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ORIGINAL PAPER

Interactive effects of cadmium and copper on metal accumulation, oxidative stress, and mineral composition in *Brassica napus*

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Abstract Heavy metals' frequent occurrence and toxicity caused considerable concerns in assessing the interactive effects of metals on exposed plants. Therefore, a hydroponic study was conducted to assess the growth response and physio-chemical changes in *Brassica napus* plants under single and combined stress of two environmentally alarming metals (Cd and Cu). Results showed that 15-day metal exposure to different metal concentrations (0, 50, 200 μM) significantly enhanced Cd accumulation, while lesser extent of Cu was observed in plant tissues. Nonetheless, Cu caused more pronounced oxidative damages and plant growth retardation. Both metals showed similar trend of changes in mineral composition, although Cu proved more damaging effect on K and Mn contents, and Cd on Zn contents. In combined treatments, Cd stimulated Cu uptake, notably at low concentration, while its own uptake was restricted by the presence of Cu. At either level of concentration, combined stress of these metals exacerbated plant growth inhibition and caused further oxidative damages compared to their individual stress. However, metals synergistic effects occurred only in conditions where Cu uptake was enhanced by Cd. A greater synergistic effect was observed in sensitive cultivar Zheda 622 as compared to the tolerant cultivar ZS 758. As to mineral composition, no metals synergistic effects were noted. This study highlighted the ecotoxicological significance of Cd-led Cu uptake in *B. napus*, which was assumed to drive metals' synergistic toxicity, and showed that the relationship between Cd-led Cu uptake and plant growth responses could vary with respect to cultivar.

Keywords Accumulation · *Brassica napus* L. · Cadmium · Copper · Interaction · Synergistic effects

Introduction

Increasing metals accumulation in soil, originated from growing industrial activity, intensive use of fertilizers and improper disposal of wastes, has become a prevalent environmental issue in the modern era (Smith 2009; Kabata-Pendias 2011). Cadmium (Cd) and copper (Cu) are among elements of most concern as they can reach high level in soil due to their frequent occurrence and high contents in common sources of soil contamination (Kikuchi et al. 2007; Nagajyoti et al. 2010). In China for instance, as a result of only two decades of industrialization and urbanization, Cd and Cu concentrations have reached higher levels than their background values in soil, even in agricultural ones (Wei and Yang 2010).

Higher concentrations of both Cd and Cu are toxic and critical constraints in limiting the crop productivity worldwide. Though, Cu is normally essential for many physiological processes in plants (Burkhead et al. 2009),

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but become strongly phytotoxic in excess amount since the redox properties that account for its essential role, also contribute to its toxicity (Cuypers et al. 2011). By contrast, Cd has no known physiological functions in plant metabolism, except for some Cd-hyperaccumulating populations that presumably require Cd for normal growth (Verbruggen et al. 2009). Nonetheless, due to its chemical similarity with essential bivalent cations, Cd is also largely absorbed by plants (Lin and Aarts 2012). Toxic levels of these metals cause over-production of reactive oxygen species (ROS) (Gill and Tuteja 2010), leading to irreversible structural damages to cellular components and severe impairment of important physiological processes in plants such as photosynthesis, respiration, and the metabolism of essential elements (Ali et al. 2013a, b; Andresen and Küpper 2013; Ravet and Pilon 2013).

Cd and Cu continuously added into the agricultural soils through common source including inorganic fertilizers, sewage sludge, irrigation water, and pesticides (de López-Camelo et al. 1997; Nagajyoti et al. 2010; Wuana and Okieimen 2011). Consequently, the toxic effects of the two metals are likely occurred simultaneously, particularly in acidic soils where their environmental availability increases with a decrease in soil pH (Guo et al. 2007; Kabata-Pendias 2011). It is thus necessary and important to assess the growth responses of plants to the combined toxic effects of Cd and Cu, which not only reflects real-life exposure of organisms (An et al. 2004), but also considers the fact that components of chemical mixture may interact in a fashion that affects each other's toxicity (Spurgeon et al. 2010). The significance of this approach drawing on such scientific attention and paying appropriate attention to the interactive toxicity of chemicals in the environment (Ali et al. 2011; Oliveira et al. 2014; Wu et al. 2014; Islam et al. 2015; Liu et al. 2015).

In the current context of widespread metals contamination, tremendous efforts have been put in identifying suitable plants to grow in contaminated soils, which highlighted the environmental importance of oilseed rape (Yadav and Srivastava 2014), a species historically grown for its edible oil (Zhou 2001). The interest in this species is derived from its properties of rapid growth, high biomass production, and relative resistance to metal stress (Yu et al. 2012), which made it a candidate plant for phytoremediation, along with species like *B. juncea*, *Helianthus annuus*, and *Zea mays* (Yadav and Srivastava 2014). Despite such importance, however, little is known about the toxicity response of *Brassica napus* plants against metal mixture stress and the interactive toxic effects of Cd and Cu, although extensive literature exist on individual response of Cd and Cu stress on plants (Burkhead et al. 2009; Ali et al. 2014; Andresen and Küpper 2013; Ravet and Pilon 2013). A few studies have previously investigated the combined toxicity of Cd and Cu in terrestrial plants including *B. chinensis* (Wong et al. 1986), *Picea sitchensis* (Burton et al. 1986), carrot (Al-Subu et al. 1993), cucumber (An et al. 2004), and more recently barley (Žaltauskaitė and Šliumpaitė 2013), but pretty varied trend of results have been obtained. This could be suggestive of species-dependent growth responses of plants to the combined stress of Cd and Cu; accordingly, specific toxicity responses could be expected for *B. napus*.

In prospect of efficient use in phytoremediation, there is an obvious need to understand the toxicological implications associated with growing *B. napus* in co-contaminated media of Cd and Cu. The present study was carried out as an effort to fill this gap, using plant growth, oxidative stress and mineral composition as toxicity endpoints in relation to the pattern of metal bioaccumulation. In this study, two *B. napus* cultivars differing in metals tolerance were selected to test the hypothesis of genotype-dependent toxicity response.

