# Cadmium and zinc induced chlorosis in Indian mustard [*Brassica juncea* (L.) Czern] involves preferential loss of chlorophyll *b*

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## Abstract

Plants of Indian mustard (*Brassica juncea*) were treated with either 50 μM Cd, 250 μM Zn, or 25 μM Cd+125 μM Zn and the progression of chlorosis in the mature leaves monitored. As relative chlorophyll (Chl) contents in the mature leaves decreased to 75, 50, and 25 % relative to controls, both mature and young leaves were harvested and the Chl pools extracted. The metal treatments caused a greater loss of Chl *b* than Chl *a*. As mature leaves underwent progressive chlorosis, the young leaves displayed a characteristic over-greening, due largely to increased content of Chl *b*. However, as the young leaves began to experience chlorosis, a greater loss of Chl *b* was also observed. Thus during metal induced chlorosis, there is a preferential turnover of the Chl *b* pool in mature and young leaves.

*Additional key words*: heavy metals.

## Introduction

One of the most common effects induced by elevated concentrations of heavy metals is the loss of chlorophyll (Chl) from leaf tissues. Heavy metals inhibit Chl synthesis at several points in the pathway (Bhattacharjee and Mukherjee 2003, Aravind and Prasad 2004). As metal exposure continues, progressive chlorosis results in the loss of the Chl present in leaves prior to metal exposure. Many studies with terrestrial plants report that the Chl *a*:*b* ratio increases as chlorosis progresses (Bhattacharyya and Choudhuri 1994, Moreno-Caselles *et al.* 2000, Bindhu and Bera 2001, Shi *et al.* 2003, Singh and Tewari 2003). An increase in this ratio would occur if the contents of Chl *a* increased and/or Chl *b* contents decreased. These observations suggest a specific pattern of Chl loss during metal-induced chlorosis, representing either a direct effect of metals on the two Chl pools or an indirect effect created by the metals.

The purpose of this study was to examine the impact of two heavy metals, zinc and cadmium, on the Chl *a* and Chl *b* pools in plant leaves in an effort to identify specific patterns of Chl loss that occur during the progression of chlorosis. These three pools were measured at discrete stages of chlorosis to determine if the same pattern of Chl loss occurs in these physiologically distinct tissues. Indian mustard (*Brassica juncea* L.) was the plant species used here because this species has been the focus of several previous heavy metal studies (Salt *et al.* 1995, 1997, Huang and Cunningham 1996, Blaylock *et al.* 1997, Ebbs and Kochian 1997, 1998, Ebbs *et al.* 1997, Begonia *et al.* 1998, Vassil *et al.* 1998, Epstein *et al.* 1999, Kayser *et al.* 2001). Additionally, the young leaves of this species respond to elevated concentrations of heavy metals with an over-production of Chl in young leaves not observed in the older leaves (Ebbs and Kochian 1997).

## Materials and methods

**Plant growth and culture**: The seeds of Indian mustard (*Brassica juncea* L. Czern) used in this study were derived through seed increase from an initial line (Accession 426308) obtained from the USDA-ARS Plant Introduction Center at Iowa State University. The seeds were germinated in a 1 : 1 perlite-vermiculite mixture, and after 5–6 d in the dark the seedlings were transferred to pots containing a nutrient solution with the following composition: 6.0 mM $KNO_3$; 4.0 M $Ca(NO_3)_2$; 0.1 mM $NH_4H_2PO_4$; 1.0 mM $MgSO_4$; 50 μM KCl; 12.5 μM $H_3BO_3$; 1.0 μM $MnSO_4$; 1.0 μM $ZnSO_4$; 0.5 μM $CuSO_4$; 0.1 μM $H_2MoO_4$; 0.1 μM $NiSO_4$ (Ebbs

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*Abbreviations*: CAO – chlorophyll *a* oxidase; Chl – chlorophyll; DMF – dimethylformamide; EDDHA – N,N'-ethylenediamine-di(O-hydroxyphenyl)acetic acid; MES – 2-[morpholino] ethanesulfonic acid.

and Kochian 1997). The solution was buffered to pH 6.0 with 1 mM MES buffer and supplemented with 5 μM Fe-EDDHA. Plants were grown in a phytotron at 24±2 ºC, under natural irradiation with supplemental light [~300 μmol(quantum) $m^{-2}$ $s^{-1}$] on a 16-h photoperiod. The pots were aerated and the nutrient solution was replaced weekly. Once plants reached the desired size (10–12 fully-expanded leaves), treatments were imposed.

