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Cadmium Toxicity and Calcium Effect on Growth and Photosynthesis of Tobacco

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This investigation was performed to study Cd toxicity and the influence of Ca on Cd toxicity in growth, and photosynthetic pigments and enzymes in tobacco. Cd inhibited both growth and level of chlorophyll, but the inhibition was compensated by the treatment of Ca. Especially, chlorophyll content was significantly increased by the combination of Cd and Ca treatment compared with Cd treatment alone. In addition, activity and content of rubisco by Cd treatment was also significantly lesser than the non-treated control. The highly reduced activity of rubisco was minimized by the combined treatment of Ca to Cd. Rubisco activase activity and content also showed a pattern of change similar to the rubisco level, suggesting that Cd- and Ca-induced changes of rubisco could be caused by rubisco activase.

Key words – cadmium, calcium, rubisco, rubisco activase, tobacco

Cadmium (Cd) has been widely used in many industrial processes. However, the high concentration of Cd has been also recognized as one of the most phytotoxic heavy-metal contaminants[33] due to its irreversible replacement of other metal ions in essential metalloenzymes[15]. These metal ions enter plant through the root system and delivered to aerial organs where they can accumulate mainly in the vacuoles to high levels, and then may cause a range of morphological and physiological disorders in plants[3]. In addition, Cd is known to modulate the activity of enzymes [45], induce changes in lipid composition and the distribution of macro and micronutrients[27], induce stress responses[4], and stimulate the oxidative burst in plants[26].

Calcium (Ca) is known to be an essential element for higher plants. Plants growing with adequate Ca in their natural habitats have shoot Ca concentrations between 0.1 and 5% d. wt. It is required for the stabilization of cell walls and membranes, as acounter-cation for inorganic and organic anions in the vacuole[22]. Moreover, Ca acts as not only an important signal in normal growth and developmental processes[35], but also an intracellular messenger in plants responding to biotic and abiotic stresses such as cold, high salinity and drought[44], increasing their adaptability to environmental changes[49].

Divalent cation (Ca^{2+}) is a major form of Ca taken up by higher plants from soil, and it translocates into shoot and leaves *via* xylem[58]. It may also traverse the root either through the cytoplasm of cells linked by plasmodesmata or through the spaces between cells[6]. Ca enters plant cells through Ca^{2+} - permeable ion channels in their plasma membrane[57].

Ca was disrupted in the presence of copper in bean plants[20]. It has been shown to ameliorate the adverse effects of salinity on wheat and barley[11]. Navarro *et al.* [25] has shown the effect of Ca on tomato grown under saline condition to investigate reduction of the negative effect by NaCl. It has been also examined that it plays a role in detoxification of Cd in higher plants[61]. Ruan *et al.*[40] investigated the effect of Ca on Fe uptake by tea plants. Sharma *et al.*[46] also reported the interference between Fe nutrition and Cd toxicity in barley seedlings grown in hydroponic culture. However, both Cd toxicity and detoxification of Cd by Ca on the photosynthesis have not been fully understood at CO_2 fixation enzyme level.

To study the toxic effect of Cd and the detoxification effect of Ca on growth and photosynthesis, chlorophyll and rubisco/rubisco activase level in tobacco was subjected after inducing from the stem of seedling *in vitro*.

Materials and Methods

Growth of Tobacco from Shoot Explant

Tobacco (*Nicotiana tabacum* L.) seeds sterilized in 70% (v/v) ethanol and 3% (v/v) sodium hypochlorite solution were germinated and grown aseptically in cell culture vessel containing MS[24] agar (0.8%) medium in the dark at 26± 1℃. Four week-old shoots were cut into 2 cm segments and used as explants. Five explants per petri dish were placed on an induction MS medium supplemented with and

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without Cd (0.2 mM $CdCl_2 \cdot 2.5H_2O$), and Cd+Ca (30 mM $CaCl_2 \cdot 2H_2O$). MS medium contained 3 mM $CaCl_2 \cdot 2H_2O$. These explants were maintained for 3 weeks on these media at 26±1℃ under a 16-h light (800 μM/m^2/s PFD) and 8-h dark photoperiod as described previously[39]. Fully expanded leaves from mature plants were used for experiments. All experiments were independently duplicated.

