ACTA SOCIETATIS
BOTANICORUM POLONIAE
Vol. XXXIX — nr 2
1970

Control of chlorophyll synthesis by coumarin and plant growth retarding chemicals

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INTRODUCTION

It has been shown that coumarin and growth retardants including AMO-1618 *, B-Nine, CCC and Phosfon D delay yellowing of the leaf discs of kale in darkness and stimulate yellowing in light (Knypl 1969a). In leaves kept in the dark only breakdown of chlorophyll takes place. In light, on the contrary, the synthesis of pigment is still continued although with a declining intensity. Hence, the rate of yellowing of the leaf tissue in the latter case is a resultant of both anabolism and catabolism of the pigment. Basing on a fact that coumarin and growth retardants effectively inhibit incorporation of labelled leucine into proteins of senescing kale leaf tissue, it has been suggested that the compounds can arrest both anabolism and catabolism of chlorophyll in plant cells by inhibition of protein synthesis. Preliminary tests have revealed that the compounds actually retard or completely block greening of etiolated cotyledons of kale (Knypl 1969 a).

The cotyledons of kale are small and sensitive to mechanical injury. Fortunately, isolated cotyledons of cucumber and pumpkin have been found to be also sensitive to growth retardants (Knypl 1969 b) and phytohormones including GA_3 and BA (*cf.* Kursanov et al. 1969). The aim of the present experiments was to study in detail the effects of growth retardants and coumarin, applied alone or in combining solutions with GA_3, BA or KCl, on both growth and greening of etiolated cotyledons of cucumber. For comparison, some analyses with isolated cotyledons of pumpkin were also carried out. Finally, the effect of the compounds on protein and RNA synthesis in cucumber cotyledons was studied.

MATERIAL AND METHODS

1. Plant material

Seeds of cucumber, *Cucumis sativus* L. var. Delicatess, were germinated for five days in darkness at 24°. Cotyledons were dissected with a razor blade from 7—8 cm long seedlings, weighed on a torsion balance and placed innerside upwards in 10-cm

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*) Abbreviations used: AMO-1618, 2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidinecarboxylate methyl chloride; B-Nine, N,N-dimethylaminosuccinamic acid; CCC, (2-chloroethyl) trimethylammonium chloride; Phosfon D, 2,4-dichlorobenzyltributylphosphonium chloride; GA_3, gibberellic acid; BA, N^6-benzyladenine (N^6-benzylaminopurine).

Petri dishes on Whatman No. 2 blotting paper discs soaked with 5 ml of distilled water containing penicillin G (100 mg/l) or solutions of the substances to be tested. Each of two cotyledons of a given seedling was placed in a different dish, with ten cotyledons in one dish (Knypl and Rennert 1970 a).

Surface-sterilized seeds of pumpkin, *Cucurbita pepo* L. var. Olbrzymia Melonowa, were soaked in water for 4 hours and germinated for four days in darkness. The cotyledons were dissected from the seeds with 25—30 mm long radicles (Knypl 1970), and handled as described for cucumber with the minor modification that 10-ml aliquots of the solutions were poured into 11-cm Petri dishes.

All solutions contained penicillin G (100 mg/l) which effectively prevents bacterial growth without effect on protein synthesis in cucumber (Venis 1967).

2. Incubation of the cotyledons

Covered dishes were incubated for two days under continuous illumination at 25°. Day-light fluorescent tubes were employed as light source; light intensity was 1300 lux at the tissue level.

All tests were carried out in triplicate and repeated 3—4 times.

3. Determination of net growth and chlorophyll content

At the end of incubation period, the cotyledons were picked out, two extreme ones from each group were discarded and the remaining eight were weighed on a torsion balance, ground with quartz sand and $CaCO_3$ in a mortar with a pestle, and extracted with 80 per cent acetone. Chlorophyll *a* and *b* in the clear extracts were immediately determined spectrophotometrically by measuring absorbancies at 645, 652 and 662 nm (Bruinsma 1963; Knypl and Rennert 1970 a).

