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Effects of Polyamines on Chlorophyll and Protein content, and δ-Aminolevulinate Dehydratase Activity in Greening Mung Bean Cotyledons

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Abstract

Effects of polyamines on chlorophyll and protein content, and δ-aminolevulinate dehydratase(ALAD) activity were investigated during the greening of mung bean cotyledons. Polyamines stimulated chlorophyll formation in greening cotyledons, and this effect was enhanced by KCl. The changes in protein content were similar to the changes for chlorophyll content. The excision entailed an increase in ALAD activity. Then a decrease appeared after 48 h incubation on water in the dark. It was more precocious in the light, but was accelerated when the cotyledons were illuminated after a dark preincubation. Putrescine had little effect on ALAD activity in the dark. In the light, putrescine prevented the decrease in ALAD activity and enhanced this activity when a dark preincubation preceded illumination. KCl had a slight stimulating effect in the dark, but was uneffective in the light. The combination putrescine+KCl was devoid of stimulating effect. The results obtained suggest that plastid development of mung bean cotyledons during greening was affected by polyamines and light and that polyamines may play a role in the regulation of plastid development.

Key Words : Chlorophyll Content, Cotyledon, δ-Aminolevulinate Dehydratase, Mung Bean, Polyamines, Potassium.

1. Introduction

Polyamines putrescine, spermidine and spermine occur in all higher eukaryotes and throughout the plant kingdom(Pegg and McCann, 1982; Smith, 1985). In plants, polyamines are involved in the regulation of growth and developmental processes(Smith, 1981; Slocum *et al.*, 1984; Feirer *et al.*, 1984; Galston and Kaur-Sawhney, 1987; Evans and Malmberg, 1989), and in cell response to stress(Priebe *et al.*, 1978, Young and Galston, 1983; Bors *et al.*, 1989; Villanueva and Santerre, 1989; Santerre *et al.*, 1990). A possible function of polyamines as hormones(Bagni, 1986; Phillips *et al.*, 1987; Masse *et al.*, 989), candidates for active regulators of plant growth(Galston, 1983), or secondary messengers that mediate phytohormone effects(Smith, 1985) have been suggested, but such a role has not been unequivocally demonstrated.

Polyamines stimulate the growth of several higher plants, suggesting that the endogenous concentrations of these amines can be growth limiting(Galston, 1983; Slocum *et al.*, 1984; Smith, 1985; Bagni, 1986; Mirza and Bagni, 1991). Application of exogenous polyamines inhibits or retards the rise in RNase and protease

activities, and a loss of chlorophyll(Cohen *et al.*, 1979; Kaur-Sawhney and Galston, 1979; Altman, 1982a; Pjon *et al.*, 1990), and induce DNA synthesis and limited protoplast mitosis (Kaur-Sawhney *et al.*, 1980). Exogenous polyamine also induce the decrease of RNase activity and solute leakage in wounded storage tissues (Altman, 1982b).

Polyamines are polycations that may easily bind to polyanions as DNA, RNA or phospholipids, therefore they can affect mitosis, meiosis and membrane permeability(Grimes *et al.*, 1986; Galston and Kaur-Sawhney, 1987). They can interact with phospholipids in the membrane bilayer and possibly stabilize cell membrane (Roberts *et al*, .1986). Altered levels of polyamines and activities of their biosynthetic enzymes have been associated with changes in growth and development in numerous plants (Evans and Malmberg, 1989; Flores *et al.*, 1989; Galston and Kaur-Sawhney, 1990). Although there is some evidence indicating that polyamines are essential for organized development in plants(Kumar and Thorpe, 1989; Tiburcio *et al.*, 1989), the role of endogenous polyamines in organ induction remains ambiquous.

During the development of chloroplasts of higher plants chlorophyll accumulation are regulated by many factors, such as light, temperature, growth regulators and age of the tissues. Cytokinines have been reported to stimulate a number of parameters related to greening, such as chlorophyll synthesis, δ -aminolevulinic acid(ALA) synthesis(Fletcher *et al.*, 1973), ALA dehydratase(ALAD) activity and rate of protochlorophyllide regeneration(Stobart *et al.*, 1972), and protein synthesis of plastid membrane(Ohya *et al.*, 1980). However, there have been little systematic investigations on the effect of exogenous application of various factors on greening and their interaction. Moreover, there have been little information on the effect of polyamines on chloroplast development involved in chlorophyll and protein synthesis and enzyme activity during greening process.

The present study was designed to elucidate the effects of polyamines on the chlorophyll and protein content, and enzymatic properties of ALAD during the greening of mung bean cotyledons.

2. Materials and Methods

2.1. Growth conditions and treatments

Seeds of mung bean(*Vigna radiata* Wilczek) obtained from a local dealer were sterilized with 1% sodium hypochlorite solution for 15 min, rinsed thoroughly with sterile distilled water and then soaked for 12 h in distilled water in the dark. The seedlings were harvested at daily intervals and the cotyledon and embryonal axis were separated. For the polyamine biosynthetic inhibitor studies, D-arginine, an competitive inhibitor of arginine decarboxylase(ADC), and methyl-glyoxal *bis*-guanylhydrazone dihydrochloride(MGBG), an inhibitor of S-adenosylmethionine decarboxylase(SAMDC), were added individually. To determine whether the effect of the inhibitors was reversible, the polyamines were added with the inhibitors.

