Ecotoxicology and Environmental Safety 94 (2013) 179–189

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Physiological and morphological responses of the root system of Indian mustard (*Brassica juncea* L. Czern.) and rapeseed (*Brassica napus* L.) to copper stress

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ARTICLE INFO

Article history:
Received 20 March 2013
Received in revised form
28 April 2013
Accepted 30 April 2013
Available online 5 June 2013

Keywords:
Brassica juncea
Brassica napus
Cell wall modifications
Nitric oxide
Reactive oxygen species
Root morphology

ABSTRACT

Copper (Cu) is an essential microelement for growth and development, but in excess it can cause toxicity in plants. In this comparative study, the uptake and accumulation of Cu as well as the morphological and physiological responses of Indian mustard (*Brassica juncea* L. Czern.) and rapeseed (*Brassica napus* L.) roots to Cu treatment were investigated. The possible involvement of redox active molecules (reactive oxygen species and nitric oxide) and modification in cell wall structure associated with Cu-induced morphological responses were also studied. In short- and long-term treatments, *B. juncea* suffered more pronounced growth inhibition as compared with *B. napus*. In addition to the shortening of primary and lateral roots, the number and the density of the laterals were also decreased by Cu. Exposure to copper induced nitric oxide generation in the root tips and this event proved to be dependent on the duration of the exposure and on the plant species. In short- and long-term treatments, Indian mustard showed more significant activation of superoxide dismutase (SOD), inhibition of ascorbate peroxidase (APX) and oxidation of ascorbate (AsA) than *B. napus*. Moreover, H_2O_2-dependent lignification was also observed in the Cu-exposed plants. In longer term, significant AsA accumulation and callose deposition were observed, reflecting serious oxidative stress in *B. juncea*. Based on the morphological and physiological results, we conclude that rapeseed tolerates Cu excess better than Indian mustard.

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1. Introduction

As an essential micronutrient, copper (Cu) is needed for the normal growth and development of plants. It is an important structural element of many proteins, takes part in photosynthetic electron transport, mitochondrial respiration, cell wall metabolism, and hormonal signaling pathways (Raven et al., 1999). Mild Cu exposure provokes symptoms of stress-induced morphogenic responses (SIMR) such as inhibition of cell elongation, local stimulation of cell division and alterations in the cell differentiation status (Pasternak et al., 2005; Potters et al., 2007). However, more serious Cu excess can be toxic causing inhibition of growth, leaf discoloration, chlorosis and necrosis (Marschner, 1995). Regarding root architecture, toxic Cu concentrations resulted in decreased elongation of primary roots (PR) and laterals (LR), thickening of the main root and inhibition of LR and root hair formation (Reichman, 2002). Copper binds to sulfhydryl groups of proteins, thereby inhibiting protein functions. It also induces nutrient deficiencies, impaired cell transport processes and disturbance in cell redox homeostasis (Yruela, 2009). Redox cycling between the two oxidation states of copper (Cu^+ and Cu^{2+}) catalyzes the formation of different types of reactive oxygen species (ROS), which subsequently damage macromolecules (Halliwell and Gutteridge, 1984). As an effect of Cu, hydrogen

Abbreviations: APF, 3′-(p-aminophenly) fluorescein; APX, ascorbate peroxidise; AsA, ascorbate; BAF, bioaccumulation factor; CAT, catalase; Cu, copper; DAF-FM DA, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; DHA, oxidized ascorbate; DHE, dihydroethidium; DTT, dithiothreitol; DW, dry weight; FDA, fluorescein diacetate; H_2O_2, Hydrogen peroxide; HM, heavy metal; LR, lateral roots; NBT, nitro blue tetrazolium; NO, nitric oxide; O_2^-, superoxide anion; OCl^-, hypochlorite; $OH\bullet$, hydroxyl radical; $ONOO^-$, peroxynitrite; POD, peroxidase; PR, primary roots; PRXs, peroxiredoxins; PVPP, polypyrrolidone; RNS, reactive nitrogen species; ROS, reactive oxygen species; SE, standard error; SIMR, stress-induced morphogenic responses; SOD, superoxide dismutase; TCA, trichloroacetic acid; U, unit.

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0147-6513/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ecoenv.2013.04.029

peroxide (H_2O_2) can be produced by the dismutation of superoxide anion ($O_2.^-$) formed by NADPH oxidases or *via* the Fenton reactions. Hydrogen peroxide can act as a signal molecule leading to the regulation of gene expression or it can cause oxidative damage of lipids through hydroxyl radical (OH•) formation (Opdenakker et al., 2012). Besides, Cu excess can indirectly lead to oxidative stress by disrupting the balance between ROS generation and detoxification (Møller et al., 2007). Scavenging of superoxide radical by superoxide dismutase (SOD) and H_2O_2 decomposition by ascorbate peroxidase (APX), peroxiredoxins (PRXs) and catalase (CAT) are predominantly responsible for the maintenance of cellular redox state. Antioxidant enzyme activities in heavy metal (HM)-exposed plants do not show a distinct pattern, since the effect depends on, *inter-alia*, the plant species, concentration and duration of exposure (Sharma and Dietz, 2008). Also, ascorbic acid (AsA) is a regulator of redox homeostasis and its oxidation leads to monodehydroascorbate and dehydroascorbate formation, which can be reduced by ascorbate-glutathione pathway (Noctor and Foyer, 1998). The major component of reactive nitrogen species (RNS) is the redox-active gas molecule, nitric oxide (NO), which plays a crucial role in stress acclimation processes of plants. It has three redox forms (nitric oxide radical, nitrosonium cation and nitroxyl anion), which can be rapidly converted to each other in biological systems. Because of its redox character, NO contributes to the maintenance of the redox state in plant cells (Potters et al., 2010). In general, short-term heavy metal treatment induces a rapid and notable NO production and a long-term treatment directly or indirectly decreases NO generation. The effect of the metal on NO production depends on many factors such as duration of treatment, metal concentration and plant age (Xiong et al., 2010). Peroxynitrite, the reaction product of NO, is responsible for the modification of lipids and/or proteins through tyrosine nitration or by interfering with phosphorylation cascades (Vandelle and Delledonne, 2011). Nitric oxide and other RNS also induce the reversible posttranslational modification of thiol-containing proteins by S-nitrosylation leading to changes in enzyme activity during signaling processes (Astier et al., 2012). Cell wall alterations such as lignification or callose deposition are protection mechanisms against heavy metal uptake and translocation, thus facilitating survival of the plant. The polymerization of lignin is catalyzed by peroxidase (POD) in the presence of H_2O_2 (Mäder and Füssl, 1982), and by laccases in the presence of O_2 (Sterjiades et al., 1993). Beyond the limitation of HM transport, lignin deposition can also interfere with cell growth (Sasaki et al., 1996), thus impacting, *e.g.*, root system architecture. Another way to prevent HM accumulation is to modify the properties of the cell wall by adding extra layers of carbohydrates, such as callose (β-1,3-glucan), synthesized by a transmembrane protein, callose synthase in the outer plasma membrane (Kartusch, 2003).

In the last few decades, Cu has become a widespread contaminant, being released to the ecosystem by several anthropogenic activities (Yruela, 2005). By documented phytoremediation processes, Indian mustard (*Brassica juncea* L.) and rapeseed (*Brassica napus* L.) are considered the most promising species for extracting HMs from contaminated soils (Gisbert et al., 2006); therefore, deciphering their responses to heavy metal exposition has great importance. Most of the earlier studies restricted to one Brassica species (*e.g.* Singh et al., 2010; Russo et al., 2008), short treatment duration (Devi and Prasad, 2005) or used soil-grown plants (Brunetti et al., 2011).