4. Measurement of protein and RNA synthesis

The cotyledons of cucumber, incubated for two days with growth regulators were rinsed briefly with water, blotted, weighed on a torsion balance after cutting of about 0.5 mm zone at the first cut, and in groups of ten transferred to two ml of L-leucine-U-^{14}C (1 μCi/ml) or uracil-2-^{14}C (5 μCi/ml) poured into small Petri dishes. Solutions of labelled precursors were supplemented with penicillin G (100 mg/l). The cotyledons were fed with leucine-^{14}C for 3 hours and with uracil-^{14}C for four hours at 22° in light (1400 lux).

The cotyledons fed with labelled leucine were subsequently washed with tap water and 0.001 M solution of leucine-^{12}C and fractionated according to previously described (Knypl 1969 a) modification of Klyachko and Kulayeva 1965) method. In this procedure the tissue is extracted several times with 5% $HClO_4$ at room temperature to remove acid soluble compounds, then leucine-tRNA complex is extracted at 90° (15 min.) with the acid, and finally the tissue debris delipided with successive washings with ethanol and ethanol:ether (3:1). White pellet is dissolved in 10% NH_4OH (5 ml) at 70° (10 min.). Triplicate 0.2 ml aliquots of the extract were dropped onto planchettes, dried at 70° and counted.

The cotyledons fed with uracil-^{14}C were fractionated according to method of Fletcher and Osborne (1965) as previously described (Knypl 1969 a).

Radioactivity was monitored using SSU-4W scintillator detector containing photomultiplier M12FS52A and a windowless plastic scintillator SPF34 (Tesla) mounted in a lead house and a planchette changing device OSZ-1. Impulses from SSU-4W were counted with an authomatic counter ZPA-1A. All devices were produced by BUTJ, Warsaw.

Using a voltage of 1360, an efficiency of 40 per cent for ^{14}C in a solid angle of 2π was achieved with a background of about 42 counts per minute.

L-leucine-U-^{14}C was purchesed from VVVR, Praha (specific activity 90 mCi/mM), and uracil-2-^{14}C (spec. activity 6.23 mCi/mM) from Institute of Nuclear Research, Świerk.

RESULTS

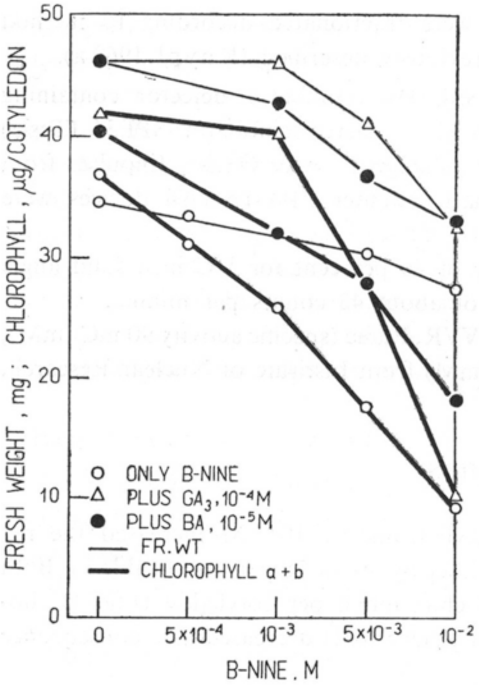
Gibberellic acid at 10^{-4} M and benzyladenine at 10^{-5} M increased the net growth of the isolated cotyledons of cucumber by about 78 per cent (Table 1). Both phytohormones increased the content of chlorophyll per cotyledon (Fig. 1); however, the level of the pigment per g fresh weight basis decreased as a consequence of accelerated growth.

B-Nine at 0.001 M inhibited net growth of cucumber cotyledons by about 40 per cent, but the content of chlorophyll decreased by about 75 per cent per cotyledon and 70 per cent per g fr. wt. (Fig. 2, Table 1). It is thus striking that inhibition of chlorophyll synthesis was greater that the degree of retardation of the growth rate. This relationship is more accentuated at 0.005 M B-Nine, at which the growth rate was reduced by about 25 per cent, whereas the chlorophyll content was lowered by more than 50 per cent per cotyledon.