**Metal treatments and Chl measurement**: The plants were transferred to pots of fresh nutrient solution containing one of four heavy metal treatments: control (no elevated metals), 250 μM $Zn^{2+}$, 50 μM $Cd^{2+}$, or 125 μM $Zn^{2+}$+25 μM $Cd^{2+}$. These concentrations have been shown in previous studies to produce significant increases in metal accumulation in Indian mustard plants and produce chlorosis (Ebbs and Kochian 1997, Ebbs *et al.* 1997). Each treatment was replicated five times. Following the treatment, the progression of chlorosis was monitored in a mature leaf ($4^{th}$ above the base) three times a week until the mature leaf on each plant reached a specified stage of chlorosis stage (25, 50, and 75 % as compared to the control plants). This monitoring was accomplished by measuring the relative Chl content of the leaves with a *Minolta SPAD-502* Chl meter (*Spectrum Technologies*, Plainfield, IL, USA). When the specified level of chlorosis was reached, the mature leaves (lowest four leaves) and young leaves (five highest leaves) were harvested, the mass and surface area determined, and the Chl pools extracted and quantified.

To extract the Chl pools, the leaves were immersed in 100 % N,N-dimethylformamide (DMF) at a 1 : 100 (m/v) ratio. The tissue was extracted with mild shaking in the dark for 18–24 h until there was no color left in the leaves. The vial was allowed to settle for 15 min and 1 $cm^3$ of the extract solution was transferred to a spectrophotometer cuvette. The absorbance of the solution was measured at 625, 647, and 663 nm using a *Cary 50* UV-VIS spectrophotometer (*Varian*, Walnut Creek, CA, USA), with 100 % DMF used as the blank. The contents of Chl *a* and Chl *b* in the fresh tissue were calculated using the equations of Moran (1982).

**Data analysis**: The Chl data obtained was normalized to both biomass (fresh mass basis) and leaf surface area to account for possible adverse effects on plant growth and development that might skew the results. Statistical analysis of the data used a two-way analysis of variance conducted using the *SPSS* software package (version *12*). In situations where a significant interaction was observed between these two factors, the ANOVA and post hoc analysis (Tukey test with a Bonferroni correction) was conducted using the interaction means. In the absence of a significant interaction between factors in a given ANOVA analysis, no *post hoc* analysis was conducted.

## Results

**Plant growth and biomass**: Regardless of metal treatment, the biomass (Fig. 1*A*,*B*) and leaf surface area (data not shown) of old and young leaves increased significantly over the course of the experiment and did not differ significantly from the control treatment. While the metal treatments did induce chlorosis in the mature leaves of Indian mustard (see below) there was clearly not an adverse effect on plant growth. This suggests that the detrimental effects of the metal treatments were restricted largely to biochemical processes that directly or indirectly influenced Chl metabolism rather than a more general response to metal-induced stress.

**Chl pools**: The metal treatments had different effects on the Chl pools of mature and young leaves (Table 1). In mature leaves, metal treatments had the most rapid effect on the Chl *b* pool. For the single metal treatments, Chl *b* contents were significantly less ($p \leq 0.001$) than the control when leaf Chl content had decreased by 25 %. The Chl *b* content of the mature leaves from the combined metal treatments was also lower than the control at this chlorosis level but this difference was not significant. In contrast, Chl *a* contents were generally not significantly different from the control at the 25 % chlorosis. As the chlorosis in the mature leaves progressed, Chl *b* contents decreased significantly ($p \leq 0.01$). Chl *a* contents in the single metal treatments at the 50 % chlorosis were not significantly different from the 25 % one, although there was a significant decrease for the combined metal treatments ($p \leq 0.01$). When chlorosis had reached the 75 % level, both Chl pools had reached their lowest contents.