Materials and methods

Plant materials and growth conditions

Seeds of two leading oilseed rape (*B. napus* L.) cultivars (cvs. Zheda 622 and ZS 758) earlier detected as differing in metals tolerance (Farooq et al. 2015; Gill et al. 2015) were obtained from the College of Agriculture and Biotechnology, Zhejiang University, and used in this study. Mature seeds were germinated in plastic pots (170 mm × 220 mm) filled with peat soil. Four-week-old seedlings were selected for morphological uniformity and transferred to 5-L black plastic pots containing 4.5 L nutrient solution prepared (in μM) as follows: 4000 $Ca(NO_3)_2{\cdot}4H_2O$, 4000 $(NH_4)_2SO_4$, 4000 K_2SO_4, 4000 KNO_3, 1300 KH_2PO_4, 1000 $MgSO_4{\cdot}7H_2O$, 50 Fe-EDTA, 10 H_3BO_3, 5 $MnSO_4{\cdot}H_2O$, 5 $ZnSO_4{\cdot}7H_2O$, 1 $CuSO_4{\cdot}5H_2O$, and 0.5 $Na_2MoO_4{\cdot}2H_2O$. Three plants per pot were plugged into evenly spaced holes on the pot cover and placed under ambient conditions (20–24 °C temperature and 55–60 % relative humidity) in a green house. After 8 days of plants acclimatization, Cd as $CdCl_2{\cdot}2.5H_2O$ and Cu as $CuCl_2{\cdot}2H_2O$ were applied to desired concentrations either individually or in equimolar mixture, to make the following treatments: (1) Control; (2) 50 μM Cd; (3) 50 μM Cu; (4) 50 μM Cd + 50 μM Cu; (5) 200 μM Cd; (6) 200 μM Cu; and (7) 200 μM Cd + 200 μM Cu. The treatment concentrations were based on findings from pre-experimental studies, in which several lower and higher levels of Cd and Cu were used, that is, 25, 50, 100, 200, and 400 μM. Both metals at concentration of 50 μM showed little damage to plant growth and 200 μM imposed significant damage to plant growth, whereas concentrations higher than 200 μM were too toxic for plant growth. The pH of solution was maintained at 5.7 ± 0.1 with 1 M NaOH or HCl solution. Aeration was given continuously through air pump in the nutrient medium. Nutrient solution was changed after every 4 days, but pH of solution was readjusted on daily basis. The study was laid out in a completely randomized design with three replicates. Fifteen days after treatment, all morphological data were measured and plants roots were thoroughly washed with deionized water to remove surface ions. Then, samples were collected for physiological and biochemical analysis as below. With respect to laboratory assay, samples were collected either fresh or immediately frozen in liquid N_2 and kept frozen until analysis. To refine the picture on how Cd and Cu interact for their respective uptake and distribution in plants, an additional and short-term experiment (3 days) was undertaken (in similar conditions) so as to minimize the influence of dilution/concentration effects associated with plant tissue growth, commonly observed in long-term experiments.

Assessment of morphological changes and elements uptake

After sampling, roots were desorbed by immersion with 20 mM EDTA-Na_2 for 3 h and then extensively washed in running deionized water for thorough metals elution (Zhou et al. 2007; Zeng et al. 2012). Then, plant samples (shoots and roots) were oven-dried at 105 °C for 3 h, followed by 80 °C for 48 h, and weighed immediately after removal from the oven until biomass become stable (Momoh and Zhou 2001).

After dry biomass determination of plants, contents of Cd, Cu, and different mineral nutrients (K, Mg, Ca, Fe, Zn, Mn) in root and shoot tissues were measured. Dried samples were wet-digested in a mixture of concentrated HNO_3:$HClO_4$ (2:1, v/v), then heated at 80 °C in water bath for about 2 h (Gill et al. 2015), and elements contents were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES, Optima 8000 DV, PerkinElmer, USA). Amounts of metal accumulated in plant tissues were obtained by multiplying the metal concentration by plant tissues dry weights.

Photosynthetic pigments analysis

Chlorophyll was extracted from upper second fully expanded leaves with 96 % (v/v) ethanol, and the concentration was estimated according to Lichtenthaler and Wellburn (1983).

Determination of indices of oxidative stress

Oxidative damage to lipids (expressed as malondialdehyde content) and contents of reactive oxygen species (hydrogen peroxide and superoxide radical) were measured in roots and upper second fully expanded leaves. Samples (0.5 g) were extracted with 5 mL of 0.1 % (w/v) trichloroacetic acid (TCA), and homogenates were centrifuged at 12,000×g for 15 min at 4 °C. The content of malondialdehyde (MDA) was estimated following the procedure of Heath and Packer (1968). A mixture made of 1 mL aliquot extract and 4 mL 0.5 % thiobarbituric acid (TBA) in 20 % TCA was incubated at 95 °C for 30 min and then quickly cooled in ice bath to terminate the reaction. The cooled mixture was centrifuged at 10,000×g for 10 min, and the absorbance of supernatant was recorded at 532 nm. Correction for non-specific absorption was made by subtracting values read at 600 nm, and the level of MDA was calculated using extinction coefficient of 155 mM^{-1} cm^{-1}. Hydrogen peroxide (H_2O_2) accumulation in leaves and roots was measured according to Velikova et al. (2004). In total 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M KI were added to 0.5 mL of the supernatant, and the absorbance of the mixture was read at 390 nm. The H_2O_2 contents were estimated from a standard curve.

For measurement of superoxide radical ($O_2^{\cdot-}$), plant material (0.5 g) were homogenized in 3 mL of 65 mM potassium phosphate buffer (pH 7.8) and extract was centrifuged at 5000×g for 10 min at 4 °C (Jiang and Zhang 2001). One milliliter of the supernatant was mixed with 0.9 mL of 65 mM potassium phosphate buffer (pH 7.8) and 0.1 mL of 10 mM hydroxylamine hydrochloride, and incubated at 25 °C for 20 min. To 1 mL of the incubated mixture, 1 mL of 17 mM sulfanilamide and 1 mL of 7 mM α-naphthylamine were added, followed by further 20 min incubation at 25 °C. Next, *n*-butanol in the same volume (3 mL) was added and centrifuged at 1500×g for 5 min. The absorbance in the aqueous solution was read at 530 nm. A standard curve was utilized to calculate the rate of superoxide generation.

Assessment of metals interactive effects on plant growth and physiological attributes

Possible interaction between Cd and Cu were assessed using Abbott's model that presumes an independent action of mixture components and considers any deviation from this hypothesis as an indication of interaction between mixture components (Gisi 1996; Teisseire et al. 1999). Based on individual toxicities of Cd (T_{Cd}) and Cu (T_{Cu}) measured experimentally, Abbott's formula helped to predict the combined toxicity of Cd and Cu (T_{pre}) assuming an independent action of metals in mixture:

$$T_{pre} = (T_{Cd} + T_{Cu}) - \frac{T_{Cd} \times T_{Cu}}{100}$$

The ratio between the mixture toxicity experimentally observed (T_{obs}) and the mixture toxicity predicted by Abbott's formula (T_{pre}) was calculated to check the existence of possible interaction between Cd and Cu: T_{obs}/T_{pre} value = 1 indicated additivity of metals toxicity, while value >1 and <1 denoted synergism and antagonism, respectively.