For the greening experiments, mung bean seeds were grown in vermiculite moistened with distilled water in the dark at 26°C. After germination for 4 days, the cotyledons were excised from etiolated seedlings under a dim green safe light and placed in Petri dishes containing filter disks moistened with distilled water or test solutions. The cotyledons were incubated in all test solutions for 18 h at 26°C in the dark and subsequently exposed to white fluorescent light

with an intensity of 2, 000 lux for 24, 48 and 72 h.

After illumination cotyledons were thoroughly rinsed with distilled water, then blotted, weighed and stored at -20°C until used.

2.2. Chlorophyll determinations

Each preweighed cotyledon was homogenized with a mortar and pestle in 80%(v/v) acetone and the homogenate was centrifuged at 10, 000 x*g* for 15 min.. These extraction procedures were performed at 0-4°C. The supernatant was made up 10ml with acetone and the absorbances of the extracts at 663 and 645 nm were determined using a spectrophotometer(Shimadzu, CS 260). Chlorophyll content was calculated according to the method of Arnon (1949) and was expressed in μg per g. fresh weight.

2.3. Protein determinations

The precipitate fraction after the chloropyll determinations was added ice-cold 10% trichloroacetic acid and treated with 1N NaOH at 80°C for 10 min. and centrifuged at 12,000 x*g* for 10 min.. The supernatant was diluted with distilled water and the amount of protein in each samples was estimated by the method of Lowry *et al.*(1951) using bovine serum albumin as a standard.

2.4. ALAD assay

The cotyledons were homogenized with a prechilled mortar and pestle in 5 ml of 50 mM Tris-HCl buffer(pH 8.2) containing 0.1 mM dithiothreitol(Naito *et al.*, 1980). The homogenate was centrifuged at 27, 000 x*g* for 20 min at 0°C. The supernatant was assayed for enzyme activity.

For the determination of ALAD activity, 1 ml of extract was incubated with 0.3 ml of 1 mg·ml^{-1} ALA, 1.3 ml of 50 mM Tris-HCl buffer (pH 8.2) containing 0.1 mM dithiothreitol and 0.8 ml of 0.2 mM $MgCl_2$ for 2.5 hr at 37°C(Naito *et al.*, 1980). The enzymatic reaction was stopped with 0.3 ml of 3M trichloroacetic acid containing 0.1 M $MgCl_2$(Schneider, 1970). Samples were then centrifuged at 400 x*g* for 10 min. PBG formed in the supernatant was measured according to Mauzerall and Granick (1956): 1 ml of supernatant was mixed with the same volume of a modified Ehrlich's reagent. The absorbance at 555 nm was determined after 15 min and the concentration of PBG was calculated using an extinction coefficient of 61 mM^{-1}·cm^{-1}. ALAD activity was expressed in nmole PBG formed per h and per cotyledon.

3. Results

3.1. Effect of polyamines on chlorophyll and protein synthesis

The effect of hypocotyls on the greening in cotyledons was observed in different situation (Fig 1). A group of excised cotyledons was preincubated with or without polyamines for 18 h in the dark, and another group of samples was not preincubated in the dark. In a third group, intact seedlings were used. These three groups of 4-day-old cotyledons or seedlings were illuminated continuously at the same time.

The rate of chlorophyll formation was lowered in both types of excised cotyledons, especially when the period of illumination exceeded 6 h. When excised cotyledons were preincubated in the dark, chlorophyll formation during the subsequent continuous illumination was considerably depressed. By contrast, rapid and large

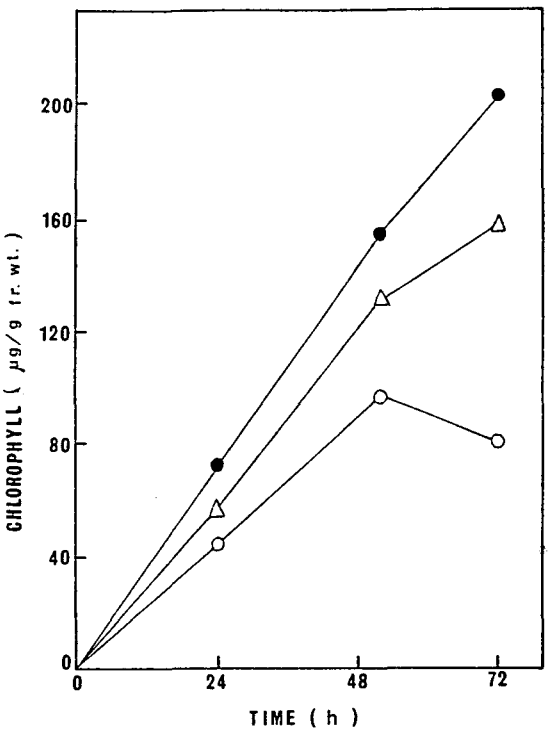

Fig.1. Time coures of chlorophyll formation during continuous illumination in attached cotyledons (●) and excised cotyledons preincubated in the dark for 18 h (O) or without preincubation in the dark(△).

chlorophyll accumulation continued at least for 72 h in the cotyledons attached to the seedlings. The patterns of chlorophyll content in excised cotyledons without dark preincubation were similar to those in attached cotyledons.

When 4-day-old etiolated mung bean cotyledons were pretreated with polyamines for 18 h in the dark and exposed to light for 72 h, it was observed that the total chlorophyll content in excised cotyledons without dark preincubation were similar to those in attached cotyledons.

