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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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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.

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; $OH^•$, 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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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.