Effect of 2-chloroethylphosphonic acid and vanillin on chlorophyll, protein and RNA synthesis in detached cucumber cotyledons, and chlorophyll degradation in senescing leaf discs of kale*

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Abstract

N⁶-benzylaminopurine (BAP) and KCl stimulated growth in detached greening cucumber cotyledons; BAP enhanced RNA synthesis whereas KCl strikingly accelerated leucine-¹⁴C incorporation into proteins.

Vanillin (0.01 M) inhibited greening and protein synthesis, and nullified the stimulatory effects of KCl. 2-chloroethylphosphonic acid (CEPA, 0.01 M) inhibited greening without any effect on protein synthesis when it was applied alone; CEPA decreased RNA synthesis, completely nullified the K⁺-induced synthesis of chlorophyll, and reduced the BAP- or KCl-induced growth.

Vanillin inhibited yellowing in senescing leaf discs of kale kept in darkness, whereas CEPA had an opposite effect.

INTRODUCTION

Exogenous auxins stimulate ethylene production in plants (Morgan and Hall 1962, Burg and Burg 1968, Maxie and Crane 1967). The same is true for gibberellin in some instances (Stewart and Freebairn 1969) and a well known growth inhibitor coumarin (Morgan and Powell 1969). It is well recognized that ethylene contributes to such the symptoms previously attributed solely to auxin as leaf senescence and abscission, growth inhibition, tissue proliferation, epinasty and geotropism. Ethylene participates in the breaking of seed dormancy (Stewart and Freebairn 1969; Ketrings and Morgan 1969) and in stimulation of rice coleoptile growth (Ku et al. 1970). It is well estab-

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lished that ethylene acts as a hormone for fruit ripening (cf. Burg and Burg 1965), and it seems that many growth regulator effects operate through an effect on ethylene production or are controlled by endogenous ethylene (cf. Pratt and Goeschl 1969; Jackson and Osborne 1970).

Numerous physiological responses characteristic of ethylene treatment may be induced by "Ethrel" (Amchem Products Inc., Ambler). The active ingredient of "Ethrel" is 2-chloroethylphosphonic acid (CEPA). This compound is converted quantitatively to ethylene by a base catalysed elimination reaction, taking place both *in vitro* and *in vivo* (Maynard and Swan 1963; Yang 1969):

Scheme I. Decomposition of 2-chloroethylphosphonic acid to produce ethylene, phosphate and chlorine.

"Ethrel" contains also 2-chloroethyl ester of CEPA which does not produce ethylene in an alkaline solution. Since the ester is biologically active, it has been postulated that the conversion of the ester to ethylene in the plant is mediated by hydrolytic enzymes (Cook and Randall 1968). CEPA ("Ethrel") is a very useful tool for studies of a physiological role of ethylene.

Vanillin is known as a stimulator of rooting and root growth (Pashkar' et al. 1969, Polesika 1968); the compound retards chlorophyll degradation in *Raphanus sativus* L. (Karantov 1969).

In this report effects of CEPA and vanillin on growth and chlorophyll, protein and RNA synthesis in detached cucumber cotyledons, and chlorophyll degradation in senescing kale leaf discs are described.

MATERIAL and METHODS

1. Plant material

Seedlings of *Cucumis sativus* L. var. "Delicatess" were grown for 5 days in darkness at 24°. The cotyledons were removed from seedlings by cutting with a razor blade at the base of the lamina, and placed inner surface uppermost in 10 cm Petri dishes on Whatman No. 2 blotting paper moistened with 5 ml of distilled water (the control) or solutions of growth regulators. The covered dishes were kept in continuous cool fluorescent light of 1300 lux at 24° (Knypl 1970).

The chlorophyll content in the cotyledons was estimated after 24 or 48 hours of incubation. In the tests with incorporation of labelled

leucine- ^{14}C into protein or uracil- ^{14}C into RNA the cotyledons were pretreated with the growth regulators for 21 or 45 hours.

Plants of kale, *Brassica oleracea* L. var. *acephala* cv. 'Niski zielony kędzierzawy', were grown in a garden. Old leaves, starting just to yellow, were gathered, thoroughly washed with dist. water and blotted. Discs, 7 mm in diameter, were punched from the interveinal portion of the leaf blade by means of a cork-borer. The discs were floated on 5 ml of the test solutions and maintained for 3 days in darkness at 25° (K n y p l 1967).

Penicillin G (50 mg/l) was included into all incubation media to prevent growth of bacteriae (V e n i s 1967).

2. Incorporation of leucine- ^{14}C into protein and uracil- ^{14}C into RNA

As a measure of the ability of the cotyledons to synthesize protein and RNA either directly (original cotyledons) or after various pretreatments, the extent of incorporation of labelled precursors was determined. The cotyledons after 21 or 45 hours of preincubation with growth regulators were thoroughly washed with distilled water and blotted. Fresh cut was then done at a distance of about 0.5 mm from the old one to ensure good penetration of solutes, and the cotyledons floated in 1.5 ml of a medium containing either 1 $\mu\text{Ci/ml}$ of L-leucine-U- ^{14}C (specific activity 90 mCi/mM) or 5 $\mu\text{Ci/ml}$ of uracil-2- ^{14}C (6 mCi/mM) and penicillin G (50 mg/l). After 3 hours of feeding in the light of 1400 lux at the tissue level at 24° , the cotyledons were rinsed quickly in water and immediately used for extraction of protein or RNA.