Chlorophyll Measurement

Frozen leaves were transferred to DMF and stored at 5℃ in darkness. Extracts were centrifuged for 5 min at 8,000*xg*. Chlorophyll contents in the supernatants were measured spectrophotometrically using its specific absorption coefficients at 664.5 nm and 647 nm[14].

Purification of Rubisco and Rubisco Activase

Rubisco and rubisco activase were purified from leaves of tobacco grown *in vitro*[56]. Frozen leaf tissue was pulverized in a mortar under liquid nitrogen and then extracted in the extraction buffer containing 50 mM BTP (pH 7.0), 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 1 mM EDTA, 0.5 mM ATP, 10 mM DTT, 1 mM PMSF, 1 mM benzamidine, 0.01 mM leupeptin, 1.5% PVPP and 3 mM MBT. The leaf slurry was filtered through four layers of cheesecloth and one layer of Miracloth. Filtered solution was centrifuged at 30,000*xg* for 40 min. $(NH_4)_2SO_4$ powder was slowly added into the supernatant to 35% saturation and stirred for 30 min. The supernatant and pellet were collected by centrifugation at 8,000*xg* for 10 min. The supernatant contains rubisco, and the re-suspended pellet contains rubisco activase.

The collected supernatant was brought to 55% saturation of $(NH_4)_2SO_4$ by addition of powder. The collected pellet by centrifugation at 8,000*xg* for 10 min was re-suspended in 5 mL of 20 mM BTP (pH 7.0) containing 0.2 mM ATP, 10 mM $MgCl_2$ and 2 mM MBT (buffer A), and 50% PEG-10K was added to a final concentration of 18%. The resulting precipitate was collected by centrifugation at 8,000*xg* for 10 min and re-suspended in buffer A.

Re-suspended solution was loaded onto a Q-Sepharose column equilibrated with 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 10 mM $NaHCO_3$. The column was washed with the same buffer containing 0.1 M NaCl before starting elution with a linear gradient from 0.1 to 0.5 M NaCl at a flow rate of 1 mL/min. 3 mL fractions were pooled, and assayed for rubisco activity and contents.

50% (w/v) PEG-10K was added into the buffer A re-suspended pellet obtained above to the final concentration to 18%, stirred 5 min, and centrifuged at 8,000*xg* for 10 min. The pellet was dissolved in 5 mL of buffer A. Solution was cleared by spinning at 20,000*xg* for 10 min. Pellet was re-suspended again in 5 mL buffer A and the solution cleared again.

The collected supernatants were loaded onto a 20 mL Q-Sepharose column equilibrated with 20 mM BTP (pH 7.0). The column was eluted with 40 mL of 20 mM BTP (pH 7.0) at a flow rate of 1 mL/min before continuing with 140 mL of a linear gradient from 0 to 0.5 M NaCl in 20 mM BTP (pH 7.0). Three mL fractions were pooled, and assayed for rubisco activase activity and contents.

All purification processings were done at 4℃ except as indicated.

Assays of Rubisco and Rubisco Activase Activity

Rubisco activity was determined spectrophotometrically at 25℃ by monitoring NADH oxidation at 340 nm[34]. The assay medium contained 1 M Tris buffer (pH 7.8), 0.006 M NADH, 0.1 M GSH, 0.5% glyceraldehyde-3-phosphate dehydrogenase, 0.025 M 3-phosphoglycerate kinase, 0.05% α-glycerophosphate dehydrogenase-triose phosphate isomerase, 0.025 M RuBP, 0.2 M ATP, 0.5 M $MgCl_2$, 0.5 M $KHCO_3$, and purified rubisco solution in a final volume of 1 mL. One unit of enzyme was defined as the amount of enzyme producing 1 μM of RuBP per min.