Statistical analysis

The experiment was carried out through a completely randomized design, and the analysis of variance (ANOVA) was carried out for statistically significant differences using the statistical package SPSS, version 16.0 (SPSS, Chicago, IL, USA). The results are the mean ± SD of at least three independent replicates, and the mean differences were compared utilizing the Duncan's multiple range test.

Results and discussion

Cd and Cu uptake and distribution in plants

Cadmium (Cd) and copper (Cu) contents in 3 and 15 days metal-treated plants are illustrated in figures (Figs. 1, 2). Concentrations of both metals in roots and shoots increased with elevation of metals exposure strength. However, Cu presented relatively low mobility, with 2- to 3- and 4- to fivefold lower contents than Cd, respectively, in root and shoot tissues. Likewise, its root-to-shoot translocation rate was twofold lower (Fig. 3), which was thought to be in relation to its higher exchange capacity than Cd (Gondar et al. 2006). Previously, Nishizono et al. (1987) also observed a higher retention of Cu at root cell wall of *Athyrium yokoscense* compared to Cd. Zhou et al. (2007) also demonstrated that Cu adsorption in maize root cell wall was such tight that a large amount of metal ions were retained in the root apoplast. Despite their varied level of accumulation in *B. napus* plants, both Cd and Cu mainly accumulated in root (TF < 100). Generally, plant ability to reduce the intracellular concentrations of metals particularly in the aboveground part, is assumed to be a tolerance trait for non-hyperaccumulator plants (Lin and Aarts 2012). Correspondingly, more tolerant genotypes are expected to accumulate fewer amounts of metal, like also demonstrated by a number of studies (Guo et al. 2004, 2007; Ali et al. 2011; Islam et al. 2015). In the current study, however, almost equivalent amounts of metals were observed in roots and shoots of Zheda 622 and ZS 758, two cultivars differing in metal tolerance (Figs. 1 and 2). These results corroborated the view that tolerant genotypes are not invariably low in metals contents (Kuboi et al. 1987; Wu and Zhang 2002; Belimov et al. 2003).

Simultaneous supply of Cd and Cu to the medium affected each other's uptake and accumulation in *B. napus* plants, suggesting their interactive relationship at root surface (Figs. 1, 2). In both cultivars, the presence of Cu decreased the tissues concentrations of Cd, whereas Cd induced an opposite effect (increase) on Cu contents. To ascertain whether the observed effects were associated with changes in metal uptake rate rather than a consequence of growth dilution/concentration effects, we examined the extent of metals accumulation in the shoots (Fig. 1c) and roots (Fig. 2c) of 15-day-treated plants that presented obvious signs of growth inhibition. Obtained results confirmed both the Cu inhibitory effects and the Cd stimulatory effects on each other's uptake, echoing the pattern recorded in the short-term experiment (Figs. 1a, 2a). It was apparent that Cd and Cu affected each other's uptake through distinct mechanisms, considering the contrast in their reciprocal effects. Normally, uptake of Cu occurred as monovalent form through specific transporters (COPTs) (Andres-Colas et al. 2010), although a marginal amount of Cu^{2+} could also be taken up through ZIP transporters (Cuypers et al. 2012). Consequently, reduction of Cu^{2+} to Cu^{+} (mediated by ferric reductase) is considered as a prerequisite for Cu uptake by plants (Burkhead et al. 2009). Therefore, the Cd stimulatory effects on Cu uptake could be explained by an improved activity of ferric reductase (FRO) with the presence of Cd ions that might enhance the pool of Cu^{+} in the rooting medium and its subsequent uptake by plants. Ghasemi et al. (2009) also observed that Ni exposure enhanced the activity of Cu^{2+} reductase at plant root surface, which was presumed to increase Cu accumulation in *Alyssum inflatum*. Likewise, Zhou et al. (2007) indicated a close relationship between the extent of Cu uptake by maize seedlings and the potential of cysteine to change the oxidation state of Cu in the rhizosphere. The Cd enhancing effects on copper uptake was also reported in barley (Lachman et al. 2004) and *Chlorella vulgaris* (Franklin et al. 2002; Qian et al. 2009). In contrast to these reports, Huang et al. (2009) did not notice any Cd stimulatory effect on Cu accumulation in rice. Besides Cd, the presence of Pb was also observed to increase Cu accumulation in *Cucumis sativus* (An et al. 2004). Converse to Cd effect, Cu restricted the accumulation of Cd in *B. napus* (Figs. 1, 2), which is arguably in relation to metal competition for binding sites at plant root surface (Gondar et al. 2006).

Current results delineated that the extent of Cd and Cu interaction varied with respect to level of metals concentration in the growth medium. Surprisingly, Cd stimulated the uptake of Cu only at lower concentration (50 μM) (Figs. 1, 2), which appeared to be indicative of limited influence of Cd on biological processes related to Cu accumulation in *B. napus*. On the contrary, the magnitude