In the young leaves, all metal treatments resulted in a significant increase in Chl pools relative to the control ($p \leq 0.001$, Table 1). This increase in Chl content occurred only when the mature leaves were experiencing a 25 % decrease in Chl content. The contents of both Chls in young leaves decreased to control values by the time the mature leaves were experiencing 50 % chlorosis. The Chl *a* pool continued to decrease, reaching a value significantly lower than the control at 75 % chlorosis ($p \leq 0.01$).

The three metal treatments also affected Chl *a*:*b* in mature and young leaves (Fig. 1*C*,*D*). In mature leaves as chlorosis progressed, there was a characteristic, albeit modest, increase in Chl *a*:*b* for all treatments (Fig. 1*C*). The ANOVA analysis of the ratios indicated that there were significant differences between the treatments as well as significant differences between the ratios at the three stages of chlorosis ($p \leq 0.01$). As the interaction between treatment and chlorosis was not significant, a *post hoc* analysis of the data was not conducted. In young leaves, the Chl *a*:*b* ratio decreased significantly when

measurements were made at the point when the mature leaves had reached the 25 % chlorosis ($p \leq 0.001$, Fig. 1*D*). While both Chl *a* and Chl *b* contents had increased relative to the control, there was a larger increase in Chl *b* pool in young leaves that for the Chl *a* pool (Table 1), hence an overall decrease in Chl *a*:*b*. As chlorosis progressed in the mature leaves to 50 and 75 %, the Chl *a*:*b* ratio in the young leaves returned to a value comparable to those in leaves of the control plants and remained there for all treatments except the Cd+Zn treatment, which showed a significant ($p \leq 0.01$) decrease.