Rubisco activase activity was determined as the ability to produce ADP in an ATP-dependent reaction in absorption at 340 nm[36]. The purified rubisco activase solution was added to a total volume of 0.4 mL of the activation reaction mixture containing 50 mM Tricine (pH 8.0), 20 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 1 mM phospho*enol*pyruvate, 0.3 mM NADH, 40 units/mL pyruvate kinase, and 40 units/mL lactate dehydrogenase. One unit was defined as the amount that catalyzed the cleavage of 1 μM ATP per min.

ELISA

The content of rubisco and rubisco activase were detected by ELISA. For coating of the antigen, 100 μL of different dilutions of two enzymes in 0.1 M sodium carbonate-bicarbonate coating buffer (pH 9.5) was added to each well of microplate. After overnight incubation at room temperature, the plate was washed with 0.01 M PBS (pH 7.4) containing 0.05% Tween 20. To eliminate nonspecific binding, 250 μL of 0.1% BSA in 0.01 M PBS (pH 7.4) was added to each

well and incubated for 1 hr at 37℃. After washing and adding of 50 μL of various dilutions of two enzymes in 0.01 M PBS (pH 7.4), 50 μL of different dilutions of a rabbit anti-rubisco and anti-rubisco activase antiserum as a primary antibody[38] was added to each well, and incubated for 30 min at 37℃. The plate was washed again as described above, then 100 μL of peroxidase-conjugated goat anti-rabbit IgG diluted to 1:20,000 in 0.01 M PBS (pH 7.4) containing 0.1% BSA was added and incubated for 30 min at 37℃. The plate was washed as previously described and 100 μL of peroxidase substrate [OPD tablets in 10 mL of 0.05 M citrate/0.1 M sodium phosphate buffer (pH 5.0) containing 30% of H_2O_2] was added. After incubation at room temperature in the dark for 20 min, the reaction was terminated by addition of 0.1 mL of 1 N HCl. Absorbance at 490 nm was determined by an ELISA microplate reader (Bio-Rad Model 3550-UV).

Results and Discussion

Growth

The optimum concentrations of Cd and Ca were determined in our preliminary study as 0.2 mM $CdCl_2 \cdot 2.5H_2O$ and 30 mM $CaCl_2 \cdot 2H_2O$.

Shoot explants of tobacco were grown on an induction MS medium (containing 3 mM $CaCl_2 \cdot 2H_2O$) supplemented with and without Cd, and Cd+Ca for 3 weeks. In comparison with non-treated control, the growth of leaves and stems was much decreased in plants treated by Cd alone, but minor decrease was observed in plants treated by Cd+Ca combination. Leaf chlorosis is one of the most commonly observed phenotype of Cd toxicity[60]. In addition to the growth, number of yellow leaves was also significantly decreased by Cd+Ca combined treatment compared with Cd treatment alone (Fig. 1 upper). Similar results were also observed in the root (Fig. 1 lower), and Ca seemed to lead better development according to the well known regulatory roles in root growth of cotton[8]. These suggest that Ca could play a role in detoxifying the effects of Cd shown in declining growth together with yellowing of leaves.

To verify the contribution of Ca to the growth (Fig. 1), the weight and length of both stem and root were determined. The fresh weight by Cd treatment alone was greatly decreased than the non-treated control whereas the addition of Ca restored the increase of fresh weight. A similar pattern was also observed in the length of stem and root as shown in Table 1. These data suggest that Cd reduced growth due to stems and roots, and that growth inhibition by Cd was recovered by Ca. These results also seem to indicate that Ca is able to act as a detoxidant against Cd stress, or triggers a number of cellular processes that influence growth. These data were in agreement with those of Choi *et al.*[5] who indicated that plants positively take up and utilize Ca for Cd detoxification. The absorbed Cd can be considered as a