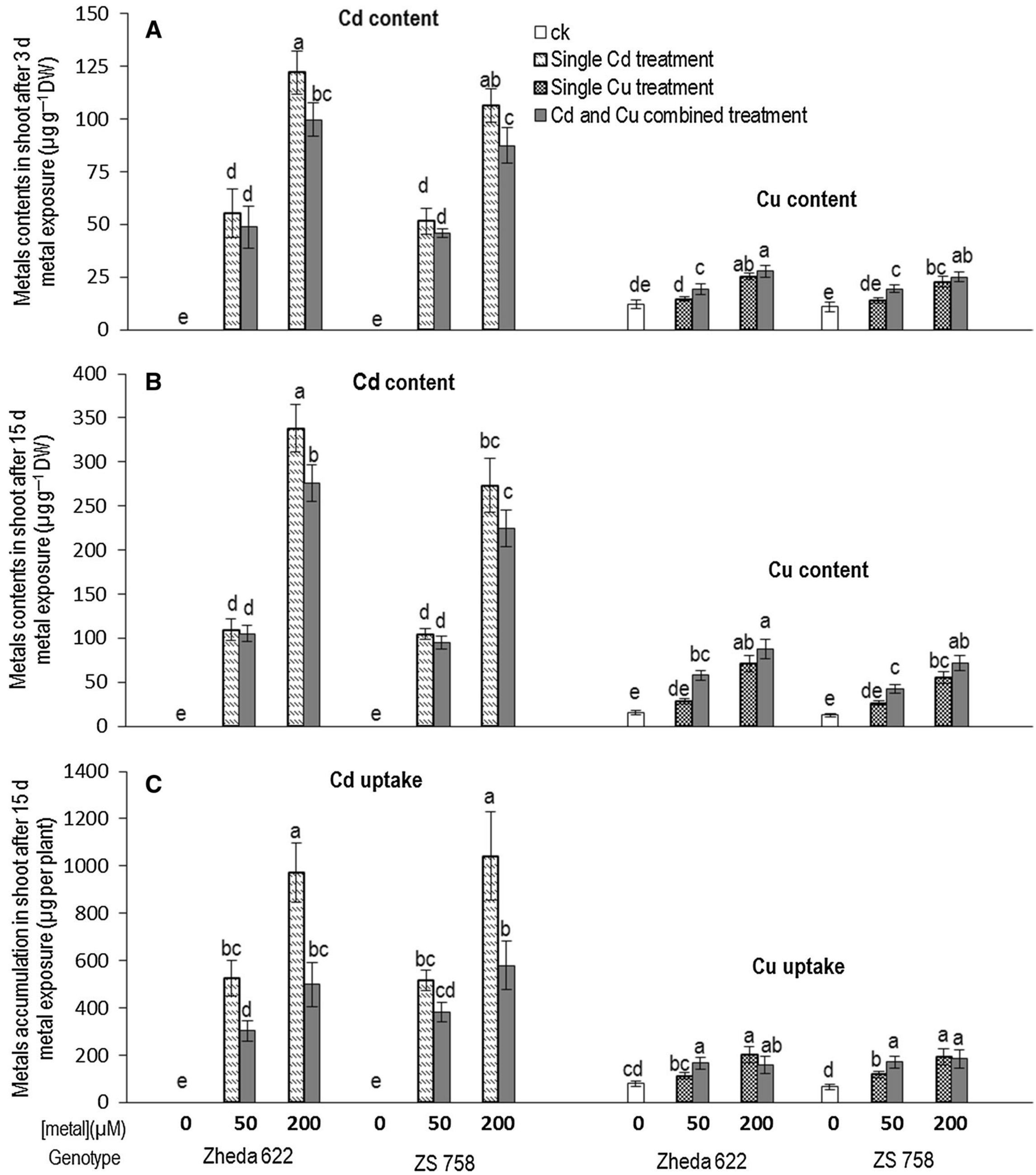

Fig. 1 Cadmium and copper contents in the shoots of *Brassica napus* plants exposed to **a** 3-day and **b** 15-day metal treatments (μM); and the amount of metals (Cd and Cu) accumulation in **c** 15-day-treated plants. *Bars* represent SD of means from at least three independent replicates. Means followed by *same letters* for the same type of metal are not significantly different by Duncan's multiple range test ($P < 0.05$)

of Cu action (on Cd uptake) increased with metal exposure strength (Figs. 1, 2), suggesting that the more the metals were present in the rooting medium, the more Cd was outcompeted by Cu for binding to root surface.

In both cultivars and at either level of metals concentrations, the presence of one metal did not affect the root-to-shoot translocation rate of the other (Fig. 3). Hence, it was thought that Cd and Cu scarcely interacted within plant.

Plant growth, chlorophyll accumulation, and oxidative stress

Single and combined effects of excess Cd and Cu, measured in terms of plant biomass reduction and decrease in chlorophyll content, are presented in Fig. 4, respectively,and their effects on ROS accumulation ($O_2^{\cdot-}$ and H_2O_2) and MDA contents are shown in Fig. 5a–f. Fifteen-day exposure of plants to Cd and Cu toxicity provoked a dose-responsive reduction in shoot and

