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Effect of cadmium on chlorophyll biosynthesis and enzymes of nitrogen assimilation in greening maize leaf segments: Role of 2-oxoglutarate

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Supply of cadmium chloride (0.5 m*M*) inhibited chlorophyll formation in greening maize leaf segments, while lower concentration of Cd (0.01 m*M)* slightly enhanced it. Inclusion of 2-oxoglutarate (2-OG, 0.1-10 m*M)* in the incubation mixture increased chlorophyll content in the absence as well as presence of Cd. Substantial inhibition of chlorophyll formation by Cd was observed at longer treatment both in the absence and presence of 2-OG. When the tissue was pre-incubated with 2-OG or Cd, the inhibition (%) of chlorophyll formation by Cd was lowered in the presence of 2-OG. Treatment with Cd inhibited ALAD activity and ALA formation and the inhibition (%) of ALA formation by Cd was strongly reduced in the presence of 2-OG. Glutamate dehydrogenase (GDH) activity was increased by the supply of Cd both in the absence as well as presence of 2-OG. In the presence of 2-OG, Cd supply significantly increased glutamate synthase (GOGAT) activity and reduced inhibition (%) of glutamine synthetase (GS) activity. The results suggested the involvement of the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway of ammonia assimilation to provide the precursor, glutamate, for ALA synthesis under Cd toxicity and 2-OG supplementation.

Keywords: Cadmium effects, Chlorophyll, Nitrogen assimilation, 2-Oxoglutarate, *Zea mays*

Cadmium (Cd) is a heavy metal toxicant for humans, animals and plants. It is a widespread trace element pollutant with a long biological half-life[1]. This metal enters agricultural soils mainly from industrial effluents, pesticides, phosphate fertilizers and atmospheric deposition, that is then transported to the food chain. Phytotoxicity of Cd is associated with growth inhibition and imbalances in macro and micronutrient levels. It affects many physiological processes, such as, nitrogen metabolism[2], membrane functions by changing the fatty acid composition of the lipids[3], oxidative stress through increased lipid peroxidation[4] and proteolytic degradation[5]. Chlorophyll biosynthesis is a physiological phenomenon which is linked to photosynthetic productivity of plants. Cd has been reported to inhibit chlorophyll content in several plant systems[6-8]. Further, the enzyme, δ-amino levulinic acid dehydratase, of the chlorophyll biosynthetic pathway is also inhibited by Cd in radish leaves[9]. Substitution of the central atom of chlorophyll, Mg, by Cd *in vivo* has been reported to be an important damage mechanism in water plants under heavy metal stress[10]. The key intermediate of the chlorophyll biosynthetic pathway, δ-amino levulinic acid, may be synthesized from 5-carbon compounds, such as, glutamate[11] or 2-OG[12]. The level of 2-OG in a plant cell reflects C/N status, and therefore, it can play a signaling role in the coordination of C and N metabolism. In the present investigation, the effect of Cd on chlorophyll formation and enzymes of ammonia assimilation in response to 2-OG supply in greening maize leaf segments is analyzed.

Materials and Methods

Plant material — Seeds of *Zea mays* L. cv. Ganga safed-2, were surface sterilized with $HgCl_2$ (0.1%) for 1-2 min and then washed thoroughly with distilled water before planting. Seedlings were raised in plastic pots containing acid washed sand in continuous dark for about 7 days at 25º ± 3 ºC. They were watered on alternate days with ½ strength Hoagland's nutrient solution modified to exclude nitrogen. For various treatments, primary leaves from uniformly grown seedlings were cut into 0.5×0.5 cm pieces and floated

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Abbreviations: 2-OG—2-oxoglutarate, ALA—δ-Amino levulinic acid, ALAD—δ-Amino levulinic acid dehydratase, GDH-glutamate dehydrogenase, GOGAT—glutamate synthase, GS—glutamine synthetase.

on 1/4th strength Hoagland's solution (*p*H 6.0) containing $HgCl_2$ and/or 2-OG for 24 h in continuous light of intensity about 40 Wm^{-2} inside the 'Newtronic growth chamber' model "NEC-216" at 26º±2ºC.

