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Reduction of Chlorophyll in Cabbage Leaf Disks Following Cu^{2+} Exposure

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Abstract. **Cabbage leaf disks (*Brassica oleracea* L., *Capitata* group) were floated (adaxial side up) in Cu^{2+} solutions (0, 0.16, and 0.40 mM Cu^{2+}) for 1–4 days. The experiments were conducted in both light and dark environments. In light, total chlorophyll, chlorophyll *a*, chlorophyll *b*, and chlorophyll *a*/*b* ratios declined linearly with increasing exposure duration and Cu^{2+} concentrations. The rate and magnitude of these declines were unaffected by the addition of 0.5 mM $CaCl_2$. Between the 2 cultivars tested, the relative chlorophyll contents in 'Market Prize' declined faster and reached lower levels than 'Resistant Danish', suggesting that 'Market Prize' is more susceptible to Cu^{2+} stress. In the absence of light, there was little difference between the chlorophyll loss in the controls and Cu^{2+}-treated tissue. For light and dark experiments, loss of chlorophyll *a* was primarily responsible for reductions in total chlorophyll content and in chlorophyll *a*/*b* ratios.**

During the last decade, interest in Cu and its relationship to agricultural systems has increased, in part due to a heightened awareness of its toxicity to plants and animals from mine and sewage waste pollutants (7). Chlorosis is a typical symptom of Cu^{2+} toxicity in algae and higher plants (4, 10). Toxic levels of Cu^{2+} have been reported to inhibit photosynthesis by interfering with electron transport in algal cells and spinach chloroplasts (9, 10). The damage to the photosynthetic apparatus is a light-dependent process (3).

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Table 1. Relative levels (compared to control of 0 Cu^{+2} at each time interval) of total chlorophyll, chlorophyll *a*, chlorophyll *b*, and chlorophyll *a/b* ratios in cabbage leaf disks of 'Market Prize' and 'Resistant Danish' after exposure to various Cu^{2+} solutions in light or dark.

		Light						Dark		
		Resitant Danish			Market Prize			Market Prize		
	Time	Cu^{2+} (mM)—Expt. I			Cu^{2+} (mM)—Expt. I + II			Cu^{2+} (mM)—Expt. II		
	(days)	0	0.16	0.40	0	0.16	0.40	0	0.16	0.40
		Relative chlorophyll levels (%)[z]								
Total chlorophyll	0	100	100	100	100	100	100	100	100	100
	1	100	92	93	97	92	86	99	99	94
	2	96	88	83	97	87	75	89	88	84
	3	93	86	80	95	79	67	70	95	92
	4	88	82	73	92	69	63	---	---	---
Chlorophyll *a*	0	100	100	100	100	100	100	100	100	100
	1	100	89	87	98	88	80	93	94	86
	2	96	82	75	97	80	68	88	84	77
	3	93	81	72	94	74	66	77	82	77
	4	87	76	63	91	67	59	---	---	---
Chlorophyll *b*	0	100	100	100	100	100	100	100	100	100
	1	102	105	112	96	110	110	101	113	115
	2	95	101	104	97	98	101	92	102	102
	3	95	100	101	98	96	91	79	102	109
	4	90	99	88	96	86	86	---	---	---
		Chlorophyll a/b ratio[y]								
	0	2.8	2.8	2.8	2.8	2.8	2.8	3.0	3.0	3.0
	1	2.7	2.2	2.0	2.9	2.3	2.0	2.9	2.4	2.2
	2	2.7	2.2	2.0	2.8	2.2	1.8	2.9	2.3	2.1
	3	2.7	2.2	1.9	2.7	2.1	1.7	2.9	2.3	2.0
	4	2.7	2.1	1.7	2.6	1.9	1.6	---	---	---

[z]Maximum SE of relative chlorophyll levels = 9, n = 4.
[y]Maximum SE of chlorophyll *a/b* ratios = 0.1, n = 4.