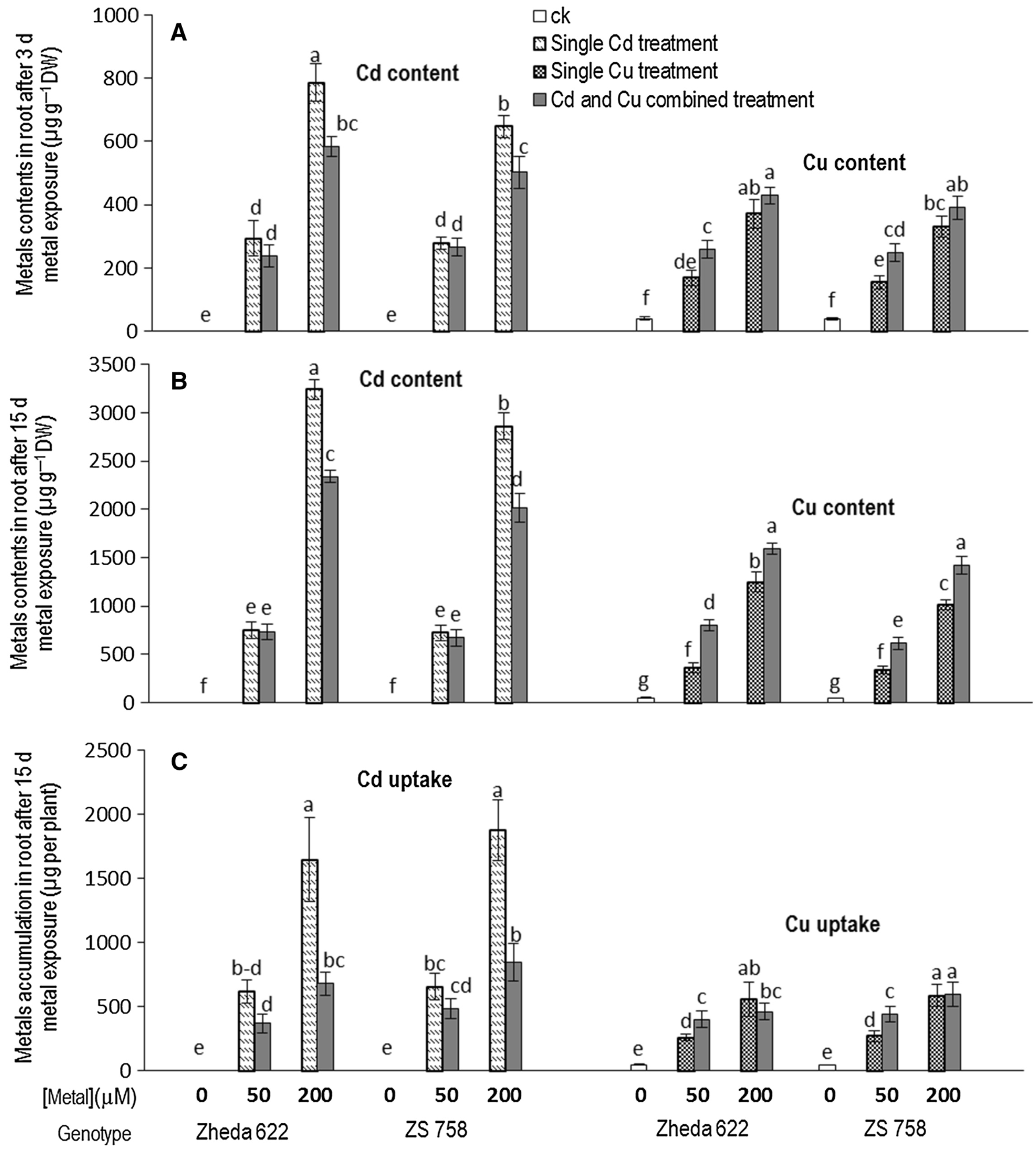

Fig. 2 Cadmium and copper contents in the roots of *Brassica napus* plants exposed to **a** 3-day and **b** 15-day metal treatments (μM); and amount of metals (Cd and Cu) accumulation in **c** 15-day-treated plants. *Bars* represent standard deviation of means from at least three independent replicates. Means followed by *same letters* for the same type of metal are not significantly different by Duncan's multiple range test ($P < 0.05$)

root dry biomass and chlorophyll contents relative to controls. Similarly, both Cd and Cu treatments induced significant accumulation of ROS and MDA in plants tissues, which are recurrent manifestations of metals phytotoxicity (Gill and Tuteja 2010). However, the extent of toxicity was not consistent with the level of metals bioaccumulation, as Cu caused more deleterious effects despite its moderate bioaccumulation compared to Cd, notably at lower level of metals concentrations (50 μM). This was believed to be in connection with the differential chemical properties between the two metals. In fact, due to its redox properties, Cu is prone to directly generate ROS through Fenton–Haber–Weiss reaction (Yadav 2010) and is considered as one of the most powerful catalysts of free radical formation (Ravet and Pilon 2013). Conversely, Cd is a non-redox active metal that can generate ROS indirectly by interaction with antioxidant molecules (Valko et al. 2005) or by disruption the electron transport chain (Andresen and Küpper 2013). Nonetheless, Cd toxicity appeared to increase faster with elevation of metals concentrations from 50 to 200 μM, producing an equistrength inhibition of plant

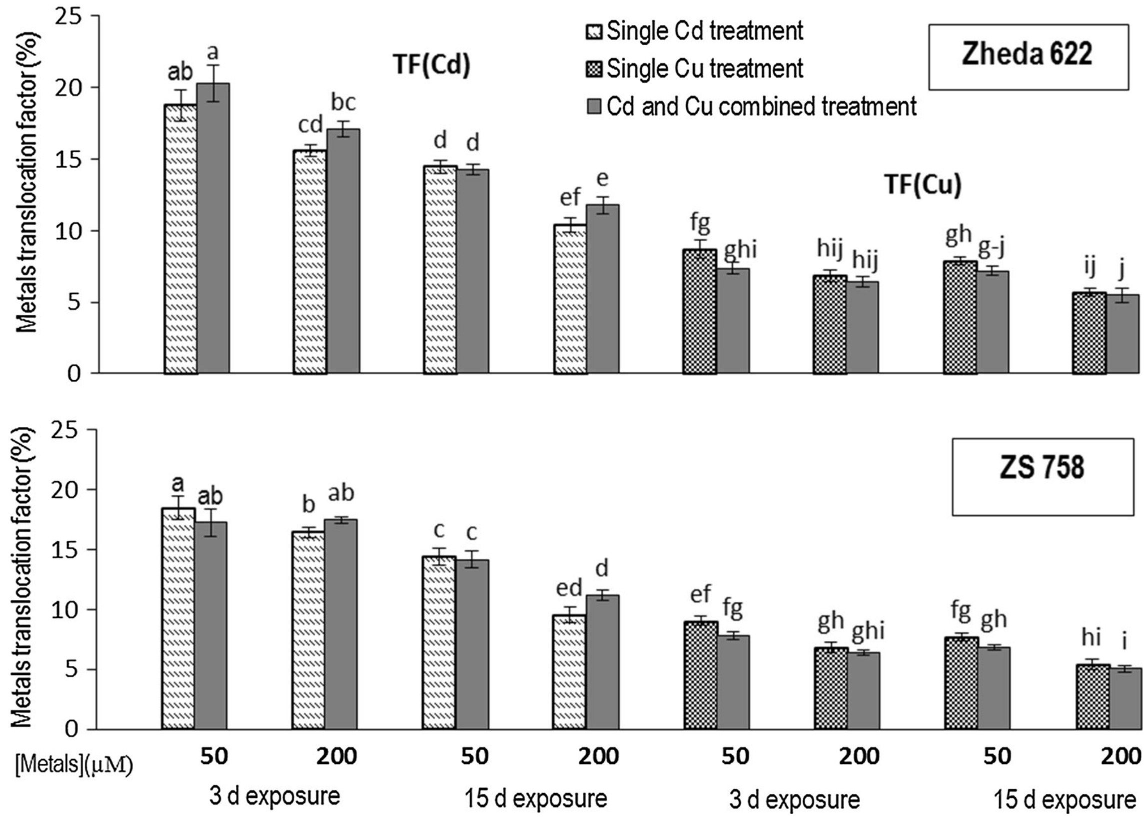

Fig. 3 Root-to-shoot translocation factors of Cd and Cu in 3 and 15 days *Brassica napus* plants exposure to different concentrations (50 and 200 μM) of both cadmium and copper. *Bars* represent standard deviation of means from at least three independent replicates. Means followed by *same letters* are not significantly different by Duncan's multiple range test ($P < 0.05$)

growth (Fig. 4a, b) and an equivalent oxidative stress compared to Cu effects (Fig. 5e, f). The toxic effects of both metals varied with respect to cultivars, and as naturally expected, much stunted plant growth (Fig. 4a, b), greater chlorophyll loss (Fig. 4c), and higher oxidative damages (Fig. 5) were observed in the sensitive cultivar Zheda 622 than cultivar ZS 758 (tolerant), confirming the earlier reported genotypic difference in metals tolerance between the two cultivars (Farooq et al. 2015; Gill et al. 2015).