3. Procedure for estimating protein

The cotyledons, in groups of 10, were plunged into 5 ml of boiling 10^{-3} M solution of leucine- ^{12}C in 80% ethanol for 60 sec., homogenized in a mortar with a pestle and centrifuged. The pellet was washed in succession with 5 ml portions of 80% ethanol (twice), ice-cold 5% TCA (once), 96% ethanol (twice), ethanol ether (3 : 1) and ether (once). The residual tissue debris was treated with 1 N NaOH on a boiling water bath for 6 min. to solubilize the protein, cooled and centrifuged (F l e t c h e r and O s b o r n e 1965). To 2 volumes of the clear protein extract 1 volume of ice-cold 4 N TCA was added and left in a refrigerator for 60 min. Precipitated protein was centrifuged and the pellet redissolved in 10% NH_4OH . This solution was used for radiometric analysis; for estimation of protein content according to Lowry et al. (1951), the NH_4OH solution was diluted 20-fold with 0.5 N NaOH.

In a case of kale leaf discs the protein was determined directly in 1 N NaOH extract, without reprecipitation with TCA.

4. Procedure for estimating RNA

Slightly modified method of Cherry (1962) was used for estimating RNA. The cotyledons (groups of 10) were homogenized in 5 ml of ice-cold 10^{-3} M solution of uracil- ^{12}C in methanol, centrifuged, and the pellet washed twice with 5 ml portions of ice-cold methanol. The tissue debris was then extracted with 5 ml of ice-cold 0.2 N HClO_4 to remove acid soluble compounds. The precipitate was next extracted with ethanol and, finally, ethanol: ether (2:1) at 50° for 30 min. to remove lipids. The remaining tissue residue was treated with 0.3 N KOH for 16 hours at 37° to hydrolyse RNA. DNA was then precipitated by acidification to pH 1 with conc. HClO_4 at 0° (60 min.) and discarded by centrifugation; the precipitate was washed once again with 4 ml of 0.1 N HClO_4 and centrifuged. Ribonucleotides in the collected supernatant fraction were determined spectrophotometrically. Absorbancy difference at 260 nm and 290 nm of the extracts, diluted 15 times with 0.2 N HClO_4 , were referred to a standard curve obtained with similarly treated yeast RNA.

5. Estimation of chlorophyll

Chlorophyll was extracted with 80% acetone and determined spectrophotometrically (Bruinsma 1963; cf. Knypl 1970).

6. Estimation of radioactivity

0.2 ml aliquots of radioactive extracts were dropped on alumina planchets, dried at 70° and counted under scintillation detector SSU-4W with a counting efficiency of approximately 20 per cent.

7. Other details

Original solution of "Ethrel" (Amchem 68—250) was diluted with water to desirable concentration and neutralized with NaOH to pH 6. Excepting special tests (cf. Table 2), the solutions were freshly prepared.

Each treatment was repeated thrice and replicates were carried out within each treatment. Other details were described previously (Knypl 1970).

RESULTS

1. Growth and chlorophyll synthesis in cucumber cotyledons

Two lots of seeds were used in the tests. Lot I was purchased in 1968, lot II in 1969 in CNOS stores. Both lots of seeds were tested in October 1969 — March 1970.

Table 1

Effect of vanillin and CEPA on chlorophyll accumulation in etiolated cucumber cotyledons, kept in light for 48 hours

Compound, M	Lot of seeds	Net increment of fresh weight, mg/cotyledon	Chlorophyll a + b, μ g	
			per cotyledon	per g fresh weight
O (Control)	I	15.5	39.4	1135
Vanillin, 0.001	I	14.6	38.6	1142
Vanillin, 0.005	I	12.7	31.6	990
Vanillin, 0.01	I	10.8	20.7	690
CEPA, 0.005	I	15.0	38.0	1111
CEPA, 0.01	I	13.2	20.4	630
O (Control)	II	15.3	33.3	957
Vanillin, 0.005	II	15.9	34.3	971
Vanillin, 0.01	II	14.1	26.1	779
CEPA, 0.005	II	14.0	27.9	853
CEPA, 0.01	II	11.7	13.0	418

I, "old" seeds; II, "young" seeds. Original fr wt of one cotyledon: I, 19.2 mg; II, 19.4 mg. Net increment of fresh weight was calculated by subtracting original fr. wt. from the final one. L.S.D. ($p = 0.01$) = 11 per cent.

