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

Gábor FEIGL[2+], Devanand KUMAR[1+], Nóra LEHOTAI[2], Andrea PETŐ[2], Árpád MOLNÁR[2], Éva RÁCZ[2], Attila ÖRDÖG[2], László ERDEI[2], Zsuzsanna KOLBERT[2*]and Gábor LASKAY[2]

[1]Department of Life Sciences and Bioinformatics, Assam University, Silchar-788 011, Assam, India
[2]Department of Plant Biology, University of Szeged, Szeged, Hungary

+ These authors contributed equally to this work.

Corresponding author: Gábor Feigl
E-mail: fglgbr@gmail.com
Tel.: +36-30-315-8126

Running title: Responses of *Brassica* seedlings to copper stress

Abstract

Hydroponic experiments were conducted to compare the effects of excess copper (Cu) on growth and photosynthesis in young Indian mustard (*Brassica juncea*) and oilseed rape (*Brassica napus*). We compared the effects of excess Cu on the two *Brassica* species at different physiological levels from antioxidant levels to photosynthetic activity. Nine-day-old plants were treated with Cu (10, 25 and 50 μM $CuSO_4$) for 7 and 14 days. Both species took up Cu from external solution to a similar degree but showed slight root-to-shoot translocation. Furthermore, after seven days of treatment, excess Cu significantly decreased other microelement content, such as iron (Fe) and manganese (Mn), especially in the shoots of *B. napus*. As a consequence, the leaves of young *Brassica napus* plants showed decreased concentrations of photosynthetic pigments and more intense growth inhibition; however, accumulation of highly reactive oxygen species (hROS) were not detected. After 14 days of Cu exposure the reduction of Fe and Mn contents and shoot growth proved to be comparable in the two species. Moreover, a significant Cu-induced hROS accumulation was observed in both *Brassica* species. The diminution in pigment contents and photosynthetic efficiency were more pronounced in *B. napus* during prolonged Cu exposure. Based on all the parameters *B. juncea* appears to be more resistant to excess Cu than *B. napus*, rendering it a species with higher potential for phytoremediation.

Keywords: *Brassica juncea*, *Brassica napus*, copper, oxidative stress, photosynthesis

1. Introduction

Among heavy metals, copper in trace amounts is essential for plant life but it becomes toxic at higher concentrations. In excess, this heavy metal seriously inhibits leaf expansion at the early growth stage and it causes changes in physiological processes such as transpiration, photosynthetic electron transport and biosynthesis of chlorophyll [27].

Excess Cu can affect photosynthetic electron transport on the reducing side of PSI at the level of ferredoxin. In addition, it can alter the PSII on the oxidising side by inhibiting the electron transport at P680 as well as by inactivating some PSII reaction centres [35]. A few studies indicate that excess Cu can impair the PSII electron transport on its reducing side by affecting the rate of electron transfer from tyrosine (Y_Z) to the oxidized primary donor $P680^+$ [15].

At molecular level, excess Cu is able to induce the formation of reactive oxygen species (ROS) *via* the Fenton- or Haber-Weiss reactions which subsequently damage proteins, nucleic acids and lipids [13]. This effect of excess Cu was supported by the positive correlation between Cu-treatment and the production of hydroxyl radicals in *Arabidopsis* [8]. Besides, excess Cu can indirectly cause oxidative stress by disrupting the balance between ROS generation and detoxification [28]. The activities of several ROS scavenging enzymes (such as superoxide dismutase, SOD; catalase, CAT and peroxidases, POD) are modulated by excess Cu but the effect depends on the plant species, the concentration and the duration of exposure [34].

Brassica species are economically important as food and oilseed plants. Indian mustard (*Brassica juncea*) is primary source of the anticarcinogenic 3-butenyl glucosynolate [18]. Oilseed rape (*Brassica napus*) is also known as an oilseed plant offering a high yield potential due to chloroplast number per unit leaf area [7]. Moreover, these species were chosen for the experiments because they possess rapid growth, high biomass, and a remarkable capacity to take up toxic metals [17; 12].

Accumulation of heavy metals in the environment and their toxic effects through the food chain can lead to serious ecological and health problems. Therefore, it is important to determine and compare the susceptibility of these promising species for phytoremediation to Cu exposure.

For that reason, the aim of the present study was to compare the effects of excess Cu at different concentrations and treatment durations on the morphological changes of young *B. juncea* and *B. napus* leaves and the background of this response such as changes in the element content and the levels of highly reactive oxygen species, furthermore the behaviour of the antioxidant defence and photosynthetic systems.

2. Materials and methods

2.1 Plant material and growth conditions

The study was conducted on *B. juncea* L. Czern. and *B. napus* L. The seeds were surface sterilised by 10% (v/v) sodium hypochlorite for 10 min, then rinsed and imbibed for 30 minutes in running water and germinated and grown in perlite supported hydroponically for 9 days under controlled condition in a greenhouse (12/12 day/night period, 150 $\mu mol/m^2/s$ light intensity, relative humidity of 55-60% and 25±2°C). Nine-day-old *Brassica* plants were grown in full-strength Hoagland solution supplemented with 10, 25 and 50 μM $CuSO_4$. As controls, untreated plants were used. Shoots were sampled for experimental analysis on the 7th and 14th days of the treatment.

2.2 Measurement of the shoot size

The plants treated with different Cu concentrations were separated carefully and washed thoroughly. Fresh and dry weights (g) of the shoots were measured on the 7th and 14th days of the treatments. Leaf area was also determined on the 7th and 14th days, on at least 10 specimens from every concentration in every case by using a grid and ImageJ (National Institute of Mental Health, Bethesda, Maryland, USA) image processing software.