The goal of this comparative study is to determine the time- and concentration-dependent physiological and morphological responses of *B. juncea* and *B. napus* to copper excess. This paper deals with; *inter-alia*, the morphological alterations of the root system, which are relevant in terms of survival, since these architectural changes can contribute to metal adaptation. Besides ROS, Cu-triggered generation of the major RNS, nitric oxide was also examined in order to get comprehensive view about the metabolism of reactive molecules.

2. Materials and methods

2.1. Plant material and growing conditions

The seeds of *Brassica juncea* L. Czern. and *Brassica napus* L. were surface sterilized using 5% (v/v) sodium hypochlorite and then placed to Eppendorf tubes filled with perlite, floating on full-strength Hoagland solution. The nutrient solution contained 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2 mM $MgSO_4$, 1 mM KH_2PO_4, 0,01 mM Fe-EDTA, 10 μM H_3BO_3, 1 μM $MnSO_4$, 5 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.1 μM $(NH_4)_6Mo_7O_{24}$ and 10 μM $CoCl_2$. Seedlings were precultivated for nine days and then treated with 0 (control) 10, 25 and 50 μM $CuSO_4$ for seven and fourteen days. The plants were grown in greenhouse at a photon flux density of 150 μmol m^{-2}/s (12/12 h light/dark cycle) at a relative humidity of 55–60% and 25 ± 2 °C.

2.2. Element content analysis

The element analysis was carried out by ICP-MS (Thermo Scientific XSeries II, Asheville, USA) according to Lehotai et al. (2012). Briefly, root and shoot material of control, 10, 25 and 50 μM Cu-treated *B. juncea* and *B. napus* plants were harvested separately and rinsed with distilled water. After drying at 70 °C for 72 h, 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 min. Values of Cu and other microelement (Fe, Zn, Mn, Mo, Co) concentrations are given in μg/g dry weight (DW) and from the data shoot: root ratios were calculated. The bioaccumulation factor (BAF) of Cu was calculated as follows:

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

2.3. Morphological measurements

Fresh and dry weights (g) of the root and shoot material were measured on the seventh and fourteenth days of the treatments. Primary root length (cm) was also measured manually using a scale. Visible lateral roots were counted manually and their number was expressed as pieces/root. Lateral root density was also calculated and is given in pieces/cm. Data are expressed as percentage of the control.

2.4. Detection of reactive oxygen species and nitric oxide

The NO levels in *Brassica* root tips were detected by 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) (Kolbert et al., 2012a). Root segments were incubated for 30 min in darkness at room temperature in 10 μM dye solution, and were washed twice with Tris–HCl buffer (10 mM, pH 7.4). For the *in situ* detection of highly reactive ROS, such as $ONOO^-$, OH• and OCl^-, 3′-(p-aminophenyl) fluorescein (APF) was applied (Kolbert et al., 2012a). Root samples were incubated in darkness at room temperature in 10 μM APF dye solution for 1 h, and were washed twice with Tris–HCl buffer (10 mM, pH 7.4). Dihydroethidium (DHE) was used for visualization of superoxide contents in the root tips, which were incubated for 30 min in darkness at 37 °C in 10 μM dye solution, and were washed twice with Tris–HCl (10 mM, pH 7.4) (Kolbert et al., 2012a). For hydrogen peroxide detection, root segments were incubated in 50 μM Ampliflu™ (10-acetyl-3,7-dihydroxyphenoxazine, ADHP or Amplex Red) solution and washed with sodium phosphate buffer (50 mM, pH 7.5) according to Lehotai et al. (2012).

2.5. In situ visualization of copper-induced membrane damage, viability and cell wall modifications in root tissues

Products of lipid peroxidation (such as malondialdehydes) were visualized using Schiff's reagent, according to Arasimowicz-Jelonek et al. (2009). Root tips were incubated in the dye solution for 20 min and then the reagent was replaced by 0.5% (w/v) $K_2S_2O_5$ (prepared in 0.05 M HCl) for a further 20 min. For the determination of cell viability in the root tips, fluorescein diacetate (FDA) staining was used according to Lehotai et al. (2011). Root segments were incubated in 10 μM dye solution (prepared in MES/KCl buffer 10/50 mM, pH 6.15), and were washed four times with MES-KCl. Callose deposition in root tissues was determined using aniline blue according to Cao et al. (2011) with slight modifications. Roots' samples were incubated in aniline blue solution (0.1%, w/v in 1 M glycine) for 5 min then washed once with distilled water. Phloroglucinol was applied for the detection of root cell wall lignification according to Rogers et al. (2005). The roots were rinsed in distilled water and incubated in 1% (w/v) phloroglucinol (prepared in 6N HCl) for 5 min. After dying, phloroglucinol–HCl was replaced by distilled water and the samples were prepared on microscopic slides.

2.6. Microscopic analysis

The roots of *Brassica* plants dyed with different fluorophores were investigated under a Zeiss Axiowert 200M inverted microscope (Carl Zeiss, Jena, Germany) equipped with a high resolution digital camera (Axiocam HR, HQ CCD, Carl Zeiss, Jena, Germany) and filter set 9 (exc.: 450–490 nm, em.: 515–∞ nm) for DHE, filter set 10 (exc.: 450–490, em.: 515–565 nm) for APF, DAF-FM and FDA, filter set 20HE (exc.: 546/12, em.: 607/80) for Amplex Red, or filter set 49 (exc.: 365 nm, em.: 445/50 nm) for aniline blue. Fluorescence intensity (pixel intensity) was measured on digital images using Axiovision Rel. 4.8 software, within circles of 100 μm radii. Non-fluorescent assays (Schiff's reagent, phloroglucinol) were carried out also using Zeiss Axiowert 200M inverted microscope, using a high resolution digital camera and a phase contrast objective (Carl Zeiss, Jena, Germany).

2.7. Measurement of enzymatic and non-enzymatic components of the antioxidant defense system

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 (Dhindsa et al., 1981). For the enzyme extract, 250 mg plant material was grinded with 10 mg polyvinyl polypyrrolidone (PVPP) and 1 ml 50 mM phosphate buffer (pH 7.0, with 1 mM EDTA added). The enzyme activity is expressed in terms of specific activity (U/(g fresh weight)); one unit (U) of SOD corresponds to the amount of enzyme causing a 50% inhibition of NBT reduction in light.

Activity of ascorbate peroxidase (APX) (EC 1.11.1.11) was measured according to a modified method by Nakano and Asada (1981) by monitoring the decrease of ascorbate content at 265 nm (E=14 mM/cm). For the enzyme extract, 250 mg plant material was grinded with 1.5 ml extraction buffer containing 1 mM EDTA, 50 mM NaCl and 900 μM ascorbate. Data are expressed as specific activity (Unit/(g fresh weight)).

For the determination of ascorbate/dehydroascorbate content the method of Law et al. (1983) was used. During sample extraction, 250 mg plant material was grinded 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, which results in pink colour with an absorption maximum at 525 nm. The amount of total ascorbate was determined by the reduction of dehydroascorbate to ascorbate by dithiothreitol (DTT). Ascorbate/dehydroascorbate contents are expressed in μmol/(g fresh weight).