As it can be seen from table 1, vanillin at 0.01 M concentration inhibited growth of the cotyledons by 30% and decreased chlorophyll content by 50% when "old" seeds of lot I were used. The compound had no effect on growth when "young" seeds of lot II were used, but the chlorophyll content was reduced by 20%. It is worth noting that in the control cotyledons of lots I and II, kept in water for 2 days, the contents of chlorophyll were 39.4 and 32.3 μ g/cotyledon, respectively. Chlorophyll accumulation in the cucumber cotyledons is thus dependent on age of the seeds. The same is true for sensitivity to vanillin.

CEPA at 0.01 M inhibited synthesis of the pigment by about 50–60 per cent per cotyledon basis whereas growth was reduced by 15–18% (Table 1).

CEPA is unstable in neutralized solutions. Special tests were then designed to test how long solutions of Ethrel (0.005 M), neutralized to pH 6.0, can be stored in a refrigerator without losing the activity. The most active were solutions freshly prepared or stored for 24 hr (Table 2). Solutions of CEPA stored for 5 days lost 50 per cent activity in comparison with the freshly prepared ones.

As it has been reported (Knypl and Rennert 1970), KCl at 0.025 M stimulated 2.5-fold the net growth of the cucumber cotyledons. Potassium ions increased the content of chlorophyll by 300 per cent per cotyledon basis and 200 per cent per g fresh weight basis (Table 3). CEPA at 0.01 M nullified the KCl-dependent stimulation of the pigment accu-

Table 2

Effectivity of 0.005 M solutions of CEPA, stored for different periods of time in a refrigerator.
(Cucumber cotyledons; 48 hr tests)

Treatment	Time of storage, days	Net increment of fresh weight, mg/cotyledon	Chlorophyll a + b, μ g	
			per cotyledon	per g fresh weight
0 (Control)		15.8	35.4	1068
CEPA	0	14.1	28.0	840
CEPA	1	14.0	26.2	789
CEPA	5	14.5	32.6	967
CEPA	30	15.8	36.8	1051

Original fr. wt. of one cotyledon = 19.2 mg. Seeds of lot I were used.

mulation, that is the both CEPA alone and CEPA + KCl treated cotyledons had about 400 μ g of chlorophyll per g fresh weight basis.

Vanillin decreased slightly stimulations dependent on KCl.

Table 3

Growth and chlorophyll synthesis in detached cucumber cotyledons, incubated for 48 hours in light with CEPA or vanillin, applied alone or in combination with KCl, BAP or GA₃

Compound, M	Net increment of fresh weight, mg/cotyledon	Chlorophyll a + b, μ g	
		per cotyledon	per g fresh weight
0 (Control)	15.4	33.0	948
CEPA, 0.005	15.3	25.0	720
CEPA, 0.01	11.7	13.0	418
Vanillin, 0.005	14.8	36.5	1040
Vanillin, 0.01	14.6	23.8	700
Plus potassium chloride, 0.025 M			
0	34.4	96.6	1795
CEPA, 0.005	28.5	43.8	940
CEPA, 0.01	22.3	16.7	401
Vanillin, 0.005	32.2	87.2	1689
Vanillin, 0.01	30.0	81.5	1648
Plus benzylaminopurine, 10^{-5} M			
0	25.8	35.2	778
CEPA, 0.005	12.8	11.7	363
CEPA, 0.01	10.8	8.2	272
Plus gibberellic acid, 10^{-5} M			
0	19.6	39.9	1023
CEPA, 0.005	16.5	26.5	738
CEPA, 0.01	11.1	12.2	400

Original fr. wt. of one cotyledon = 19.4 mg. Seeds of lot II were used. L.S.D. ($p = 0.01$) between any two values = 12 per cent.

Fig. 1. Synergistic effect of KCl and benzylaminopurine on growth of detached cucumber cotyledons. From top to bottom: original etiolated cotyledons; water treated control; KCl, 0.025 M; KCl, 0.025 M and benzylaminopurine, 10^{-5} M. Incubation: 2 days.

CEPA erased completely the growth stimulatory effect of either N^6 -benzylaminopurine (BAP) or gibberellic acid (GA_3). It is of interest to note that the level of chlorophyll in CEPA+BAP treated cotyledons was significantly reduced in comparison with the CEPA alone treated samples (Table 3).

In the tests summed up in tables 1—3 the chlorophyll was estimated after 48 hours of incubation in light. In shorter, 24 hours tests, both CEPA and vanillin retarded chlorophyll accumulation by about 50 per cent. The effect of vanillin was completely reversed by BAP or KCl. BAP, in contrast to 48-hr tests, increased also a content of chlorophyll in the cotyledons treated with CEPA (Table 4). It is thus evident that [1] the chlorophyll synthesis inhibitory effect of vanillin is relatively short living, and [2] CEPA counteracts the effect of BAP.

Benzylaminopurine in combination with KCl synergistically stimulated growth of the cotyledons. In the control sample the net increment of fresh weight was 7.6 mg, in KCl alone treated sample 17.9 mg and in BAP alone treated one 12.3 mg. When KCl was applied in a combined solution with BAP, the net growth was increased to 26.7 mg. The increased growth rate was strictly correlated with the increased synthesis of chlorophyll (Table 1, Fig. 1).