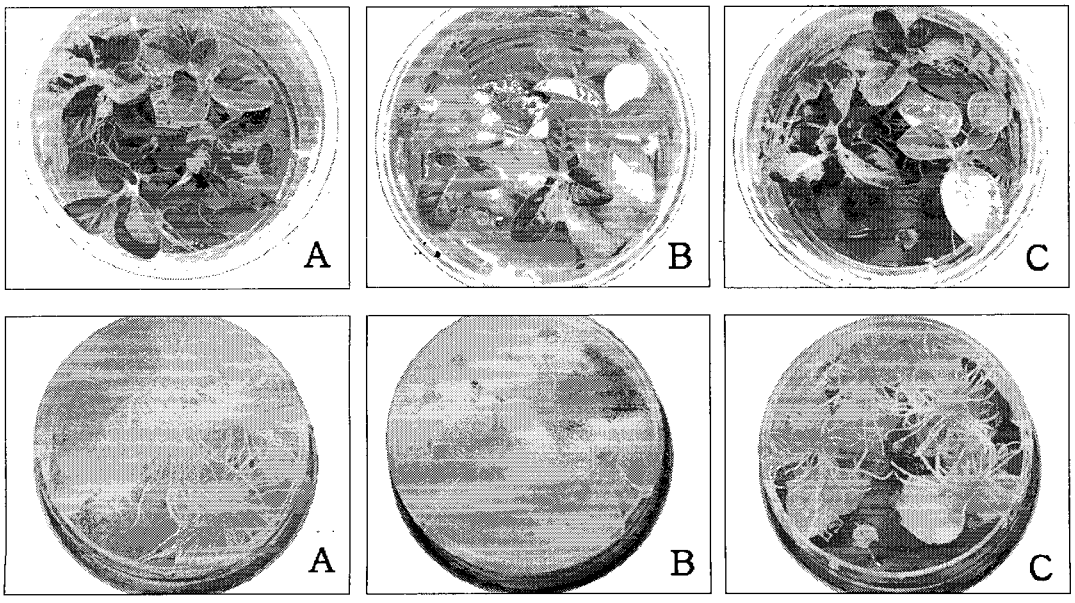

Fig. 1. Influence of cadmium and calcium on leaves and shoots (upper), and roots (lower) growth of tobacco *in vitro*. Plants were grown on MS medium without Cd (A), with 0.2 mM Cd (B), and with 0.2 mM Cd plus 30 mM Ca (C) for 3 weeks, respectively.

Table 1. Effects of cadmium and calcium on fresh weight, and length of shoot and root in tobacco.

Treatment	Fresh weight (g)	Length (cm)	
		Shoot	Root
Control	1.620	4.32	3.04
0.2 mM Cd	1.221	3.40	1.18
0.2 m Cd + 30 mM Ca	1.383	3.66	2.28

Plants were grown for 3 weeks *in vitro*.

Table 2. Effects of cadmium and calcium on chlorophyll content in tobacco leaves.

Treatment	Chl. a	Chl. b	Chl. a/b	Total chl.
	(mg/g fr. wt)			
Control	16.82	15.44	1.06	31.76
0.2 mM Cd	5.29	5.76	0.92	11.05
0.2 mM Cd + 30 mM Ca	7.16	8.78	0.82	15.94

Plants were grown for 3 weeks *in vitro*.

toxic element leading to growth inhibition[28]. Clarkson and Lüttge[7] observed that Cd interfere with the root uptake of Ca by competition for the membrane channel. Ca is actively secreted together with Cd[5]. Unlike, when excessive Ca is present in the rhizosphere solution, plants may suffer Ca tocixity. This may present the germination of seeds and reduce plant growth rate[59]. Lauchli[18] have proposed that an increase in the concentration of Ca can often ameliorate the inhibitory effects on growth to salt stress.

