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Chelate mediated phytoextraction of copper (II) involves changes in metal uptake, osmoprotectant and photosynthetic parameters in *Brassica juncea* L.

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Abstract : Chelate mediated phytoextraction of copper (II) was studied in 7 day old seedlings of *Brassica juncea* L. (var. RLC 1) grown as controls and binary combinations of various copper (Cu) and tartaric acid (TA) concentrations. The seedlings from effective combinations were measured for percent seedling emergence, fresh weight, total seedling length and percent metal tolerance index as growth parameters and analysed via spectrophotometric techniques for various physiological parameters including photosynthetic pigments (total chlorophylls, Chlorophyll ratio (a/b), carotenoids, xanthophylls, flavanoids and anthocyanins), osmoprotectants (carbohydrates, proline, glycine betaine) and copper uptake. Growth parameters showed a steady decline with increasing Cu stress however tartaric acid to an extent seemed to shield the seedlings grown in controls and binary combination with Cu. In lieu of exposure to facilitated metal uptake, the hyperaccumulator responded to toxicity with a fall in most pigment levels with the exception of flavanoids and anthocyanins and heightened osmoprotectant accumulation. With an increase in Cu and tartaric acid exposure, high values for metal accumulation were recorded, indicating the enhanced chelate mediated hyperaccumulative potential of *B. juncea* plants.

Key Words: ***Brassica juncea,*** **Chelating agents, Copper, Hyperaccumulators, Osmoprotectants, Phytoextraction.**

INTRODUCTION

An array of anthropogenic activities and incessant industrial growth rates have contributed to mass scale up of non biodegradable and toxic heavy metals in air, soil and water leading to growth inhibition and death in plants [1,2]. Heavy metals (atomic number>20) such as cadmium, lead, copper, zinc, nickel etc are now an integral part of the food webs and pose a massive threat to human health and livestock well being[3]. Phytoremediation comes across as an effective and eco friendly solution to this problem [(4)]. Its defined as the use of special plants called the hyperaccumulators (accumulate metals up to 100 times more as compared to a non hyperaccumulator with scant toxicity symptoms) to leach out or extract heavy metals from the substrate over a number of cropping cycles[5,6]. Out of 45 hyperaccumulator plant families, Brassicaceae boasts of maximum number of hyperaccumulators that is about 87 species[7, 8]. Out of these species, *Brassica juncea* L. has great accumulative potential for copper[9]. Copper (II) is an essential element which plays key role in photosynthetic and respiratory transport chains, oxidative stress, homeostasis and metabolism in plants[10]. However, it has been identified as moderately toxic when present beyond an optimum range[11,12]. To fortify the cleanup process, use of soil amendments such as chelating/ reducing agents is known to enhance the phytoextraction potential of hyperaccumulators. LMWOA(s) or Low Molecular Weight Organic Acids such as ascorbic acid,

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citric acid, oxalic acid, tartaric acid etc are biodegradable in nature and are a preferred choice over non biodegradable chelators such as EDTA, GEDTA, HEDTA, NTA, etc.[13, 14]. The aim of our work was to compare the impact of individually applied Cu and tartaric acid concentrations as well as their most suitable combinations on various growth and physiological parameters and metal extraction potential of *B. juncea L.* under controlled conditions.

MATERIALS AND METHODS

Certified seeds of *B. juncea* L. var. RLC 1 were procured from the Department of Seed technology, Punjab Agriculture University, Ludhiana. The seeds were washed and rinsed thoroughly with double distilled water followed by surface sterilization with 0.1 % $HgCl_2$. Seeds (30 per Petri plate) were then cultured on Whatmann No.1 filter paper lined Petri plates, soaked with different aqueous solutions of Cu and tartaric acid (individual or binary concentrations). The solutions were prepared using AR grade $CuSO_4.5H_2O$ and tartaric acid procured from Merck and Loba Chemie respectively. Sterilized seeds grown in double distilled water were used as control. The triplicate Petri plate sets were monitored for a 7 d growth period at 25±2 ºC temperature and 16:8 h dark: light photoperiod (1700 lux).

Measurement of growth parameters: After 7 days of controlled growth, the seedlings were harvested and measured for percent seedling emergence, fresh biomass (g), total seedling length (cm) and percent metal tolerance index [15].