2.3 Measurement of Cu concentration by ICP-MS

For determination of Cu concentrations in the plant tissues, inductively-coupled plasma mass spectrometer (Thermo Scientific XSeries II, Asheville, USA) was applied. Shoots of control, 10, 25 and 50 μM Cu-treated *B. juncea* and *B. napus* plants were rinsed with distilled water. After drying at 70°C for 72 hours, nitric acid (65%, w/v) and H_2O_2 (30%, w/v) was added to the samples, which were destructed at 200°C and 1.600 W for 15 minutes. Values of Cu concentrations are given in μg/g dry weight (DW) and the bioaccumulation factor (BAF) was calculated as follows:

BAF= Cu concentration in plant tissues (μg/g)/ Initial Cu concentration in the nutrient solution (μg/g).

2.4 *In situ* detection of highly reactive ROS and measurement of antioxidants

Highly reactive ROS, such as peroxynitrite ($ONOO^-$), hydroxyl radical ($OH^\cdot$) and hypochlorite anion (OCl^-) were visualized by 3’-(p-aminophenyl) fluorescein (APF) [16]. Leaf discs with a diameter of 7.5 mm were cut representatively from the fully developed leaves. They were incubated in 10 μM APF solution for 1 hour at room temperature in darkness, and were washed twice with TRIS-HCl buffer (10 mM, pH 7.4). Experiments were carried out using a Zeiss Axiovert 200M inverted-fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a high resolution digital camera (Axiocam HR, HQ CCD) with filter set 10 (excitation: 450-490 nm, emission: 515-565 nm). Fluorescence intensities (pixel intensities) were measured on digital images within circular areas of 600 μm radii using Axiovision Rel. 4.8 software. The radii of circles were not modified during the experiments.

SOD (EC 1.15.1.1) activity was determined by measuring the ability of the enzyme to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) in the presence of riboflavin in light [6]. The enzyme activity was expressed in terms of U/mg fresh weight; one unit (U) of SOD corresponds to the amount of enzyme causing 50% inhibition of NBT reduction in light.

CAT (EC 1.11.1.6) activity was determined by the decomposition of H_2O_2 and was measured spectrophotometrically by following the decrease in absorbance at 240 nm [38]. One U = the amount of H_2O_2 (in μmol) decomposed in 1 min. For measuring SOD and CAT the same crude extract was used. 250 mg plant material was ground with 10 mg polyvinyl polyvinylpyrrolidone (PVPP) and 1 ml 50 mM phosphate buffer (pH 7.0, with 1mM EDTA added).

Activity of ascorbate peroxidase (APX) (EC 1.11.1.11) was measured according to a modified method by Nakano and Asada [29] by monitoring the decrease in ascorbate content at 265 nm (E=14 mM cm^{-1}). For the enzyme extract, 250 mg plant material was ground with 1.5 ml extraction buffer containing 1mM EDTA, 50mM NaCl and 900 μM ascorbate. Data are expressed as specific activity (Unit/g fresh weight).

Determination of ascorbate (ASA)/dehydroascorbate (DHA) contents was carried out by the method of Law et al. [21]. Plant material (250 mg) was ground in 1 ml 5% (w/v) trichloroacetic acid (TCA). The measurement is based on the reduction of Fe^{3+} to Fe^{2+} by ascorbate and then Fe^{2+} forms a complex with bipyridyl resulting in pink colour with an absorption maximum at 525 nm. The amount of total ASA was determined by the reduction of dehydroascorbate to ascorbate by dithiothreitol (DTT). ASA/DHA contents were expressed in μmol/g fresh weight.

2.5 Measurement of pigment composition and chlorophyll fluorescence

The amount of chlorophyll a, b and total carotenoids were determined using the method described by Lichtenthaler [23]. The pigments were extracted with 80% acetone and then the extract's absorbance was measured at 663, 646 and 470 nm. The calculated amounts of the pigments are expressed as μg pigment/g fresh weight.

Chlorophyll fluorescence parameters were measured using a Pulse Amplitude-Modulated Fluorometer (Program "Run 8", PAM 200 Chlorophyll Fluorometer, Heinz Walz GmbH, Effeltrich, Germany). Leaves of treated and control plants were first dark adapted for 30 minutes and Fm, Fm', Ft and Fo' parameters were measured in the function of increasing light intensity (PAR = Photosynthetic Active Radiation) from 60 to 850 μmol photons/m/s. From these parameters the effective quantum yield of PSII (Yield = $(Fm'-Ft)/Fm'$), electron transport rate (ETR = Yield x PAR x 0.5 x 0.84), photochemical quenching (qP = $(Fm'-Ft)/(Fm'-Fo')$) and non-photochemical quenching (NPQ = $(Fm-Fm')/Fm'$) were calculated and recorded. All measurements were carried out on leaves from five different plants in three parallel experiments.

2.6 Statistical analysis

The results are expressed as mean ± SE. Multiple comparison analyses were performed with SigmaStat 12 software using analysis of variance (ANOVA, $P<0.05$) and Duncan's test. In some cases, Microsoft Excel 2010 and Student's t-test were used ($^{*}P\leq0.05$, $^{**}P\leq0.01$, $^{***}P\leq0.001$). All experiments were carried out at least two times. In each treatment at least 10 samples were measured.

3. Results and discussion

3.1 Morphological changes, Cu accumulation capability and tolerance

Regarding all the three examined shoot growth parameters, a remarkable and concentration-dependent reduction was found in the *Brassica* plants. In the short-term treatments (up to 7 days) both fresh and dry weight was reduced more significantly in *B. napus*. However, by the 14th day of the treatment this difference disappeared and both species exhibited a significant reduction in the shoot growth. The highest doses of Cu treatment resulted in 80% decrease in both fresh and dry mass (Figure 1ab). On the contrary, the biggest reduction in leaf area was observed in *B. juncea* and this was caused by 25 and 50 μM Cu at both short- and long-term treatments (Figure 1c; Fig 2). According to Maksimyec [27], the serious shoot growth inhibition induced by copper is characteristic in the early developmental stage of the plants. Despite the growth inhibition, no visible symptoms of cell death (e.g. necrotic spots, lesions) were observed on the leaves during the treatment period.