In the present study, loss of chlorophyll in cabbage leaf disks following Cu^{2+} exposure was studied. Copper toxicity was induced by varying the concentration of Cu^{2+} in the solution and by varying the exposure time. The influence of light on Cu^{2+}-induced chlorophyll loss was studied by comparing light- and dark-treated samples. Calcium has been reported to reduce heavy metal toxicity symptoms in soil and solution culture (5). In the present study, the effect of Cu^{2+} in the presence and absence of 0.5 mM $CaCl_2$ also was examined.

Two cabbage cultivars were compared for differential response to Cu^{2+} toxicity. Chlorophyll levels were measured in whole tissue rather than organelles in order to mimic the elevated internal Cu^{2+} concentrations in leaf tissue of Cu^{2+}-sensitive and tolerant plants. Using leaf tissue dissociated from root tissue eliminated the complex variable of root to shoot interactions and any exclusionary mechanisms present in the roots.

Plant material. Cabbage seeds of 'Resistant Danish' and 'Market Prize' were germinated between fabric strips and kept there until transplanting (8). The fabric strips were kept in contact with full-strength Hoagland's solution containing 0.05 μM Cu^{2+}. At 2 weeks, seedlings were transplanted to 1-liter aerated pots containing the same solution. The solutions were changed at 2-week intervals. Plants were grown in a walk-in growth chamber (Sherer Gillett model CEL 512-37) and maintained at 23°C day/night temperature and 330 $\mu mol \cdot s^{-1} \cdot m^{-2}$ light supplied by cool-white fluorescent bulbs for 16 hr per day.

Sample preparation. Leaf disks, 1-cm in diameter, were punched from fully expanded (about 10 × 15 cm) leaves of 4- to 6-week-old plants. Preliminary experiments showed that this age difference was inconsequential. About 25 disks were cut from the interveinal regions at least 1-cm from the margins of each leaf. Five disks were weighed and floated adaxially over 25 ml of $CuSO_4$ solutions (0, 0.16, and 0.40 mM Cu^{2+}). The samples were incubated at room temperature (20°C for 1 to 4 days and received 80 $\mu mol \cdot s^{-1} \cdot m^{-2}$ of continuous light provided by cool-white fluorescent bulbs. Two series of experiments (I and II) with 4 replications per treatment were conducted to measure the effects of Cu^{2+} on chlorophyll levels. The first compared the response of 2 cultivars ('Resistant Danish' and 'Market Prize') under light conditions to Cu^{2+} in the presence and absence of 0.5 mM $CaCl_2$. The 2nd, using only 'Market Prize', compared the effects of light vs. dark on chlorophyll loss after exposure to Cu^{2+}.

Chlorophyll measurements. The disks were removed from the treatment solution, rinsed 3 times in 5 ml of distilled H_2O, and sliced into 2-mm-wide strips. Chlorophyll was extracted in 10 ml of 96% ethanol (v/v) and determined spectrophotometrically. The chlorophyll *a* and chlorophyll *b* concentrations were calculated accordingly to the procedure of Wintermans and Demots (11).

Visible injury. After incubation in the light for 4 days, the control disks showed no visible injury, whereas Cu^{2+}-treated disks showed chlorosis and necrosis. Necrosis first appeared in the 0.40-mM treatment at 24 hr and subsequently developed in the 0.16 mM treatment. Necrosis intensified over time but was confined primarily to the outer 1.0 mm of the disk. All of the tissue was still floating after 4 days at each Cu^{2+} level. Chlorosis first appeared after 3 days in 0.40 mM Cu^{2+} at the outside margin of the disk and progressed toward the center. On the 4th day, chlorosis also was seen in the 0.16 mM treatment. There were no visible differences between cultivars or Ca^{2+} treatments.

In the dark-treated disks, the controls were not chlorotic after 3 days. The necrotic symptoms were less severe in the dark treatment than light and could be discerned only after chlorophyll extraction in ethanol. In contrast to the light experiments, chlorosis first appeared in the center of the Cu^{2+}-treated disks and then moved towards the margin.

Chlorophyll loss in 2 cultivars (in light). Original chlorophyll *a* and *b* levels were slightly higher in 'Market Prize' (1.20 and 0.43 $\mu g \cdot mg^{-1}$ fresh weight, respectively) than 'Resistant Danish' (1.04 and 0.38 $\mu g \cdot mg^{-1}$ fresh weight, respectively). To minimize the effect of senescence on the measurement of chlorophyll decline, the relative content of chlorophyll was determined in comparison to the control at each time interval. Since no Ca^{2+} differences were detected, the Ca^{2+} treatments were pooled.