Determination of photosynthetic pigments: Chlorophyll and Carotenoid content

Total Chlorophylls, Chlorophyll ratio (a/b) ratio and Carotenoids were determined by homogenizing 1g fresh plant tissue in 4ml of 80 % acetone and followed by centrifugation at 13000 rpm, 4ºC temperature for 20 minutes using Eltek cooling centrifuge . The supernatant was measured for absorbance at 645 and 663nm for chlorophylls and 483 and 510 nm for carotenoids [16].

Xanthophylls

These were determined by AOAC method in which, 0.05g of dried plant material was transferred to 100ml flask followed by addition of 30ml of extractant (Hexane+Acetone+Abs. Alcohol+ Toluene in the ratio 10:7:6:7) into it. The mixture was shook vigorously for 10-15 minutes. 2ml of 40 % methanolic KOH was added next. The flask was then refluxed in water bath at 56°C and then incubated for an hour in darkness. Next, 30ml of hexane was poured to the sample and shook for 1 minute. The total volume was made up to 50 ml with 10 % sodium sulphate solution. The samples were again kept in dark for an hour. Separation of distinct phases was visible. The upper phase was collected in volumetric flask and the volume was again made up to 50 ml with hexane. Absorbance was measured at 474 nm[17].

Total flavanoid content

Flavanoids were estimated by homogenizing 1g of fresh plant tissue in 3ml of absolute methanol followed by centrifugation. 1ml of this plant extract was diluted with 4ml of double distilled water. 0.3ml of sodium nitrite ($NaNO_2$) and aluminum chloride ($AlCl_3$) were added to it. This was followed by incubation for 5 minutes. Next, 2ml sodium hydroxide (NaOH) was added to the reaction mixture and pink color was developed. Absorbance for the mixture was recorded at 510nm[18].

Anthocyanins

For estimating anthocyanins, 1gm of fresh plant tissue was homogenized in 3ml of extraction mixture i.e. acidified methanol (methanol: water: HCl, 79:20:1).This mixture was incubated overnight at 4ºC followed by centrifugation at 13,000 rpm for 20 minutes. The absorbance of the supernatant was recorded at 530nm and 657nm[19].

Determination of osmoprotectants:

Carbohydrates

For carbohydrate estimation, 100 mg of dried plant sample was hydrolysed in 5 ml of 2.5 N HCl in a boiling water bath for 3 hours. The mixture was neutralized with sodium carbonate till the effervescence stopped. The total volume was made up to 100 ml with distilled water. Took I ml of this mixture, added 4 ml of anthrone reagent (200 mg anthrone in 95% H_2SO_4) to it and boiled it for 10 minutes. Recorded absorbance at 630 nm. Estimated carbohydrate content from standard glucose curve[20].

Proline

For proline estimation, 0.5 g of plant material was homogenized in 10 ml of 3% sulphosalicylic acid. 2 ml each of glacial acetic acid and ninhydrin were added to 2 ml of plant extract followed by heating at 100ºC for an

hour and immediate cooling in ice. 4 ml of toluene was added in the reaction mixture, vortexed for a minute followed by separation of layers. Red colored toluene layer was checked for absorbance at 520 nm[21]. Proline Content was calculated from the standard proline curve.

Glycine Betaine

It was determined by homogenizing 0.5 g of plant material in 5 ml of water and toluene extract followed by 24 h incubation at room temperature. Plant extract was filtered and 1 ml of 2 N HCL and 0.1 ml of potassium tri-iodide respectively were added to 0.5 ml of filterate. This was followed by incubation in ice bath for 90 minutes and vigorous shaking. 2 ml of ice cold water and 10 ml of 1, 2-Dichloroethane were added to reaction mixture followed by vortexing. Upper aqueous layer was discarded and lower pink colored organic layer was subjected to absorbance recording at 365 nm[22]. Glycine Betaine content was measured from standard betaine hydrochloride curve.

Determination of Cu content: Dried seedlings were ground and digested in H_2SO_4: HNO_3: $HClO_4$ (1:5:1) digestion mixture. Digests were diluted with double distilled water and filtered[23]. Metal analysis was done using atomic absorption spectrophotometer (Model 6200, Shimadzu, Japan).

Statistical Analysis: The data was evaluated by Sigma Stat 3.5 and MS Excel programs for means, standard error and two way analysis of variance (ANOVA). Comparison and significance was at measured at $P<0.05$. The experimental trials were conducted in triplicates.