2.8. 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. Copper disturbs the microelement homeostasis of Brassica species

Concentrations of Cu and other micronutrients were analyzed in the root and shoot system of *Brassica* plants, and shoot:root ratios were calculated in order to draw conclusions about element distribution at the whole-plant-level (Table 1A). Both species showed similar Cu accumulation rates and the enhancement of Cu content in both organs proved to be dependent on the metal concentration in the nutrient solution. In case of 50 μM Cu treatment, *B. juncea* roots accumulated more of this metal in longer term than those of *B. napus*. Most of the Cu taken up by the plants was retained in the roots, since this organ is in a direct contact with the metal-containing solutions and is known to act as a storage for heavy metals and can restrict their translocation to the shoot system (Mazhoudi et al., 1997). However, the increment of Cu content within the *Brassica* shoots indicates an efficient translocation. Presumably, Cu makes complexes with nicotinamine and is transported *via* xylem vessels into the shoot (Burkhead et al., 2009). The bioaccumulation factor (BAF) gives information about the metal accumulation potential, and so about the phytoremediation ability of plants (Zhao et al., 2003). The BAF value for Cu was higher in the roots than in the shoots of both *Brassica* species. Cu accumulation was higher in the root system than the enhancement of Cu content in the external solution, therefore, BAF significantly increased in both species. Interestingly, the highest BAF value was determined in the case of 25 μM Cu exposure, which means that *Brassica* roots treated with this concentration were able to uptake and accumulate Cu in the highest quantity. BAF of the shoot system notably decreased as the effect of increasing Cu concentrations in the nutrient solution, which indicates a lower accumulation in the shoot. Based on these results, both *Brassica* species can be used for rhizofiltration purposes, since they possess high root volume, which is able to uptake large amount of copper. Moreover, Cu is slightly transported into their shoot system (Dushenkov et al., 1995). Copper excess markedly modified the microelement homeostasis of *Brassica* plants. The concentrations of Zn, Fe, Mn and Co were significantly reduced in both organs, but this effect did not depend on the intensity of the applied Cu treatment (Table 1B). Iron depletion reflects the antagonism between Cu and Fe due to their competition during the uptake (Lequeux et al., 2010). The main symptom of iron deficiency is intervenial chlorosis (Taylor and Foy, 1985), which was clearly visible on the leaves of both *Brassica* species (see Fig. 1A). Root and shoot zinc status was negatively affected by Cu excess, since both ions use the same transporter molecules, Zinc-regulated transporters, Iron-regulated transporter-like Proteins (ZIP, Wintz et al., 2003). The reducing effect of Cu on manganese and cobalt contents can be explained by the fact that Cu may change their uptake rates (Lidon and Henriques, 1993). However, it has to be mentioned that because of the high ionic strength, *e.g.* Cu–Zn antagonism may occur in nutrient solutions (Luo and Rimmer, 1995) and this can also result in Cu-induced Zn (or Fe) deficiency. Mild copper exposure increased Mo contents of the shoot and root tissues in both species. With respect to shoot:root ratios, in non-stressed plants roots contained higher microelement concentrations than the shoot material. In general, this distribution pattern of microelements was not notably affected by Cu exposure, similarly to *Arabidopsis* (Lequeux et al., 2010). Albeit, in the case of rapeseed exposed to long-term Cu treatment the shoot:root ratio of manganese was heavily increased. This suggests that beyond the disturbances in manganese homeostasis, long-term copper excess is able to modify also its distribution within *B. napus* plants.

3.2. Copper impacts on root system architecture of Indian mustard and rapeseed

Both of the two examined species showed serious morphological changes induced by Cu excess. As shown in Fig. 1A, a Cu concentration-dependent reduction of the stem size can be seen, and even the lowest applied Cu concentration affected stem development seriously. Nevertheless, visible signs of cell death, as for example necrotic lesions, could not be noticed. In addition to the growth inhibition, on the seventh and fourteenth days, chlorotic symptoms were also visible on the leaves of both species due to iron depletion (see Table 1B). Detailed investigation of the root system revealed lower fresh weights in both species, and this effect proved to be dependent on the concentration of copper (Fig. 1B). Moreover, *B. napus* showed a slighter response to 10 and 25 μM Cu on both days and the threshold toxicity for *B. juncea* was lower (25 μM Cu) than that of *B. napus* (50 μM Cu). Regarding root length, the effect of Cu excess was independent of the applied concentrations, since all treatments significantly inhibited root elongation. Consequently, growth of the main root was more sensitive to copper stress than the biomass of the whole root system. However, the reduction in root growth in *B. juncea* was more pronounced than in *B. napus*, especially on the fourteenth

Table 1
(A) Copper concentration (μg/g DW) and bioaccumulation factor of *Brassica* species. (B) Microelement concentrations (μg/g DW) and shoot:root ratios in control and copper-treated *Brassica juncea* and *Brassica napus*. Significant differences according to Student's t-test ($n=10$, $^{*}P\le0.05$, $^{**}P\le0.01$, $^{***}P\le0.001$) are indicated.

A		Initial Cu content **in solution** (μg/ml)	Cu concentration **in plant tissue** (μg/g) after seven days		Bioaccumulation factor after seven days		Cu concentration **in plant tissue** (μg/g) after fourteen days		Bioaccumulation factor after fourteen days	
			Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
B. juncea	Control	0.03 ± 0.00	24.50 ± 0.28	12.93 ± 0.66	742.42	391.82	21.60 ± 0.44	12.41 ± 0.42	654.55	376.06
	10 μM Cu	0.66 ± 0.01	475.30 ± 7.97***	45.35 ± 1.52***	717.98	68.50	686.10 ± 37.01***	49.79 ± 1.33***	1036.40	75.21
	25 μM Cu	1.60 ± 0.00	1666.00 ± 37.22***	65.22 ± 1.28***	1043.21	40.84	1821.00 ± 7.09***	79.56 ± 3.30***	1140.26	49.82
	50 μM Cu	4.01 ± 0.02	3531.00 ± 11.00***	162.20 ± 4.15***	880.99	40.47	3667.00 ± 5.49***	88.29 ± 2.87***	914.92	22.03
B. napus	Control	0.03 ± 0.00	27.68 ± 0.13	18.52 ± 0.33	838.79	561.21	23.74 ± 0.19	12.47 ± 0.15	719.39	377.88
	10 μM Cu	0.66 ± 0.01	713.20 ± 5.23***	40.50 ± 1.75***	1077.34	61.18	740.40 ± 8.45***	57.66 ± 0.36***	1118.43	87.10
	25 μM Cu	1.60 ± 0.00	1849.00 ± 10.38***	66.60 ± 1.01***	1157.80	41.70	1994.00 ± 34.80***	74.74 ± 4.66***	1248.59	46.80
	50 μM Cu	4.01 ± 0.02	3260.00 ± 35.09***	85.34 ± 2.32***	813.37	21.29	2478.00 ± 13.61***	82.01 ± 5.15***	618.26	20.46