Benzyladenine and gibberellic acid markedly reduced the B-Nine induced retardation of growth. GA_3 and BA reduced also, in part, the inhibition of chlorophyll accumulation per cotyledon, BA being more effective against the effect brought about by a high concentration of the retardant (0.01 M) than GA_3 (Fig. 1). Nevertheless, GA_3 had no effect on the B-Nine inhibitory effect on chlorophyll synthesis per g fresh weight basis, whereas cytokinin decreased it significantly (Table 1).

CCC even at the highest concentration of 0.01 M had no effect on growth of the cotyledons, either applied alone or in combined solutions with phytohormones. This growth retardant, however, very effectively inhibited accumulation of the pigment. GA_3 did not decrease this effect. BA completely reversed the inhibitory effect of CCC on chlorophyll synthesis per cotyledon (Fig. 2) and markedly reduced it per g fr. wt. basis (Table 1).

Phosfon D like CCC had little effect on growth (Fig. 3) and at 10^{-4} M decreased the content of chlorophyll by about 50 per cent per cotyledon. The latter effect was not modified either by GA_3 or BA as calculated per g fr. wt. basis (Table 1), but was reduced by gibberellic acid more effectively than by BA when calculated per cotyledon (Fig. 3).



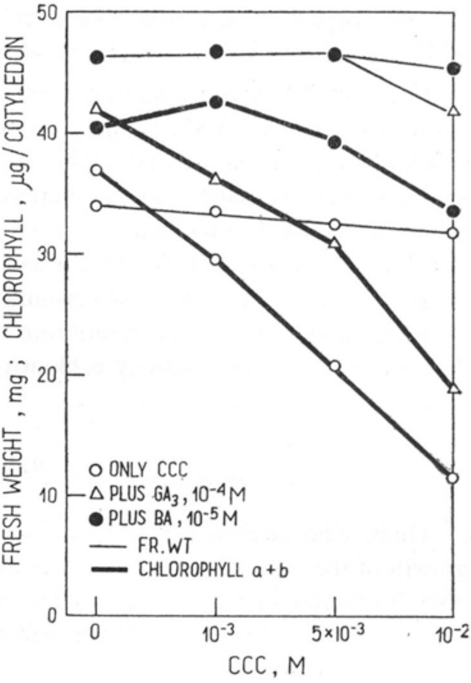


Fig. 1. Growth and chlorophyll synthesis in detached cotyledons of cucumber as affected by B-Nine applied alone or in combined solution with GA_3 or benzyladenine.

Fresh wt. of one cotyledon after two days of incubation is presented without substracting the original weight. Original fr. wt. of one cotyledon = 18.3 mg. L.S.D. at $p = 0.01$ = 10 per cent

Fig. 2. Effect of CCC on growth and chlorophyll synthesis in detached cotyledons of cucumber. Other details as in Fig. 1.

It has been reported that potassium ions markedly stimulate both the growth of the isolated cotyledons of cucumber and the synthesis of chlorophyll in them (Knypl and Rennert 1970 b). As can be seen from the data of Table 2, KCl at 0.01 M increased net growth of the cotyledons by 256 per cent, and the content of chlorophyll by 76 and 300 per cent per g fr. wt. and cotyledon, respectively, as compared with the water treated control taken as 100 per cent (Fig. 4). KCl very effectively reduced or completely reversed the growth inhibition caused by B-Nine, CCC and Phosfon D, and reduced the inhibitory effect of growth retardants on the accumulation of chlorophyll. Only in a case of Phosfon D, applied at a supra-optimal concentration of 5×10^{-4} M, KCl did not reduce the degree of retardant-dependent inhibition of pigment synthesis per g fr. wt. It means that in this sample the KCl-dependent reversal of growth inhibition was the same as KCl-dependent reversal of pigment accumulation.

Potassium was particularly effective in reversing the CCC-induced effects.