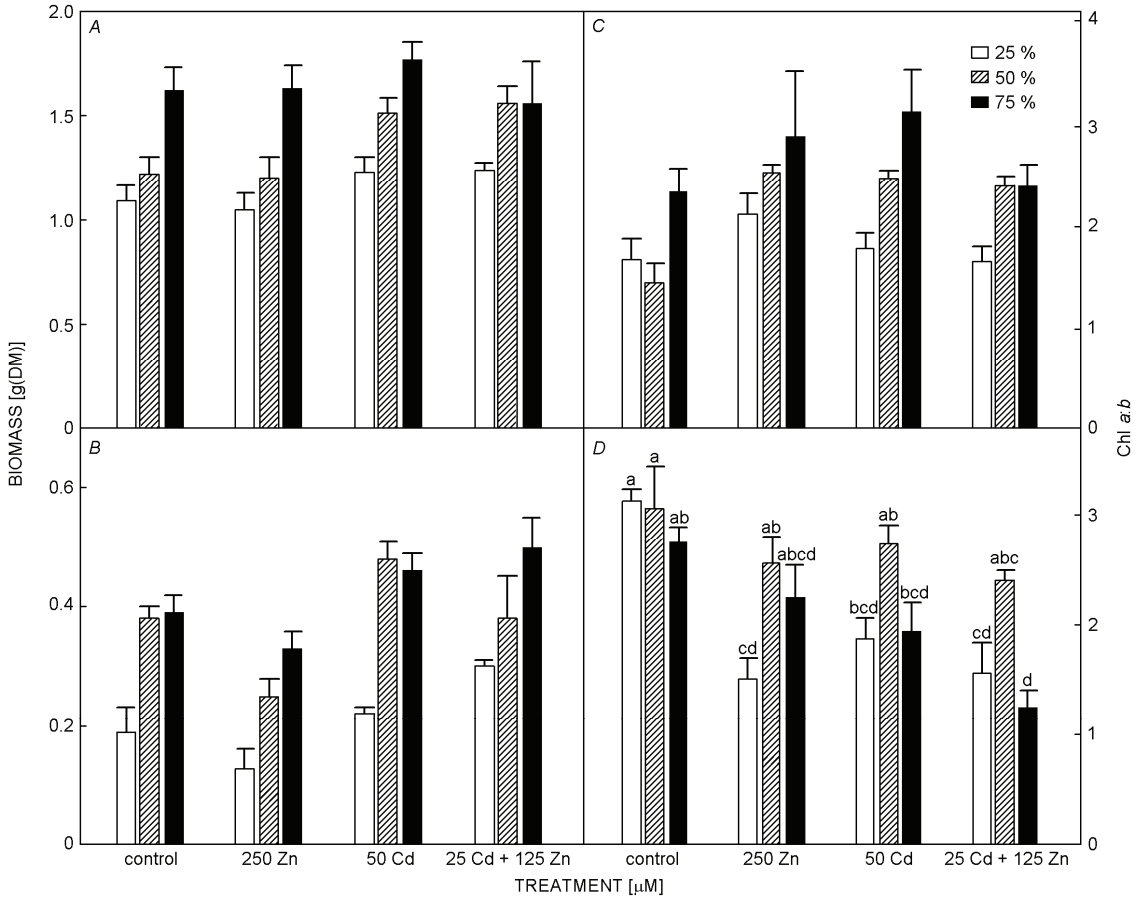


Fig. 1. Biomass (*A*, *B*) and chlorophyll (Chl) *a*:*b* (*C*, *D*) of mature (*A*, *C*) and young (B, *D*) leaves of Indian mustard sampled from plants treated with either Zn, Cd, or Cd+Zn. Both young and mature leaves were harvested as chlorosis in the mature leaves progressed through three stages (25, 50, or 75 % chlorosis relative to control plants). Data for control plants represent values for untreated plants harvested when metal-treated plants had reached the indicated chlorosis. Means and standard errors.

Thus in the presence of Zn and/or Cd, there is a more rapid and extensive turnover of the Chl *b* pool. To further illustrate this lability of the Chl *b* pool, Chl *a* and *b* values for each of the metal treatments from both the mature and young leaves were expressed as percent of the respective controls (Fig. 2). In mature leaves, Chl *b* contents decreased continually as chlorosis progressed. However, Chl *a* contents did not begin to decrease until the chlorosis exceeded 50 % (Fig. 2*A*). The over-production of Chl *a* and *b* in leaves of young leaves was also evident, although there was a greater increase in the Chl *b* content (300 % of control) as compared to the Chl *a* content (150 % of control) for all three treatments (Fig. 2*B*). As chlorosis progressed in the mature leaves, the Chl *b* content in the young leaves for all treatments dropped much more drastically than the Chl *a* contents, reaching a level comparable to the Chl *a* pool at the 75 % chlorosis in mature leaves. By the time the chlorosis in the mature leaves had reached 75 %, the young leaves were beginning to lose Chl *a* and *b*. These Chls had decreased to 50–75 % of the control values for both pools generally for all treatments. The only exception was the Chl *b* pool in young leaves from plants treated with Cd+Zn, which showed only a 25 % decrease relative to the control.

## Discussion

The inhibition of Chl synthesis by heavy metals is well documented. Cadmium, for example, can inhibit both Δ-aminolevulinic acid dehydratase and protochlorophyllide reductase (Aravind and Prasad 2004). Elevated concentrations of Cd or Zn can also interfere with Chl synthesis indirectly by inducing micronutrient (*e.g*. Fe) deficiencies (Balsberg Påhlsson 1989, Van Assche and Clijsters 1990). No doubt one component of the chlorosis observed in the mature leaves of Indian mustard was due to such effects on Chl synthesis. An induction of Fe deficiency was observed previously for Indian mustard following exposure to Zn or Cu (Ebbs and Kochian 1997, 1998). In contrast, there has been little work examining the turnover of existing Chl *a* and *b* molecules following exposure to elevated concentrations of heavy metals. Our study sought to address this question by examining the effect of Cd, Zn, or Cd+Zn on the loss of Chl *a* and *b* pools in mature and young leaves.