Combined effects of Cd and Cu caused further plant growth inhibition, chlorophyll loss and oxidative stress compared to alone Cd and Cu stress. Importantly, as revealed by Abbott's model, the resulting mixture toxicity was different from the individual metal effects (Tables 1, 2). Synergistic effects (more than additive effects) were noted at low metal concentrations, while converse effects (antagonistic) occurred at higher level of metals exposure, denoting varied toxicity responses of *B. napus* plants with respect to the level of metal exposure. The extent of metal toxicity is generally associated with the amount of metals likely to accumulate in plant tissues (Davis and Becker 1978). In the present study, Cu and Cu synergistic effects on plant growth, chlorophyll content and oxidative stress were recorded only in conditions where the Cu uptake was effectively stimulated by the presence of Cd (at low metals concentrations). Likewise, their antagonistic effects at high metals concentrations could be ascribed to the reduced Cd effect on Cu bioaccumulation, coupled with the Cu-restricting effect on Cd uptake (Figs. 1, 2). Different from current results, Qian et al. (2009) found no direct relationship between the Cd-led Cu uptake and the algal (*C. vulgaris*) growth responses; which is most probably due to differences in metals concentrations used. Synergism is the interaction of most concern from toxicological point of view, since it increases the overall toxicity of chemicals mixture (Teisseire et al. 1999). Correspondingly, the Cd-led Cu bioaccumulation observed in this study, which is assumed to drive metals synergistic toxicity to *B. napus* plants, constitutes a matter of toxicological significance, for which underlying molecular basis and relevant environmental factors need to be clarified. Cd and Cu synergistic effects on plant growth inhibition, chlorophyll loss and oxidative stress were observed in both cultivars Zheda 622 and ZS 758, but a stronger extent changes were noted in the sensitive cultivar Zheda 622. Previously, significant genotypic differences have been detected between cultivars ZS 758 and Zheda 622 regarding their ability to modulate the intracellular activity of metals ions (data not shown);

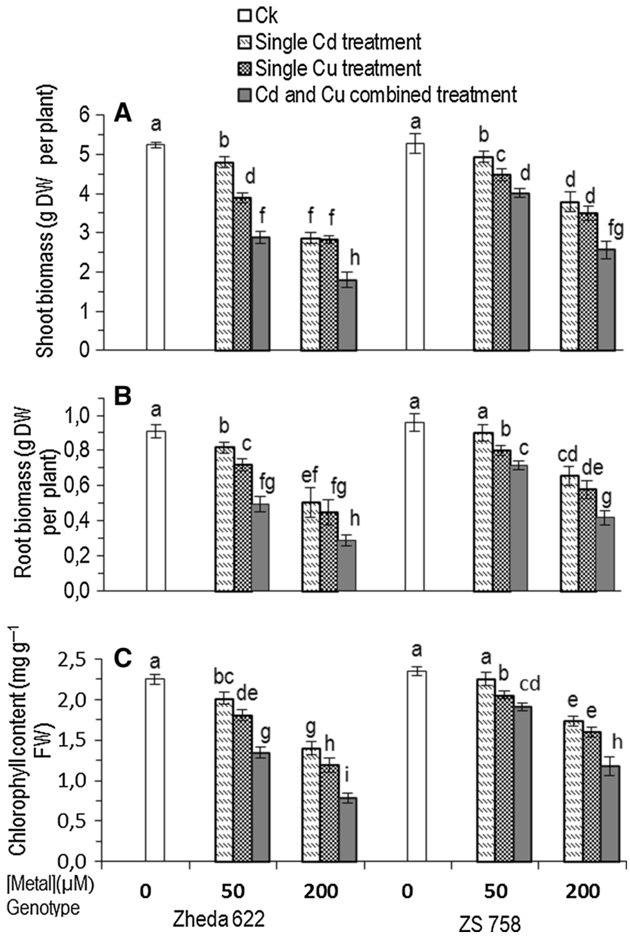

Fig. 4 Effects of different concentrations (50 and 200 μM) of both cadmium and copper on **a**, **b** shoot and root dry weight and **c** chlorophyll contents in 15-day-treated *Brassica napus* plants. Bars represent standard deviation of means from at least three independent replicates. Means followed by *same letters* are not significantly different by Duncan's multiple range test ($P < 0.05$)

this could be a possible reason accounting for the greater synergistic effects noted in the sensitive cultivar Zheda 622, which presented a lesser growth. Guo et al. (2007) also detected an important synergistic effect of Al, Cd, and Cu in a sensitive cultivar of barley, which was not noticed with the tolerant cultivar. However, both individual and combined treatments of Cd and Cu significantly affected plant growth, chlorophyll accumulation and oxidative stress, although in some cases, the low level of Cd treatment did not induce significant effects.

Nutrients contents

Effects of different concentrations of both Cd and Cu on nutrients contents in the leaves of 15-day-treated *B. napus* plants have been shown in Table 3. Addition of Cd or Cu to the growth medium affected the mineral composition in a similar fashion. Both metals decreased the content of K, Fe, Zn, and Mn in a dose-responsive trend in the two cultivars (Zheda 622 and ZS 758), but no significant effects were detected on Ca and Mg contents. Cu induced significant reductions of K, Fe, Zn and Mn contents at either level of concentration, while the deleterious effects of Cd were mostly noticeable at higher concentration, except for Zn contents (Table 3). It was evident that Cu stress was much more critical for K and Mn nutrition than Cd stress; conversely, Cd stress showed more deleterious effects on Zn content in comparison with Cu. A number of reports also suggested a strong reduction of Mn uptake as a common manifestation of Cu phytotoxicity (Lidon and Henriques 1992; Lin and Wu 1994), which is not necessarily the case for Cd (Kabata-Pendias, 2011); likewise, Cd similarity with Zn is considered as a recurrent cause of its phytotoxicity (Verbruggen et al. 2009). Combined effects of metals on plant mineral nutrition have received little attention from researchers. Changes in mineral composition have been investigated under combined stresses of Al and Cd in barley (Guo et al. 2004), Al and Cr in barley (Ali et al. 2011), As and Cr in jute (Islam et al. 2015), and observed results have shown a further decrease in nutrient content compared to effects of single metal. Similarly, current results indicated that Cd and Cu stress together caused marked reduction in nutrients relative to control. However, when compared to Cd and Cu stress alone, the overall effects were not overly exacerbated in mixture treatments (Table 3), and contrary to combined effects of metals on plant growth and oxidative stress, additivity (on Fe and Zn contents) and antagonism (on K and Mn contents) best described the interactive effects of Cd and Cu on mineral composition in *B. napus* plants (Table 4). Similar to their single effects, the combined effects of Cd and Cu did not affect Ca and Mg uptake (Table 4). Both levels of Cu and Cd treatments, individually and in combination significantly reduced K, Fe and Zn contents, but no effects were detected on Ca and Mg nutrition. Mn contents were significantly affected only at high concentration of Cd, while a significant reduction was recorded at either level of Cu concentration.

