SURVIVAL TO EARLY TOXIC COPPER EXCESS: BIOCHEMICAL AND ANATOMICAL CHANGES DURING GERMINATION OF INDIAN MUSTARD

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It is well-known that essential heavy metals like copper (Cu), mainly at higher concentrations usually cause overproduction of reactive oxygen species (ROS) resulting in oxidative stress in plants. Till date many experiments were carried out to evaluate how Cu toxicity influences in adult plants but only a few reports are available about the effects during germination. Since this is a very sensitive period and the effects of heavy metal stress are more serious. The aims of our study were to investigate potential oxidative stress and antioxidative defense mechanisms beside potential morphological and/or anatomical alterations in germinating seeds of Indian mustard (*Brassica juncea L.*) exposed to excess Cu. The following parameters were evaluated to describe oxidative stress: FRAP (ferric reducing ability of plasma), lipid peroxidation (LP), reduced glutathione content (GSH), total protein content and the activity of glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPOX) and glutathione reductase (GR). We also made an assessement of histochemically LP and the loss of plasma membrane integrity in the root tips, the production of callose and the lignification of cell walls. Our results showed that Cu treatments were followed by notable GSH-depletion. We could detect LP histochemically in the root tips. The application of Cu increased the activity of SOD in time and dose-dependent manner. The activity of CAT and GPOX increased after 48-96h Cu excess. Morphological symptoms of metal toxicity occurred such as stunted, hooked-formed and brownish root tips. Production of callose and lignification of cell walls could be visualized, too.

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Introduction

It is well-known fact that heavy metals due to their common presence in soil-water-plant- animal- human system may affect development, growth, basic metabolisms etc. in living organisms. Essential heavy metals like copper (Cu) which are often co-factors of various enzymes such as oxidases or components of the photosynthetic system, also might act as pollutants at higher concentrations in the environment due to industrial, municipial or agricultural activities.[1, 2, 3, 4] Similarly to toxic heavy metals (e.g. Pb, Cd), essential heavy metals at higher levels may also provoke overproduction of reactive oxygen species (ROS) resulting in oxidative stress in plants, and consequently lipid peroxidation in the membranes, disorders in various metabolic processes and visible morphological and/or histological changes.[4, 5, 6] Though Cu is an important biometal and its average amount concentration in natural soils is 2-40 mg kg^{-1} DM (dry matter), it can accumulate in agricultural soils 10-15-fold higher than the recommended levels due to emission of sewage sludge and fertilizers, and may trigger remarkable changes in the photosynthetic activity.[1, 2, 3, 7]

Numerous plants can survive with the excess of Cu, taken up and allocate it to different plant parts, generally to root or shoot. These tolerant plants can also accumulate this metal, hence are called 'hyperaccumulators' because of amassing metals at levels even 100-fold higher than nonaccumulator plants.[8] Indian mustard (*Brassica juncea* L., Brassicaceae, Mustard family) is known as Cd-hyperaccumulator but it can tolerate Cu. Beacuse of its fast growth and relatively small size it can be a good model.

Most of the earlier studies reported oxidative stress only in adult plants (including Indian mustard). After heavy metal stress there are not many reports on the effects in the early stages of ontogenesis in *Brassica juncea* L., which was exposed to heavy metal stress, since germination, which is a very sensitive period of development and the effects of heavy metal stress are more expressed and visual than later.[4,9, 10]

Aims

The aims of our study were to investigate potential oxidative stress and antioxidative defense mechanisms in germinating seeds of Indian mustard exposed to copper excess. We wanted to know whether copper as an essential heavy metal can cause changes similar to cadmium, which is basicly toxic for the plants. We intended to answer the following questions:

-Does heavy metal treatment cause oxidative stress during the germinative stage ?

-Which biochemical parameters are the most appropriate to decribe this oxidative stress ?

-How can the duration and heavy metal concentration influence oxidative stress ?

-How can oxidative stress be proved and visualized histochemically ?

-What morphological and/or histological alterations occur in the root tips after heavy metal exposure ?

Experimental

Plant material and germination conditions

Seeds of Indian mustard (*Brassica juncea* L.) derived from Research Institute of the Hungarian Academy of Sciences, Budakalász, Hungary, were germinated in sterilized Petri dishes at 0, 50, 100 and 200 mg L^{-1} Cu concentrations (indicated as Control, Cu50, Cu100, Cu200, Table 1).

Table 1. Parameters of Cu concentrations applied

Salt form	Concentration, mg L^{-1}	Concentration, mM	pH at the beginning
	0	0.00	7.07
$CuSO_4$	50	0.78	4.93
	100	1.57	4.72
	200	3.15	4.57

The effect of each concentration of copper was investigated on 0.5-0.5 g seeds with 8 repetitions. The seeds were sown on Petri dishes with four-layered filter paper wetted with 10 mL of metal solutions. Then the Petri dishes were closed and were kept in dark for 12, 24, 48 and 96 h, at 24±1°C. The control group of seeds was germinated in the same way by the addition of distilled water only.