When 4-day-old etiolated mung bean cotyledons were pretreated with polyamines for 18 h in the dark and exposed to light for 72 h, it was observed that the total chlorophyll content was dose-dependent and decreased with increasing polyamine concentration(Table 1). In the present study, when cotyledons were incubated in the presence of low concentrations of polyamines, chlorophyll formation was enhanced. Concentration ranging from 0.1 to 1 mM of polyamines had a tendency towards stimulation, but became inhibitory at 10 mM of polyamines. The data shows that polyamines give the maximum stimulation of chlorophyll synthesis at 1 mM concentration. This indicates the chloroplasts become almost fully developed under the conditions employed.

Table 1. Effect of polyamines on chlorophyll and protein content in excised cotyledons exposed to light after 18 h dark preincubation

Treatment	Conc.	Chl content(μg/g fr.wt.)			Protein content(mg/g fr.wt.)		
		h			h		
	(mM)	24	48	72	24	48	72
Control	0	43	93	80	30	36	35
Putrescine	0.1	47	90	78	25	32	34
	1.0	45	106	95	36	38	37
	10.0	32	75	63	35	39	36
Spermidine	0.1	43	95	82	29	33	30
	1.0	49	110	98	36	38	39
	10.0	40	74	72	30	32	36
Spermine	0.1	42	102	94	34	37	38
	1.0	50	113	102	33	38	40
	10.0	38	66	60	32	36	34

When the cotyledons were incubated in low concentrations of polyamines, cotyledons appeared much greener than the controls, while browning of the cotyledons appeared at the high concentrations. The concentration of 1mM for polyamines were applied in all further experiments. The diamines, as well as the triamine and the tetraamine were all active in chlorophyll formation. Spermine was more effective than putrescine and spermidine at 1 mM concentration.

The changes in protein level in the cotyledon tissue were similar to the changes occurring in the cotyledons for chlorophyll contents. Considerable increase of protein was also found after

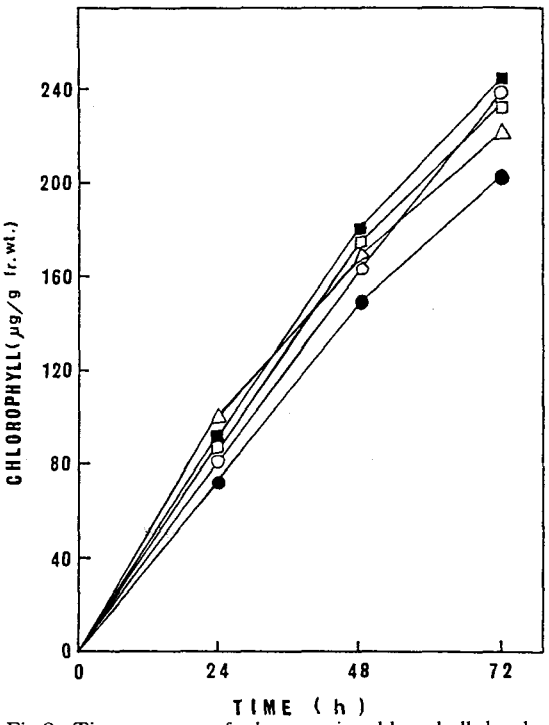

Fig.2. Time course of changes in chlorophyll levels in attached cotyledons exposed to light for 72 h. ● , control; O, putrescine; ■, cadaverine; □ , spermidine; △ , spermine.

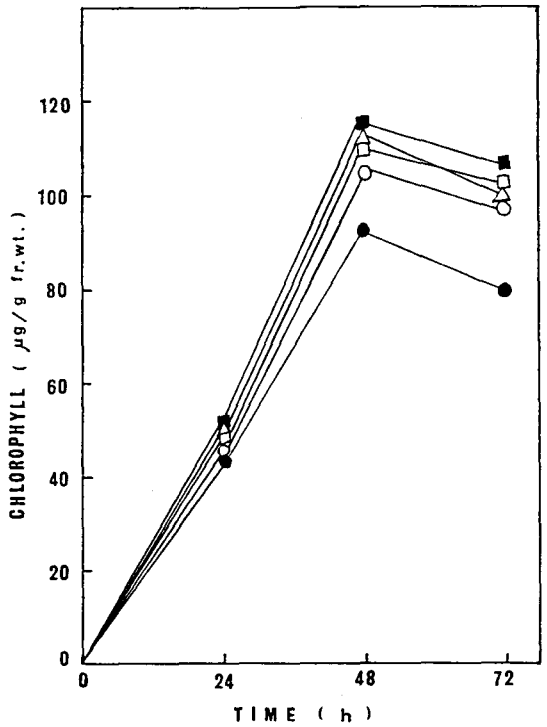

Fig.3. Time course of changes in chlorophyll levels in excised cotyledons exposed to light after 18 h dark preincubation. ●, control; O , putrescine; ■, cadaverine; □, spermidine; △, spermine.

48 h of exposure to light, indicating that in the polyamine-treated cotyledons the protein-synthesizing mechanisms were operating at maximum efficiency during this period. At all times the level of proteins in polyamine-treated cotyledons at 1 mM polyamines was higher than the controls.