Table 4

Effect of CEPA, vanillin, KCl and BAP on growth and chlorophyll synthesis in detached cucumber cotyledons, kept in light for 24 hours

Compound, M	Net increment of fresh weight, mg/cotyledon	Chlorophyll a + b, μ g		Chl a Chl b
		per cotyledon	per g fresh weight	
0 (Control)	7.6	17.5	662	2.4
CEPA, 0.01	6.2	7.8	312	2.5
Vanillin, 0.01	5.9	7.9	320	2.0
Plus potassium chloride, 0.025 M				
0	17.9	37.4	1019	2.4
CEPA, 0.01	11.2	9.2	305	2.7
Vanillin, 0.01	10.7	17.5	593	2.5
Plus benzylaminopurine, 10^{-5} M				
0	12.3	20.0	643	2.1
CEPA, 0.01	9.8	12.6	441	2.0
Vanillin, 0.01	10.7	18.0	610	2.2
KCl, 0.025	26.7	47.6	1046	2.0

Original fresh weight of one cotyledon = 18.8 mg. Seeds of lot II were used. L.S.D. ($p = 0.01$) = 0.9 mg for growth and 58 μ g for chlorophyll content.

2. Chlorophyll degradation in senescing leaf discs of kale

Vanillin at 0.01 M strikingly retarded yellowing of the kale leaf discs, kept in darkness for 3 days (Fig. 2, Table 5). During this time the chlorophyll content in the control discs, treated with water, decreased from original 17.66 to final 5.45 μ g per disc basis. Under the influence of

Table 5

Retardation and acceleration of chlorophyll degradation in senescing leaf discs of kale by vanillin and CEPA, respectively

Treatment, M	Time of incubation, days	Chlorophyll a+b, μ g/10 discs	Protein mg/100 mg original dry wt	Protein, mg/10 discs,
0	0*)	176.6	15.1	3.08
H ₂ O (Control)	3	54.7	9.6	1.90
Vanillin, 0.01	3	145.7	11.1	2.20
Vanillin, 0.01 plus CEPA, 0.005	3	124.5		
CEPA, 0.005	3	19.0		

*) The discs were analysed immediately after punching out. Protein was estimated in 1 N NaOH extracts, without reprecipitation with TCA.

vanillin the discs remained dark green and showed no visible symptoms of intoxication. Protein degradation was also postponed.

Fig. 2. Inhibition and stimulation of chlorophyll degradation in senescing leaf discs of kale, *Brassica oleracea* L. var. *acephala*, by vanillin and CEPA, respectively.

CEPA accelerated yellowing of kale leaf discs. Despite this fact, CEPA did not erase the retarding effect of vanillin on yellowing (Table 5).

3. Incorporation of leucine-¹⁴C

As it can be seen from table 6, protein content in the control cotyledons decreased from original 1.12 mg to 1.09 and 1.02 mg per cotyledon basis after 21+3 and 45+3 hours of incubation, respectively. Original cotyledons, etiolated, showed a relatively low protein synthesizing ability. Specific activity of proteins, extracted from the cotyledons fed with labelled leucine for 3 hours immediately following cutting, equalled 540 counts/min./mg protein. Light stimulated leucine-¹⁴C incorporation into protein by about 6-fold (spec. activity = 3010 and 3550 counts/min./mg protein after 21+3 and 45+3 hr, respectively).

Since the content of total protein in the green cotyledons was lower than in the original etiolated ones, it should be concluded that the light-dependent hydrolysis of storage proteins prevailed over the induced synthesis of protein.

CEPA at 0.01 M had no effect on protein synthesis. Vanillin inhibited leucine-¹⁴C incorporation by about 50% in the shorter, 21+3 hr lasting tests. This effect was transient and in the longer tests, 45+3 hr lasting

ones, vanillin treated cotyledons incorporated leucine- ^{14}C into proteins even more intensively than the control ones (Table 6).

KCl stimulated protein synthesis by about 250 per cent. This stimulation was decreased by CEPA and vanillin despite a fact that these compounds, when applied alone, did not inhibit protein synthesis in 48 hr tests.

Benzylaminopurine had no effect on protein synthesis when applied alone or in combination with CEPA; it significantly decreased the inhibitory effect of vanillin on this process (Table 6).

Table 6

Effects of CEPA and vanillin, applied alone or in combination with KCl or BAP, on protein synthesis in detached cucumber cotyledons

	Original values*)	After 21 hours in (M):			After 45 hours in (M):				
		H ₂ O (Control)	CEPA 0.01	Vanillin 0.01	H ₂ O (Control)	CEPA 0.005	CEPA 0.01	Vanillin 0.005	Vanillin 0.01
Fresh weight, mg/cotyledon	19.0	25.0	23.7	23.4	35.7	34.3	31.3	35.4	35.4
Protein, mg/cotyledon	1.12	1.09	1.09	1.10	1.02	1.06	1.06	1.04	1.08
Protein, counts/min./cotyledon	605	3281	3453	1485	3620	4210	3400	3880	4140
Specific activity of protein, counts/min./mg	540	3010	3170	1350	3550	3970	3120	3730	3830
Plus potassium chloride, 0.025 M									
Fresh weight, mg/cotyledon					57.3	51.1	43.8	57.7	52.4
Protein, mg/cotyledon					1.16	1.09	0.98	1.12	1.09
Protein, counts/min/cotyledon					10060	7520	5850	7990	5460
Specific activity of protein, counts/min./mg					8670	6900	5970	6870	5010
Plus benzylaminopurine, 10^{-5} M									
Fresh weight, mg/cotyledon		31.6	28.0	28.8					
Protein, mg/cotyledon		1.05	1.06	1.10					
Protein, counts/min./cotyledon		3400	3540	2266					
Specific activity of protein, counts/min./mg		3240	3340	2060					