Determination of Cu content in seeds

In order to detect the heavy metal content in the germinating seeds, about 0.5-0.5 g fresh plant material from each treatment was separated for drying at room temperature until at least 5 days. The real copper content in Indian mustard seeds was determined by atomic absorption spectrophotometry (AAS) (Hitachi, Z-8200, Japan). Values of Cu concentration in seeds are given in mM g^{-1} dry weight (DW).

Preparation of the samples

At each metal concentration 8 replicates of 0.3 g fresh material (germinated seeds) were homogenised with 1.2 ml cool phosphate buffer using quartz sand in a cold mortar and centrifuged for 10 min at 12.000. x *g*, then the supernatant layer was used for all assays. In the assays for the spectrophotometric measurements, Thermo Spectronic Biomate 5 was used.

Determination of total antioxidant capacity

To evaluate the antioxidant power of the homogenates a simple and quick method was used called ferric reducing ability of plasma (FRAP).[11] The procedure described in the literature was modified and applied for plant material.[12] The total antioxidant capacity was expressed in units of μmol g^{-1} fresh weight (FW).

Determination of lipid peroxidation

Since malonyldialdehyde (MDA) is one of the end products of lipid peroxidation, the assay of MDA was applied to estimate lipid peroxidation (LP; modified method).[13] MDA content was determined and is expressed in units nmol g^{-1} fresh weight (FW).

Glutathione (GSH) evaluation

Glutathione content was measured using the method of Sedlak and Lindsay[14]. Data are expressed in μmol GSH g^{-1} fresh weight (FW).

Estimation of total protein content

Protein content of plant homogenate was measured spectrophotometrically at 675 nm using the method of Lowry et al.[15] These data were used to calculate the enzyme activities.

Activities of enzymes

The activity of glutathione-S-transferase (GST, EC 2.5.1.18.) was determined according to Mannervik and Guthenberg.[16] Guaiacol peroxidase (GPOX, EC 1.11.1.7) activity was evaluated by the modified method of Singh et al., while for catalase (CAT, EC 1.11.1.6) we applied the assay of Beers and Sizer.[17, 18] Total superoxide dismutase (SOD, EC 1.15.1.1) activity assayed as described by Misra and Fridovich.[19] Glutathione reductase (GR, EC 1.6.4.2) activity was measured applying the method of Pinto and Bartley.[20] The values were calculated as the production of 1 mM conjugate per min and expressed as unit mg^{-1} protein.

Histochemical detection of oxidative stress and its consequences

We assessed the loss of plasma membrane integrity in the root tips *in vivo* using Trypan blue, Aniline blue was applied for callose staining, Schiff's reagent was used for the detection of LP, and phloroglucinol-HCl for visualization of the lignification of cell walls as described by Yamamoto et al., Arduini et al. and Lequeux et al.[21, 22, 23]

Statistics

Statistical analysis of the results was carried out using STATISTICA 9.0 software. We used Kruskal-Wallis ANOVA to test the differences between the mean values. In order to determine the relationship between the metal concentration and the four biochemical parameters, Spearman's Rank Order Correlation was used. Data are given in mean values ± standard deviation (SD) and calculated for fresh homogenate. The level of significance was generally $p<0.05$.

Results and discussion

Parameters of oxidative stress

Oxidative stress occurred in the seeds due to heavy metal treatments and all parameters were significantly affected by time duration and metal concentration used. Increase of time duration and/or metal concentration resulted in higher metal uptake in germinating seeds of *Brassica juncea* L. (Table 2A, B) similar to the results of Mihoub et al.[24] The data of the Tables show that the changes of all parameters depended either on the Cu concentration or the time of exposure, or both.

FRAP showed to be a good and quick semi-quantitative parameter to prove that oxidative stress caused by heavy metals is followed by the rapid activation of antioxidant defence system including e.g. ascorbic acid and phenolic components.[25] Cu-treatment was also followed by GSH-depletion which was probably due to the increased activity of GST and the enzymes of Halliwell-Asada cycle and the elavated synthesis of phytochelatins, as well (Fig. 1).[25, 26]

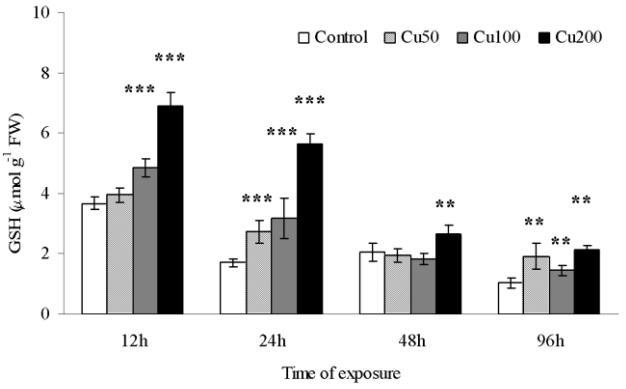

Figure 1. Time and dose-dependent changes of GSH-level in Cu-treated *B. juncea* seeds. Asterisks indicate significant differences between control and treated plants at *p< 0.05, **p< 0.01 and ***p< 0.001.