Table 1. The change in the chlorophyll (Chl) *a* and *b* pools [g $m^{-3}$] with progressing chlorosis [%] in the mature leaves of Indian mustard plants treated with Zn, Cd, or Cd+Zn. Data for control plants represent values for untreated plants harvested when metal-treated plants had reached the indicated level of chlorosis. These data were included to show that Chl content of untreated plants remained constant. Means ± standard errors. For each Chl pool in a given leaf type (*e.g*. Chl *a* in mature leaves), letters are used to indicate significant differences between the treatments across all three levels of chlorosis.

| Leaves | | Chlorosis [%] | Control | [μM] 250 Zn | 50 Cu | 25 Cd+125 Zn |
|---|---|---|---|---|---|---|
| mature | Chl *a* | 25 | 2.6 (0.12) ab | 2.2 (0.07) bcd | 2.0 (0.03) cde | 2.1 (0.10) bcde |
| | | 50 | 2.3 (0.09) abc | 1.7 (0.17) def | 1.8 (0.06) cde | 1.3 (0.17) fg |
| | | 75 | 2.7 (0.16) a | 0.5 (0.04) h | 0.9 (0.01) gh | 0.8 (0.03) gh |
| | Chl *b* | 25 | 1.6 (0.13) ab | 1.1 (0.12) cd | 1.2 (0.08) c | 1.3 (0.05) abc |
| | | 50 | 1.6 (0.13) a | 0.7 (0.08) def | 0.8 (0.04) de | 0.5 (0.06) efg |
| | | 75 | 1.2 (0.10) c | 0.2 (0.02) g | 0.3 (0.05) fg | 0.4 (0.02) efg |
| young | Chl *a* | 25 | 1.7 (0.18) cd | 2.5 (0.02) a | 2.5 (0.02) ab | 2.5 (0.03) ab |
| | | 50 | 1.8 (0.16) cd | 1.0 (0.05) e | 1.7 (0.01) cd | 2.1 (0.10) bc |
| | | 75 | 1.8 (0.15) cd | 0.5 (0.06) f | 0.5 (0.04) f | 0.7 (0.07) ef |
| | Chl *b* | 25 | 0.6 (0.07) cd | 1.8 (0.20) a | 1.4 (0.16) a | 1.8 (0.03) a |
| | | 50 | 0.6 (0.07) cd | 0.4 (0.05) cd | 0.6 (0.04) bcd | 0.9 (0.06) cd |
| | | 75 | 0.7 (0.05) cd | 0.2 (0.05) d | 0.3 (0.03) cd | 0.6 (0.06) cd |

Cd, Zn, and the combination of the two metals had generally similar effects on the Chl *a* and *b* pools (Table 1, Fig. 2). Despite reports that Indian mustard is a moderate metal accumulator (Huang and Cunningham 1996, Blaylock *et al.* 1997, Ebbs *et al.* 1997, Begonia *et al.* 1998, Ebbs and Kochian 1998, Epstein *et al.* 1999, Elless *et al.* 2000, Kayser *et al.* 2001), the metal concentrations used here were sufficiently high to cause chlorosis and enhance the turnover of both Chl *a* and *b* in mature leaves, although more so for the latter. When plants were treated simultaneously with Cd and Zn, even at half the concentration of the single metal treatments, the rate of Chl *a* and *b* loss was greater than for the single metal treatment (Table 1, Fig. 2). This suggests that there may have been an initial additive effect of the two metals. Nevertheless, since the extent of Chl *a* and *b* loss in mature leaves from the last measurement was similar to the single metal treatments, the combination of the two metals was essentially an equivalent toxic unit, perhaps inducing Chl loss by a common mechanism involving preferential loss of Chl *b* from mature leaves.