Coumarin at 250 and 500 mg/l concentrations inhibited net growth of cucumber cotyledons by about 35 and 54 per cent, respectively (Fig. 5), and almost completely

Table 1

Chlorophyll content in detached cotyledons of cucumber incubated for two days with growth regulators

Compound, M *	Net increment of one cotyledon fresh weight **		Chlorophyll $a+b$		$\frac{\text{Chl } a}{\text{Chl } b}$
	mg	%	µg/g fr. wt.	%	
0 (control)	15.7	100	1088.2	100	2.46
B-Nine, 0.001	13.6	86.6	808.8	74.3	2.48
B-Nine, 0.005	11.9	75.8	579.5	53.3	2.48
B-Nine, 0.01	8.9	56.7	341.9	31.4	2.48
CCC, 0.001	15.1	96.2	886.2	81.4	2.48
CCC, 0.005	14.4	91.7	636.1	58.5	2.50
CCC, 0.01	13.5	86.0	358.5	32.9	2.68
Phosfon D, 0.00001	14.9	94.9	858.4	78.9	2.49
Phosfon D, 0.00005	14.4	91.7	611.6	56.2	2.52
Phosfon D, 0.0001	12.5	79.6	590.9	54.3	2.50
PLUS GIBBERELLIC ACID, 0.0001 M					
0	27.9	177.7	902.6	82.9	2.39
B-Nine, 0.001	27.6	175.8	873.6	80.3	2.46
B-Nine, 0.005	22.5	143.3	683.8	62.8	2.48
B-Nine, 0.01	14.4	91.7	296.6	27.3	2.48
CCC, 0.001	28.1	179.0	773.7	71.1	2.45
CCC, 0.005	28.3	180.3	658.8	60.5	2.41
CCC, 0.01	23.5	149.7	227.4	41.1	2.34
Phosfon D, 0.00001	27.6	175.8	701.5	64.5	2.46
Phosfon D, 0.00005	26.2	166.2	707.2	65.0	2.45
Phosfon D, 0.0001	24.8	158.0	573.1	52.7	2.48
PLUS BENZYLADENINE, 0.00001 M					
0	27.9	177.7	872.3	80.2	2.36
B-Nine, 0.001	24.5	156.1	738.3	67.8	2.36
B-Nine, 0.005	18.1	115.3	766.5	70.4	2.36
B-Nine, 0.001	14.5	92.4	551.8	50.7	2.48
CCC, 0.001	28.4	180.9	912.2	83.8	2.38
CCC, 0.005	27.9	177.7	848.5	78.0	2.38
CCC, 0.01	27.0	172.0	737.3	67.8	2.52
Phosfon D, 0.00001	28.9	184.1	658.9	60.5	2.34
Phosfon D, 0.00005	26.6	169.4	559.2	60.6	2.36
Phosfon D, 0.0001	25.2	160.5	446.0	41.0	2.34

* Final concentrations, the same in sole and combined solutions.

** Original fr. wt. of one cotyledon = 18.3 mg. A difference of 10 per cent between any two values is significant at $p = 0.01$.

inhibited greening of the tissue. The coumarin induced inhibition of chlorophyll synthesis was little affected by phytohormones (data not shown); it was, however, markedly reduced by KCl (Fig. 4, Table 3). The KCl reversal was well accentuated when coumarin was applied at a concentration of 250 mg/l; the compound at 500 mg/l

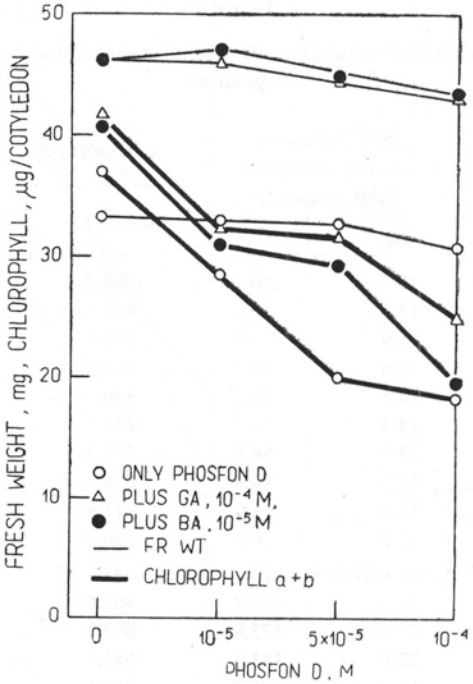


Fig. 3. Effect of Phosfon D on growth and chlorophyll synthesis in detached cotyledons of cucumber. Other details as in Fig. 1.