The level of lipid peroxidation (LP) determined as malondialdehyde (MDA) content showed time- and dose-dependence, too. Generally, lipid peroxidation was higher in the beginning of germination at all concentrations, and then attenuated. Significant differences were observed between the control and the treated seeds after each period. The reduction of LP in the second period of the germination was probably due to the activation of antioxidants and the elimination of lipid peroxides.[25]

The application of excess Cu increased the activity of SOD in time-dependent manner in good agreement with the results of Wang et al.[10] The activity of CAT and GPOX increased after 48-96h Cu treatment and was in significantly positive correlation with the changes of SOD activity (Spearman's r= 0.63 and r= 0.44; p< 0.001).[10, 27]

Histochemical detection of oxidative stress and morhological changes

LP as one the markers of oxidative stress was detected after staining with Schiff's reagent in root tips (Fig. 2). Typical morphological symptoms of metal toxicity occurred in Cu-stressed plants (stunted, hook-formed and brownish root tips).[23, 24]

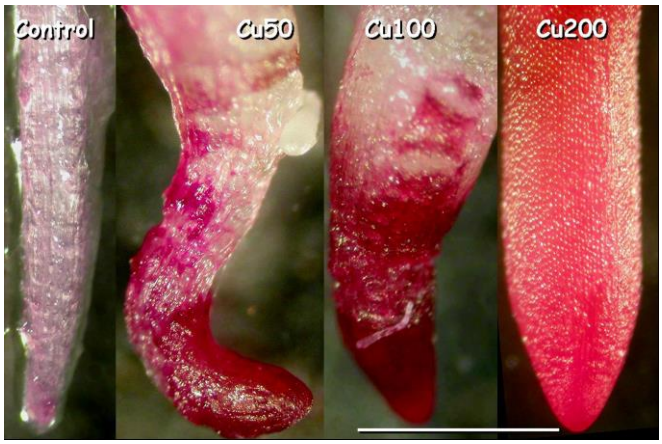

Figure 2. Histochemical detection if LP in the root apices of germinating *B. juncea* seeds after 96 h Cu treatment. Bars indicate 1 mm.

Table 2A. The effect of Cu concentration and duration on the parameters (AAS, FRAP, GSH and GR) testing by two-way ANOVA. The level of significance: * p<0.05, ** p<0.01 and *** p<0.001.

Effect	AAS	FRAP	GSH	GR
Cu concentration	$F_{3, 89}$ = 109.44***	$F_{3, 110}$ = 92.96***	$F_{3, 111}$ = 616.51***	$F_{3, 80}$ = 104.89***
Time	$F_{3, 89}$ = 297.97***	$F_{3, 110}$ = 13.63***	$F_{3, 111}$ = 270.67***	$F_{3, 80}$ = 71.45***
Conc. x time	$F_{9, 89}$ = 28.14***	$F_{9, 110}$ = 11.17***	$F_{9, 111}$ = 42.37**	$F_{9, 80}$ = 26.46***

Table 2B. The effect of Cu concentration and duration on the parameters (GST, GPOX, CAT and SOD) testing by two-way ANOVA. The level of significance: * p<0.05, ** p<0.01 and *** p<0.001

Effect	GST	GPOX	CAT	SOD
Cu conc.	$F_{3, 93}$ = 66.37***	$F_{3, 90}$ = 124.40***	$F_{3, 88}$ = 127.85***	$F_{3, 76}$ = 242.80***
Time	$F_{3, 93}$ = 27.57***	$F_{3, 90}$ = 10.63***	$F_{3, 88}$ = 15.25***	$F_{3, 76}$ = 19.73***
Conc. x time	$F_{9, 93}$ = 27.91***	$F_{9, 90}$ = 13.74***	$F_{9, 88}$ = 10.91***	$F_{9, 76}$ = 24.60***

After 96 h using Trypan blue we found remarkable disruption of the rhizodermal and the cortical cells mainly the elongation zone due to increased level of LP. [21, 23]

Decreased root elongation and induced production of callose could be visualized using Anilin blue as a fast reaction of the cells to avoid Cu toxicity, while lignification of cell walls was detected by phloroglucinol-HCl which refers to the long-term adaptation of the root tips.[5, 21, 23, 27]

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