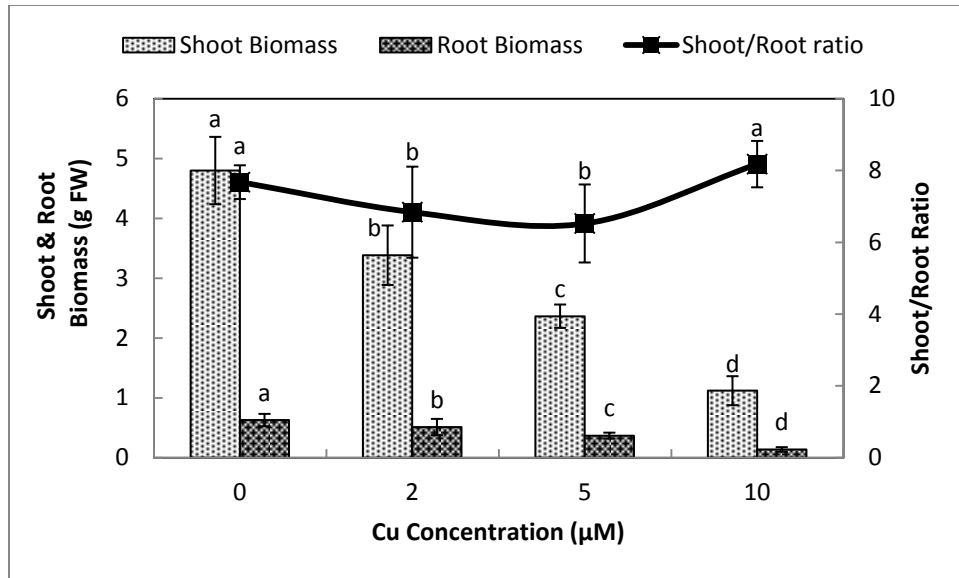
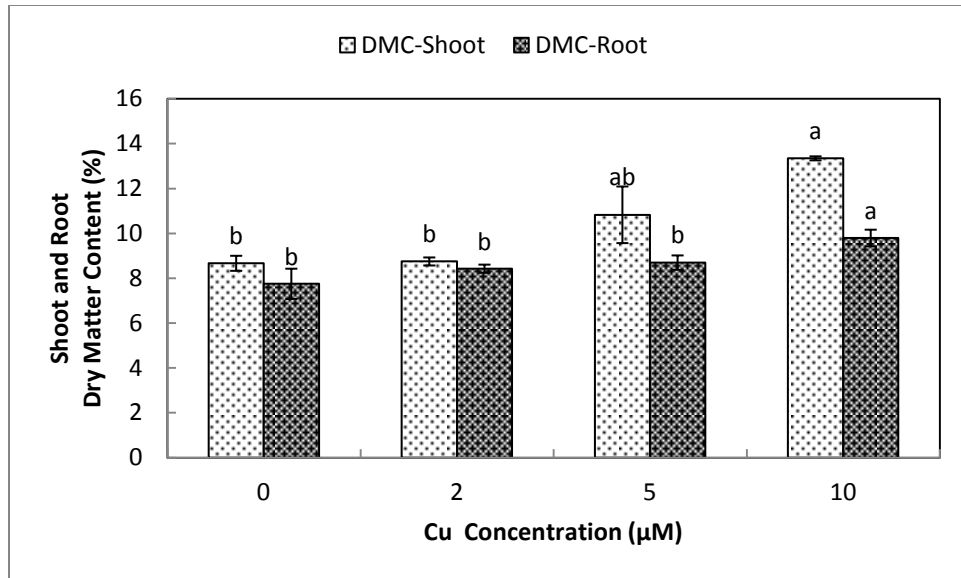
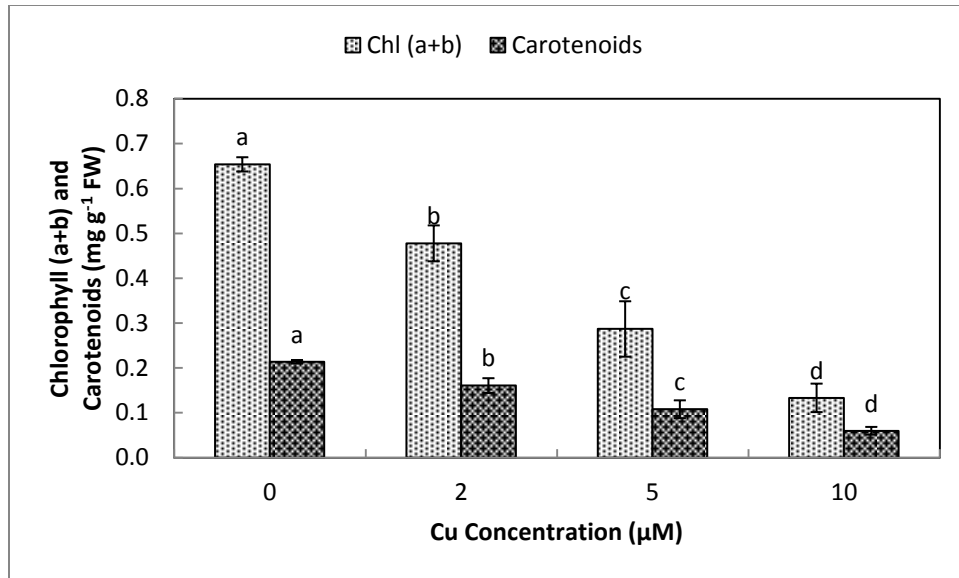
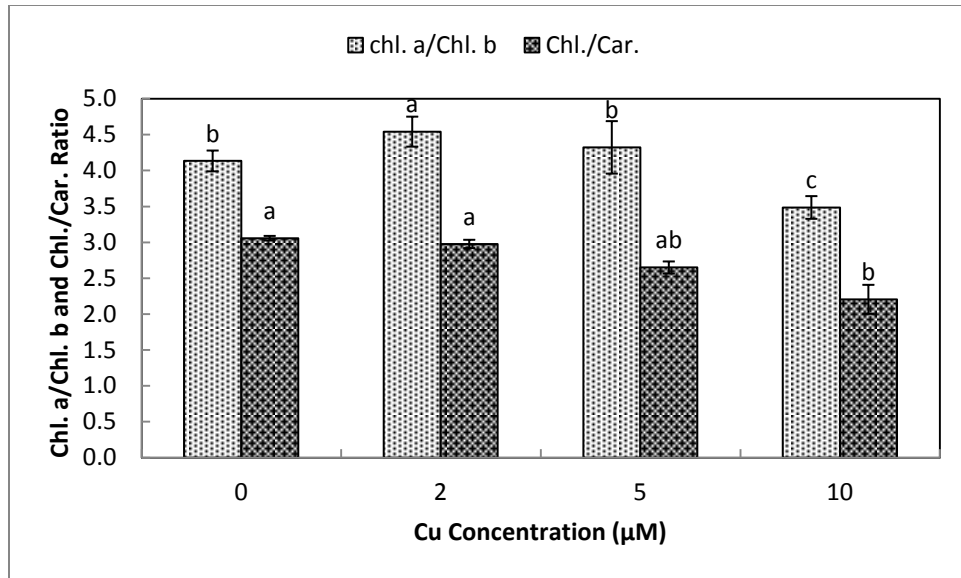
268