Both *Brassica* species possessed similar basal Cu contents (~12-18 μg/g DW) in their shoots, which is in agreement with the results published by Russo et al. [32], where the Cu content in the control leaves of *B. napus* was determined as 18 μg/g DW. Both species showed similar Cu accumulation rates and the enhancement of Cu contents in the shoots depends on the metal concentration in the nutrient solution. A notable increment in Cu content was measured already in *Brassica* shoots treated with the lowest Cu concentration. However, in cases of 25 and 50 μM Cu treatments the enhancement of Cu content in the shoot tissues is lower, compared to the lowest external Cu concentration (Table 1). The root-to-shoot Cu transport can be conceived by the movement of nicotiamine complexes via the xylem vessels [5].

7th day

		Control	10 μM Cu		25 μM Cu		50 μM Cu	
B. juncea	**Cu**	12.93 ± 0.66	45.35 ± 1.52	***	65.22 ± 1.28	***	162.2 ± 4.15	***
	Cu BAF	391.81 ± 7.25	68.50 ± 2.56	***	40.83 ± 1.97	***	40.46 ± 1.88	***
	Fe	90.99 ± 0.75	61.95 ± 1.45	***	37.04 ± 0.42	***	73.03 ± 1.22	***
	Mn	48.66 ± 0.36	25.86 ± 1.33	***	17.79 ± 0.20	***	19.85 ± 0.33	***
B. napus	**Cu**	18.52 ± 0.33	40.50 ± 1.75	***	66.60 ± 1.01	***	85.34 ± 2.32	***
	Cu BAF	561.21 ± 9.58	61.17 ± 2.33	***	41.70 ± 2.01	***	21.29 ± 0.32	***
	Fe	104.20 ± 1.03	37.02 ± 1.50	***	85.69 ± 0.50	***	48.63 ± 1.12	***
	Mn	82.52 ± 0.42	21.86 ± 0.17	***	20.08 ± 0.13	***	19.88 ± 0.05	***

14th day

		Control	10 μM Cu		25 μM Cu		50 μM Cu	
B. juncea	**Cu**	12.41 ± 0.42	49.79 ± 1.33	***	79.56 ± 3.30	***	88.29 ± 2.87	***
	Cu BAF	376.1 ± 8.11	75.21 ± 3.02	***	49.81 ± 2.18	***	22.28 ± 0.56	***
	Fe	83.66 ± 0.99	30.43 ± 0.71	***	32.73 ± 0.88	***	48.44 ± 0.44	***
	Mn	60.25 ± 0.41	27.78 ± 1.02	***	15.40 ± 0.14	***	13.81 ± 0.08	***
B. napus	**Cu**	12.47 ± 0.15	57.66 ± 0.36	***	74.74 ± 4.66	***	82.01 ± 5.15	***
	Cu BAF	377.87 ± 7.98	87.09 ± 3.35	***	46.80 ± 2.04	***	20.46 ± 0.78	***
	Fe	74.26 ± 0.75	43.03 ± 0.84	***	37.62 ± 1.35	***	39.44 ± 0.79	***
	Mn	60.01 ± 0.16	35.36 ± 0.10	***	15.77 ± 0.33	***	15.96 ± 0.04	***

Table 1 Concentrations (µg/g DW) of Cu, Fe, Mn and bioaccumulation factor for Cu in the shoots of B. juncea and B. napus after 7 and 14 days of Cu treatment. Significant differences according to Student’ s t-test (n = 10, ***P ⩽ 0.001) are indicated.

Similarly to the results of Ebbs and Kochian [10], excess Cu induced deficiency in other microelements such as Fe and Mn in the shoots. In the short term, Fe and Mn contents were lower in Cu-treated *B. napus* than in *B. juncea*. As the effect of 14 days of Cu exposure, the difference between Fe and Mn contents between the species disappeared. Iron depletion in the shoot derives from the competition between Fe and Cu during the uptake [22]. Intervenial chlorosis as the major symptom of iron deficiency [37] was very conspicuous and visible on the leaves of both *Brassica* species treated with Cu (see Fig 2). According to Lidon and Henriques [24], excess Cu changes the uptake rate of manganese, which can explain the Cu-induced reduction in its content.

The bioaccumulation factor (BAF) gives information about the metal accumulation potential, as well as the phytoremediation ability of plants [40]. The BAF values of the shoot system significantly decreased in both species, since the rate of Cu accumulation was lower in the shoot system than the enhancement in Cu content in the external solution (Table 1). On the contrary, BAF of the root enhanced in the function of increasing external Cu concentrations [11]. These suggest the strong ability of these plants to extract Cu from the medium and to effectively accumulate it in their root system; however, they possess a slighter root-to-shoot translocation of Cu. Similarly, Cd was shown to accumulate preferentially in the roots and part of it was translocated to the shoot of *B. juncea* [33]. Therefore, the application of these *Brassica* species in rhizofiltration purposes can be suggestible [9;11].

3.2 Oxidative damage and activation of the antioxidant defence system

Oxidative processes taking place in the leaves can be inferred by measuring the levels of highly reactive ROS (hydroxyl radical, peroxynitrite and hypochlorite radical) and the activities of antioxidants. In *B. juncea* 7-day exposure to excess Cu caused a significant accumulation of ROS, but not in *B. napus*. In the long term (14 days) both species exhibited significant ROS production which was elicited by 25 and 50 µM Cu in *B. juncea*, whereas in *B. napus* 10 µM was effective, too (Fig 3a).