Several studies (Angadi and Mathad 1998, Moreno-Caselles *et al.* 2000, Fargaová 2001, Bhattacharjee and Mukherjee 2003) have shown that the Chl *b* pool is more dramatically affected during chlorosis than the Chl *a* pool. A preferential loss such as this could ostensibly be due to either a decreased conversion of Chl *a* to Chl *b* or the converse, an increased reversion of Chl *b* to Chl *a*. Chl *a* is the primary Chl synthesized by photosynthetic organisms, comprising the reaction centers of the photosystems. The accessory Chl *b* pigment is formed *via* a subsequent reaction, mediated by Chl *a* oxygenase (CAO), which converts a methyl group on the porphyrin ring (ring 2) to a formyl group (Schneegurt and Beale 1992, Porra *et al.* 1993, 1994, Espineda *et al.* 1999). This enzyme is regulated by light, with increased CAO mRNA observed in the green alga *Dunaliella salina* when cultures were transferred from high to low irradiance (Masuda *et al.* 2002, 2003). There is no direct evidence, however, that the expression or activity of this enzyme is affected by metal stress nor has a change in CAO activity been associated with metal chlorosis. Nevertheless, the importance of Chl *a* relative to Chl *b* would make down-regulation of CAO a logical response to Cd or Zn toxicity, particularly in response to damaged chloroplast membranes or photosystems.

A more plausible explanation for the preferential loss of Chl *b* in the presence of heavy metals is the conversion

of Chl *b* to Chl *a*. Although the synthesis of Chl *b* was initially assumed to be a committed reaction step, the conversion of Chl *b* to Chl *a* is now known to occur in plants (Ito *et al.* 1993, 1994, 1996, Ito and Tanaka 1996, Ohtsuka *et al.* 1997) and is mediated by a ferredoxin-dependent Chl *b* reductase that forms an hydroxymethyl intermediate similar to the one formed during the synthesis of Chl *b* (Scheumann *et al.* 1998). This reaction is an important component of the adaptation to changing irradiance (*i.e.* the shift from low to high irradiance), with the Chl *b* released from protein complexes in photosystem 2 and then converted to Chl *a* for the formation of reaction center Chl *a* molecules (Ohtsuka *et al.* 1997).

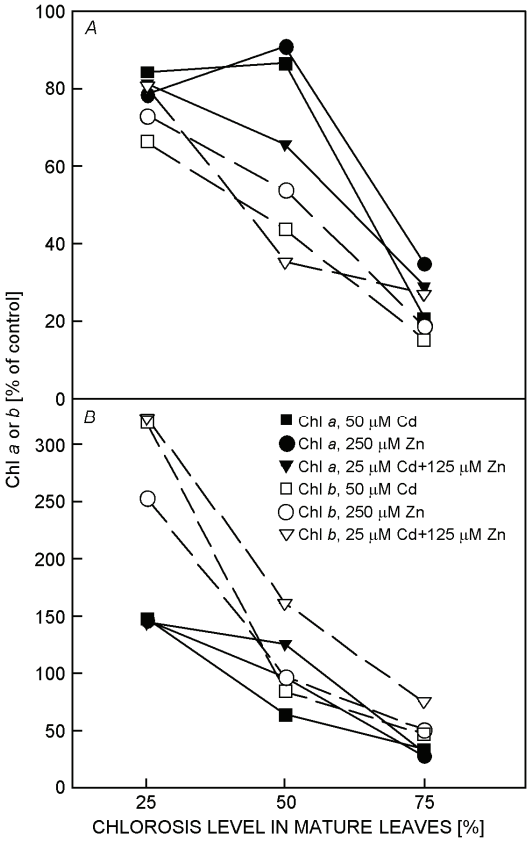


Fig. 2. Chlorophyll (Chl) *a* and *b* contents of mature (*A*) and young (*B*) leaves from Indian mustard sampled from plants treated with either Zn, Cd, or Cd+Zn expressed as percent of control over the progression of chlorosis in the mature leaves.