RESULTS AND DISCUSSION

Growth parameters form the most basic, yet indicative benchmarks for understanding metal stress exposure and plant responses to the same[24]. Our results revealed that the percent seedling emergence, fresh weight, total seedling length significantly decreased with an increase in the level of Cu treatments. The least values were recorded at 100 ppm Cu in the order of 24.66%, 0.033 g and 5.23 cm for percent seedling emergence, fresh weight, and total seedling length respectively. As indicated in Table 1, the presence of 75 ppm tartaric acid seemed to significantly alleviate Cu stress in all the binary combinations with Cu. There was a steady decrease in percent metal tolerance index with rising Cu stress, however tartaric acid seemed to increase the tolerance index in binary combinations though this was not statically significant. We found our results in coherence with similar work reported for Cu toxicity in *Cajanus cajan* L and *Crassula helmsii*[25,26]. Similar observations were found for the reduction in Metal Tolerance Index in response to Zn and Cu stress in *Triticum aestivum* and *Oryza sativa*[27].

Table 2 describes the extent of osmoprotectant accumulation in terms of carbohydrates (mainly disaccharides), proline and glycine betaine contents under Cu, tartarate and binary treatments. Osmoprotectants are a very stress sensitive group of signalling molecules which offer defence against various abiotic stresses including heavy metal stress[28, 29]. Statistically significant increase in all the three mentioned osmoprotectants was observed as Cu exposure was elevated. Maximum sugar levels were recorded at 50 ppm Cu, whereas proline and glycine betaine contents of 0.0533mg/g FW and 4.28 mg/g FW respectively were recorded for 100 ppm Cu. As seen in Table 2, in case of Cu treatments in combination with tartaric acid, all the three osmoprotectants showed a steady and significant fall in accumulation as compared to their parallel single metal treatments. Hence, it's safe to say that tartaric acid played a stress shielding role in *B. juncea* seedlings under Cu stress by decreasing osmoprotectant levels to a significant extent. Earlier too, similar results were reported in rice seedlings and runner bean plants exposed to Cd, Ni and Cu stress[30,31,32]. The plants showed a rise in carbohydrate content and simultaneous decrease in photosynthesis as discussed later, which lead to the linking of these processes to growth inhibition as already discussed previously. As for rising proline and glycine betaine contents under Cu stress, our results support previous claims. Proline as the first accumulated osmoprotectant in response to Cu stress is well studied in sunflower, wheat and rice[33,34,35]. Similar reports exist for glycine betaine accumulation in various plants under Cu stress[36].

Another important physiological group which operates in unison with osmoprotectants when plants are exposed to heavy metal stress is that of the photosynthetic pigments[30]. The quantitative analysis of chlorophylls, carotenoids and xanthophylls represents the variation in photosynthetic efficiency and flavanoids and anthocyanins

in particular are well known metal chelators as well as antioxidants. Hence, pigments show diverse spectra of trends in response to similar metal stress and chelate treatments. Table 3 indicates these variations in detail. Total Chlorophyll content and carotenoid contents seemed to decrease with increase in Cu concentrations; in binary combination with 75 ppm tartaric acid also similar trend was observed, however these results were not statistically significant. Interestingly, Chlorophyll (a/b) ratio suffered a statistically significant decline beyond 50 ppm Cu as compared to control. This is proved previously in *Phaseolus vulgaris, Lolium perene* L. and *Triticum aestivum* seedlings[37,38,39]. Xanthophylls showed a significant and sharp decline with elevated Cu stress; the least recording being 14.59 mg/g FW for 75 ppm Cu. On the other side, tartaric acid when present in combination with Cu was seen to significantly increase xanthophyll content; the maximum calculated as 30.04 mg/g FW for 50 Cu+ 75 TA. It's important to mention that Cu is a well known photosynthesis inhibitor and reduces thylakoid surface area resulting in overall depression of these pigments[40]. Flavanoids and their subclass; the anthocyanins however showed opposite trends. Their contents increased significantly with rise in Cu stress exposure. 0.159 mg/g FW and 0.515 mg/g FW were the maximum flavanoid and anthocyanin contents recorded for 100 ppm Cu as denoted in Table 3. The presence of tartaric acid seemed to significantly add on to their contents as seen for 100 Cu + 75 TA and 75 Cu+ 75 TA (0.107 mg/g FW-flavanoids and 0.594mg/g FW- anthocyanins respectively). Our findings drew focus on flavanoids and anthocyanins as the most significant photosynthetic pigments under Cu stress as these seem to increase with the efficacy of stress given. Probably, flavanoids are known to acts as strong metal chelators and anthocyanins have an antioxidant role to play as ROS scavengers which explains this behaviour [41,42].