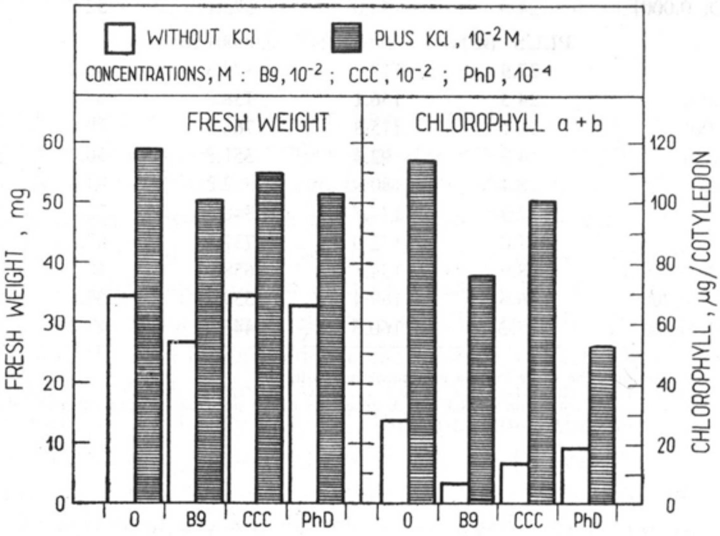


Fig. 4. Reversal by potassium of inhibitory effect of B-Nine, CCC and Phosfon D on chlorophyll synthesis in detached cucumber cotyledons. B9 and PhD = B-Nine and Phosfon D, respectively. Other details as in Fig. 1.

Table 2

Reversal by potassium of inhibitory effect of B-Nine, CCC and Phosfon D on growth and chlorophyll synthesis in detached cotyledons of cucumber

Compound, M	Net increment of fresh weight		Chlorophyll $a+b$		Chl a / Chl b
	mg	%	μg/g fr. wt.	%	
0 (control)	15.6	100	1101.4	100	2.45
B-Nine, 0.01	7.9	50.6	268.7	24.4	2.60
CCC, 0.005	17.2	110.3	639.9	58.1	2.45
CCC, 0.01	15.6	100.0	400.0	36.3	2.54
Phosfon D, 0.0001	14.1	90.4	575.8	52.3	2.45
Phosfon D, 0.0005	8.8	56.4	371.8	33.8	2.45
PLUS POTASSIUM CHLORIDE, 0.01 M					
0	40.0	256.4	1935.4	175.7	2.45
B-Nine, 0.01	31.5	201.9	1506.0	136.7	2.50
CCC, 0.005	37.9	242.9	1735.9	157.6	2.45
CCC, 0.01	35.9	230.1	1835.8	166.7	2.46
Phosfon D, 0.0001	32.7	209.6	1015.5	92.2	2.45
Phosfon D, 0.0005	18.6	119.2	368.0	33.4	2.45

Original fr. wt. of one cotyledon = 18.9 mg. Other details as in Table 1.

concentration almost completely neutralized the stimulatory effect of potassium ions on both growth and chlorophyll accumulation.

The growth retardants and coumarin affected relatively little growth of the pumpkin cotyledons, and very effectively inhibited the synthesis of chlorophyll in them (Fig. 6). Benzyladenine at 10^{-5} M concentration increased the content of chlorophyll per cotyledon about twofold. In spite of this stimulation, cytokinin had no effect on the activity of growth retarding compounds and coumarin as inhibitors of chlorophyll synthesis in pumpkin cotyledons.

Table 3

Reversal by potassium of inhibitory effect of coumarin on growth and chlorophyll synthesis in detached cotyledons of cucumber

Concentration of coumarin, mg/l	Net increment of fresh weight		Chlorophyll $a+b$		Chl a / Chl b
	mg	%	μg/g fr. wt.	%	
0 (control)	15.2	100	1020.6	100	2.47
100	15.2	100	588.2	57.6	2.56
250	9.5	62.5	204.9	20.1	2.90
500	6.8	44.7	128.9	12.6	2.90
PLUS POTASSIUM CHLORIDE, 0.01 M					
0	37.6	247.4	1883.0	184.5	2.45
100	46.2	303.9	1641.6	160.8	2.45
250	24.7	162.5	901.1	88.3	2.45
500	8.5	55.9	219.8	21.5	2.50

Original fr. wt. of one cotyledon = 18.8 Other details as in Table 1.