B	**Control**			**10 μM Cu**			**25 μM Cu**			**50 μM Cu**		
	Root	Shoot	Shoot:Root	Root	Shoot	Shoot:Root	Root	Shoot	Shoot:Root	Root	Shoot	Shoot:Root
B. juncea seven days												
Zn	212.80 ± 1.01	89.25 ± 0.75	0.42	94.16 ± 1.22***	51.47 ± 0.37***	0.55	109.60 ± 1.10***	47.03 ± 1.16***	0.43	109.50 ± 1.50***	61.55 ± 1.78***	0.56
Fe	519.10 ± 8.23	90.99 ± 0.75	0.18	786.60 ± 2.25***	61.95 ± 1.45***	0.08	580.10 ± 2.04***	37.04 ± 0.42***	0.06	403.40 ± 4.57***	73.30 ± 1.22***	0.18
Mn	250.00 ± 2.01	48.66 ± 0.36	0.19	38.60 ± 0.40***	25.86 ± 1.33***	0.67	34.03 ± 1.68***	17.79 ± 0.20***	0.52	41.46 ± 0.13***	19.58 ± 0.33***	0.47
Co	19.25 ± 0.28	2.69 ± 0.03	0.14	11.57 ± 0.09***	1.09 ± 0.01***	0.09	7.64 ± 0.02***	0.87 ± 0.01***	0.11	4.13 ± 0.03***	0.90 ± 0.01***	0.22
Mo	20.31 ± 0.08	9.04 ± 0.10	0.45	24.95 ± 0.05***	6.87 ± 0.05***	0.28	18.64 ± 0.08***	5.79 ± 0.03***	0.31	18.60 ± 0.26***	7.11 ± 0.01***	0.38
B. juncea fourteen days												
Zn	232.00 ± 0.79	105.80 ± 0.46	0.46	100.80 ± 0.88***	47.39 ± 1.07***	0.47	88.37 ± 1.49***	37.64 ± 0.29***	0.43	120.80 ± 1.73***	39.10 ± 0.49***	0.32
Fe	500.60 ± 5.02	83.66 ± 0.99	0.17	398.60 ± 2.05***	30.43 ± 0.71***	0.08	310.70 ± 1.30***	42.73 ± 0.88***	0.14	335.80 ± 6.24***	48.44 ± 0.44***	0.14
Mn	214.90 ± 2.14	60.25 ± 0.41	0.28	30.92 ± 0.03***	27.78 ± 1.02***	0.90	35.83 ± 0.14***	15.40 ± 0.14***	0.43	41.03 ± 0.50***	13.81 ± 0.08***	0.34
Co	14.06 ± 0.14	1.43 ± 0.01	0.10	21.70 ± 0.10***	1.14 ± 0.01***	0.05	10.40 ± 0.12***	0.56 ± 0.01***	0.05	6.82 ± 0.11***	0.58 ± 0.01***	0.09
Mo	15.92 ± 0.04	8.86 ± 0.05	0.56	37.51 ± 0.26***	7.70 ± 0.02***	0.21	28.97 ± 0.41***	9.52 ± 0.09***	0.33	20.13 ± 0.11***	7.66 ± 0.03***	0.38
B. napus seven days												
Zn	258.40 ± 2.60	155.70 ± 0.45	0.60	120.70 ± 1.34***	56.85 ± 0.43***	0.47	104.60 ± 1.16***	48.90 ± 1.06***	0.47	89.05 ± 1.45***	53.61 ± 0.41***	0.60
Fe	1051.00 ± 7.78	104.20 ± 1.03	0.10	750.10 ± 2.28***	37.02 ± 1.50***	0.05	556.80 ± 3.28***	85.69 ± 0.50***	0.15	548.60 ± 3.44***	48.63 ± 1.12***	0.09
Mn	145.10 ± 0.92	82.52 ± 0.42	0.57	46.21 ± 0.39***	21.86 ± 0.17***	0.47	35.91 ± 1.50***	20.18 ± 0.13***	0.56	33.64 ± 0.21***	19.88 ± 0.05***	0.59
Co	43.10 ± 0.30	2.77 ± 0.01	0.06	11.40 ± 0.08***	1.18 ± 0.01***	0.10	4.96 ± 0.04***	0.95 ± 0.00***	0.19	4.11 ± 0.04***	0.91 ± 0.01***	0.22
Mo	19.24 ± 0.05	7.65 ± 0.04	0.40	25.17 ± 0.10***	5.53 ± 0.04***	0.22	17.78 ± 0.13***	4.72 ± 0.03***	0.27	14.24 ± 0.09***	4.54 ± 0.05***	0.32
B. napus fourteen days												
Zn	195.70 ± 0.39	217.20 ± 91.53	1.11	154.00 ± 1.37	65.68 ± 0.84*	0.43	107.30 ± 1.71***	42.43 ± 0.67*	0.40	96.53 ± 0.55***	40.29 ± 0.38*	0.42
Fe	761.70 ± 2.70	74.26 ± 0.75	0.10	657.80 ± 4.63	43.03 ± 0.84***	0.07	402.10 ± 0.35***	37.62 ± 1.35***	0.09	338.70 ± 10.34***	39.44 ± 0.79***	0.12
Mn	108.60 ± 0.36	60.01 ± 0.16	0.55	31.64 ± 0.17	35.36 ± 0.10***	1.12	35.92 ± 0.40***	15.77 ± 0.33***	0.44	30.74 ± 0.50***	15.96 ± 0.04***	0.52
Co	20.93 ± 0.07	1.04 ± 0.00	0.05	22.97 ± 0.12	1.60 ± 0.02***	0.07	7.64 ± 0.02***	0.83 ± 0.01***	0.11	6.31 ± 0.15***	0.87 ± 0.00***	0.14
Mo	12.67 ± 0.12	6.55 ± 0.02	0.52	33.69 ± 0.18	10.65 ± 0.04***	0.32	21.81 ± 0.15***	7.65 ± 0.04***	0.35	19.59 ± 0.05***	8.55 ± 0.04***	0.44