Being a transition metal, Cu in excess induces the formation of reactive oxygen species (ROS) based on the Fenton- or Haber–Weiss reactions [13] and it changes the activities or transcription levels of antioxidants [26; 19; 25]. In the short term, the activity of the H_2O_2 decomposing enzyme CAT decreased by ~40% as the effect of excess Cu in *B. juncea* shoots. In contrast, a significant and concentration-dependent increase in CAT activity was observed in *B. napus*. In long term, no significant alteration of CAT activity was observed (Fig 3b). In the case of APX enzyme, the 7-day-long Cu exposure did not lead to activity changes, while in long term the activity of this antioxidant enzyme decreased in both species (Fig 3c). Similarly, in the work of Luna et al. [26] reduced APX activity was detected in Cu-exposed oat plants, which can contribute to ROS accumulation. However, in our previous work a significant Cu-induced induction of SOD activity was observed in the root system of *Brassica* [11], in the shoot the activity of this enzyme did not show any significant change during the experimental period (Fig 3d). Similarly, Singh et al. [36] observed an enhancement of SOD activities in *Brassica* shoots only caused by 50 µM or higher Cu concentration. The amount of ascorbate and dehydroascorbate (Fig 4) and their ratios (ASA/DHA, Table 2) were also determined in the shoot system. The concentration of the reduced ascorbate form (ASA) did not show significant changes in none of the examined species during the experimental period. On the 7th day, Cu treatments caused a concentration- and time-dependent accumulation of oxidized ascorbate (DHA) in both species; however this effect of excess Cu proved to be slighter in the shoot system of *B. juncea*. In case of long Cu exposure, the enhancement of DHA content was significant only in *B. juncea* treated with 50 µM Cu. Under control conditions, *B. napus* showed more reduced ascorbate pool (lower ASA/DHA ratios) in the shoot system on both sampling days than *B. juncea*. As the effect of the increasing applied Cu concentrations, the reduced ASA ratio remarkably decreased in both species after 7-day-long exposure. In long term, the diminution of the ASA/DHA ratio was more pronounced in *B. napus*, which can be explained by the higher APX activity. Based on these observations we can conclude that highly reactive ROS (e.g. hydroxyl radical, peroxynitrite) are formed in both *Brassica* species in the long-term treatment and furthermore in *B. juncea* after seven days as well. Parallel with the accumulation of ROS the activity of antioxidants decreased, which can contribute to the increased efficiency of toxic oxygen radicals [26].

	AsA/DHA ratios			
	***B. juncea* 7th day**	***B. napus* 7th day**	***B. juncea* 14th day**	***B. napus* 14th day**
Control	3.55	5.42	1.39	4.09
10 µM Cu	1.51	2.71	1.22	1.35
25 µM Cu	1.57	2.29	1.09	1.41
50 µM Cu	1.58	1.76	1.13	1.32

Table 2 Ratios of ASA and DHA in the shoots of control and Cu-treated B. juncea and B. napus.

3.3 Pigment composition and photosynthetic capacity

Copper treatment decreased both chlorophyll and carotenoid contents significantly in these two *Brassica* species, more or less in a dose-dependent manner, but in *B. juncea* the effects were less pronounced (Table 3). Namely, excess Cu inhibits chlorophyll and carotenoid biosynthesis and retards the incorporation of these pigments in photosystems [3; 4]. In addition, the substitution of the central magnesium ion in the porphyrin ring of chlorophyll by excess Cu may also damage the chlorophyll synthesizing system [20].

		Chl a		**Chl b**		**Chl a/b**	**Total chl**		**Carotenoids**	
B. juncea **7th day**	Control	11,1907 ± 0,0046		3,1180 ± 0,0049		3,589	14,3081 ± 0,0093		3,0664 ± 0,0045	
	10 μM Cu	5,8517 ± 0,0048		1,3959 ± 0,0072		4,192	7,2484 ± 0,0119		1,9772 ± 0,0013	
	25 μM Cu	4,3665 ± 0,0050	***	1,0617 ± 0,0054	***	4,113	5,4274 ± 0,0104	***	1,5175 ± 0,0004	***
	50 μM Cu	3,0658 ± 0,1221		0,0886 ± 0,8970		34,588	3,2384 ± 0,7748		1,3802 ± 0,3867	
B. juncea **14th day**	Control	10,3395 ± 0,0027		4,1937 ± 0,0056		2,465	14,5343 ± 0,0083		3,3623 ± 0,0047	
	10 μM Cu	5,0749 ± 0,0029		1,8118 ± 0,0080		2,801	6,8857 ± 0,0107		1,6174 ± 0,0010	
	25 μM Cu	2,9784 ± 0,0128	***	0,9924 ± 0,0067	***	3,001	3,9753 ± 0,0099	***	1,0397 ± 0,0126	***
	50 μM Cu	2,4494 ± 0,0040		0,4693 ± 0,0093		5,219	2,9180 ± 0,0132		1,0900 ± 0,0030	
B. napus **7th day**	Control	10,8251 ± 0,0028		3,1109 ± 0,0080		3,480	13,9370 ± 0,0107		3,2593 ± 0,0014	
	10 μM Cu	8,8388 ± 0,0051		2,6375 ± 0,0047		3,351	11,4774 ± 0,0096		2,7681 ± 0,0006	
	25 μM Cu	4,3885 ± 0,0528	***	1,2287 ± 0,0175	***	3,572	5,6215 ± 0,0361	***	1,4429 ± 0,0084	***
	50 μM Cu	5,4949 ± 0,0028		1,6088 ± 0,0080		3,416	7,1030 ± 0,0108		1,8564 ± 0,0010	
B. napus **14th day**	Control	14,0376 ± 0,0057		4,0549 ± 0,0036		3,462	18,0916 ± 0,0093		4,2415 ± 0,0015	
	10 μM Cu	7,4806 ± 0,0021		1,6289 ± 0,0100		4,592	9,1086 ± 0,0121		2,5135 ± 0,0031	
	25 μM Cu	3,5589 ± 0,0058	***	0,6393 ± 0,0031	***	5,567	4,1974 ± 0,0087	***	1,2139 ± 0,0016	***
	50 μM Cu	7,1903 ± 0,0029		1,8830 ± 0,0106		3,819	9,0732 ± 0,0135		2,3191 ± 0,0030	