The conversion of Chl *b* to Chl *a* is also a necessary first step for the catabolism of Chl during senescence (Scheumann *et al.* 1998, 1999, Lu *et al.* 2001, Mukherjee and Ponmeni 2004) because the enzymes involved are specific for Chl *a* catabolites, but not Chl *b* (Matile *et al.* 1999, Dangl *et al.* 2000). Leaf senescence, and therefore the associated loss of Chl *b*, can also be initiated by the presence of oxygen radicals generated by stresses such as ozone, drought, high irradiance in combination with nutrient (K, Mg, Zn) deficiency (Marschner and Cakmak 1989, Kim and Lee 1995, Marschner 1995). In rape (*Brassica napus*), for instance, expression of the gene for catalase increased during senescence while in maize (*Zea mays*), glutathione *S*-transferase expression increased (Dangl *et al.* 2000). The heavy metals Cd (Singh and Tewari 2003, Qadir *et al.* 2004, Lang *et al.* 2005) and Zn (Prasad *et al.* 1999, Chatterjee *et al.* 2005) increase the expression of these two enzymes in Indian mustard, as well as other species. An increase in Chl *a*:*b* concomitant with an increase in catalase activity was specifically reported for Indian mustard in response to Cd exposure (Singh and Tewari 2003). A speculative model that could therefore be proposed is that the preferential decrease in the Chl *b* during the exposure of low-irradiance adapted plants to high irradiance, the onset of senescence and metal-induced chlorosis all involve a similar metabolic modification of the Chl pools in response to oxygen radicals: increased conversion of Chl *b* to Chl *a*, perhaps with concomitant with down-regulation of CAO. Given that the conversion of Chl *b* to Chl *a* is an integral component of the adaptation to high irradiance and that Chl degradation during senescence is a programmed response meant to resorb components of the photosynthetic apparatus into primary metabolism, both represent situations where Chl *b* is being metabolized for another biochemical purpose. The presence of excess Cd or Zn could induce a proactive reduction of Chl *b* to Chl *a*, perhaps to offset both the inhibition of Chl synthesis and the direct damage to the photosynthetic machinery caused by the metals. In the mature leaves of Indian mustard here, Chl *a* contents were maintained from 25 to 50 % chlorosis coincident with a sharp decrease of Chl *b* content (Fig. 2*A*). This could indicate that Chl *b* reduction was utilized to maintain the Chl *a* pool. As chlorosis progressed with continued metal exposure and accumulation in leaf cells, the damage created by the metals may have exceeded the ability of the leaves to maintain Chl levels, resulting in a decrease in both Chl *a* and *b*.

A similar explanation could hold true for young leaves, albeit delayed due to the initial over-greening that was observed. In the young leaves, despite a substantial increase in the Chl *b* content (250–300 % of control), this Chl pool was rapidly depleted as chlorosis progressed (Fig. 2*B*). Why the Chl *b* pool increased more dramatically during the over-greening of the young leaves in response to metals than the Chl *a* pool is unclear, but could suggest a change in CAO activity and assembly of photosystems following metal exposure. Nevertheless, as chlorosis in the mature leaves indicated a progressive detrimental effect of the metal exposure, the same pattern of preferential Chl *b* loss was observed in the young leaves. One interesting observation was that the rate of Chl loss for the single metal treatments was greater than that for the combined metal treatments, although the same Chl contents were reached at the last measurement. This is particularly obvious for Chl *a* and the Zn treatment (Table 1, Fig. 2). This is opposite of the observation in

mature leaves and requires further study to explain.

Studies of Chl metabolism have shown that Chl metabolism is dynamic, responding to make necessary adjustments to the Chl *a*:*b* ratio to adapt to the external irradiance. Similarly, senescence involves the programmed conversion of Chl *b* to Chl *a* prior to degradation. Chlorosis is a symptom of heavy metal toxicity and also involves the loss of Chl from leaves. Our results suggest that in addition to an inhibition of Chl synthesis, both Cd and Zn trigger a conversion of Chl *b* to Chl *a* in Indian mustard similar to that of a senescence-mediated response. This is a potentially novel observation that suggests a crosstalk between the pathways that control senescence and the response to irradiance, heavy metals, and other stresses. Additional study of this phenomenon is warranted to determine the relative contribution of CAO inhibition and Chl *b* reductase activity to the Chl pools in metal-treated plants. Future studies should also follow the progression of chlorosis in the young leaves as those tissues themselves continue to lose Chl to confirm that the pattern of Chl *a* and Chl *b* loss continues as in the mature leaves. The results from such studies will add to the knowledge of the specific metabolic responses of plants to heavy metal exposure.

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