In the absence of Cu^{2+}, both cultivars maintained about the same level of relative total chlorophyll content over time (Table 1, Expt. I). Regression analyses showed that relative total chlorophyll levels declined linearly over time at both 0.16 and 0.40 mM Cu^{2+} at the 0.01 level of significance. The relative total chlorophyll content was re-

duced in 'Market Prize' at each sampling time for both Cu^{2+} levels tested as were the final levels of total chlorophyll after 4 days (Table 1, Expt. I).

After 4 days, chlorophyll *a* and *b* of the control for both cultivars declined <13% (Table 1, Expt. I). However, after exposure to Cu^{2+}, chlorophyll *a* declined more rapidly than chlorophyll *b* in both cultivars. For example, after 4 days in the 0.40 mM Cu^{2+} solution, there was about a 13% loss of relative chlorophyll *b* content while there was a 40% loss of relative chlorophyll *a*.

The chlorophyll *a/b* ratio decreased rapidly for both the cultivars during the first 24 hr of Cu^{2+} treatments (Table 1, Expt. I). This decline appears to be due to a rapid reduction in chlorophyll *a* during the first 24 hr while chlorophyll *b* increased during the same period (Table 1). At 0.40 mM Cu^{2+} the decline was greater than at 0.16 mM Cu^{2+} in both the cultivars. 'Resistant Danish' had a higher chlorophyll *a/b* ratio at 0.40 mM Cu^{2+} than 'Market Prize'. During the same period of treatments, the control samples maintained relatively constant chlorophyll *a/b* ratios.

Chlorophyll loss in the presence and absence of light ('Market Prize'). After 3 days in darkness, the control had lost 30% of the original total chlorophyll content (Table 1, Expt. II). In the absence of Cu^{2+}, chlorophyll *a* and chlorophyll *b* each declined to 90% after 3 days in the light and to 77% after 3 days in the dark (Table 1, Expt. II). However, chlorophyll *a* was not as sensitive to Cu^{2+} treatment in the dark. Although chlorophyll *b* remained above 90% of the control in both light and dark treatments, the relative levels of chlorophyll *a* declined to 59% in the light but remained at 77% in the dark. These higher relative levels of chlorophyll a resulted in a higher chlorophyll *a/b* ratio in the dark than light.

The intervarietal differences in total chlorophyll levels were due primarily to greater declines of chlorophyll *a* in 'Market Prize' than in 'Resistant Danish'. Therefore, reductions in chlorophyll *b* were perhaps due largely to senescense, whereas reductions in chlorophyll *a* were due mainly to Cu^{2+} treatments. The preferential destruction of chlorophyll *a* rather than *b* suggests that the light-harvesting chlorophyll *a/b* protein (LHCP) largely was unaffected by Cu^{2+} treatment. Over 83% of the chlorophyll *b* has been reported to be located in the LHCP and the chlorophyll *a/b* ratio in LHCP is known to be one (1). If *b* remains unchanged, as in this study (Table 1), changes in chlorophyll *a/b* ratios are due primarily to changes in chlorophyll complexes other than the LHCP (6). Copper ions have been reported to inhibit electron transport on the oxidizing side of PSII and the reducing side of PSI (2). The cultivar differences in the decline of chlorophyll *a* and *a/b* ratios during Cu^{2+} treatment (Table 1, Expt. I) may be attributed to differences in PSI and PSII chlorophyll complexes rather than LHCP.

The higher relative total chlorophyll levels, after Cu^{2+} treatment, in the dark than in the light (Table 1, Expt II) are consistent with previous work and suggest that Cu^{2+}-induced chlorophyll reduction is a light-dependent process (3). Although Ca^{2+} has been reported to reduce heavy metal toxicity (5), the decline of chlorophyll in the presence of Cu^{2+} in the present study was unaffected by 0.5 mM $CaCl_2$.

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