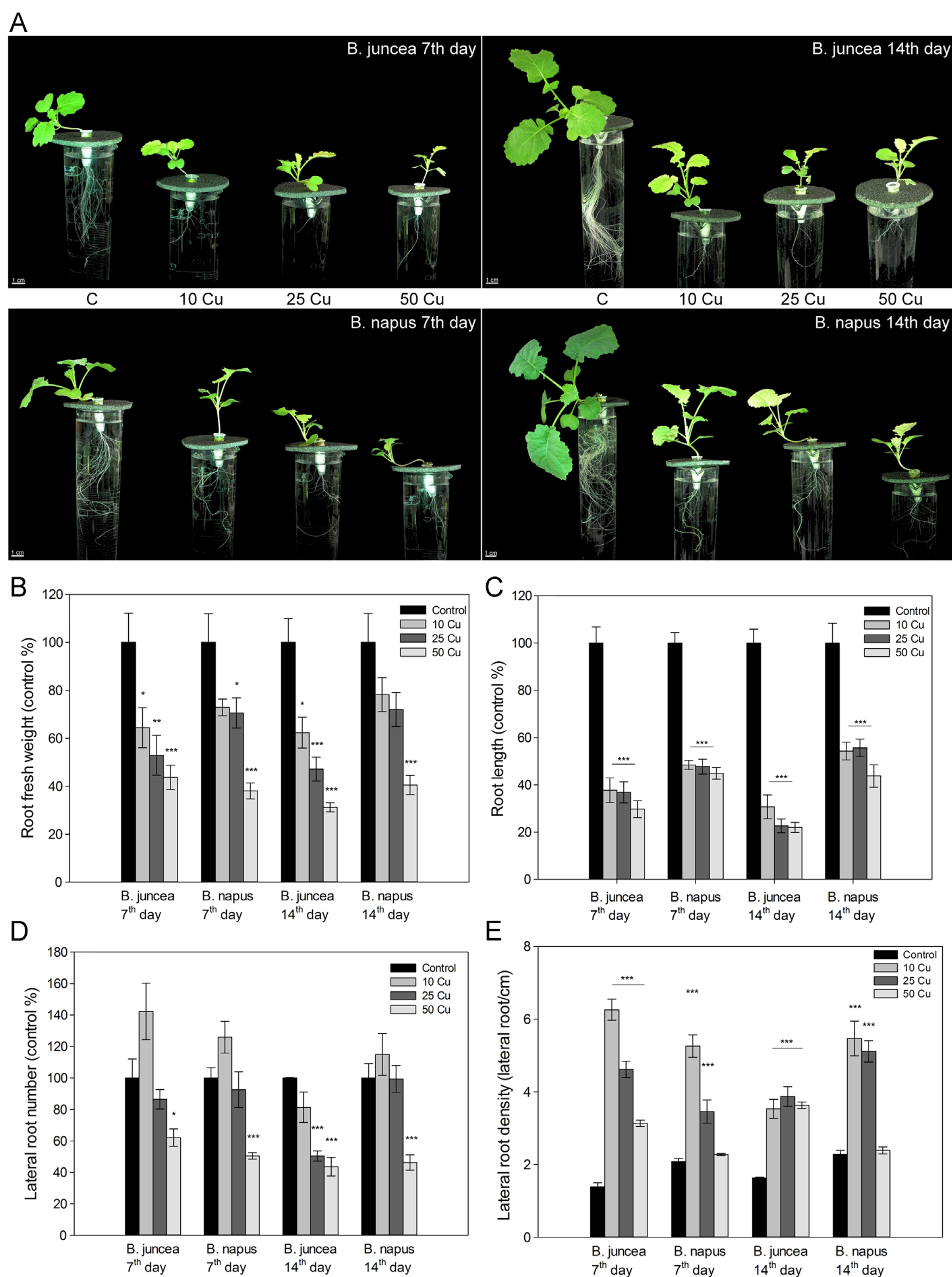


Fig. 1. (A) Representative images of *Brassica* plants after seven and fourteen day copper treatment. Bars = 1 cm. Fresh weight (panel B) and length (panel C) of roots, lateral root number (panel D) and density (panel E) of Cu-treated *Brassica* plants. Values are expressed as percentage of the control. Significant differences according to Student's *t*-test ($n = 10$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$) are indicated.

day of treatment (Fig. 1C). After seven days of mild Cu exposure (10 μM), the number of lateral roots increased by 33% in rapeseed and by 25% in Indian mustard as compared with the control (Fig. 1D). This means that a mild copper stress induces a positive growth response allowing better nutrient and water uptake, thereby facilitating the survival of the metal-exposed plant. Moreover, the size reduction of the stem can contribute to the maintenance of the appropriate water balance. Similar copper-induced morphogenic responses (SIMR) were found in the root system of *Arabidopsis* (Kolbert et al., 2012b). Excessive amounts of other elements, such as selenium or the non-essential cadmium, can trigger LR development depending on the concentration and duration of the exposure (Lehotai et al., 2012; Yang et al., 2004). The 10 μM copper-induced LR formation could not be observed

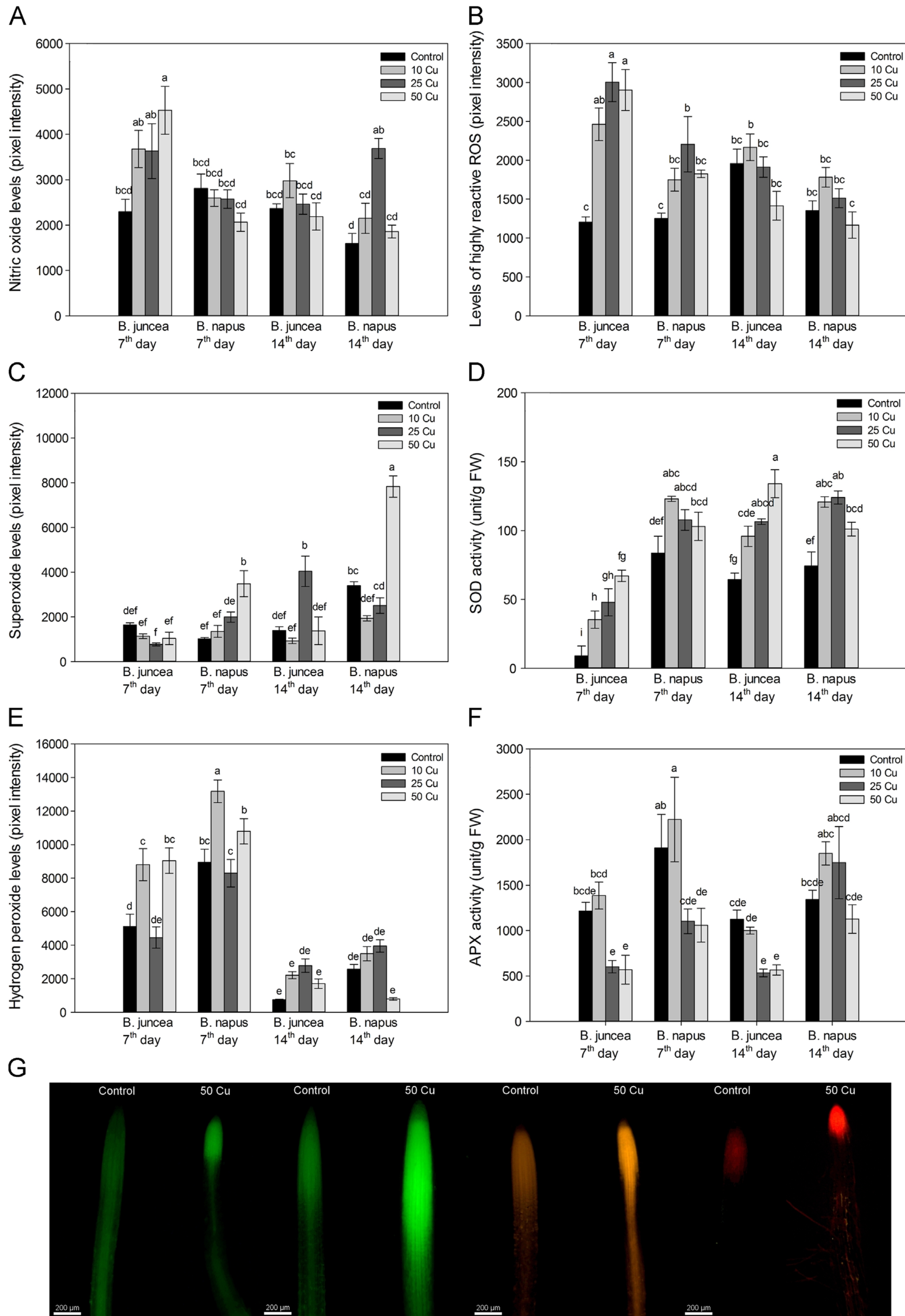


Fig. 2. Values of NO- (A), highly reactive ROS- (B) and superoxide-dependent fluorescence (C, pixel intensity) in root tips of Cu-treated *Brassica* plants. (D) Activity of SOD (Unit/g FW) in the root system. (E) Values of H_2O_2-specific fluorescence (pixel intensity). (F) Ascorbate peroxidase activity (Unit/g FW) in the root system. Different letters indicate significant differences according to Duncan-test ($n=10$, $P\leq0.05$). (G) Representative fluorescence microscopic images of control and 50 μM Cu-exposed *Brassica* root tips stained with different fluorophores (from left: DAF-FM DA for NO, *B. juncea* seventh day; APF for hROS, *B. juncea* seventh day; DHE for superoxide, *B. napus* fourteenth day; Amplifluᵀᴹ for H_2O_2, *B. juncea* seventh day). Bars = 200 μm.