In a time course study, the effect of polyamines was further elucidated by following the kinetics of chlorophyll for up to 72 h of greening(Fig. 2). Chlorophyll levels in the intact cotyledons rose sharply during whole period of the excised cotyledons preincubated with polyamines in the dark continued to rise with the onset of greening up to 48 h. From the 2 days after illumination, the chlorophyll content remained constant or decreased slightly, indicating that the chlorophyll became almost fully developed on the 3rd day under the conditions employed(Fig. 3)

Under the present incubation conditions, the time lag for chlorophyll synthesis were 2 to 3 h and was the same for three types of specimens (data not shown). After a lag phase the cotyledons accumulated chlorophyll in a linear manner. During this period the chloroplasts are differentiating and synthesizing the necessary enzymes for chlorophyll synthesis and photosynthesis. Treatment of polyamines reduced the lag time before appearance of the stimulating effect.

The effects of KCl(40 mM) on the fresh weight and chlorophyll synthesis of cotyledons incubated in the light were determined. The results obtained when polyamines, KCl or their combination were added are summerized in

Table 2. Effect of polyamines and KCl alone and in combination on fresh weight and chlorophyll levels in excised cotyledons exposed to light for 48 h after 18 h dark preincubation.

Treatment	Fresh weight (mg/cotyledon)	Chl content (μg/g fr.wt.)
Control	61.8	92.7
KCl	70.2	94.3
Putrescine	62.9	100.2
Cadaverine	64.2	100.4
Spermidine	63.5	105.0
Spermine	67.4	109.7
Putrescine + KCl	75.0	110.3
Cadaverine + KCl	77.8	128.2
Spermidine + KCl	75.7	117.5
Spermine + KCl	76.5	114.9

Table 2 and Fig. 4. The illumination of 4-day-old etiolated cotyledons for 48 h with putrescine, KCl alone had slight effect on their fresh weights(Table 2). However, a combination of putrescine + KCl increased the fresh weights.

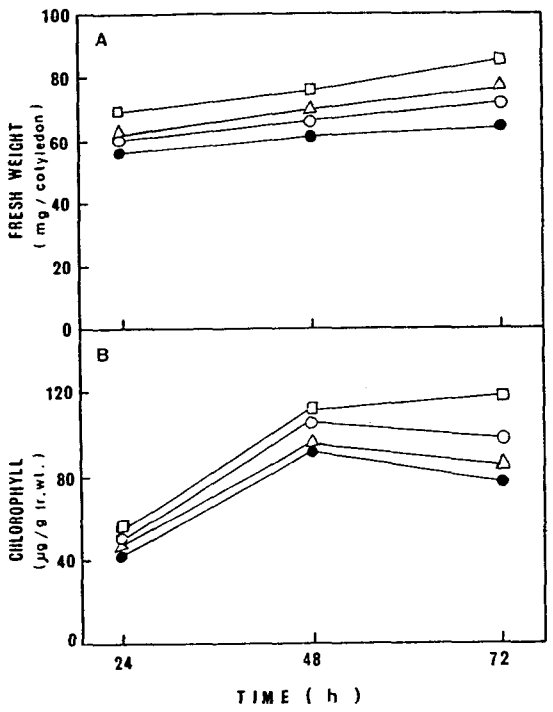

Fig.4. Time course of changes in the levels of fresh weight(A) and chlorophyll(B) in excised cotyledons incubated in spermine and KCl alone and in combination. ●, control; O , spermine; △, KCl; □, spermine+KCl.

The stimulation of chlorophyll formation by polyamine and KCl alone was observed during 48 h of illumination, and this effect was further amplified by addition of potassium to polyamines.

During the greening, the stimulation of chlorophyll formation induced by KCl andpolyamine was very similar(Fig. 4). The addition of polyamine and KCl simultaneously induced more chlorophyll synthesis than polyamine or KCl alone. The significant increse in chlorophyll levels might be ascribed to an enhanced uptake of potassium by the cotyledons and the stimulatory effect of polyamines is enhanced by addition of potassium.

The inclusions of polyamine biosynthetic inhibitors that contained polyamines did not have any significant effect on the greening response(Table 3). Nevertheless, the chlorophyll levels were higher than the controls but less than polyamine alone, suggesting an antagonistic effect between polyamine and inhibitors. D-arginine reduced the putrescine-stimulated rise in excised cotyledons by 35% and MGBG also inhibited putrescine-induced chlorophyll formation.

However, inhibitors of polyamine biosynthesis were applied alone or simultaneously with polyamines did not have significant effect on the protein content.

Table 3. Effect of putrescine and polyamine biosynthetic inhibitors on chlorophyll and protein contents in excised cotyledons exposed to light for 72 h after 18 h dark preincubation

Treatment	Chl content (μg/g fr.wt.)	Protein content (mg/g fr.wt.)
Control	73.6	35.8
Putrescine(1 mM)	95.9	37.9
MGBG(1 mM)	71.2	36.1
D-arginine(10 mM)	62.8	35.9
Putrescine + MGBG	79.7	37.2
Putrescine + D-arginine	77.3	36.3

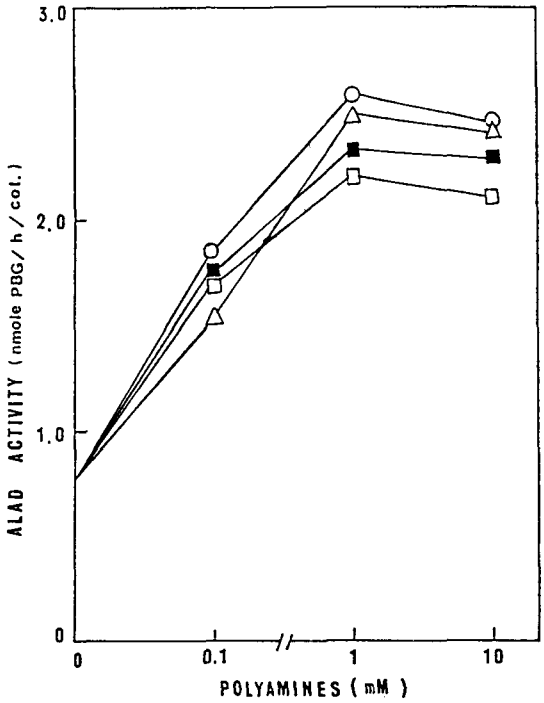

Fig.5. Effect of polyamine concentration on ALAD activity in excised cotyledons illuminated for 48 h after 18 h dark preincubation. O, putrescine; ■, cadaverine; □, spermidine; △, spermine.