Chlorophyll determination—Chlorophyll was extracted from the treated samples with acetone (80%) and its quantity was calculated from its extinction at 645 and 663 nm according to Strain and Svec[13].

ALA content determination—ALA content was measured by the method of Kruse *et al*[14]. The treated leaf segments were incubated with KH_2PO_4 (10 m*M*), *p*H 7.2 containing levulinic acid (80 m*M)* for 2 h and then homogenized with 1 N TCA containing SDS (1%) and the extract was condensed with ethylacetoacetate followed by treatment with modified Ehrlich's reagent. Porphobilinogen formed from δ-ALA was estimated spectrophotometrically at 553 nm and its amount was calculated using standard curve prepared with ALA.

Enzymatic analyses—δ-Aminolevulinic acid dehydratase activity was assayed by estimating colorimetrically the amount of porphobilinogen formed using modified Ehrlich's reagent as described earlier in Jain and Gadre[15]. Desalted preparations of NADH-GDH and NADH-GOGAT were obtained and assayed spectrophotometrically by monitoring NADH oxidation at 340 nm according to the method of Duke and Ham[16]. Glutamine synthetase preparation was obtained according to the method of Sawhney *et al*[17] and was assayed by the method of Boland *et al* [18].

Data presented in the paper are average values of at least three independent experiments. One way ANOVA was performed and F values, calculated variance ratio, are given in the Tables.

Results

Supply of $CdCl_2$ (0.5 m*M*) to excised maize leaf segments during greening decreased the chlorophyll content substantially, however, it was slightly increased at 0.01 m*M* of $CdCl_2$ (Table 1). Inclusion of 2-OG (0.1 to 10 m*M*) increased the chlorophyll content in the absence as well as presence of 0.01 m*M* and 0.5 m*M* $CdCl_2$ (Table 1).

When leaf segments were floated on 1/4th strength Hoagland solution for different time periods, the chlorophyll level increased slowly up to 8 h, followed by a substantial increase up to 24 h (Fig. 1). Inclusion of 2-OG (5 m*M*) exhibited almost similar patterns of increase with a higher level being maintained after 4 h (Fig. 1). However, supply of Cd (0.5 m*M*) gradually increased the chlorophyll content up to 8 h followed by a decrease at 24 h in the absence as well as presence of 2 OG (Fig. 1).

When excised leaf segments pre-incubated for 8 h with $CdCl_2$ (0.5 m*M*) were transferred to 1/4th strength Hoagland solution in the absence and presence of 2-OG (5.0 m*M*), chlorophyll content increased (Table 2). However, transfer to $CdCl_2$ (0.5 m*M*) caused an increase in chlorophyll content in the

Table 1—Effect of supply of $CdCl_2$ and 2-OG on chlorophyll formation in excised greening maize leaf segments

[Values are mean ± SE of 7 experiments]

2-OG conc. (m*M*)	Total chlorophylls (μg g^{-1} fresh wt) $CdCl_2$ conc. (m*M*) 0.0	0.01	0.5	F value
0.0	170 ± 10 (100)	179 ± 19 (105)	51 ± 04 (30)	9.23 (19.46)
0.1	186 ± 22 (100)	219 ± 25 (118)	62 ± 13 (33)	5.27 (19.44)
1.0	184 ± 18 (100)	207 ± 31 (111)	70 ± 18 (39)	8.77 (19.45)
5.0	235 ± 23 (100)	241 ± 34 (102)	65 ± 08 (29)	18.78 (19.45)
10.0	217 ± 25 (100)	255 ± 29 (117)	67 ± 05 (31)	16.48 (19.45)
F value	2.05 (5.70)	1.27 (5.72)	0.37 (5.76)	

Critical variance ratio from F-distribution table at 5% level of significance is given in parentheses.