Table 3 Concentration of photosynthetic pigments (ug/g FW) and ratios of Chl/ and b in the leaves of control and Cu-treated B. juncea and B. napus after 7 and 14 days. Significant differences according to Student’s t-test (n = 10, ***P ≤ 0.001) are indicated.

Chlorophyll fluorescence parameters are recognized as powerful tools to study physiological responses of plants to metal-induced stress and offer a reliable method for assessing photosynthetic activity [30]. Exposure to excess Cu induced inhibition of photosynthesis in both species after 14 days, but the pattern of inhibition was different. Interestingly, the Yield, ETR and qP parameters of *B. juncea* were not effected by 25 μM Cu treatment significantly, but they were equally inhibited by 10 and 50 μM Cu (Fig 5a). On the other hand, in *B. napus* clear dose-response relationships were seen in all these three parameters (Yield, ETR, qP; Fig 5b), indicating that excess Cu is an effective blocker of PSII function. Indeed, through binding to both donor and acceptor sides of PSII, excess Cu inhibits electron transfer processes of PSII *in vitro* [14; 39]. Moreover, Cu in excess is more efficient at inactivating the ferredoxin-dependent reactions of the photosynthetic electron transport (such as $NADP^+$ photoreduction) than the light reactions in isolated chloroplasts of spinach [35]. No significant alteration in NPQ was seen in either species (Fig 5ab), indicating that Cu treatment did not increase the probability of dissipating the excess excitation energy via this alternative route. Based on these, the photosynthesis of *B. juncea* was more sensitive to low applied Cu concentration, while *B. napus* showed susceptibility to more serious excess Cu (25 and 50 μM Cu). Considering the remarkable ROS accumulation detected in the leaves we can assume that chloroplast membranes may suffer Cu-induced peroxidation, which can partially explain the diminution of photosynthetic efficiency [2]. Furthermore, free radicals can damage the photosynthetic apparatus [1] and may also catalyse protein degradation through oxidative modification and increased proteolytic activity [31], which may also lead to the decrease in photosynthesis.

4. Conclusions

Cu in excess became toxic and notably inhibited the growth of young *Brassica* shoots. This hindrance was more pronounced in *B. napus* in short treatment period. Excess Cu reduced the iron and manganese contents of *B. napus* shoots to a larger extent than those of *B. juncea* to levels associated with Fe and Mn deficiencies, which definitely contributed to growth inhibition. Highly reactive ROS (e.g. hydroxyl radical, peroxynitrite) were formed in both species with a parallel decrease in the activity of antioxidants, which is indicative of oxidative stress induced by Cu excess. Summarizing, the extent of Cu-induced oxidative stress was greater in *B. napus* compared to *B. juncea*. In addition, decreases in the amounts of photosynthetic pigments along with a decrease in photosynthetic activity were more pronounced in *B. napus*. The decrease in pigment concentrations (or increase in their degradation), the consequent negative effect on photosynthetic electron transport and the oxidative damage to photosynthetic membranes could lead to a decrease in the photosynthetic activity of young *Brassica* leaves during Cu toxicity. Taken the parameters together, the growth and the photosynthesis of *B. juncea* plants were comparatively less affected by excess

Cu compared to *B. napus*, indicating the species-specific nature of Cu tolerance in the early growth stage.

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List of figure captions

Fig 1 Shoot fresh weight (in control %, a), shoot dry weight (in control % , b) and leaf area (c) of untreated and Cu-treated *B. juncea* and *B. napus* on the 7th and the 14th day. Significant differences according to Student's t-test (n = 10, *P≤0.05, **P≤0.01, ***P ≤ 0.001) are indicated.

Fig 2 Representative photographs of *Brassica* shoots in case of control and 10, 25 and 50 µM Cu treatment.

Fig 3 Intensity of hROS-dependent fluorescence (a), activity of CAT (b), APX (c) and SOD (d) in the leaves of *B. juncea* and *B. napus*. Different letters indicate significant differences according to Duncan-test (n = 10, P≤0.05).

Fig 4 Ascorbate contents in the shoot system of *B. juncea* and *B. napus*. Significant differences according to Student's t-test (n = 10, *P≤0.05, ***P ≤ 0.001) are indicated.

Fig 5 Chlorophyll fluorescence parameters (Yield, ETR, qP, NPQ) of *B. juncea* (a) and *B. napus* (b) leaves after 14-days-long Cu exposure.

Table 1 Concentrations (µg/g DW) of Cu, Fe, Mn and bioaccumulation factor for Cu in the shoots of *B. juncea* and *B. napus* after 7 and 14 days of Cu treatment. Significant differences according to Student's t-test (n = 10, ***P ≤ 0.001) are indicated.

Table 2 Ratios of ASA and DHA in the shoots of control and Cu-treated *B. juncea* and *B. napus.*

Table 3 Concentration of photosynthetic pigments (ug/g FW) and ratios of Chl/ and b in the leaves of control and Cu-treated *B. juncea* and *B. napus* after 7 and 14 days. Significant differences according to Student's t-test (n = 10, ***P ≤ 0.001) are indicated.

Fig 1.