Chlorophyll

Chlorophyll content was determined from leaves of tobacco treated with and without Cd, and Cd+Ca. Chlorophyll *a* and *b* content, chlorophyll *a/b* ratio, and total chlorophyll at the non-treated control was significantly higher than those at Cd treatment and Cd+Ca treatment. The most significant inhibition was found by Cd treatment (Table 2), and it could be due to inhibition of aminolevulinic acid synthesis[52] and of protochlorophyllide reduction[2], leading to destruction of chlorophyll, and of a direct action on the pigment-protein complexes of the photosystems[16]. Dubé and Bornman[10], and Larsson *et al.*[17] observed a decrease in chlorophyll content in the presence of Cd in leaves of spruce and oilseed rape, respectively. Sandalio *et al.*[43] also found that chlorophyll concentration in leaves of pea were reduced by Cd.

Chlorophyll *a* and *b* content was significantly increased by Cd+Ca treatment compared with Cd treatment alone. In addition, total chlorophyll at Cd+Ca treatment was also increased as shown in Table 2, suggesting that inhibition by Cd was modified by Ca addition. These data imply that Cd toxicity and detoxification by Ca could be mediated by a reduction and induction of chlorophyll level. Therefore, Cd has a negative effect on chlorophyll content, while Ca has a positive effect against Cd toxicity. In contrast to our results, however, Milivojevic and Stojanovic[41] reported that Ca had no effect on the reduction of chlorophyll *a* and *b* content by alumium toxicity in soybean leaves. Copper excess reduces the quantum yield of PSII photochemistry assessed by chlorophyll fluorescence[21].

Rubisco and Rubisco Activase

For effect of Cd and Ca on rubisco, it was purified from leaves of tobacco grown on an induction MS medium supplemented with and without Cd, and Cd+Ca by anion exchange chromatography on Q-Sepharose column (data not shown). Its activity and content were detected using the fraction with the highest activity.

Rubisco activity was reduced in Cd and Cd+Ca treated plants compared with the non-treated control (Fig. 2). These results agree with those of Stiborova *et al.*[51] who showed that rubisco activity was significantly decreased by Cd. The inhibition of rubisco activity has been considered to be the primary plant response to Cd stress[48]. Cd is a potent

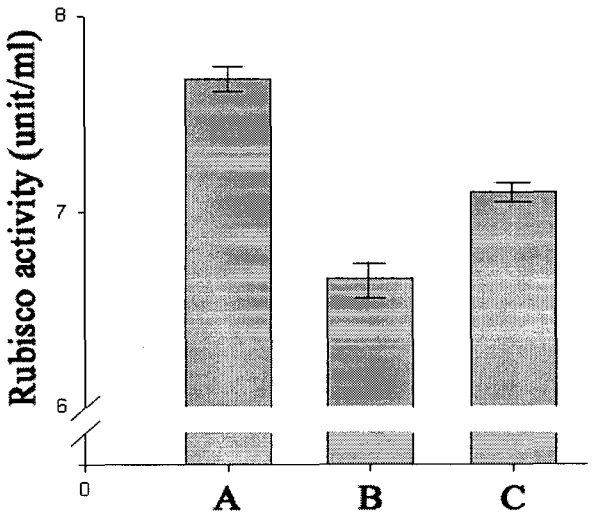

Fig. 2. Effects of cadmium and calcium on the activity of rubisco in tobacco leaves. Plants were grown on MS medium without Cd (A), with 0.2 mM Cd (B), and with 0.2 mM Cd plus 30 mM Ca (C) for 3 weeks, respectively.

inhibitor on the photochemical activity of photosynthesis [12,47]. It was suggested that Cd could irreversibly bind to SH group on the active site of rubisco, thus lowering its activity[50]. Van Assche and Clijsters[54] also reported that metal ions might directly interfere with the metabolic activities by altering the conformation of enzymes owing to their strong affinities as ligands to carboxylic group. In addition, the inhibition of photosynthesis in Cu-stressed cucumber leaves is more likely a consequence of an altered source-sink relationship, rather than due to toxic effects of copper on the photosynthetic apparatus[55].