L.S.D. ($p = 0.01$) between any two values: 1.0 mg for fresh weight; 0.046 mg for protein content; 330 counts./min./cotyledon for protein radioactivity. Seeds of lot II were used for tests.

*) Original values: The cotyledons were fed with leucine- ^{14}C for 3 hr in light immediately following cutting and analysed.

Radioactivity in 80 per cent ethanol soluble fraction can be taken as a measure of absorption of leucine from the medium. In the control and vanillin treated samples for 21 + 3 hr, radioactivity in this fraction was the same as in the original cotyledons. After 2 days of pretreatment radioactivity in the control doubled. CEPA at 0.01 M markedly (50%) increased radioactivity of 80% ethanol soluble fraction after 2 days of pretreatment. A slight stimulatory effect of CEPA after 21+3 hr of incubation was additive with the same effect of BAP (Table 7).

Table 7

Distribution of leucine- ^{14}C label between protein and 80 per cent ethanol soluble fractions of cucumber cotyledons treated with CEPA, vanillin, KCl and BAP

Fraction	Original values	Radioactivity, counts/min./cotyledon							
		After 21 hours in (M):			After 45 hours in (M):				
		H ₂ O (Control)	CEPA 0.01	Vanillin 0.01	H ₂ O (Control)	CEPA 0.005	CEPA 0.01	Vanillin 0.005	Vanillin 0.01
Ethanol 80%	1220	1350	1600	1190	3030	4290	4730	3140	3630
Protein	610	3280	3450	1490	3620	4210	3400	3880	4140
Total	1810	4630	5050	2680	6750	8500	8130	7020	7770
Plus potassium, chloride, 0.025 M									
Ethanol 80%					3000	3490	6180	2460	2240
Protein					10060	7520	5850	7990	5460
Total					13060	11010	12030	10450	7700
Plus benzylaminopurine, 10^{-5} M									
Ethanol 80%		1540	2000	1470					
Protein		3400	3540	2270					
Total		4940	5540	3740					

Fresh weight of the cotyledons is presented in Table 6.

KCl, in spite of a striking stimulation of protein synthesis, had no effect on activity in 80% ethanol soluble fraction when compared with the proper water treated control. In the sample pretreated with KCl+BAP radioactivity of this fraction was twice as much as in the control.

KCl markedly decreased radioactivity in 80% ethanol extracts of the cotyledons pretreated with vanillin (Table 7).

It has been reported that the detached cotyledon of cucumber contained 1.90—1.70 mg of protein (Knypl and Rennert 1970). In the tests summed up in Table 6 the content of protein was markedly lower. Radioactivity of protein fraction per cotyledon basis in Table 6 is also lower than it has been reported earlier (Knypl 1970). Special tests revealed that the differences were caused by modification of the procedure of extraction of protein. In previous experiments (Knypl 1970, Knypl and Rennert 1970) the protein was not re-precipitated with

4 N TCA from the 1 N NaOH-extracts. Table 8 shows that 1 N NaOH extract of the tissue (Fletcher and Osborne 1965) contains [1] protein which can be precipitated with 4 N TCA, and [2] non-precipitable supernatant material which is radioactive and positively reacts with the Folin phenol reagent in the method of Lowry et al. (1951) for protein estimation. This material gave no positive reaction in other tests for protein (thermal denaturation, salt precipitation); chromatography on Sephadex G-75 gels revealed that it was a low molecular weight material (data not shown). "Specific activity" of this TCA non precipitable fraction, that is counts per minute per mg of "protein" determined according to Lowry et al. (1951) colorimetric method, was 3 times lower in comparison with the specific activity of the protein (e.g. 1000 and 3260 counts/min./mg, respectively, after 21+3 hr of incubation).

Table 8

Distribution of the leucine- ^{14}C label between the protein and TCA non precipitable fraction of 1 N NaOH extract of cucumber cotyledons

Time of incubation in light, hours	Protein			TCA non precipitable material		
	mg/cotyledon	Counts/min./cotyledon	Specific activity*)	mg/cotyledon**)	Counts/min./cotyledon	"Specific activity"
21	1.08	3 260	3 020	0.48	480	1 000
45	1.01	3 560	3 525	0.41	540	1 317

*) Specific activity is expressed in counts/min./mg protein.

**) Since the TCA non precipitable material reacts positively with the Folin phenol reagent, A_{750} values were referred to a standard calibration curve for protein (bovine serum albumin).