3.2. Effect of polyamines on ALAD activity

The increase in growth and chlorophyll levels produced by polyamines suggest that they may act by affecting the enzymes of reserve mobilization in the cotyledons. Studies were, therefore, carried out on the enzymes in presence of these compounds in the similar experimental arrangement.

Exicised cotyledons of mung bean were treated with different concentrations of polyamines, respectively(Fig. 5). ALAD stimulation in cotyledons was concentration-dependent. Only moderate stimulation was noticed at concentrations of up to 1 mM, and polyamines were slightly inhibitory at concentrations above 10 mM. There was parallel progressive enhance

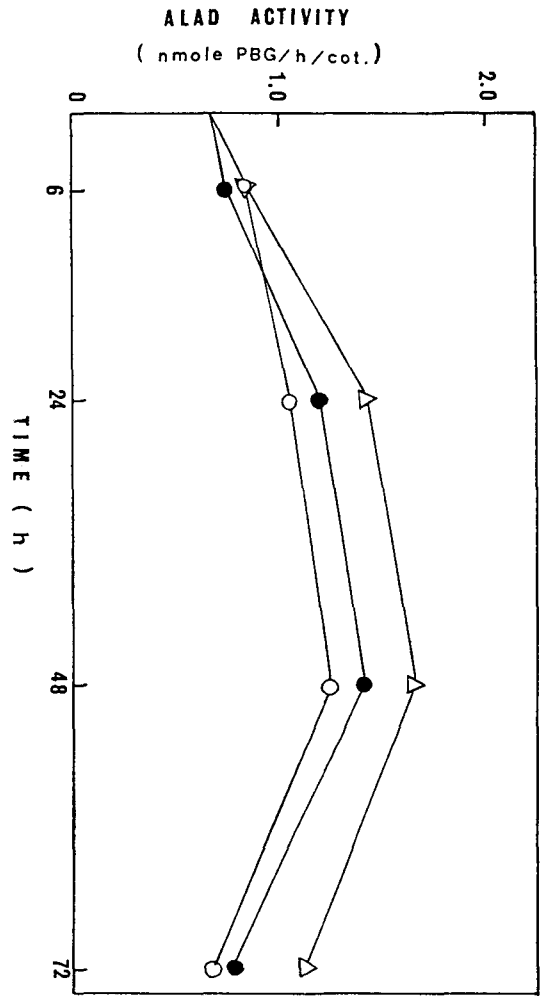

Fig.6. ALAD activity in excised cotyledons incubated on water in the dark(●), in the light(O) or in the light after 18 h dark preincubation(△).

-ment of the ALAD activity and chlorophyll content(Table 1) with initial increase in the polyamine concentrations. The degree of stimulation varied with the amines. Thus putres-cine was the most potent among these.

ALAD activity in control cotyledons-Whatever the conditions, the excision and incubation of the cotyledons led to an increase in ALAD activity(Fig. 6). In the dark, this increase lasted up to 48 h, then ALAD activity slowly decreased. In the light, the decrease in ALAD activity was more precocious. After 72 h, it was

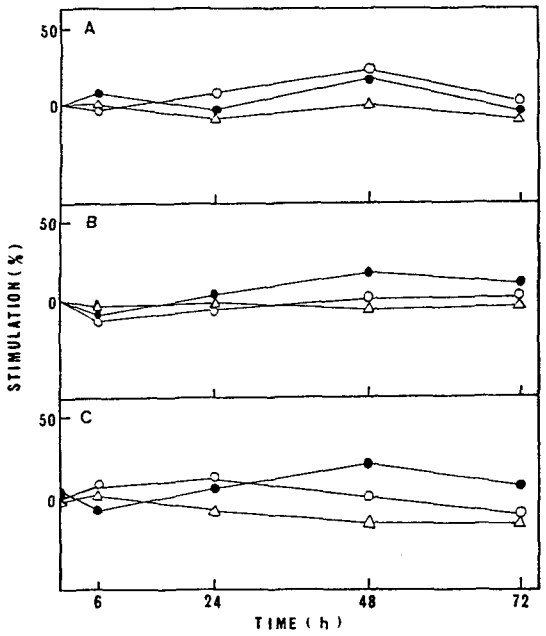

Fig.7. ALAD activity in excised cotyledons treated with putrescine(●), KCl(○) or putrescine+ KCl(△). A: cotyledons incubated in the dark; B: cotyledons incubated in the light: C: cotyledons incubated in the light after 18 h dark preincubation. Stimulation expressed as % of control.

approximately the same as before excision. Except during the first 6 h of illumination, ALAD activity was lower in the light than in the dark. When the cotyledons were illuminated after a dark preincubation, a slight increase in ALAD activity was observed in 24 h illuminated cotyledons and entailed an continuous increase which became higher than in the dark. A gradual decrease was also observed between 48 and 72 h illumination. It was delayed as compared to that of cotyledons maintained in the dark.