after fourteen days exposure, which proves the time-dependence of the appearance of SIMRs. In the long term, differences in the behavior of the two *Brassica* species treated with 25 μM Cu were observed, since LR number was halved in *B. juncea*, while it was not affected in *B. napus*. The highest Cu concentration caused a reduction in the LR number in both species on both days. In order to get a more accurate view about the root architectural changes, lateral root densities were calculated. In the case of 10 and 25 μM Cu, the number of lateral roots per centimeter of the main root significantly increased in both *Brassica* species on both days. This was the consequence of the PR shortening and/or the intensified LR formation. However, in *B. napus* the highest Cu concentration did not result in an alteration of LR density, since both parameters notably decreased (Fig. 1E). And moreover, copper exposure resulted in a remarkable shortening of laterals in both species (data not shown, see Fig. 1). These results show that Cu exerts more pronounced inhibitory effects on root elongation than on lateral root formation. This suggests a higher sensitivity of cell elongation to Cu as compared with cell division and differentiation processes (Arduini et al., 1994). Based on the morphological observations, the structure of the root system suffered serious changes, since Cu triggered PR and LR shortening and it also affected LR formation. The morphological adaptation strategy of the two *Brassica* species proved to be different. In short term, *B. juncea* reduced the growth of the root system, while *B. napus* showed more significant reduction in shoot size. Furthermore, stress-induced LR formation may contribute to the adaptation of both *Brassica* species. However, in the case of longer exposure time both the root and shoot growth of *B. juncea* showed more pronounced responses to Cu excess.

3.3. Copper alters the metabolism of reactive molecules in *Brassica* root tips

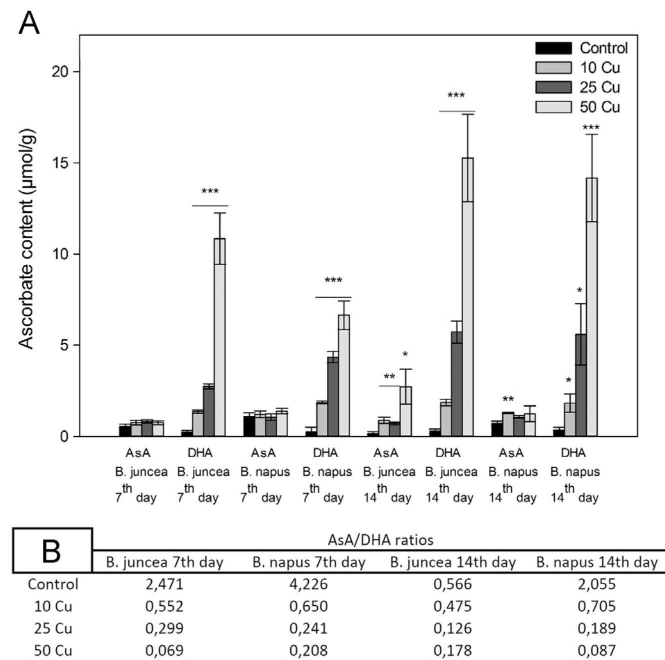
Imbalances in the metabolism of reactive molecules, such as reactive oxygen- and nitrogen-species, causing a disorder in the cell redox status may influence the growth responses. The levels of the main RNS, nitric oxide, were examined *in situ* by fluorescence microscopy (Fig. 2A). Nitric oxide contents of control root tips were similar in both species, but under copper stress NO metabolism was differentially affected. In the case of a shorter duration of Cu treatment (seventh day), root meristems of Indian mustard showed almost two-fold NO accumulation, while in rapeseed only a slight decrease of NO content could be detected. The copper-induced NO may originate from the activity of nitrate reductase, as it was shown in the roots of *Arabidopsis* (Kolbert et al., 2012b). This could be confirmed by the involvement of Cu in the biosynthesis of molybdenum cofactor for, *e.g.*, nitrate reductase (Pilon et al., 2006). On the contrary, in the root tips of *Vicia faba*, a NOS-like enzyme was found to be responsible for the Cu-induced NO generation (Zou et al., 2012). On the fourteenth day of the treatment a notable NO formation was found in *B. napus* roots, while *B. juncea* showed only non-significant changes. In addition to the duration and concentration of the exposure, the effect of copper on NO metabolism also depended on the plant species, since NO homeostasis in the two *Brassica* species was affected differently by Cu. We observed a tissue specificity of the Cu-triggered NO production in *B. juncea*, since the homogenous fluorescence of the control roots changed; it increased in the meristematic cells and decreased far from the root tip (Fig. 2G). Also, in the case of cadmium-stressed pea plants, the root tips showed elevated NO content (Rodríguez-Serrano et al., 2006). The accumulated NO may play a pro- or an antioxidative role during copper stress. As a pro-oxidant it can contribute to cell death (Arasimowicz-Jelonek et al., 2012), thus inhibiting growth, or it can activate defense-related signaling and gene expression. Moreover, NO plays a vital role in the enhancement of antioxidant enzymes activities and alleviates the toxicity of heavy metals (Kopyra and Gwóźdź, 2003).