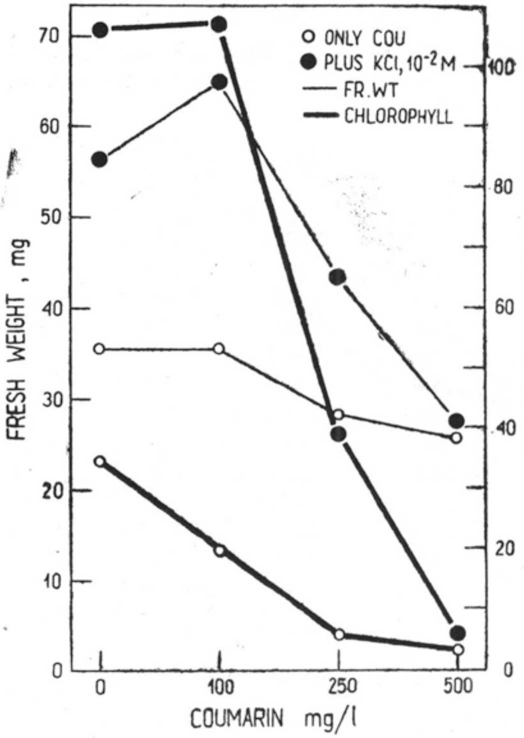


Fig. 5. Effect of coumarin and KCl on growth and chlorophyll synthesis in detached cotyledons of cucumber. Original fr. wt. of one cotyledon = 18.8 mg. Other details as in Fig. 1.

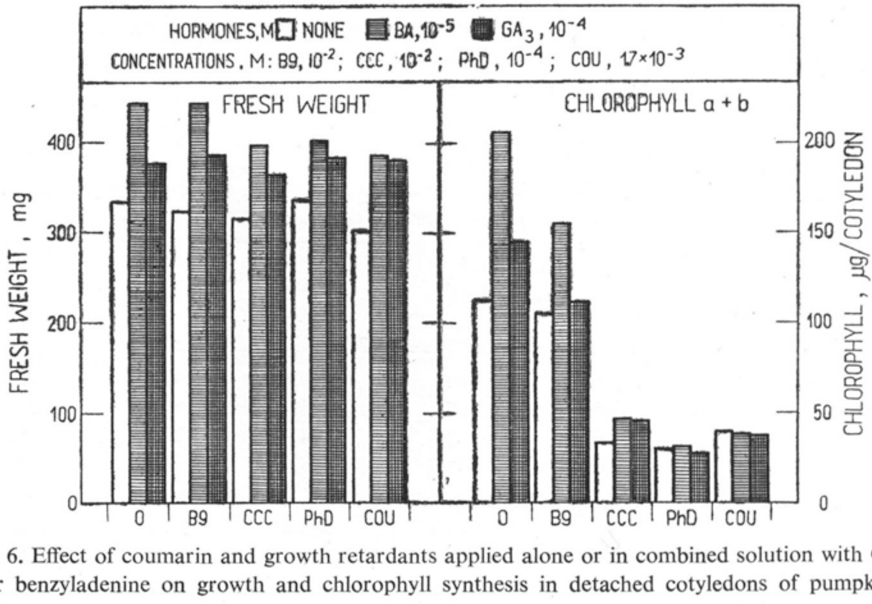


Fig. 6. Effect of coumarin and growth retardants applied alone or in combined solution with GA_3 or benzyladenine on growth and chlorophyll synthesis in detached cotyledons of pumpkin

Fresh weight of one cotyledon after two days of incubation is presented without subtracting the original weight. Original fr. wt. of one cotyledon = 172 mg. L.S.D. at $p = 0.01$ = 10 per cent. B9, PhD and COU = B-Nine, Phosfon D and coumarin, respectively

Table 4

Effect of KCl on growth and chlorophyll synthesis in detached cotyledons of pumpkin

KCl, M	Fresh weight of one cotyledon, mg	Chlorophyll $a+b$		$\frac{\text{Chl } a}{\text{Chl } b}$
		µg/g fr. wt.	%	
0	472.9	535.2	100	2.50
0.001	496.8	482.0	90.1	2.50
0.05	480.6	456.6	85.3	2.55
0.1	432.4	355.9	66.5	2.65

Original fresh weight of one cotyledon = 170 mg. Time of incubation = 3 days.