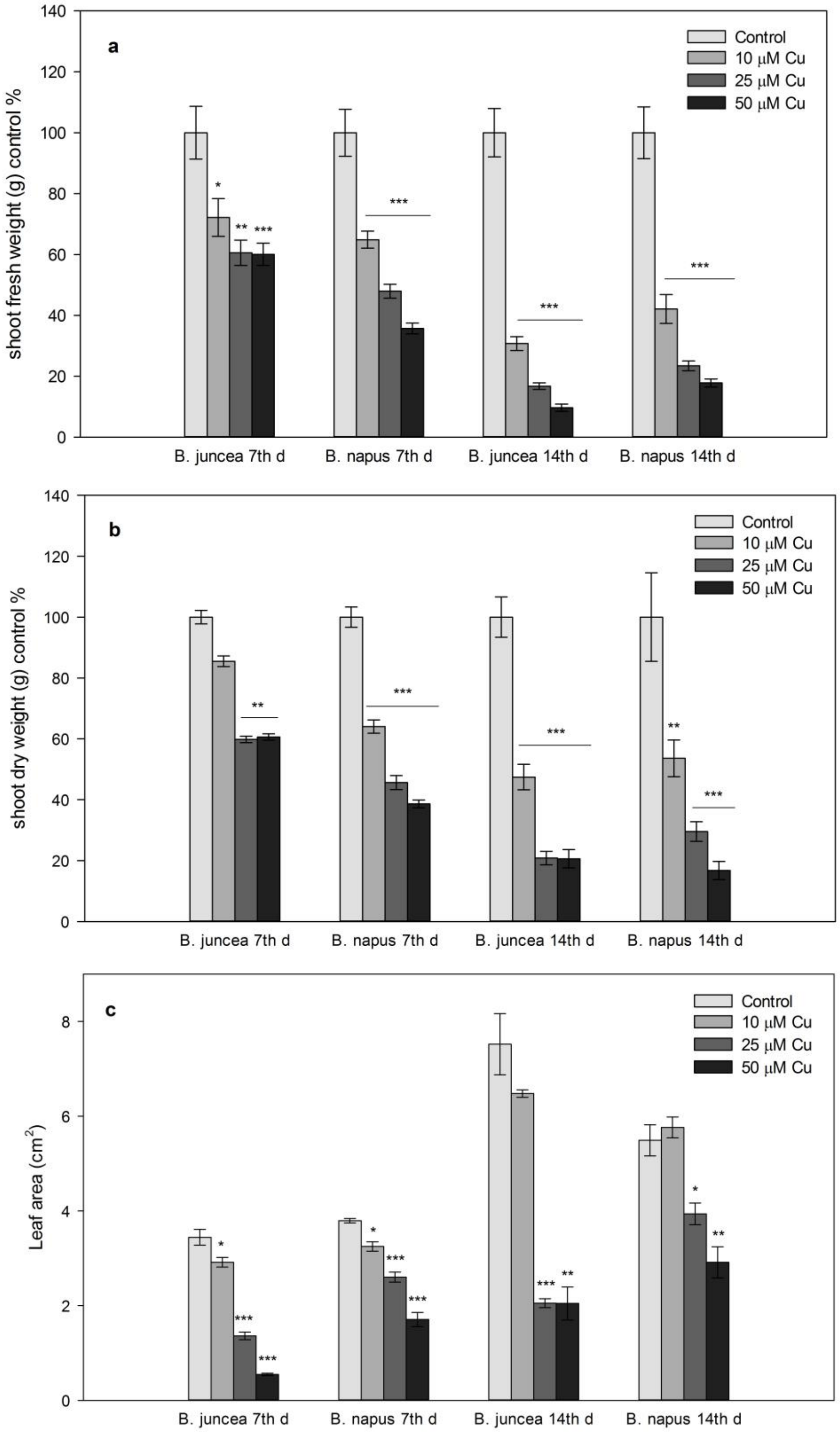

a
shoot fresh weight (g) control %
0
20
40
60
80
100
120
140
Control
10 μM Cu
25 μM Cu
50 μM Cu
*
**
***
***
***
***
B. juncea 7th d
B. napus 7th d
B. juncea 14th d
B. napus 14th d
b
shoot dry weight (g) control %
0
20
40
60
80
100
120
140
Control
10 μM Cu
25 μM Cu
50 μM Cu
**
***
***
**
***
B. juncea 7th d
B. napus 7th d
B. juncea 14th d
B. napus 14th d
c
Leaf area (cm²)
0
2
4
6
8
Control
10 μM Cu
25 μM Cu
50 μM Cu
*
***
***
*
***
***
***
**
*
**
B. juncea 7th d
B. napus 7th d
B. juncea 14th d
B. napus 14th d

Fig 2.