After seven days, all Cu concentrations applied significantly induced the formation of highly reactive oxygen species (especially $ONOO^-$, OH• and OCl^-) in root apexes of *B. juncea*, while only 25 μM Cu had significant effect in case of *B. napus*. In the longer term, a non-significant reduction in hROS levels was detected in both species treated with copper (Fig. 2B). These suggest the occurrence of early oxidative and nitrosative processes, while in the longer term ROS detoxification may be activated. In addition to the meristematic region, the highly reactive ROS-dependent fluorescence was elevated in the elongation zone, as well (Fig. 2G). Similarly, Cu- or Cd-induced enhancement of hROS-dependent fluorescence was detected in pea or lupine root tips (Lehotai et al., 2011; Arasimowicz-Jelonek et al., 2011). Apart from the reactive oxygen species, the levels of superoxide radical and H_2O_2 were investigated by fluorescence microscopy. As far as superoxide is concerned, a slight and concentration-dependent decrease was observed in copper-treated *B. juncea*, while it was accumulated in *B. napus* root tips. The NADPH oxidases were found to be responsible for superoxide generation in copper-stressed plants (Opdenakker et al., 2012). On the fourteenth day, 25 μM Cu caused superoxide generation in *B. juncea*, while in *B. napus* 50 μM Cu resulted in a similar response (Fig. 2C). The superoxide-dependent fluorescence signal was localized in the meristematic and elongation zones of the *Brassica* roots (Fig. 2G). Superoxide dismutase constitutes the first line of enzymatic defense against oxidative stress. The activity of this enzyme was significantly increased in both species on both days, but it proved to be concentration-dependent only in Indian mustard roots (Fig. 2D). The results are in agreement with those of, *e.g.*, Singh et al. (2010), who observed an increased SOD activity in Cu-treated *B. juncea* roots. Copper exposure led to an enhancement in *in situ* hydrogen peroxide levels in root apices; however, no obvious tendency could be determined (Fig. 2E). Interestingly, Cu-induced H_2O_2 generation was restricted to the root apex (Fig. 2G). APX activities were significantly reduced by 25 and 50 μM Cu in both species after seven days of treatment and in *B. juncea* on the fourteenth day (Fig. 2F), which may contribute to the H_2O_2 accumulation. Similarly, in the work of Luna et al. (1994) reduced APX activity was detected in copper-exposed oat plants. Interestingly, in the longer term *B. napus* was able to slightly enhance the activity of APX under mild Cu stress, which indicates the activation of an acclimatization process. Superoxide and H_2O_2 levels were not always in accordance with the activities of the antioxidant enzymes. The reason for this could be that the levels of the ROS species were detected in the root apices, while the enzyme activities were measured in the whole root system. The amount of ascorbate and dehydroascorbate (Fig. 3A) and their ratios (AsA/DHA, Fig. 3B), indicators of the redox homeostasis, were determined in the root system. Copper treatment strongly increased the amount of oxidized ascorbate (DHA) in a concentration- and time-dependent manner in both *Brassica* species. The concentration of the reduced ascorbate form (AsA) did not change significantly in either *B. juncea* or in *B. napus* on the seventh day. In contrast, after fourteen days both species showed AsA accumulation, but it proved this to be significant and concentration-dependent only in the case of *B. juncea* (Fig. 3A). Accumulation of AsA refers to strong oxidative stress after the long-term copper exposure in *B. juncea*. Regarding the AsA/DHA ratios, the roots of non-stressed *B. napus* possessed more reduced ascorbate pool on both days compared with *B. juncea* (Fig. 3B). Copper concentration-dependent diminution of reduced AsA ratio was observed in both species. In case of long-term Cu exposure of oilseed rape, the ratio of reduced ascorbate decreased more significantly than in *B. juncea*, which can be explained by the higher APX activity.

3.4. Lipid peroxidation and cell death in the root apex of Cu-treated *Brassica* species

Copper is able to induce the production of radicals (hydroxyl, peroxyl and alkoxyl), which cause peroxidative damage to membrane lipids by binding their thiol groups and by disrupting the redox status of the cell (Dietz et al., 1999). Lipid peroxidation serves as an indicator of the extent of oxidative damage under stress (Halliwell and Gutteridge, 1992). This process, being a direct consequence of the oxidative stress, was investigated *in situ* in *Brassica* roots by microscopic methods. In the root samples stained with the Schiff's reagent, the appearance of pink coloration indicates accumulation of lipid peroxidation products, such as malondialdehyde. Based on this qualitative assay, oxidation of membrane lipids occurred in every copper treatment in both species, although it was especially pronounced in *B. juncea* root tips on the fourteenth day of treatment (Fig. 4A). Accumulation of lipid peroxidation products was the least significant in rapeseed root tips after long-term Cu exposure. Similar results were published by Tian et al. (2011), where cadmium-induced lipid peroxidation was found in the root tips of *Sedum alfredii*. As it is shown in Fig. 4A, the cells of the root cap, and those of the meristem, the transition and the elongation zones were also affected.

Cell viability was examined in the meristematic zone of root tips dyed with fluorescein diacetate, and from the viability loss the extent of cell death was evaluated. All copper concentrations caused serious decrease in cell viability in both short- and long term and in both species. However, the mildest copper concentration (10 μM) used in this study caused slighter diminution in viability in *B. juncea* root meristem compared with *B. napus* (Fig. 4B). The reasons for Cu-induced cell death can be the imbalance in redox homeostasis and the occurrence of oxidative and nitrosative stress (Mullineaux and Baker, 2011; Corpas et al., 2011). The loss of cell viability in the meristem can lead to the inhibition of root elongation.



	AsA/DHA ratios			
	B. juncea 7th day	B. napus 7th day	B. juncea 14th day	B. napus 14th day
Control	2,471	4,226	0,566	2,055
10 Cu	0,552	0,650	0,475	0,705
25 Cu	0,299	0,241	0,126	0,189
50 Cu	0,069	0,208	0,178	0,087

Fig. 3. (A) Contents of oxidized (DHA) and reduced ascorbate (AsA) in the root system of *B. juncea* and *B. napus* treated with 0, 10, 25 and 50 μM Cu for seven and fourteen days. Significant differences according to Student's *t*-test ($n=10$, *$P\leq0.05$, **$P\leq0.01$, ***$P\leq0.001$) are indicated. (B) Ratios of AsA and DHA in the roots of control and copper-treated *Brassica juncea* and *napus*.

3.5. Copper-induced cell wall modifications in the root system of *Brassica*

Of the heavy metal-induced cell wall modifications, *in situ* examinations of lignification and callose deposition were carried out in the root system of *Brassica* species. Phloroglucinol staining indicating lignin in the walls was visible at the base of the lateral roots already after seven day Cu treatment in both species (Fig. 5A). The *in situ* lignin formation showed co-localization with hydrogen peroxide, since both molecules were localized in the cells at the offset point of LRs (Fig. 5B), which suggests the involvement of H_2O_2 in the lignification process. Similarly, Olson and Varner (1993) detected H_2O_2 in cells undergoing lignification. Lignified roots lose their capacity for nutrient uptake and consequently the ability for growth. This would result in growth inhibition at the whole-plant level (Schützendübel and Polle, 2002).

Deposition of the polysaccharide callose was investigated in aniline blue-stained root tips, and callose content in the cell walls was estimated by measuring the intensity of fluorescence emission. After seven days, no significant changes in callose contents were found in *Brassica* roots. On the fourteenth day of Cu exposure, *B. napus* did not show any callose accumulation, while in 25 μM Cu-treated *B. juncea* a significant increase in the callose content was measured (Fig. 5C). Of the metals, copper was found one of the most effective inducers of callose formation in onion epidermal cells (Kartusch, 2003). The deposited callose could inhibit cell wall loosening, thus preventing the passage of signal

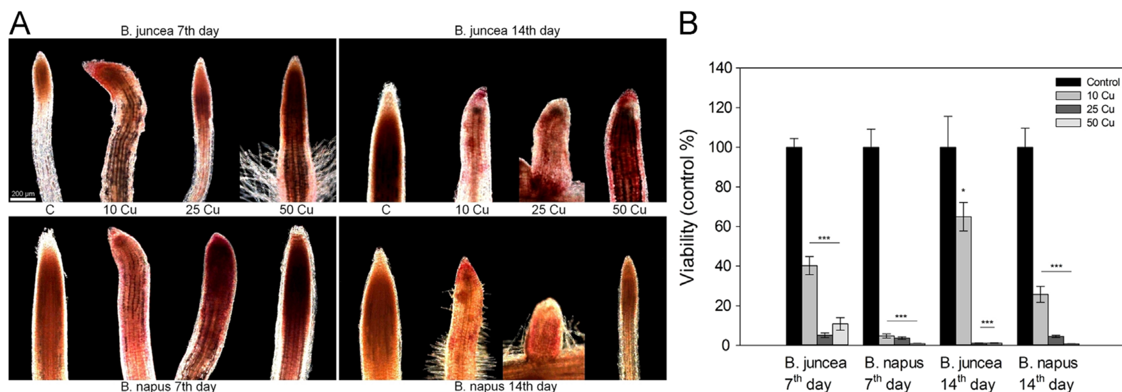


Fig. 4. (A) Microscopic images of Brassica root tips stained with Schiff's reagent indicating lipid peroxidation. Bar = 200 μm. (B) Cell viability (FDA fluorescence, control %) in root meristems of copper-exposed Brassica plants. Significant differences according to Student's *t*-test ($n=10$, *$P\leq0.05$, **$P\leq0.01$, ***$P\leq0.001$) are indicated.