The rubisco activity was highly reduced in the plants treated only with Cd but a little reduced when Ca was added to Cd (Fig. 2). These data suggest that the Cd-induced inactivation of rubisco could be modulated by Ca. The modulation by Ca might be caused by blocking Cd binding to rubisco *via* irreversible combining with rubisco after converting Ca to a very reactive compound or by competing between Cd and Ca for binding on the active site of rubisco. The inactivation of rubisco is likely to lower the photosynthetic activity[1]. In the report of Pierce[30], magnesium plays a key role in the modulation of RuBP carboxylase in the stroma of the chloroplasts.

In comparing with the non-treated control, the treatment of Cd decreased the content of rubisco detected by immunological assay. However, the combining treatment of Ca to the Cd increased its content compared to that in plants exposed only to Cd (Fig. 3). Decrease or increase of rubisco content means a change of rubisco induction. Therefore, these results indicate that the inhibition of rubisco by Cd was recovered by Ca. The similarity between content and activity pattern of rubisco suggests that content was associated with the activity of rubisco protein[9], and that induction and activation were directly correlated with Cd and Ca. The activation of rubisco involves formation of a carbamate which is binding of an activator CO_2 to the ε -amino group of lys-201 within the active site on the large subunit, followed by addition of Mg^{2+}[19]. Pankovic *et al.*[29] have reported that Cd affected the RuBP regeneration capacity of the Calvin cycle. The multiplicity of Cd effects on the photosynthesis can be explained by the variety of plant species and the concentration of Cd.

The activation of rubisco *in vivo* is mediated by rubisco activase[42] in the presence of ATP[53] and RuBP[31]. This action serves to "activate" rubisco by freeing the rubisco active site of compounds that either hinder carbamylation

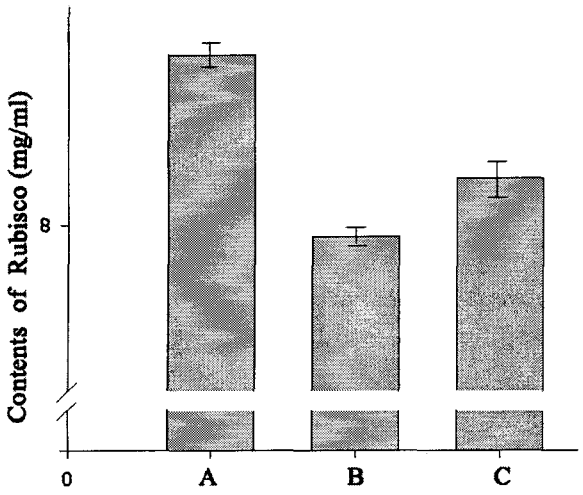

Fig. 3. Effects of cadmium and calcium on the content of rubisco in tobacco leaves. Plants were grown on MS medium without Cd (A), with 0.2 mM Cd (B), and with 0.2 mM Cd plus 30 mM Ca (C) for 3 weeks, respectively.

of the activator lysine or block RuBP from binding to the carbamylated rubisco[13]. In the absence of rubisco activase, only 20 to 40% of rubisco catalytic sites are carbamylated, leading to a significant inhibition of photosynthesis[31,41]. Therefore, the carbamylation of rubisco was inhibited under the condition in which RuBP was present but rubisco activase was absent[37]. Because of these reasons, we postulated that Cd- and Ca-induced changes of rubisco activity and content might be related to rubisco activase. The rubisco activase activity at Cd treatment was more decreased than that at the non-treated control. The combination of Cd and Ca increased its activity compared to that in plants exposed only to Cd (Fig. 4). A similar change patterns were also observed in content of rubisco activase (Fig. 5). As a result of the findings in our work, the effects in rubisco activase are similar to that found in rubisco. Therefore, it proposes that Cd- and Ca-induced changes of rubisco activity and content are caused by the level of rubisco activase. Regulation of rubisco activase in species containing both isoforms can occur via *redox* changes in the carboxy-terminus of the larger isoform, mediated by thioredoxin-f, which alters the response of activase to the ratio of ADP to ATP in the stroma[32].