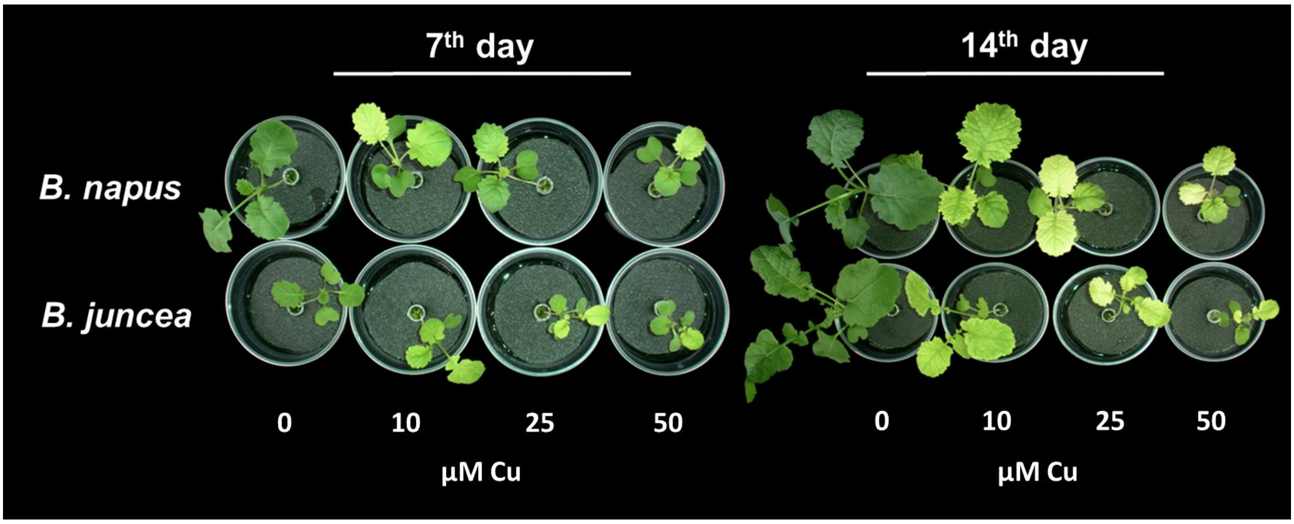

7th day
14th day
B. napus
B. juncea
0
10
25
50
µM Cu
0
10
25
50
µM Cu

Fig 3.

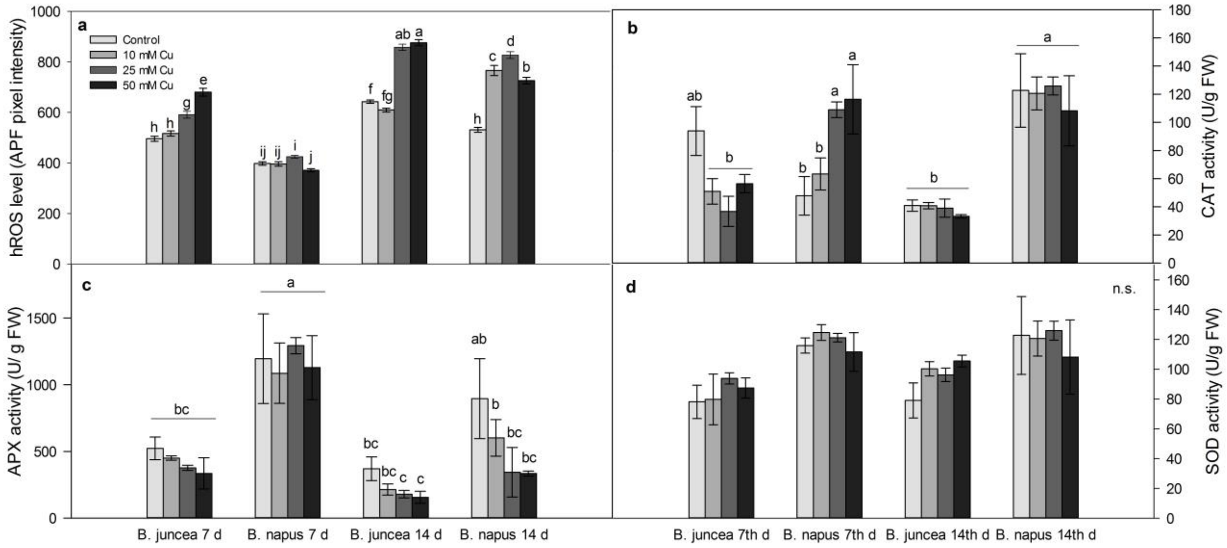

a
Control
10 mM Cu
25 mM Cu
50 mM Cu
hROS level (APF pixel intensity)
0
200
400
600
800
1000
h
h
g
e
ij
ij
i
j
f
fg
ab
a
h
c
d
b
b
CAT activity (U/g FW)
0
20
40
60
80
100
120
140
160
180
ab
b
b
b
a
a
b
a
c
APX activity (U/ g FW)
0
500
1000
1500
bc
a
bc
bc
c
c
ab
b
bc
bc
B. juncea 7 d
B. napus 7 d
B. juncea 14 d
B. napus 14 d
d
n.s.
SOD activity (U/g FW)
0
20
40
60
80
100
120
140
160
B. juncea 7th d
B. napus 7th d
B. juncea 14th d
B. napus 14th d

Fig 4.