On the basis of these data it can be concluded that [1] the step of re-precipitation with TCA is necessary since it purifies the protein fraction, and [2] the radioactive material remaining in TCA supernatant is not the protein. It is quite possible that this material represents leucine- ^{14}C originally bound in N-acetyl-leu-tRNA complex and/or in low molecular weight peptides (cf. Lipmann 1969).

4. Incorporation of uracil- ^{14}C

In the detached cotyledons of cucumber the content of protein dropped during the period of light incubation (Table 6). In contrast, the level of RNA after 21+3 and 45+3 hours of treatment rose in the control from original 93 μg /cotyledon to 135.0 and 147.8 μg /cotyledon, respectively. The RNA radioactivity per cotyledon basis increased from 148 counts per

Table 9

Effect of CEPA and vanillin, applied alone or in combination with KCl and BAP, on RNA synthesis in detached cucumber cotyledons

	Original values	After 21 hours in (M):			After 45 hours in (M):				
		H ₂ O (Control)	CEPA 0.01	Vanillin 0.01	H ₂ O (Control)	CEPA 0.005	CEPA 0.01	Vanillin 0.005	Vanillin 0.01
RNA, µg/cotyledon	93.0	135.0	118.7	110.7	147.8	151.6	77.0	135.6	133.4
RNA, counts/min./cotyledon	148	1200	1120	927	1508	1734	654	1365	1200
Specific activity of RNA, counts/min./µg	1.60	8.90	9.44	8.37	10.20	11.44	8.49	10.07	9.00
Plus potassium chloride, 0.025 M									
RNA, µg/cotyledon		169.0	143.6	167.6	173.2	190.4	149.9	186.7	186.4
RNA, counts/min./cotyledon		860	1330	1170	1300	1500	770	1120	1090
Specific activity of RNA, counts/min. µg		5.09	9.26	6.98	7.51	7.88	5.13	6.00	5.85
Plus benzylaminopurine, 10 ⁻⁵ M									
RNA, µg/cotyledon		198.3	153.0	168.8					
RNA, counts/min./cotyledon		2130	1530	1880					
Specific activity of RNA, counts/min./µg		10.74	10.00	11.37					

L.S.D. at $p = 0.01$: 10.0 µg for RNA; 160 counts/min./cotyledon for RNA radioactivity.

Fresh weight of the cotyledons is presented in Table 6. Original values: the cotyledons were fed with uracil-¹⁴C for 3 hours in light immediately following cutting, and analysed.

min. for original cotyledons (fed with uracil-¹⁴C for 3 hr in light immediately after cutting) to 1200 and 1510 counts/min. after 21+3 and 45+3 hours of incubation in light. Because of a rise of RNA content, the increment of specific activity of RNA was lower (1.60, 8.90 and 10.20 counts/min./µg RNA for 0, 21 and 45 hr pretreatment periods, respectively). There is no doubt that in the cucumber cotyledons the synthesis of RNA, on the contrary to synthesis of proteins, prevails over the RNA degradation in light (Table 9).

CEPA at 0.01 M markedly, and vanillin slightly, prevented the rise of RNA level. It is worth to note that in the CEPA treated cotyledons the content of RNA after 2 days of pretreatment was lower than in the original, etiolated ones. Owing to this 50 per cent reduction of the RNA content, the specific activity of ribonucleic acid fraction was only little diminished in comparison with the water treated control. Nevertheless, the total RNA activity per cotyledon basis was lowered even more than 50% (654 and 1508 counts/min./cotyledon for CEPA and water treated

samples, respectively). CEPA at a lower concentration of 0.005 M had no effect on the level of RNA, and slightly stimulated its synthesis (Table 9).

Specific activity of RNA in vanillin treated samples was of the same range as in the control. However, total RNA radioactivity per cotyledon basis was slightly but significantly reduced.

KCl significantly enhanced the content of RNA per cotyledon, but slightly reduced incorporation of uracil- ^{14}C into RNA. In consequence, KCl markedly reduced the specific activity of RNA. This was incompatible with results of previous tests (Knypl 1970) in which a marked stimulatory effect of potassium ions on RNA synthesis had been recorded. There is no doubt that the discrepancy was caused by application of different procedures of RNA extraction and estimation. The method applied in the present study seemed to be more satisfactory than the previously applied one.

KCl markedly increased the level of RNA in the samples treated with vanillin and CEPA, but the total RNA radioactivity per cotyledon basis was not markedly affected. It seems, thus, that KCl reduces and CEPA accelerates the process of RNA degradation (cf. Table 9).

Under the influence of BAP the radioactivity of RNA per cotyledon basis was doubled in comparison with the water treated control. The level of RNA was also markedly increased as well in the BAP alone treated samples as in the BAP+CEPA or BAP+Vanillin treated ones (Table 9).