ALAD activity in cotyledons treated by putrescine(PUT), KCl or PUT+KCl—When cotyledons were treated with putrescine(PUT) in darkness, a slight stimulation of ALAD activity was observed after 6 h incubation on PUT (Fig. 7A). At the same time, KCl and the combination of PUT+KCl had no effect. During 48 h of incubation, a slight stimulation occurred under PUT treatment(+18%) and KCl treatment (+22%), the treatment with PUT+KCl being practically uneffective.

In the light, no stimulation was observed during the first 6 h of illumination(Fig. 7B). After 24 h illumination, PUT only enhanced ALAD activity(+12% after 72 h). ALAD activities in cotyledons treated by KCl and PUT +KCl remained approximately the same as in the control. The similar effects observed when the cotyledons were illuminated after a dark preincubation(Fig. 7C). The PUT stimulation in ALAD activity which occurred after 48 h illumination was, however, decreased after 72 h illumination. A slight stimulation observed after 24 h illumination in KCl-treated cotyledons (about 10%). In contrast, the combination PUT+ KCl induced a slight inhibition of ALAD activity.

Under continuous illumination in both control and polyamine-treated cotyledons, the ALAD activity and chlorophyll levels increased in a similar manner. ALAD activity increased with growth up to 48 h, as did the chlorophyll accumulated. Interestingly, after the chlorophyll content decreased slightly, the ALAD activities also tended to decrease.

ALAD activity in cycloheximide(CHI)-treated cotyledons—To test whether the augmented total ALAD activity were due to a *de novo* synthesis of the enzyme protein, CHI, the usual inhibitor of cytoplasmic protein synthesis, was treated along with the polyamines. CHI(10 $\mu g. ml^{-1}$) effectively checked the increase in enzyme activity in excised cotyledons treated for 48 h under incubation conditions.

When the cotyledons were incubated on water in the dark for 48 h, CHI entailed a very slight inhibition(less than 15%)(Table 4A). CHI inhibition was greater when the cotyledons were

Table 4. ALAD activity in excised cotyledons treated with putrescine, KCl and putrescine + KCl for 48 h under different incubation conditions

Treatment	CHI ($\mu g \cdot ml^{-1}$)	ALAD activity(nmole PBG·h^{-1}·cot^{-1})			
		Water	PUT	KCl	PUT + KCl
A: Dark	0	1.32	1.51	1.74	1.32
	10	1.12	1.06	1.20	0.92
B: Light	0	1.23	1.42	1.15	1.13
	10	0.98	1.25	1.13	1.15
C: Light after 18h dark preincubation	0	1.60	1.93	1.48	1.21
	10	1.47	1.52	1.15	0.92

treated by PUT, KCl or PUT+KCl. In all cases, it reached about 30% as compared to control. In the light a slight CHI inhibition was observed on water(about 20%) and PUT-treated cotyledons(-12%), while CHI did not have significant effect on ALAD activity in cotyledons treated by KCl or PUT+KCl(Table 4B).

When the cotyledons were illuminated after a dark period(Table 4C), a slight CHI inhibition occurred after 48 h illumination(-8%). In treated cotyledons, CHI inhibited more significantly ALAD activity in the presence of PUT, KCl and PUT+KCl. Morphologically there was no greening of cotyledons on treatment with the inhibitor even in the presence of PUT.

4. Discussion

4.1. Effect of polyamines on chlorophyll synthesis

The effect of polyamines of cotyledons that are attached to the seedling can be expected to be quite different from that on excised cotyledons since in whole seedlings the cotyledons are likely to have higher endogenous levels of polyamines that may affect the exogenously applied polyamines. Moreover, the presence of the axis can modify the effects of polyamines on reserve degradation.

Chlorophyll formation in 4-day-old excised cotyledons during the subsequent continuous illumination after 18 h dark preincubation was considerably depressed(Fig.1). The situation was even more obvious in the 6-day-old cotyledons (Dei, 1978). The physiological significance of these declines is still obscure. It would be more conceivable that the levels in the cotyledons of "some endogenous factor(s)" which has the ability to maintain the rapid chlorophyll formation, fell as the dark period after the excision of cotyledons lengthened. The magnitude of the promotive effect of the excision of cotyledon on chlorophyll formation seemed to depend on the age of the cotyledon as well as the dark preincubation period. The longer incubation period of 18 h did not promote as great a response, possibly because of the partially anaerobic conditions during incubation(Dei, 1978).

The rapid loss of chlorophyll-forming capacity of excised cotyledons during dark preincubation suggest a stimulatory effect of hypocotyl on the greening in the cotyledons. The difference in stimulation by polyamines between attached cotyledons and detached cotyledons could be due either to the presence of the hypocotyl that acts as a sink for products derived from reserve breakdown or to differences in endogenous polyamine content.

The data presented in Figs.2 and 3 show that polyamines have a stimulatory effect on chlorophyll formation, and that each effect requires a different incubation conditions for manifestation. Therefore, the effects are separable. When polyamines were applied to attached cotyledons, the cholrophyll formation increased up to 72 h of illumination. With treated excised cotyledons, cholrophyll contents were lowered than intact cotyledons. This was true in

particular for excised cotyledons with dark preincubation for 18 h. The decrease in chlorophyll content was observed at late phase of greening and this inhibition might result from supraoptimal concentrations of polyamines, particularly if endogenous levels were already high. Since the effects of the polyamines on cotyledons do not seem to depend on the absolute concentration of the polyamine present in the organ, rather on the concentration ratio between cotyledon and hypocotyl(Longo *et al.*, 1984).