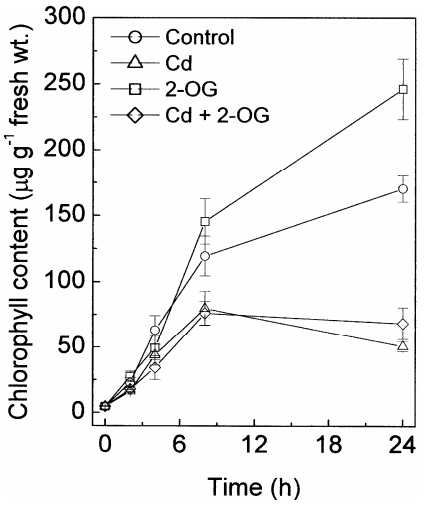

Fig. 1—Chlorophyll formation during cadmium and 2-OG supply at different time periods [(○–○) – Control; (Δ–Δ) – Cadmium; (□–□) – 2-OG; and (◊–◊) – Cd + 2-OG]

Table 2—Effect of supply of 2-OG on inhibition of chlorophyll formation by Cd after pre-incubation with $CdCl_2$ and 2-OG

[Values are mean ± SE of 5 experiments]

Total chlorophyll content (μg g^{-1} fresh wt)

Treatment	- $CdCl_2$	+ $CdCl_2$	% Inhibition	F value
After preincubation with $CdCl_2$ (0.5 m*M*)				
	64 ± 13			
After incubation with				
- 2 OG	133 ± 06	58 ± 02	56	14.48 (4.96)
+ 2 OG	127 ± 07	72 ± 03	33	10.53 (4.54)
F value	0.14 (4.54)	0.41 (4.96)		
After preincubation with 2-OG (5.0 m*M*)				
	146 ± 17			
After incubation with				
- 2 OG	224 ± 22	173 ± 20	23	2.75 (4.19)
+ 2 OG	193 ± 04	170 ± 05	12	0.19 (4.66)
F value	0.58 (4.32)	0.00 (4.35)		

Critical variance ratio from F-distribution table at 5% level of significance is given in parentheses.

presence of 2-OG and a decrease in its absence. Hence, the % inhibition by Cd was lower in the presence of 2-OG. Similarly, on transferring the maize leaf segments pre-incubated for 8 h with 2-OG (5.0 m*M*) to 1/4th strength Hoagland solution in the absence and presence of Cd and/or 2-OG, the chlorophyll content increased in all the cases (Table 2). However, per cent inhibition of chlorophyll formation by Cd was reduced with 2-OG supply.

Treatment with $CdCl_2$ (0.5 m*M*) decreased the ALA content substantially both in the absence and presence of 2-OG (5.0 m*M*) (Table 3). However, the per cent inhibition by Cd was more pronounced in the absence of 2-OG. Supply of $CdCl_2$ (0.5 m*M*) inhibited the ALAD activity both in the absence and presence of 2-OG (5.0 m*M*) with % inhibition of enzyme activity by Cd being with later (Table 3).

Incubation of leaf segments with $CdCl_2$ (0.5 m*M*) increased the NADH-GDH activity both in the absence and in the presence of 2-OG (5.0 m*M*) with the increase being more pronounced for the former (Table 4). NADH-GOGAT activity was slightly decreased by Cd, however, inclusion of 2-OG, increased the enzyme activity in presence of Cd (Table 4). Substantial decrease in glutamine synthetase activity was caused by Cd, however, with the addition of 2-OG, the decrease in enzyme activity by Cd was less pronounced (Table 4).

Table 3—Effect of supply of $CdCl_2$ and 2-OG on ALAD activity and ALA content in excised maize leaf segments during greening

[Values are mean ± SE of 5 experiments]

Treatment	- $CdCl_2$	+ $CdCl_2$	% Inhibition	F value
***δ*-Amino levulinic acid dehydratase activity, nmole porphobilinogen formed h^{-1} g^{-1} fresh wt**				
- 2 OG	73 ± 05	45 ± 04	38	18.57 (4.32)
+ 2 OG	64 ± 02	35 ± 02	45	78.42 (4.27)
F value	2.55 (4.27)	4.26 (4.32)		
***δ*-Amino levulinic acid content, nmole amino levulinic acid g^{-1} fresh wt**				
- 2 OG	36 ± 05	11 ± 02	69	64.52 (4.35)
+ 2 OG	50 ± 06	32 ± 02	36	21.40 (4.35)
F value	0.86 (4.3)	10.81 (4.41)		

Critical variance ratio from F-distribution table at 5% level of significance is given in parentheses.