Impact of elevated levels of Cu^{2+} on biomass production of Stone-head cabbage (*Brassica oleracea* var. *capitata*).

10-day-old seedlings of Stone-head cabbage (*Brassica oleracea* var. *capitata*) were grown on a 25% Hoagland solution containing 0, 2, 5 and 10 µM $CuCl_2$ in the root environment. Data on biomass production (g FW) and shoot/root ratio represent the mean of 2 independent experiments with 9 measurements having 3 plants in each treatment (± SD). Means with different letters differ significantly at $p \leq 0.01$ (Student's t-test).

Impact of elevated levels of Cu^{2+} on dry matter content of Stone-head cabbage (*Brassica oleracea* var. *capitata*).

10-day-old seedlings of Stone-head cabbage (*Brassica oleracea* var. *capitata*) were grown on a 25% Hoagland solution containing 0, 2, 5 and 10 μM $CuCl_2$ in the root environment. Data on dry matter content (%) represent the mean of 2 independent experiments with 9 measurements having 3 plants in each treatment (± SD). Means with different letters differ significantly at p ≤ 0.01 (Student's t-test).

Impact of elevated levels of Cu^{2+} on pigment content (chl a+b & carotenoids) of Stone-head cabbage (*Brassica oleracea var. capitata*).

10-day-old seedlings of Stone-head cabbage (*Brassica oleracea* var. *capitata*) were grown on a 25% Hoagland solution containing 0, 2, 5 and 10 µM $CuCl_2$ in the root environment. Data on chlorophyll content (chl a+b; mg g^{-1} FW) and carotenoids content (mg g^{-1} FW)represent the mean of 2 independent experiments with 9 measurements having 3 plants in each treatment (± SD). Means with different letters differ significantly at $p \leq 0.01$ (Student's t-test).

Impact of elevated levels of Cu^{2+} on pigment content (chla/chlb & chl/car. ratio) of Stone-head cabbage (*Brassica oleracea var. capitata*).

10-day-old seedlings of Stone-head cabbage (*Brassica oleracea* var. *capitata*) were grown on a 25% Hoagland solution containing 0, 2, 5 and 10 μM CuCl$_2$ in the root environment. Data on chlorophyll content (chl a/Chlb and chl/carotenoid ratio) represent the mean of 2 independent experiments with 9 measurements having 3 plants in each treatment (± SD). Means with different letters differ significantly at p ≤ 0.01 (Student's t-test).

Impact of elevated levels of Cu^{2+} on nitrate and sulfate content of Stone-head cabbage (*Brassica oleracea* var. *capitata*).

10-day-old seedlings of Stone-head cabbage (*Brassica oleracea* var. *capitata*) were grown on a 25% Hoagland solution containing 0, 2, 5 and 10 μM $CuCl_2$ in the root environment. Data on nitrate and sulfate content (μmol g^{-1} FW) represent the mean of 2 independent experiments with 9 measurements having 3 plants in each treatment (± SD). Means with different letters differ significantly at $p \leq 0.01$ (Student's t-test).