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Full Length Research Paper

Copper Scavenging Potential and its Effect on Chlorophyll in Seedlings of *Brassica Juncea* (L.) Czern.

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Heavy metals are of particular importance from the agricultural and eco-toxicological point of view. Information on their content in plant and animal is of great botanical, nutritional and environmental interest. This study was designed, to investigate the effect of copper on Chlorophyll a, Chlorophyll b and total chlorophyll contents and results were correlated with results of accumulation of intracellular copper estimated by atomic absorption spectroscopy (AAS), in leaves of *Brassica juncea* (L.) Czern. Seedlings were treated with copper at 0.5, 1.0, 5.0, 10.0, 20.0, 40.0, 50.0, 75.0 and 100.0 µmol concentrations on day 5 and 10. The varying trends were observed for Chl a, Chl b and total chlorophyll content with respect to corresponding accumulated intracellular copper.

Keywords: *Brassica juncea* (L.) Czern., Chlorophyll, Intracellular copper, Scavenging activity, Seedlings.

INTRODUCTION

Heavy metals make a significant contribution to environmental pollution as a result of anthropogenic activities such as mining, smelting, electroplating, energy and fuel production, power transmission, intensive agriculture, sludge dumping and military operations (Nedelkoska and Doran, 2000). They present a risk for primary and secondary consumers and ultimately humans through a complex food chain (Zeller and Feller, 1999).Heavy metals such as Cu and Zn are essential for normal plant growth and development since they are constituents of many enzymes and other proteins. However, elevated concentrations of both essential and nonessential heavy metals in the soil can lead to toxicity symptoms and growth inhibition in most plants (Hall, 2002). Toxicity may result from the binding of metals to sulphydryl groups in proteins, leading to inhibition of activity or disruption of structure, or from displacement of an essential element, resulting in deficiency effects (van Assche and Clijsters, 1990). In addition, a heavy metal excess may stimulate the formation of free radicals and reactive oxygen species, perhaps resulting in oxidative stress (Dietz et al., 1999). The lifetime of active oxygen species within the cellular environment is determined by the antioxidant system, which provides crucial protection against oxidative damage. The antioxidative defense system comprises numerous enzymes and compounds of low molecular weight (Noctor and Foyer, 1998). The antioxidant properties of plants exposed to various stress factors have been studied (Havaux and Kloppstech, 2001), but studies related to the effect of heavy metal-induced stress on vitamin levels in plants are limited. Lead and mercury were reported to cause an increase in ascorbic acid and α-tocopherol levels in two *Oryza sativa* cultivars (Mishra and Choudhuri, 1999), and mercury exposure was found to increase the ascorbic acid levels in *Bacopa monnieri* (Sarita et al., 1996).

Detailed studies indicate that heavy metals have effects on chlorophyll content in plants. Heavy metals are known to interfere with chlorophyll synthesis either through direct inhibition of an enzymatic step or by inducing deficiency of an essential nutrient (Van Assche and Clijsters, 1990). The amount of chlorophyll was reduced in *Triticum aestivum* cv. Vergina grown on Cu-enriched soil (Lanaras et al., 1993), and in *Brassica oleracea* var. Botrytis cv. Maghi exposed to Cu^{2+}, Co^{2+} and Cr^{2+} (Chatterjee and Chatterjee, 2000).

Cu acquisition and transport into and within cells is relatively little known in plants. However, recently rapid progress has been made, particularly with the application of the knowledge of transport processes in yeast to other eukaryote organisms (Eide, 1998; Nelson,

1999). The present work involved following experimental steps:-

i) Growing of seed of *Brassica juncea* (L.) Czern. in Hoagland's nutrient solution with and without different concentration of copper.

ii) Impact of varying concentrations of copper on Chlorophyll a, Chlorophyll b and total chlorophyll content.

iii) Quantification of intracellular copper accumulated in leaves of *Brassica juncea* (L.) Czern. seedlings by AAS.

MATERIALS AND METHODS

Brassica juncea (L.) Czern. seeds were procured from the Krishi Vigyan Kendra, Banasthali University, Banasthali, Rajasthan, India. These seeds were stored in airtight container. Before use, seeds were surface sterilized with 5 % sodium hypochlorite solution for 15 minutes and washed thoroughly by using the plenty of distilled water. These seeds were then germinated in glass Petri dishes (90 mm) lined with two sheets of sterilized blotting paper soaked with 10 ml of distilled water at 30 ºC in the dark. Each petridish contained 30-40 seeds. The covers of petridishes were removed on day 3rd and germinated seeds were transferred to hydroponic culture medium in plastic containers of 10×10 cm (height x width) and kept in thermo-statistically controlled culture room maintained at 25 ± 2 ºC and less than 50 % relative humidity. The Hoagland's nutrient solution having composition 16 mM KNO_3, 4 mM $Ca(NO_3)_2.4H_2O$, 2 mM $NH_4H_2PO_4$, 1 mM $MgSO_4.7H_2O$, 50 µM KCl, 25 µM H_3BO_3, 2 µM $MnSO_4.H_2O$, 2 µM $ZnSO_4.7H_2O$, 0.5 µM $CuSO_4.5H_2O$, 0.5 µM H_2MoO_4, 30 µM NaFeEDTA was used. The pH of the Hoagland's nutrient solution was adjusted to 6.4 ± 6.6 using 1N HCl. Seedlings were placed in light (500 µmol m^{-2} s^{-1}) for 12/12 hours day/night daily. The nutrient solution was bubbled with glass rod to provide sufficient oxygen and mixing of nutrients. Further, medium was changed on every 3rd day to avoid any nutrient deficiencies to seedlings.