Distribution of carbon-14 label of uracil between 4 fractions of cucumber cotyledons is presented in Table 10. Cherry (1962) has shown that uracil containing nucleotides were present in both methanol and 0.2 N HClO_4 extracts of peanut cotyledons. Methanol and perchloric acid soluble compounds of cucumber cotyledons showed high radioactivity: 1470 and 220 counts/min./cotyledon in the case of original cotyledons, 3240 and 690 counts/min./cotyledons after 21 hours of preincubation in water, and 13960 and 1125 counts/min./cotyledon after 45 hours of preincubation in water (Table 10). In contrast to results noted with leucine (Table 7), light already during the first 21 hr intensified the absorption of uracil from the medium by more than 2 times; the process was intensified by about 10 times during the second day of incubation. It can be thus concluded that each precursor was absorbed selectively. Leucine was readily incorporated into protein, and it did not accumulate in the cells. On the contrary, only a little portion of absorbed uracil was incorporated into the RNA, the major one remaining in a methanol soluble form (possibly free base or uridylic acid).

KCl and benzylaminopurine increased radioactivity in methanol and PCA extracts in comparison with the proper, water treated control. The effect of KCl is of special interest, because potassium ions decreased the radioactivity in RNA (Table 10). It seems, therefore, that absorption of

Table 10

Distribution of uracil-¹⁴C label between four fractions of cucumber cotyledons, incubated with CEPA, Vanillin, KCl and BAP

Fraction	Original values	Radioactivity, counts/min./cotyledon							
		After 21 hours in (M):			After 45 hours in (M):				
		H ₂ O (Con- trol)	CEPA 0.01	Vanil- lin 0.01	H ₂ O (Con- trol)	CEPA 0.005	CEPA 0.01	Vanil- lin 0.005	Vanil- lin 0.01
Methanol	1470	3240	4760	3000	13960	12530	7030	9000	9040
HClO ₄ , 0.2 N	220	690	1020	550	1125	1320	700	1380	1130
RNA	150	1200	1120	930	1510	1730	655	1365	1200
Tissue debris	30	410	350	280	1135	1100	230	1290	900
Total	1870	5540	7250	4750	17730	16680	8615	13035	12270
Plus potassium chloride, 0.025 M									
Methanol		4400	5720	2930	19330	19620	20500	19840	15160
HClO ₄ , 0.2N		730	1030	720	970	1190	980	810	980
RNA		860	1330	1170	1300	1500	770	1120	1090
Tissue debris		420	330	290	1110	880	390	1510	1300
Total		6410	8410	5110	22710	23190	22640	23280	18530
Plus benzylaminopurine, 10 ⁻⁵ M									
Methanol		4200	6420	4130					
HClO ₄ , 0.2N		890	1200	1060					
RNA		2130	1530	1880					
Tissue debris		500	440	520					
Total		7720	9590	7590					

This table is directly comparable with Table 9.

uracil from the medium is not strikingly correlated with the actual ability of cucumber cotyledons to synthesize RNA.

CEPA at 0.01 M increased radioactivity in both methanol and PCA fractions after 21 hr, and markedly decreased it after 45 hr of pretreatment. The latter effect was completely reversed by KCl. Potassium ions reversed also the inhibitory effect of vanillin (Table 10).

Table 10 shows that in the tissue debris, remaining after extraction with 0.3 N KOH, a relatively high radioactivity was included. This activity could not be extracted with hot (70°) 5% HClO₄; it was thus not bound with DNA (cf. Cherry 1962).

DISCUSSION

Experiments reported here have shown that CEPA and vanillin can inhibit growth and chlorophyll synthesis, the synthesis of chlorophyll being more affected than growth. The quantitative results, especially

those induced by vanillin, were markedly dependent on a lot of seeds and time of incubation of the detached cotyledons.

It is well established that greening of any etiolated tissue is dependent on continuous synthesis of proteins and nucleic acids. If these syntheses are disturbed, the greening is retarded (cf. B o g o r a d 1966; K h a v k i n 1969). In the case of etiolated cucumber cotyledons light accelerated indeed incorporation of leucine- ^{14}C into proteins and uracil- ^{14}C into RNA (Table 6). Since KCl stimulated selectively incorporation of labelled leucine into protein, it can be concluded that K^+ -dependent acceleration of chlorophyll synthesis and growth follow the stimulation of protein synthesis. KCl, in addition, retards possibly degradation of RNA.

N^6 -benzylaminopurine, in contrast to KCl, stimulated selectively incorporation of labelled uracil into RNA, being without effect on protein synthesis. It seems to be clear that BAP-induced growth of cucumber cotyledons follows the accelerated synthesis of RNA. The cotyledons treated with KCl in a combined solution with BAP showed the highest growth rate and have the highest content of chlorophyll per cotyledon basis (Fig. 1). It can be suggested that these effects follow simultaneous stimulation of synthesis of both RNA and protein by BAP and KCl, respectively.

CEPA had slight effect on protein synthesis. The compound markedly [1] inhibited synthesis of RNA, [2] reduced the stimulatory effect of BAP on uracil- ^{14}C incorporation into RNA, and [3] decreased the content of RNA in the cucumber cotyledons. It seems that CEPA can arrest the RNA synthesis as well as accelerate RNA breakdown. Both events can be responsible for arrested greening of the etiolated cotyledons on one hand, and stimulated yellowing of senescing leaf discs of kale on the other hand.