The excised cotyledons of pretreatment with polyamines produced a stimulatory effect on chlorophyll formation under subsequent illumination(Fig. 3), contrary to the findings of Walker *et al.*(1988). This discrepancy is probably the differences in experimental schemes rather than in the plant species used. This material used for the early phase of greening was 24 h illuminated mung bean cotyledons which showed increased chlorophyll content, while theirs was 3 h illuminated cucumber cotyledons which induced decreased synthesis of chlorophyll.

The mechanical shock caused by cotyledon excision seems to prevent the polyamine stimulation of chlorophyll formation. This probably coincides with the excision-induced temporary increase in chlorophyll-forming activity (Dei, 1978).

Differences in activity of various polyamines during greening have been ascribed to the number of free amine groups, which can affect their cationic properties(Altman and Bachrach, 1981; Kaur-Sawhney and Galston, 1979; Tabor, 1984). In the present study(Table 1), diamines and polyamines were similarly active, indicating that either putrescine is rapidly metabolized to spermidine and spermine or a free amine groups are sufficient to saturate the system.

Protein content increased till the 2nd day of illumination and declined gradually(Table 1) in both control and treated cotyledons. The major function of the polyamines is to facilitate protein synthesis(Smith, 1982), and polyamines may affect the protease activity either by activating the preexisting enzyme or by effecting its release(Srivastava *et al.*, 1985). Polyamines also have been reported to prevent senescence by inhibiting RNase and protease activity (Altman, 1982; Altman and Bachrach, 1981; Kaur-Sawhney *et al.*, 1982). The increase in the protein levels was not due to a *de novo* synthesis of the enzyme since the activating effect was observed even when the cotyledons were incubated in the presence of polyamine synthesis inhibitors(Table 3).

Potassium is known to exert an activity upon chlorophyll synthesis of cotyledons and to be an important or in the regulation of the greening of cotyledons (Haru *et al.*, 1982). Potassium was found to be effective in stimulating expansion and chlorophyll production in cucumber cotyledons(Fletcher *et al.*, 1982: Green and Muir, 1978) and to modify the growth response to cytokinins(Green and Muir, 1979;Le Pabic *et al.*, 1983).

The results presented in Fig. 4 show that chlorophyll synthesis in mung bean cotyledons is slightly stimulated in the presence of SPM or KCl alone during greening. With the combination of SPM+KCl, the resulting effect was higher than in the case of each compound used separately and appears to be approximately additive. The absence of a synergistic interaction between polyamine and KCl suggests that the two compounds influence different mechanisms implicated in chlorophyll synthesis. Moreover, polyamine and KCl together seems to have a longer effect than polyamine or KCl alone. As the chlorophyll content is influenced to a lesser extent by polyamine than by KCl, it is suggested that potassium is the first limiting

factor for pigment synthesis in the cotyledons. The role of K^+ in the greening process and its relationships to putrescine are not clear. Putrescine levels have been shown to increase up to 90-fold in K^+ deficient plants, and it has been suggested that divalent amine may substitute for K^+ in one or more physiological processes(Smith, 1975). The decrease in putrescine in the cucumber cotyledons following application of K^+ is consistent with that view, but complete inhibition of putrescine synthesis in the absence of added K^+ did not retard chlorophyll synthesis (Walker *et al.*, 1988). Thus, the K^+ effect, i.e. the fall in putrescine and subsequent rise in chlorophyll, does not constitute evidence that K^+ substitutes for putrescine in the greening process. This similarity between the effects of K^+ and polyamines has been noted in other plant systems(Cho, 1983) and during tests of their effects on the physical properties of plant membranes(Roberts *et al.* 1986). The observation suggest that polyamine is chiefly active in the presence of exogenous K^+ or when endogenous K^+ content is sufficiently high.

4.2. Effect of polyamines on ALAD activity

There is good evidence during the transition, upon illumination, of etioplasts into cholroplasts, the chloroplasts are synthesizing the necessary enzyme for chlorophyll synthesis and photosynthesis(Harvey *et al.* 1974) and the enzyme ALAD is synthesized *de novo* in the cytoplasm (Balangé and Lambert, 1980; Shibata and Ochiai, 1976). It was found that ALAD situated in the pathway for chlorophyll synthesis is located in proplastids or chloroplasts(Rebeiz and Castelfranco, 1971; Shibata and Ochiai, 1976).

In the dark, the increase in ALAD activity observed after excision and during the 48 h incubation of the cotyledons on water(Fig. 6). The stimulation was probably due to the inhibition of a protease formation(Knypl and Mazurczyk, 1972) or to the inhibition of an ALAD inactivator synthesis. The mechanical shock and the sudden osmotic changes given to cotyledons by excision and incubation, which seemed to influence the cotyledon metabolism (Dei, 1982), could be the cause of an ALAD activation. A decrease in ALAD activation was observed in aging cotyledons: the breakdown of ALAD molecules would be then higher than their synthesis. A treatment of cotyledons by polyamines or KCl entailed a slight stimulation of ALAD activity after 48 h in the dark(Fig. 7A). After 72 h, the level of ALAD activity was about the same as in the control. ALAD turnover seemed therefore to be slightly accelerated by these treatments.