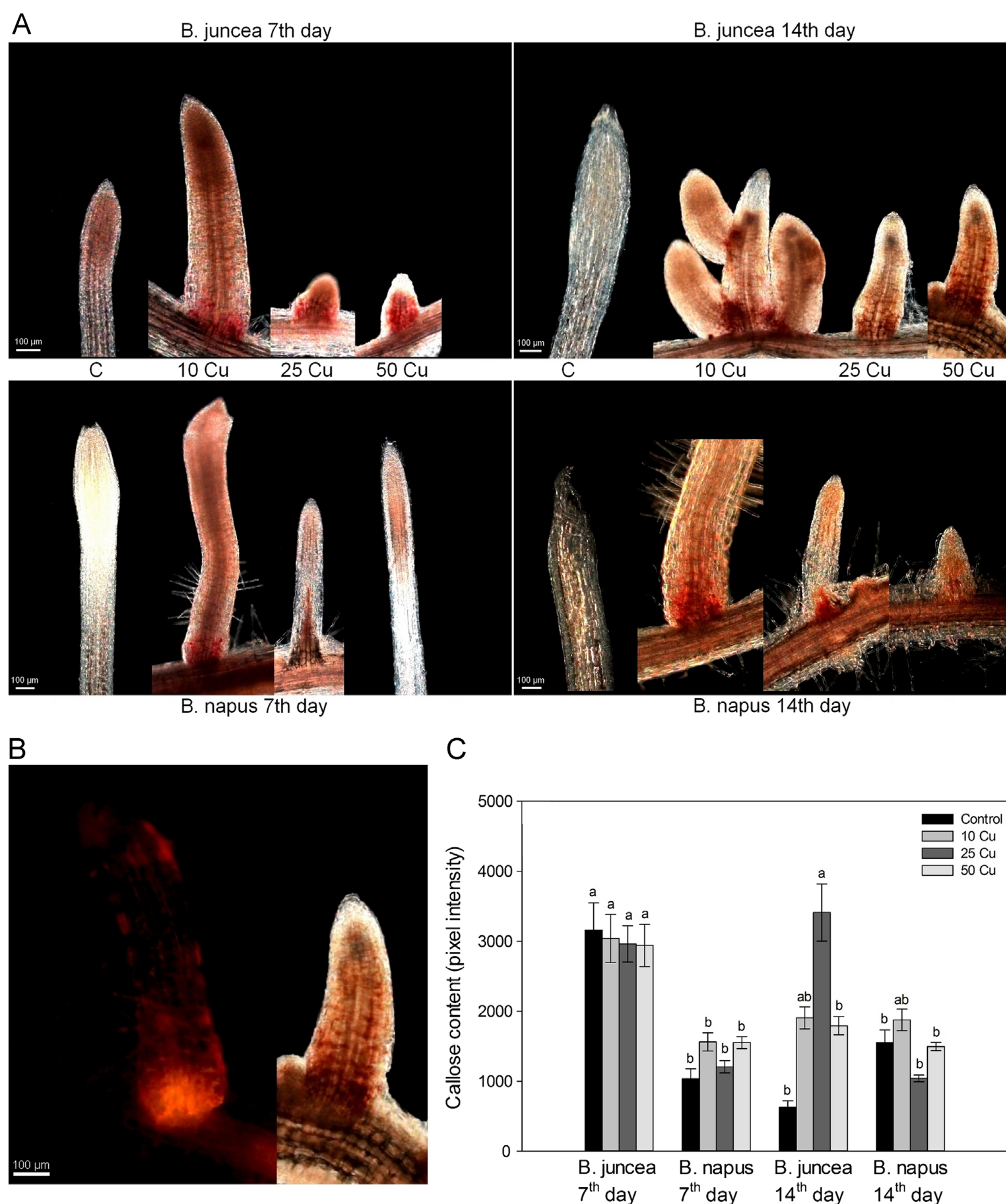


Fig. 5. (A) Microscopic images of Brassica roots stained with phloroglucinol for lignin. Bar = 100 μm. (B) Localization of H_2O_2 (left) and lignin (right) in the root system of copper-stressed Brassica. (C) Values of callose-dependent aniline blue fluorescence (pixel intensity) in the root tips of Brassica treated with 0, 10, 25 and 50 μM Cu. Different letters indicate significant differences according to Duncan-test ($n = 10$, $P \leq 0.05$).

molecules or inhibit the symplastic supply of carbon required for root growth (Jones et al., 2006).

4. Conclusions

Copper excess modified the microelement homeostasis, since it induced depletion of iron, zinc, manganese and cobalt. The excessive amount of this metal triggered serious morphological alterations of the root system. Apart from PR and LR shortening, the number and the density of the laterals were also affected by Cu. Interestingly, mild Cu exposure induced LR formation, which is a characteristic symptom of morphogenetic responses. This enhanced LR number, together with the reduced shoot size, may contribute to the maintenance of a better water balance of the metal-exposed plants. In both short- and long-term exposures, *B. juncea* suffered more pronounced growth modifications. Copper exposure resulted in NO production in the root tips of *Brassica* plants, but this event was dependent on the duration of Cu exposure and on the plant species. Moreover, meristem-specific NO accumulation was detected in the Cu-treated *Brassica* roots. In both short- and long-term treatments, *B. juncea* showed a more significant activation of SOD, inhibition of APX and oxidation of ascorbate than *B. napus.* Also, hydrogen peroxide accumulated in the root tips leading to lipid peroxidation. These events caused the disturbance of redox homeostasis, which can result in cell death and growth inhibition. Moreover, H_2O_2-dependent lignification was also observed in copper-exposed roots. In a longer term, the

significant AsA accumulation and the callose deposition reflects the occurrence of acute oxidative stress in *B. juncea* roots. Despite these, in agreement with the results of other reports (*e.g.* Marchiol et al., 2004; Wu et al., 2004; Turan and Esringü, 2007), we can conclude that both *Brassica* species tolerate excessive amount of Cu well, but rapeseed appears to be more tolerant to Cu than Indian mustard.

Acknowledgments

This study was carried out during a 1-year stay by Devanand Kumar at the Department of Plant Biology, University of Szeged, supported by the C2 type Hungarian Balassi Fellowship. The authors wish to thank "Scientia Amabilis Foundation for Hungarian Plant Physiology" for the financial support. This work was supported by the Hungarian Scientific Research Fund (Grant no. OTKA PD100504). We acknowledge HURO/0901/147/2.2.2 SZETISA1. project for the ICP-MS instrumental background. This research was realized in the frames of TÁMOP 4.2.4. A/2-11-1-2012-0001 "National Excellence Program—Elaborating and operating an inland student and researcher personal support system". The project was subsidized by the European Union and co-financed by the European Social Fund.

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