Vanillin at 0.01 M in one day tests markedly inhibited protein synthesis (Table 6) and prevented the rise of RNA level (Table 9), the effects being transient. Moreover, the compound in contrast to CEPA inhibited chlorophyll degradation in the leaf discs of kale. Since it has been revealed that arrested yellowing of senescing kale leaf tissue is dependent on arrested synthesis and degradation of proteins (K n y p l 1969), it can be suggested that vanillin affects, first of all, the protein metabolism.

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SUMMARY

Effects of 2-chloroethylphosphonic acid (CEPA) and vanillin applied alone or in combination with KCl, N⁶-benzylaminopurine (BAP) or gibberellic acid (GA₃) on growth, RNA, protein and chlorophyll synthesis in detached cucumber cotyledons have been investigated. Effect of vanillin and CEPA on chlorophyll breakdown in senescing leaf discs of kale (*Brassica oleracea* L. var. *acephala*) has also been studied.

1. Vanillin (0.01 M) and CEPA (0.01 M) retarded greening of the etiolated cucumber cotyledons more effectively than the net growth. The effect of vanillin was transient and markedly dependent on a lot of seeds used for tests.

2. The chlorophyll synthesis inhibitory effect of CEPA was not reduced by BAP or GA₃. CEPA completely nullified the K⁺-induced synthesis of chlorophyll, and reduced the BAP- or KCl-induced growth.

3. Vanillin at the highest concentration of 0.01 M inhibited yellowing of the leaf discs of kale; CEPA had an opposite effect.

4. BAP and KCl synergistically stimulated growth of the cotyledons. BAP markedly increased the content of RNA and accelerated incorporation of uracil-¹⁴C into RNA; the cytokinin had no effect on protein synthesis. KCl (0.025 M) stimulated incorporation of leucine-¹⁴C into protein by about 250%, and slightly decreased RNA synthesis. The stimulatory effect of KCl on protein synthesis in the cucumber cotyledons was reduced by CEPA and vanillin.

5. CEPA had no effect on protein synthesis. It inhibited synthesis of RNA and decreased RNA content per cotyledon basis, especially in prolonged 2 day tests. Vanillin in 1 day lasting tests markedly inhibited incorporation of leucine-¹⁴C into protein, and prevented the rise in RNA content in the detached cucumber cotyledons.

It is suggested that CEPA acts primarily on RNA metabolism whereas vanillin on protein turnover.

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*Wpływ kwasu 2-chloroetylofosfoniowego i waniliny na syntezę chlorofilu,
białek i RNA w odciętych liściach ogórka*

Streszczenie

Badano wpływ kwasu 2-chloroetylofosfoniowego (CEPA) i waniliny — stosowanych pojedynczo i w mieszaninach z KCl, benzyloaminopuryną (BAP) lub kwasem gibberelowym (GA_3), na wzrost oraz syntezę RNA, białek i chlorofilu w etiolowanych liściach ogórka. Zbadano również wpływ waniliny i CEPA na rozpad chlorofilu w starzejących się krążkach liści jarmużu. Stwierdzono co następuje:

1. Wanilina (0.01 M) i CEPA (0.01 M) hamują bardziej efektywnie proces zielenienia się etiolowanych liści ogórka, niż ich wzrost. Skutki działania waniliny przemijają w czasie i w dużej mierze zależą od serii nasion używanych do hodowli siewek.

2. BAP i GA_3 nie zmniejszają hamującego wpływu CEPA na syntezę chlorofilu. CEPA całkowicie znosi pobudzający wpływ jonów potasu na proces zielenienia się liści, oraz silnie obniża stopień pobudzenia wzrostu liści przez KCl i BAP.

3. Wanilina w stężeniu 0.01 M silnie hamuje rozpad chlorofilu w krążkach liści jarmużu, hodowanych w ciemności; CEPA przyspiesza żółknięcie krążków lecz nie zmienia efektywności waniliny.

4. BAP i KCl pobudzają synergistycznie wzrost izolowanych liści ogórka. BAP silnie przyspiesza syntezę RNA (włączanie uracylu- ^{14}C) oraz zwiększa poziom RNA w przeliczeniu na jeden liść, a nie wpływa na syntezę białek. KCl (0.025 M) przyspiesza włączanie leucyny- ^{14}C do białek liści o 250%, oraz słabo hamuje syntezę RNA. CEPA i wanilina zmniejszają stopień pobudzającego wpływu jonów potasu na syntezę białek.

5. CEPA nie wpływa na syntezę białek, ale silnie hamuje włączanie uracylu- ^{14}C do RNA i obniża poziom RNA w liściach ogórka, zwłaszcza w doświadczeniach dwudniowych. Wanilina w doświadczeniach jednodniowych silnie hamuje syntezę białek, oraz obniża wzrost poziomu RNA w liściach.

Przypuszcza się, że CEPA działa przede wszystkim na przemiany RNA, a wanilina zaburza syntezę białek.