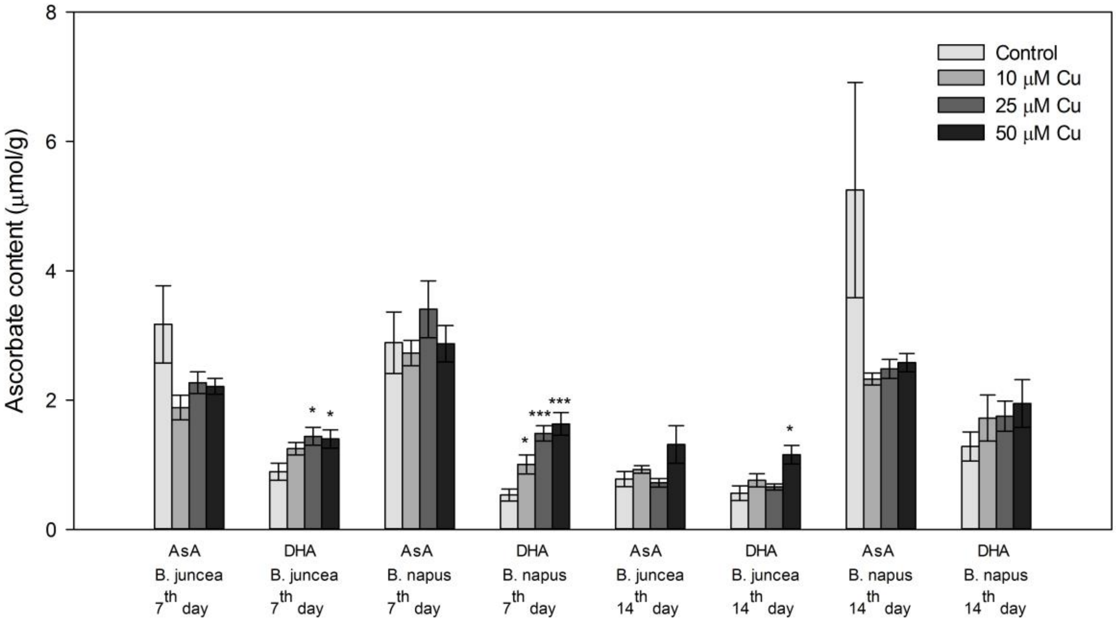
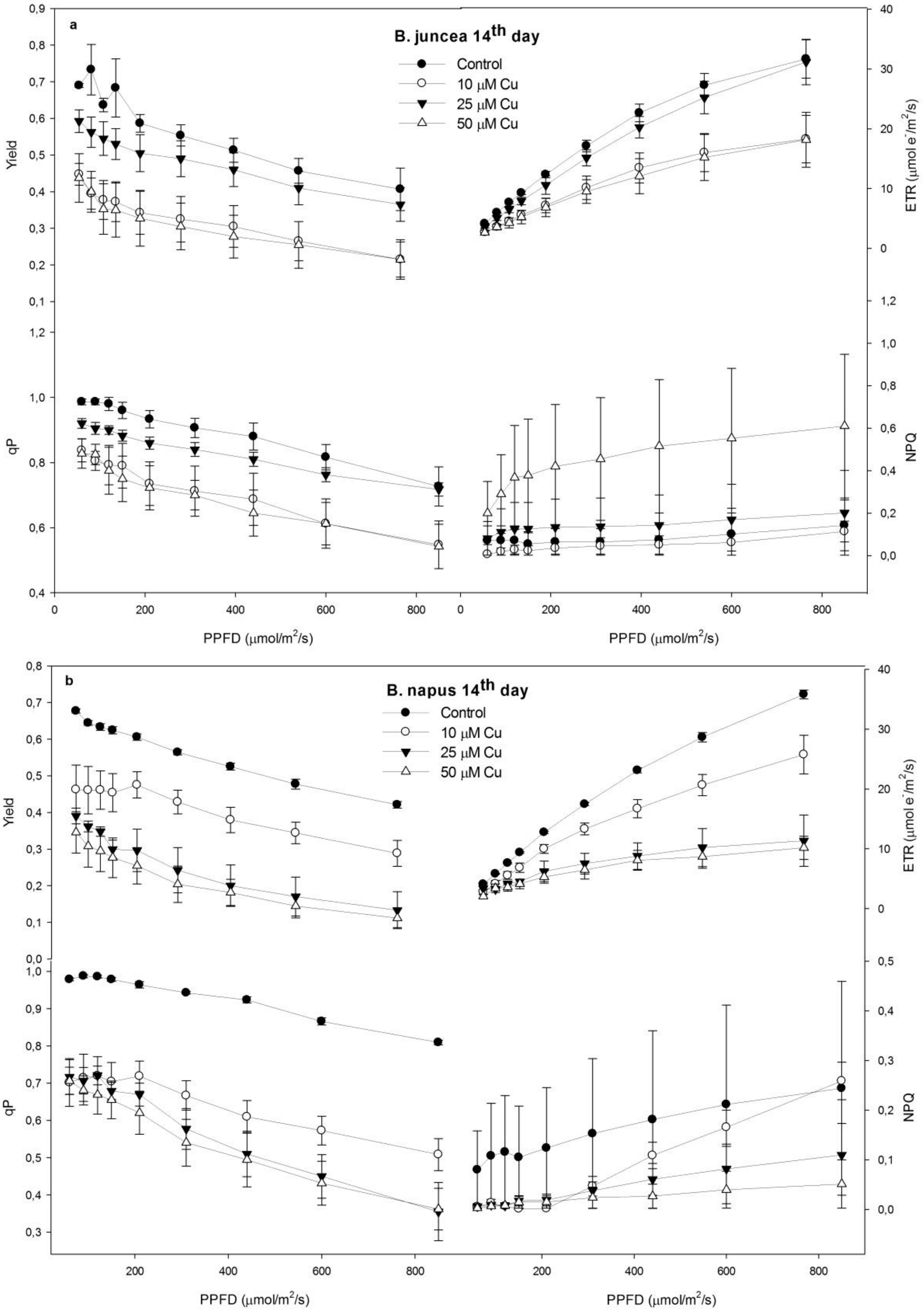

Ascorbate content (μmol/g)
0
2
4
6
8
Control
10 μM Cu
25 μM Cu
50 μM Cu
*
*
*
***
***
*
AsA B. juncea 7th day
DHA B. juncea 7th day
AsA B. napus 7th day
DHA B. napus 7th day
AsA B. juncea 14th day
DHA B. juncea 14th day
AsA B. napus 14th day
DHA B. napus 14th day

Fig 5.


a
B. juncea 14th day
Control
10 μM Cu
25 μM Cu
50 μM Cu
Yield
ETR (μmol e⁻/m²/s)
qP
NPQ
PPFD (μmol/m²/s)
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
1,2
1,0
0,8
0,6
0,4
40
30
20
10
0
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
200
400
600
800
b
B. napus 14th day
Control
10 μM Cu
25 μM Cu
50 μM Cu
Yield
ETR (μmol e⁻/m²/s)
qP
NPQ
PPFD (μmol/m²/s)
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
40
30
20
10
0
0,5
0,4
0,3
0,2
0,1
0,0
200
400
600
800