The copper concentration in Hoagland's nutrient solution was maintained 0.5, 1.0, 5.0, 10.0, 20.0, 40.0, 50.0, 75.0, and 100.0 µmol ,seedlings of uniform size were sorted out and leaves taken for the analysis in day 5 and 10 after transferring to the Hoagland's nutrient solution, simultaneously controlled seedling (seedling without copper treatment) were also analyzed.

Estimation of chlorophyll content

For chlorophyll estimation fresh leaves of *Brassica juncea* (L.) Czern. seedlings were collected and processed for determination of pigments using the method of Arnon (1949). The chlorophyll content was determined at different Cu concentrations. 100 mg of leaves were ground in cold conditions using 80 % chilled acetone in dark. The homogenate was centrifuged at 10,000 g for 10 minutes at 4ºC. Absorbance of supernatant was taken at 480, 645 and 663 nm and pigments (Chlorophyll a, Chlorophyll b and total chlorophyll) were calculated by using the formula given by Arnon (1949) as follows:

Chlorophyll a (mg g^{-1} FW) = $[(12.7)\,A_{663} - (2.69)\,A_{645}] \times \frac{V}{W \times 1000}$

Chlorophyll b (mg g^{-1} FW) = $[(22.9)\,A_{645} - (4.68)\,A_{663}] \times \frac{V}{W \times 1000}$

Total chlorophyll (mg g^{-1} FW) = $[(8.02)\,A_{663} + (20.2)\,A_{645}] \times \frac{V}{W \times 1000}$

Intracellular Cu content determination

Intracellular copper accumulation was determined by the method of Bates et al. (1982). Plants were harvested and washed thoroughly with 20 ml of 2 mM EDTA solution and roots were separated. After oven drying at 80 ºC for overnight dry weight was determined and 100 mg of dried plant material was digested in 5 ml of digestion mixture containing HNO_3 (70 %) + H_2O_2 (30 %) + deionized water in 1:1:3 ratio until the solution become colorless. Residues were dissolved in 2 % (v/v) nitric acid to a final volume of 5 ml and Cu concentration was determined by atomic absorption spectrophotometer (Varian, Model No. 240 FS). The calculation of intracellular cu was done by weight by weight and results are reported in mg/100 mg of sample. Merck Cu standard was used for quantitation of intracellular Cu content .The operational parameter of the instrument was set as below.

Instrument type	Flame
In Flame type	Air: Acetylene (13.50:2.0 liter/minute)
Sampling mode	Manual
Replicate standard	3
Replicate sample	3
Concentration unit	mg/l
Weave length	324.8 nm
Back ground correction on	Correlation coefficient 0.9900

Data analysis

All experiments were performed thrice (three replicates of each). Similar results and identical trends were observed each time and the data presented here is mean of three replicates ± SD. The significance of

Table 1: Results of Chl a, Chl b, Total Chl and intracellular copper accumulation on day 5.

Cu stress (µ mol)	Chl. A (mg g-1FW)	Chl. B (mg g-1 FW)	Total Chl. (mg g-1 FW)	Cu accumulation (mg/100mg)
00	1.5±0.114	0.235±0.191	1.467±0.063	0.857±0.054
0.5	0.853±0.183*	0.491±0.089	1.322±0.224	0.950±0.048
1	0.327±0.176*	0.309±0.083	0.407±0.039*	1.115±0.119*
5	0.660±0.115*	0.414±0.163	0.867±0.105*	1.889±0.089*
10	1.773±0.106*	2.487±0.364*	4.455±0.291*	2.395±0.152*
20	1.739±0.398*	1.342±0.190*	2.448±0.088*	2.763±0.177*
40	1.83±0.145*	1.503±0.138*	2.550±0.330*	2.721±0.165*
50	1.204±0.103*	1.284±0.324*	2.083±0.080*	2.292±0.238*
75	1.122±0.108*	1.520±0.139*	1.493±0.111*	2.953±0.055*
100	1.082±0.133*	0.634±0.157*	2.227±0.133*	2.793±0.101*

Values are means ± SD (n=3)

* Indicate significant difference from control at p≤ 0.05

Table 2: Results of Chl a, Chl b, Total chl and intracellular copper accumulation on day 10.