Cu uptake and accumulation in the seven day old seedlings was significantly increased with rising Cu stress as compared to the controls. As seen from Table 2, highest Cu levels were observed at 100 ppm (9.08 Cu mg/g DW). With tartaric acid exposure in combination to Cu stress, statistically significant results indicated a sharp increase in Cu accumulation in plant material in presence of chelate. At binary combination, 100 Cu + 75 TA we recorded 9.53 mg/g DW as most significant Cu level as compared to 9.08 mg/g DW for 100 ppm Cu treatment and 0.0064 mg/g DW for control. Since *B. juncea* is a hyperaccumulator itself, Cu accumulation rose with increasing Cu treatments, however tartaric acid significantly escalated Cu uptake further with decreased phytotoxicity in the plants. Similar results have been reported previously in *Triticum aestivum* and *Vicia faba* as well[43,44].

The results of the present study concluded that though Cu is a moderately toxic metal, it hampers the growth parameters and many a photosynthetic pigments at high levels and activates the osmoprotectant and antioxidant system in the stressed *B. juncea* seedlings. However, in binary combination with a LMWOA like tartaric acid, the seedlings were able to bear the phytotoxic effect of Cu to a great extent.

Table 1: The effect of copper (Cu) metal and tartaric acid (TA) treatments on growth parameters in 7 day old seedlings of *Brassica juncea* L.

Treatment	Percent Seedling Emergence (Mean ± S.E)	Fresh Weight (Mean ± S.E) g	Total Seedling Length (Mean ± S.E) cm	Percent Metal Tolerance Index (Mean ± S.E)
0 (Control)	82.66 ± 1.763	0.088 ± 0.0041	17.53 ± 1.29	100 ± 0
25 Cu	63.33 ± 0.666	0.084 ± 0.0013	13.23 ± 0.43	76.83 ± 8.691
50 Cu	52 ± 2.309	0.076 ± 0.0031	11.56 ± 0.38	67.03 ± 7.639
75 Cu	41.33 ± 1.333	0.053 ± 0.0021	8.10 ± 0.47	47.23 ± 6.081
100 Cu	24.66 ± 1.763	0.033 ± 0.0027	5.23 ± 0.35	30.05 ± 2.438
F-Ratio $_{(df1,4)}$ P= 0.05	**16.27***	**177.82***	**21.76***	**4.372**
75 TA	76.33 ± 2.603	0.092 ± 0.0008	15.9 ± 0.230	85.27 ± 0.706
F-Ratio $_{(df1,4)}$ P =0.05	**212.15***	**142.65***	**84.50***	**19.35***
25Cu + 75 TA	68 ± 1.527	0.096±0.00063	13.66 ± 0.42	79.20 ± 8.720
50 Cu + 75 TA	60 ± 1.527	0.092 ±0.00049	12.2 ± 0.21	70.38 ± 5.807
75 Cu + 75 TA	45.33 ± 1.763	0.076 ±0.00108	11.86 ± 0.14	68.34 ±4.661
100 Cu + 75 TA	37.66 ± 1.453	0.067 ±0.00183	9.9 ± 0.416	57.64 ± 7.178
F-Ratio $_{(df1,4)}$ P= 0.05	**75.75***	**14.55***	**11.80***	**3.91**

Table 2: The effect of copper (Cu) metal and tartaric acid (TA) treatments on osmoprotectant levels and copper uptake in 7 day old seedlings of *Brassica juncea* L.