In the light, stimulation of ALAD activity was also observed after cotyledon excision(Fig. 6). No ALAD synthesis occurred at the beginning of the incubation on water, which suggest an ALAD activation as in the dark. Then, a decrease in ALAD activity, more precocious than in the dark, occurred at the same time as a slight ALAD synthesis. ALAD turnover seemed to be accelerated in the light, and ALAD molecules could also be more unstable in the light than in the dark(Balangé and Rollin, 1979). Putrescine, KCl or their combination had no effect on ALAD activity at the beginning of incubation(Fig. 7B). During a slight increase in ALAD activity in the control, putrescine maintained the level of the enzyme activity. The induction of the synthesis of new enzyme molecules could be only one of the effects of putrescine, another being a decrease of ALAD breakdown. In cotyledons preincubated in the dark(Fig. 6), illumination induced considerable increase in ALAD activity.

Putrescine stimulating effect was also observed and increased by the dark preincubation. KCl had a slight stimulating effect on ALAD activity as observed after 24 and 48 h incubation in the dark. Then, KCl and the combination putrescine + KCl decreased ALAD activity in spite of a stimulation of ALAD synthesis. It can be concluded that putrescine enhanced ALAD activity of mung bean cotyledons in the light and maintained it after a dark preincubation.

KCl stimulation on ALAD activity was rather slight and occurred only in the dark or in the light after a dark period. As for putrescine, it seemed that its stimulation was not the result of an ALAD activation. Moreover, it has been reported that K^+ was not required as a cofactor for the activity of the plant enzyme(Schneider, 1970; Sluiters-Sholten *et al.*, 1973).

Le Pabic *et al.*(1987) showed that different K^+ concentrations added to an enzyme extract of cotyledons had no effect on the *in vitro* ALAD activity. KCl probably enhanced ALAD turnover. This result agrees with those of Kynpl and Rennert(1970) which suggest that K^+ ions stimulate protein turnover in cucumber cotyledons. In the light, KCl induced no stimulation of ALAD activity or sometimes a slight decrease. To explain these facts, the hypothesis might be advanced of the presence of an ALAD inactivator stimulated by KCl in the light. Balangé and Rollin(1979) had suggested the presence of such an inhibitor in radish seedlings, located in the plastids, which synthesis is promoted by light. The assumption could also explain that the combination of putrescine+KCl caused approximately the same effects as KCl only, the presence of KCl preventing the effect of putrescine on ALAD activity.

ALAD activity on a protein level is closely correlated with the chlorophyll level in etiolated cotyledons exposed to light. Increase of ALAD activity in the course of light induced synthesis of chlorophyll has been reported for bean leaves (Steer and Gibbs, 1969) and tobacco tissue culture (Schneider, 1970). ALAD attained maximum at 48 h and decreased thereafter, indicating that its pattern was qualitatively uninfluenced by the presence of polyamines.

There was parallel progressive enhancement of ALAD activity and chlorophyll content. These observations support that there is a regulatory system for ALAD activity which may participate in regulating chlorophyll synthesis, and most of the ALAD activity is localized in the plastids. The constant relationship between ALAD activity and chlorophyll content also suggest a common intracellular site and common control over development for the 2 systems (Steer and Gibbs, 1969). Naito *et al.* (1980) assumed that chlorophyll synthesis might be regulated not only by ALA synthetic ability but also ALAD activity.

ALAD activity appeared to be inhibited by cycloheximide, a well known inhibitor of cytoplasmic translational activity(Table 4). The inhibitor interferes with the synthesis of membrane proteins to which the electron transfer proteins are bound(Hovenkamp-Obbema *et al.*, 1974), and inhibits the synthesis of structural proteins of the chloroplast thylakoids in *Euglena*(Kirk, 1968). CHI inhibition indicates that the increased ALAD activity is dependent upon both concomitant RNA and enzyme synthesis. The experiment with CHI also shows that a small amount of ALAD may still occur leading to a reasonable chlorophyll synthesis, which means that there must be enough structural protein to act as building sites for the chlorophyll molecules.

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녹두자엽에서 엽록소 및 단백질함량과 δ-Aminolevulinate Dehydratase활성에 미치는 Polyamine의 영향

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녹두 유식물의 자엽에서 엽록소 및 단백질 함량과 δ-aminolevulinate dehydratase(ALAD)활성의 변화에 미치는 polyamine의 영향을 조사하였다. Polyamine은 녹화과정에서 자엽내의 엽록소 생성을 촉진하였으며. 이 효과는 KCl에 의해서 상승되었다. 자엽내의 단백질 함량의 변화 또한 엽록소 함량의 변화와 유사하였다. ALAD활성은 암하에서보다 광선하에서 억제되었으나, 18시간 암처리후의 광조사는 ALAD활성을 증가시켰다. Putre- scine처리에 의한 ALAD활성은 암하에서 촉진효과가 낮았으나 광선하에는 그 활성이 증가되었다. KCl은 암하에서 ALAD활성을 촉진시켰으나 광선하에서는 그 효과가 감소되었다. 또한 polyamine과 KCl의 복합처리에서는 촉진효과가 없었다. 이와같은 결과에서 녹두자엽에서의 색소체발달은 polyamine과 광선에 의해 영향을 받으며, polya- mine은 색소체발달에 중요한 작용을 하는 것으로 사료된다.