Cu stress (µ mol)	Chl. A (mg g-1FW)	Chl. B (mg g-1 FW)	Total Chl. (mg g-1 FW)	Cu accumulation (mg/100mg)
00	0.392±0.243	0.209±0.089	0.959±0.168	0.296±0.029
0.5	0.799±0.035*	1.102±0.538*	1.743±0.215*	0.855±0118
1	0.797±0.050*	0.845±0.371*	1.338±0.117*	1.026±0.086*
5	1.290±0.020*	1.211±0.279*	1.984±0.221*	1.353±0.223*
10	1.685±0.365*	2.734±0.186*	4.349±0.223*	1.455±0.271*
20	1.576±0.211*	1.026±0.189*	2.443±0.222*	1.604±0.130*
40	1.350±0.321*	1.673±0.220	2.280±0.176*	1.809±0.219*
50	0.416±0.106	0.341±0.061	1.167±0.250	1.994±0.167*
75	0.578±0.070	0.598±0.038	1.156±0.233	2.459±0.267*
100	0.686±0.085	0.330±0.033	1.494±0.192*	2.738±0.324*

Values are means ± SD (n=3)

*Indicate significant difference from control at p≤ 0.05

difference between control and each treatment was analyzed by using Students *t* test.

RESULTS

The effects of copper on Chlorophyll content and intracellular accumulation of Cu were summarized in Table 1 and 2 above ,on day 5 and 10 at 0.5, 1.0, 5.0, 10.0, 20.0, 40.0, 50.0, 75.0, and 100.0 µ mol concentrations along with untreated control in leaves of *Brassica juncea* (L.) Czern. Seedlings.

A decrease in chl a content on day 5 (Table-1 above) was found to be significant on increasing copper concentration from 0.5 to 5 µ mol (p≤ 0.05), while the increase in chl a content was found to be significant on increasing copper concentration from 10 to 100 µ mol (p≤ 0.05). On day 10 chl a content results (Table-2

above) reveal that from 0.5 to 40µ mol copper stress increased chl a content significantly (p≤ 0.05).

Chl b content results on day 5 (Table1 above)shows significant (p≤ 0.05) increment from 10 to 100 µ mol copper stress and same pattern was found on day10 (Table 2 above) from 0.5 to 20 µ mol copper stress.

The results of total chlorophyll content on day 5 (Table 1 above) decreased significantly (p≤ 0.05) at 1.0 and 5 µ mol concentration of copper while increased significantly (p≤ 0.05) at 10 µ mol and 10 to 100 µ mol copper concentration. Change in total chlorophyll content on day 10 (Table 2 above) revels significant increment (p≤ 0.05) at 0.5 to 40 µ mol and at 100 µ mol copper concentrations.

The intracellular copper accumulation results on day 5 (Table 1 above) and day 10 (Table 2 above), shows significant (p≤ 0.05) accumulation by leaves of *Brassica juncea* (L.) Czern. Seedlings with increasing the copper stress concentration at 1.0 to 100 µ mol on day 10.

DISCUSSION

Photosynthetic pigments such as Chlorophyll a, chlorophyll b and total chlorophyll contents of *Brassica juncea* (L.) Czern. Leaves increased at lower concentration of copper stress , while decreased with increasing copper concentration in growth medium .Similar changes in the content by various heavy metals were recorded (Schlegel et al.,1987).The formation of chlorophyll pigment depends on the adequate supply of iron and protophorphyrin is a precursor for chlorophyll synthesis (Granic,1951).Where Ganesh et al.(2006) suggested that reduced chlorophyll content observed in the plants grown in presence of very high copper content due to iron deficiency. Hence, the trends of our results are in line with the findings of number of researches.

The present study so far suggests the *Brassica juncea* (L.) Czern. Under stress of Cu concentrations shows a considerable decrease of the photosynthetic pigment after long time incubation due to the toxicity of copper. Similar trends were found for chlorophyll a content of *Chaetoceros radicans* under copper stress (Wagdy et al., 1995). The reduction of chlorophyll content in higher concentration of copper stress could be the result of the inhibition of metabolic activity through the copper effect.

The findings of the present investigation will act as stimuli for new phyto-extraction campaigns in order to have the selectivity of copper concentration and its assimilation. *In vitro* copper stress results of *Brassica juncea* (L.) Czern. That is chlorophyll a, chlorophyll b, Total chlorophyll content and intracellular accumulation of cooper suggest that *Brassica juncea* (L.) Czern. Is a good accumulator of intracellular copper and could also be used as a model plant for phyto-remedian campaigns.

The chlorophyll contents indices over the varying concentration of copper play a pivotal role and would be helpful for design, development and validation of phyto-extraction campaigns according to the requisite copper contaminated soil.

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