Treatment	Carbohydrate Content (Mean ± S.E) (mg/g FW)	Proline Content (Mean ± S.E) (mg/g FW)	Glycine Betaine Content (Mean ± S.E) (mg/g FW)	Copper Uptake (Mean ± S.E) (mg/g DW)
0 (Control)	0.269 ± 0.0037	0.0201 ± 0.0035	1.83 ± 0.069	0.0064 ± 0.0003
25 Cu	0.315 ± 0.0042	0.0163 ± 0.0013	1.92 ± 0.024	2.63 ± 0.0673
50 Cu	0.477 ± 0.0032	0.0223 ± 0.0016	2.072 ± 0.027	4.28 ± 0.3424
75 Cu	0.401 ± 0.0041	0.0303 ± 0.0011	2.21 ± 0.033	7.91 ± 0.0927
100 Cu	0.298 ± 0.0057	0.0533 ± 0.0007	4.28 ± 0.049	9.08 ± 0.0843
F-Ratio $_{(df1,4)}$ P= 0.05	**238.57***	**20.05***	**242.49***	**56.51***
75 TA	0.384 ± 0.0168	0.0235 ± 0.0013	0.74 ± 0.145	0.0068 ± 0.0028
F-Ratio $_{(df1,4)}$ P = 0.05	**301.14***	**78.01***	**334.09***	**926.46***
25Cu + 75 TA	0.635 ± 0.0081	0.0154 ± 0.0015	1.55 ± 0.037	3.57 ± 0.1704
50 Cu + 75 TA	0.463 ± 0.0042	0.0225 ± 0.0024	1.92 ± 0.063	6.28 ± 0.3165
75 Cu + 75 TA	0.518 ± 0.0147	0.0200 ± 0.0009	2.104 ± 0.051	8.75 ± 0.1631
100 Cu + 75 TA	0.181 ± 0.0082	0.0359 ± 0.0012	2.77 ± 0.062	9.53 ± 0.1418
F-Ratio $_{(df1,4)}$ P = 0.05	**179.54***	**12.07***	**45.93***	**8.703***

Table 3: The effect of copper metal and tartaric acid (TA) treatments on photosynthetic pigments in 7 day old seedlings of *Brassica juncea* L.

Treatment	Total Chlorophyll Content(Mean ± S.E) (mg/g FW)	Chlorophyll (a/b) (Mean ± S.E)	Total Carotenoid Content (Mean ± S.E) (mg/g FW)	Xanthophyll Content (Mean ± S.E) (mg/g FW)	Flavanoid Content (Mean ± S.E) (mg/g FW)	Anthocyanin Content (Mean ± S.E) (mg/g DW)
0 (Control)	0.132 ± 0.002	1.46 ± 0.174	0.047± 0.0004	25.8 ± 1.061	0.0125 ±0.0031	0.558 ±0.0037
25 Cu	0.089 ± 0.002	1.69 ± 0.146	0.041± 0.0014	21.36 ± 0.659	0.0173 ±0.0008	0.277 ±0.0020
50 Cu	0.060 ± 0.002	2.16 ± 0.118	0.032± 0.0004	18.16 ± 0.737	0.0217 ±0.0017	0.396 ±0.0037
75 Cu	0.046 ± 0.002	1.54 ± 0.123	0.025± 0.0002	14.59 ± 0.570	0.0519 ±0.0018	0.441 ±0.0027
100 Cu	0.043 ± 0.001	1.01± 0.054	0.020 ±0.0012	14.68 ± 0.652	0.159± 0.0032	0.515 ±0.0127
F-Ratio $_{(df1,4)}$ P =0.05	**2.938**	**14.31***	**6.512**	**132.83***	**390.82***	**58.44***
75 TA	0.124 ± 0.001	1.44 ± 0.056	0.044 ±0.0001	25.04± 0.570	0.2166 ± 0.0104	0.326 ±0.0014
F-Ratio $_{(df1,4)}$ P =0.05	**4.05**	**12.08***	**64.18***	**7.79***	**208.93***	**257.85***
25Cu + 75 TA	0.077 ± 0.009	2.55 ± 0.449	0.047 ±0.0051	25.98 ± 0.978	0.061± 0.0026	0.329 ±0.0056
50 Cu + 75 TA	0.046 ± 0.003	3.22 ± 0.055	0.034 ±0.0005	30.04 ± 2.37	0.065 ± 0.0040	0.268± 0.0040
75 Cu + 75 TA	0.332 ± 0.137	1.76 ± 0.475	0.030 ±0.0007	27.39 ± 1.018	0.072 ± 0.0044	0.594 ±0.0206
100 Cu + 75 TA	0.026 ± 0.002	1.62 ± 0.053	0.025 ±0.0003	25.98 ± 1.068	0.107 ± 0.0017	0.469± 0.0061
F-Ratio $_{(df1,4)}$ P =0.05	**4.67**	**1.94**	**2.22**	**14.34***	**259.37***	**156.72***

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