Although both single and combined stresses of Cd and Cu significantly altered mineral composition in plants, the reduction extent did not differ between the sensitive cultivar (Zheda 622) and the tolerant cultivar (ZS 758) relative to their respective controls (Table 3). Hence it may be assumed that reduction in nutrients contents was less accounted for the greater sensitivity of Zheda 622 to Cd and Cu stresses than ZS 758.

Conclusion

The findings of present study revealed an interactive but contrasting relationship between Cd and Cu for their uptake and accumulation in *B. napus* plants; Cd enhanced Cu bioaccumulation while its own uptake was restricted by

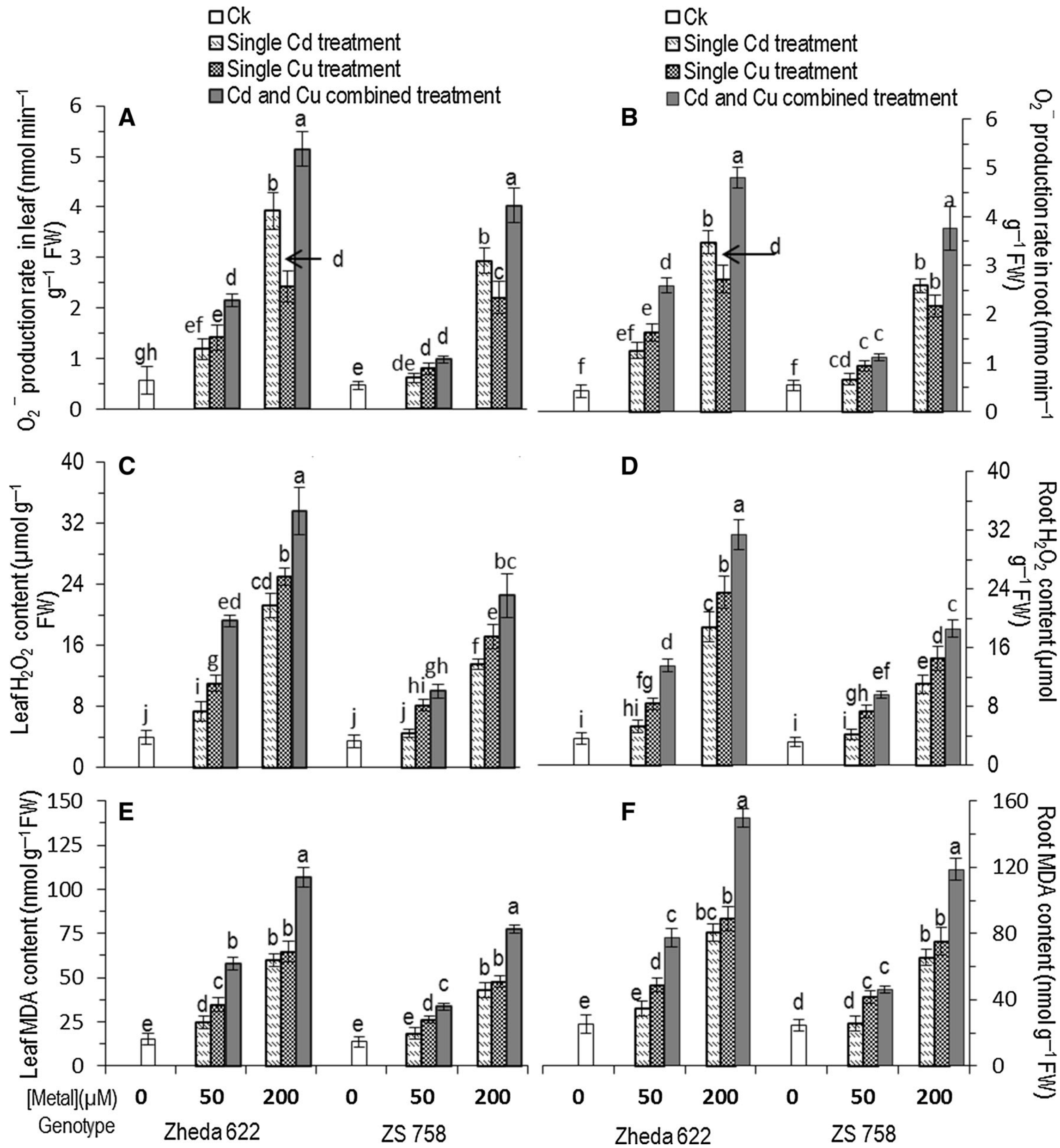

Fig. 5 Effects of different concentrations (0, 50 and 200 μM) of both cadmium and copper on the levels of (**a**, **b**) O_2^{-}, (**c**, **d**) H_2O_2 and MDA (**e**, **f**) in leaves and root of 15-day-treated *Brassica napus*. *Bars* represent standard deviation of means from at least three independent replicates. Means followed by *same letters* are not significantly different by Duncan's multiple range test ($P < 0.05$)

Table 1 Interactive effects of cadmium and copper on biomass accumulation and chlorophyll content in 15 days treated in two *Brassica napus* cultivars

Treatment	Cultivar	Toxicity ratio (T_{obs}/T_{pre})[a]		
		Shoot DW	Root DW	Chlorophyll
50 μM Cd + 50 μM Cu	Zheda 622	1.33 ± 0.09a	1.44 ± 0.16a	1.35 ± 0.11a
	ZS 758	1.12 ± 0.09b	1.13 ± 0.12b	1.11 ± 0.12b
200 μM Cd + 200 μM Cu	Zheda 622	0.73 ± 0.04c	0.72 ± 0.03c	0.77 ± 0.03c
	ZS 758	0.84 ± 0.07c	0.79 ± 0.06c	0.86 ± 0.09c

Data are mean ± SD of at least three independent replicates. Values within column followed by the same letter are not significantly different by Duncan's multiple range test ($P < 0.05$)

[a] Toxic effects of metals mixture were measured experimentally (T_{obs}) and were predicted with Abbot's model (T_{pre}). T_{obs}/T_{pre} value = 1 indicated additivity of Cd and Cu individual effects, value >1 synergism and value <1 antagonism

Table 2 Interactive effects of cadmium and copper on ROS ($O_2^{\cdot-}$ and H_2O_2) and MDA accumulation in leaf and root of two cultivars of *Brassica napus*