Table 4—Effect of supply of $CdCl_2$ and 2-OG on NADH-GDH, NADH-GOGAT and GS activities

[Values are mean ± SE of 4 experiments]

Treatment	- $CdCl_2$	+ $CdCl_2$	% Increase/ Decrease	F value
NADH-GDH activity, nmole NADH oxidized min^{-1} g^{-1} fresh wt				
- 2 OG	820 ± 370	939 ± 424	14↑	1.73 (4.96)
+ 2 OG	648 ± 298	701 ± 325	08↑	1.22 (4.96)
F value	8.96 (4.96)	2.01 (4.96)		
NADH-GOGAT activity, nmole NADH oxidized min^{-1} g^{-1} fresh wt				
- 2 OG	52 ± 09	51 ± 12	02↓	0.00 (7.70)
+ 2 OG	26 ± 13	56 ± 10	115↑	3.15 (7.70)
F value	2.74 (7.70)	0.07 (7.70)		
GS activity, nmole γ-glutamyl hydroxamate formed h^{-1} g^{-1} fresh wt				
- 2 OG	109 ± 14	62 ± 08	43↓	8.74 (4.30)
+ 2 OG	82 ± 08	69 ± 07	15↓	1.53 (4.30)
F value	2.94 (4.30)	0.36 (4.30)		

Critical variance ratio from F-distribution table at 5% level of significance is given in parentheses.

Discussion

The present results demonstrated a concentration dependent effect of Cd on chlorophyll formation, being stimulated by lower concentration and inhibited by higher concentration in the absence as well as presence of varying concentrations of 2-OG. Low

dose stimulation and high dose inhibition by Cd, termed as 'hormesis' has been observed in a number of organisms[19,20]. Further, the effect of Cd and 2-OG on chlorophyll formation is time-dependent. Thus, lesser degree of inhibition by Cd during earlier period of incubation indicated the time required for the absorption and accumulation of metal at the site of inhibition. Vacuolar accumulation of heavy metals after high dose exposure of plants has been suggested earlier[21]. Further, 2-OG appears to check the uptake and accumulation of Cd, as the per cent inhibition of chlorophyll formation due to Cd supply is least (12%) in the presence of 2-OG (Table 2) and increase in chlorophyll content by 2-OG is more pronounced at longer duration (Fig. 1). The experiments suggested that exogenously supplied 2-OG might be involved in affecting the levels of accumulated Cd by increasing vacuolar mobilization and thereby reducing the per cent inhibition.

Inhibitory effect of Cd on chlorophyll synthesis may involve inhibition of the enzyme(s) of chlorophyll biosynthetic pathway, thus, limiting the availability of various intermediates. In the present system, Cd seemed to be involved in limiting the availability of ALA, as inhibitory effect of Cd on ALA formation was more substantial than inhibition of ALAD activity (Table 3). However, inhibition of porphobilinogen synthase activity by Se without any effect on ALA synthesizing activity has been reported in mungbean seedlings[22]. In radish leaves, inhibition of ALAD activity by Cd has been reported[9]. Supply of 2-OG increased ALA content and also reduced the inhibitory effect of Cd on it, while ALAD activity reduced (Table 3). Hence, it was likely that 2-OG favoured ALA formation being a precursor of it and inhibited ALAD activity due to excessive ALA accumulation.

Cadmium affected the key enzymes of N assimilation utilizing 2-OG and maintaining glutamate supply in different manner. Thus, NADH-GDH activity was increased by Cd in the absence as well as presence of 2-OG (Table 4). Increase in NADH-GDH activity has been reported under Cd stress in other systems also[23-25]. Parallel increase in NADH-GOGAT and NADH-GDH activities due to Cd toxicity has also been reported in *Lycopersicon esculentum*[26]. However, in the presence of 2-OG, prominent increase in NADH-GOGAT activity by Cd and also less pronounced inhibition of GS activity by Cd suggested that glutamate for ALA synthesis could be furnished by NH_4^+ assimilation via GS/GOGAT pathway rather than GDH pathway, under condition of Cd toxicity and 2-OG supplementation.

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