In conclusion, Cd may depress growth due to inhibition of chlorophyll synthesis depending upon a direct action on enzymes of the chlorophyll biosynthesis. Rubisco levels were reduced by Cd, and Cd tocixity was recovered by detoxification effect of Ca. Ca reduced the negative effect of Cd stress, possibly through the activation and induction in rubisco activase leads to a large change in rubisco activity

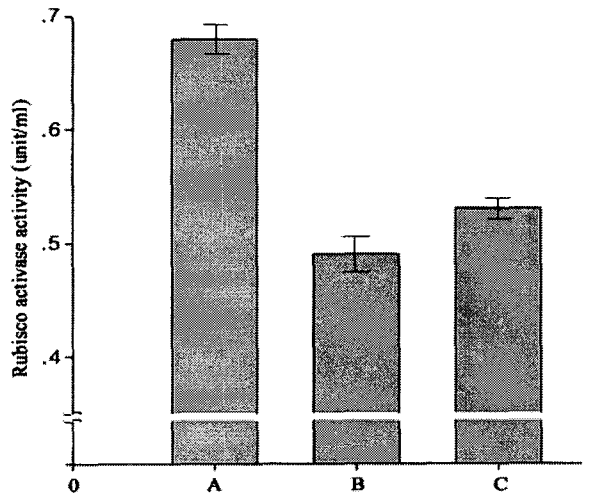

Fig. 4. Effects of cadmium and calcium on the activity of rubisco activase in tobacco leaves. Plants were grown on MS medium without Cd (A), with 0.2 mM Cd (B), and with 0.2 mM Cd plus 30 mM Ca (C) for 3 weeks, respectively.

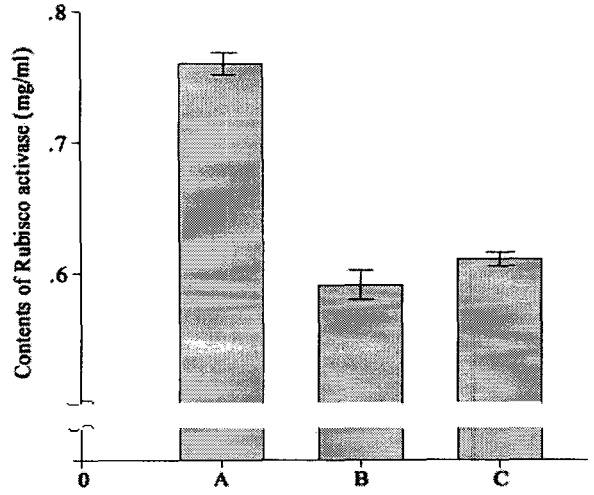

Fig. 5. Effects of cadmium and calcium on the content of rubisco activase in tobacco leaves. Plants were grown on MS medium without Cd (A), with 0.2 mM Cd (B), and with 0.2 mM Cd plus 30 mM Ca (C) for 3 weeks, respectively.

and content, leading to a processing of growth and photosynthesis.

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초록 : 담배의 생장과 광합성에 미치는 카드뮴의 독성과 이에 대한 칼슘의 효과

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담배의 생장과 광합성 색소 및 효소에 대한 카드뮴의 독성과 이에 대한 칼슘의 영향을 연구하였다. 카드뮴에 의한 생장과 엽록소 수준에서의 억제 현상은 칼슘에 의해 회복되었다. 특히, 엽록소의 함량은 카드뮴 만 처리한 것 보다 카드뮴과 칼슘을 같이 처리하였을 때 현저하게 증가하였다. 또한 rubisco의 활성과 함량은 카드뮴을 처리한 것 보다 처리하지 않았을 때 현저하게 감소하였으며, 카드뮴의 이러한 효과는 칼슘에 의해 환원되었다. Rubisco activase의 활성과 함량도 rubisco와 같은 경향을 보였다. Rubisco에서 카드뮴과 칼슘에 의해 유도되는 이와 같은 변화는 rubisco activase에 의해 기인됨을 의미한다.