Treatment	Cultivar	Toxicity ratio (T_{obs}/T_{pre})[a]					
		$O_2^{\cdot-}$		H_2O_2		MDA	
		Leaf	Root	Leaf	Root	Leaf	Root
50 μM Cd + 50 μM Cu	Zheda 622	1.09 ± 0.09ab	1.06 ± 0.08a	1.42 ± 0.07a	1.53 ± 0.13a	1.36 ± 0.12a	1.52 ± 0.16a
	ZS 758	1.10 ± 0.13a	1.11 ± 0.12a	1.14 ± 0.16a	1.25 ± 0.09b	1.10 ± 0.10b	1.19 ± 0.11b
200 μM Cd + 200 μM Cu	Zheda 622	0.87 ± 0.06ab	0.81 ± 0.04b	0.71 ± 0.08b	0.73 ± 0.05c	0.79 ± 0.05c	0.81 ± 0.04c
	ZS 758	0.84 ± 0.08b	0.87 ± 0.12ab	0.76 ± 0.11b	0.77 ± 0.06c	0.87 ± 0.03c	0.84 ± 0.06c

Data are mean ± SD of at least three independent replicates. Values within column followed by the same letter are not significantly different by Duncan's multiple range test ($P < 0.05$)

[a] Toxic effects of metals mixture were measured experimentally (T_{obs}) and were predicted with Abbot's model (T_{pre}). T_{obs}/T_{pre} value = 1 indicated additivity of Cd and Cu individual effects, value >1 synergism and value <1 antagonism

Table 3 Effects of different concentrations of both cadmium and copper on the uptake of mineral nutrients in the leaves of two cultivars of *Brassica napus*

Treatment	K (mg g^{-1} DW)	Ca	Mg	Fe (μg g^{-1} DW)	Zn	Mn
Zheda 622						
0 μM						
Ck	80.6 ± 2.7a	27.3 ± 1.8a	7.38 ± 0.48a	352.0 ± 20.7a	142.5 ± 13.4a	35.6 ± 3.2a
50 μM						
Cd	67.4 ± 4.6b	27.3 ± 2.4a	7.41 ± 0.54a	332.3 ± 16.5a	114.1 ± 8.3bc	31.3 ± 2.6ab
Cu	64.3 ± 5.0b	26.3 ± 0.9a	7.10 ± 0.11a	263.7 ± 25.1b	127.0 ± 5.8b	21.6 ± 2.0c
Cd + Cu	59.5 ± 4.1b	25.1 ± 1.6a	6.82 ± 0.41a	262.3 ± 11.7b	106.2 ± 6.0c	21.9 ± 2.9c
200 μM						
Cd	64.1 ± 3.4b	25.0 ± 1.6a	6.94 ± 0.50a	259.7 ± 18.0b	97.5 ± 7.4d	28.8 ± 2.1b
Cu	53.5 ± 5.4c	24.3 ± 1.8a	6.76 ± 0.26a	247.0 ± 18.5b	112.2 ± 7.8bc	21.5 ± 2.7c
Cd + Cu	52.1 ± 4.7c	24.1 ± 1.5a	6.93 ± 0.40a	249.0 ± 17.6b	86.9 ± 6.8d	21.4 ± 3.7c
ZS 758						
0 μM						
Ck	73.5 ± 3.3a	27.1 ± 2.0a	7.42 ± 0.90a	336.3 ± 17.4a	151.4 ± 9.7a	34.4 ± 3.4a
50 μM						
Cd	70.6 ± 3.3ab	27.1 ± 2.3a	7.27 ± 0.58a	311.0 ± 14.5b	132.1 ± 7.2b	29.9 ± 3.2a
Cu	64.3 ± 4.1bc	24.9 ± 1.7a	7.22 ± 0.04a	307.7 ± 8.5b	133.5 ± 6.5b	19.8 ± 2.3c
Cd + Cu	63.8 ± 2.7c	26.1 ± 1.3a	6.98 ± 0.33a	284.3 ± 6.7c	117.5 ± 5.5c	21.1 ± 2.7c
200 μM						
Cd	67.1 ± 2.4bc	23.2 ± 2.4a	6.93 ± 0.58a	271.7 ± 8.7cd	95.1 ± 6.0d	26.8 ± 3.3b
Cu	55.6 ± 4.1d	23.1 ± 2.5a	6.86 ± 0.56a	258.0 ± 7.6d	118.3 ± 5.6c	19.2 ± 2.6c
Cd + Cu	55.3 ± 2.8d	22.8 ± 2.4a	6.60 ± 0.69a	250.3 ± 7.0d	97.8 ± 5.5d	19.1 ± 3.3c

Data are mean ± SD of at least three independent replicates. Different letters within column for the same cultivar indicate significant differences by Duncan's multiple range test ($P < 0.05$)

the presence of Cu in the medium. Consequently, their mixture toxicity gave rise to effects different from additively. However, synergistic effect was observed only at low metal concentrations, where Cd effectively induced Cu bioaccumulation. This demonstrates the ecotoxicological significance of the Cd-led Cu uptake in *B. napus* and highlights the interest to clarify the molecular basis underlying this phenomenon. While the reduction extent of nutrients uptake was similar between cultivars, much stunted plant growth and oxidative stress were recorded with Zheda 622 as compared to ZS 758, confirming their varied level of tolerance to metal toxicity. Moreover, a

Table 4 Interactive effects of cadmium and copper on the uptake of different mineral nutrients in the leaves of two cultivars of *Brassica napus*

Treatment	Cultivar	Toxicity ratio (T_{obs}/T_{pre})[a]					
		K	Ca	Mg	Fe	Zn	Mn
50 μM Cd + 50 μM Cu	Zheda 622	0.77 ± 0.15a	–	–	0.99 ± 0.13a	0.92 ± 0.15a	0.77 ± 0.17a
	ZS 758	0.82 ± 0.17a	–	–	0.98 ± 0.14a	1.01 ± 0.16a	0.72 ± 0.15a
200 μM Cd + 200 μM Cu	Zheda 622	0.71 ± 0.12a	–	–	1.11 ± 0.14a	0.90 ± 0.11a	0.71 ± 0.18a
	ZS 758	0.78 ± 0.12a	–	–	0.93 ± 0.08a	0.93 ± 0.10a	0.71 ± 0.15a

Data are mean ± SD of at least three independent replicates. Values within column followed by the same letter are not significantly different by Duncan's multiple range test ($P < 0.05$)

– Indicates that Ca and Mg did not show any interactive efforts at combined Cd and Cu treatments

[a] Toxic effects of metals mixture were measured experimentally (T_{obs}) and were predicted with Abbot's model (T_{pre}). T_{obs}/T_{pre} value = 1 indicated additivity of Cd and Cu individual effects, value >1 synergism and value <1 antagonism

stronger synergism was recorded in the sensitive cultivar Zheda 622 than the tolerant cultivar ZS 758, which was ascribed to the genotypic difference in their ability to modulate the intracellular activity of metals ions. However, Cd and Cu synergistic toxicity was noted on plant growth and oxidative stress, while their joint effects were characterized by additively or antagonism.

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