In contrast to the results noted with cucumber cotyledons, potassium ions stimulated neither growth nor chlorophyll synthesis in the cotyledons of pumpkin. On the contrary, KCl at higher concentrations decreased the content of chlorophyll in this tissue (Table 4).

Phosfon D at 10^{-4} M decreased the synthesis of proteins and RNA by about 50 per cent in cucumber cotyledons (Table 5). CCC at 0.005 M decreased the synthesis of protein by 25 per cent, but synthesis of RNA decreased by about 65 per cent. B-Nine had no effect on incorporation of labelled uracil to RNA in cucumber cotyledons, but incorporation of labelled leucine to proteins decreased by about 30 per cent. Potassium ions stimulated the synthesis of both protein (by about 95 per cent) and RNA (by about 70 per cent). KCl completely reversed the inhibitory effect of Phosfon D on protein synthesis, and markedly reduced the effect on RNA synthesis. KCl reversed the CCC-dependent inhibition of RNA synthesis; it is striking that cotyledons treated with CCC+KCl incorporated leucine to protein more intensively than did that ones treated with KCl alone. Potassium ions reversed also the inhibitory effect of B-Nine on protein synthesis (Table 5).

Table 5

Effect of growth retarding chemicals and KCl on protein and RNA synthesis in detached cucumber cotyledons, preincubated for two days with the regulators and than fed with labelled leucine or uracil

Precursor	counts/min./cotyledon			
	Compound, M			
	0 (Control)	CCC 0.005	B-Nine 0.005	Phosfon D 0.0001
Leucine-^{14}C	4300	3 225	3083	1849
Uracil-^{14}C	3050	1 193	3294	1525
PLUS POTASSIUM CHLORIDE, 0.025 M				
Leucine-^{14}C	8385	10 800	6716	7142
Uracil-^{14}C	5173	4 550		2507

A difference of 14 per cent between any 2 values is significant at $p = 0.01$.

DISCUSSION

This study showed that growth retarding chemicals and coumarin inhibit more effectively chlorophyll synthesis than growth of etiolated cotyledons. This relationship is best observed in the case of pumpkin (Fig. 6) and of cucumber cotyledons treated with CCC and Phosfon D. Phytohormones generally did not reverse the effect of the compounds on chlorophyll synthesis. On the contrary, CCC, Phosfon D and coumarin neutralized the stimulatory effect of benzyladenine on greening of the cotyledons of pumpkin.

Some growth retarding compounds have been shown to inhibit the biosynthesis of gibberellins (*cf.* Dennis et al. 1965; Kende et al. 1963; Barnes et al. 1969). According to a current view, growth inhibition caused by CCC and Phosfon D follows the inhibition of GA synthesis. There are, however, several lines of evidence showing that these compounds can inhibit the growth processes stimulated by cytokinins (Rennert and Knypl 1967), auxins (Kuraishi and Muir 1963; Knypl 1964; Šebánek 1967) and coumarin (Knypl 1964). Besides inhibition of gibberellin biosynthesis, some growth retardants inhibit also synthesis of diterpene steviol (Ruddat 1966) and control synthesis of carotenoids (Knypl 1969 c), tocopherols (Michniewicz and Kamieńska 1969) and, as shown in this study, of chlorophyll. Thus, it would be an over-simplification to regard the growth retardants only as inhibitors of gibberellin biosynthesis.

Greening of etiolated leaves is dependent on continuous synthesis of proteins with preceding synthesis of RNA, necessary for maturation of proplastids to chloroplasts (Bogorad 1966). It has recently been shown that coumarin, B-Nine, CCC and Phosfon D strongly inhibit both degradation and synthesis of proteins in kale leaves (Knypl 1969 a). This study showed that B-Nine, CCC and Phosfon D inhibit incorporation of labelled leucine to proteins in cucumber cotyledons; CCC and Phosfon D strongly inhibit synthesis of RNA (Table 5). It can thus be suggested that delayed greening of the cotyledons treated with coumarin or growth retarding chemicals is caused by partial inhibition of protein and/or RNA synthesis in the tissue.

Potassium ions strikingly stimulate both growth and chlorophyll synthesis in the cotyledons of cucumber, being ineffective in the cotyledons of pumpkin. This fact seems to indicate that cucumber cotyledons, in contrast to those of pumpkin, suffer a deficit of potassium. Potassium ions stimulate both growth and chlorophyll synthesis in cucumber, and reverse the symptoms of action of growth retardants owing to a striking stimulation of RNA and protein synthesis (Table 5).

ACKNOWLEDGEMENTS

I wish to express my thanks to the State Board for Peaceful Use of Atomic Energy, Warsaw, for a grant providing equipment for research with isotopes. I thank Calbiochem AG, Luzerne, for a sample of B-Nine, and Industrial Chemical and Dye Co., Inc., New York for a sample of Phosfon D.

SUMMARY

1. Coumarin, B-Nine, CCC and Phosfon D at concentrations little affecting growth of detached cotyledons of cucumber and pumpkin very effectively inhibited chlorophyll synthesis in them.

2. Gibberellic acid and benzyladenine stimulated growth of detached cotyledons of both cucumber and pumpkin; BA markedly increased the content of chlorophyll in pumpkin cotyledons. Both phytohormones had generally no effect on the activity of coumarin and growth retardants as inhibitors of chlorophyll synthesis.

3. Potassium ions at 0.01 M concentration strikingly stimulated both growth and chlorophyll synthesis in detached cotyledons of cucumber, and completely reversed or markedly reduced the inhibitory influence of coumarin and growth retardants on chlorophyll synthesis.

4. KCl had no effect on the growth of detached cotyledons of pumpkin and decreased accumulation of chlorophyll in them.

5. Phosfon D and CCC inhibited incorporation of uracil-^{14}C to RNA and leucine-^{14}C to proteins of detached cotyledons of cucumber. B-Nine inhibited synthesis of proteins and had no effect on synthesis of RNA.

6. Potassium ions strikingly stimulated synthesis of both protein and RNA in cucumber cotyledons, and reversed the inhibitory effect of growth retardants on incorporation of labelled precursors to RNA and protein.

7. It is suggested that coumarin and growth retardants inhibit synthesis of chlorophyll as the consequence of inhibition of protein synthesis.

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(Entered: November 28, 1969)

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Hamujący wpływ kumaryny i retardantów wzrostu na syntezę chlorofilu

Streszczenie

1. Kumaryna, B-9, CCC i Phosfon D, zastosowane w stężeniach nieznacznie wpływających na wzrost, silnie hamują syntezę chlorofilu w izolowanych, etiolowanych liścieniach ogórka i dyni.

2. Kwas giberelowy i benzyloadenina pobudzają wzrost izolowanych liścieni; BA silnie zwiększa poziom chlorofilu w liścieniach dyni. Oba fitohormony zasadniczo nie zmieniają aktywności kumaryny i retardantów jako inhibitorów syntezy chlorofilu; wymienione substancje znoszą pobudzający wpływ cytokininy na zielenienie się liścieni dyni.

3. Jony potasu w stężeniu 0.01 M silnie pobudzają zarówno wzrost izolowanych liścieni ogórka jak i nagromadzanie się w nich chlorofilu. KCl całkowicie znosi lub silnie obniża symptomy hamującego działania retardantów wzrostu i kumaryny na syntezę chlorofilu u ogórka.

4. KCl nie wpływa na wzrost liścieni dyni, a zastosowany w wyższych stężeniach obniża w nich poziom chlorofilu.

5. Phosfon D i CCC hamują włączanie uracylu-^{14}C do RNA i leucyny-^{14}C do białek izolowanych liścieni ogórka. B-9 hamuje syntezę białek, lecz nie wpływa na syntezę RNA.

6. KCl silnie przyśpiesza zarówno syntezę białek jak i RNA w izolowanych liścieniach ogórka, oraz odwraca skutki hamującego wpływu retardantów wzrostu na te procesy.

7. Przypuszcza się, że kumaryna i retardanty wzrostu hamują syntezę chlorofilu dzięki zahamowaniu syntezy białek.

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