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THE INFLUENCE OF LIGHT OF DIFFERENT SPECTRAL REGIONS ON THE SYNTHESIS OF PHENOLIC COMPOUNDS IN GHERKIN SEEDLINGS IN RELATION TO PHOTOMORPHOGENESIS

I Biosynthesis of phenolic compounds

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ABSTRACT

1) Dark-grown gherkin seedlings contain the sugar derivatives of p-coumaric acid and ferulic acid, present in both the cotyledons and hypocotyls, and a number of flavonols, occurring only in the cotyledons. Exposure of these seedlings to light results in an increase in the accumulation of these phenolic compounds after a lag of 1½ to 2 hours.

2) The responsivity of this effect to radiation in the blue and far-red wavelength regions is higher than to red light. Both photoresponse I, the "high-energy reaction", and photoresponse II, the phytochrome reaction, seem to be involved.

3) The distribution pattern of the phenolics in the hypocotyl changes in the course of the irradiation: An initial increase in the concentration of hydroxycinnamic acids is followed by a decrease, particularly in the apical part. The ratio of ferulic acid to p-coumaric acid increases and is always higher in the lower part of the hypocotyl than in the apical part.

4) The accumulation of hydroxycinnamic acids in the hypocotyl in red and far-red light is largely dependent on the presence of the cotyledons. In blue light there is also a considerable synthesis when the cotyledons are excised.

INTRODUCTION

Evidence that changes in the auxin metabolism play an important role in photomorphogenesis has been obtained in a number of investigations. In experiments with gherkin seedlings and young tomato plants MEIJER (1958b, 1959) has demonstrated that the characteristic effects of certain light treatments could be imitated by the application of synthetic auxins or antiauxins. BLAAUW-JANSEN (1959) and BRIGGS (1963) have shown that irradiation with red light causes a lowering of the level of auxin or auxinlike substances in *Avena* and corn coleoptiles. In studies with *Phaseolus vulgaris* FLETCHER and ZALIK (1964) found this to be the case for other spectral regions as well. They obtained a direct relationship between the indoleacetic acid (IAA) content after one light cycle and plant height after 7 cycles.

A mechanism to account for these changes in IAA concentration is given in the IAA oxidase hypothesis according to which light

regulates the concentration of IAA through a lightdependent synthesis of phenolic cofactors and inhibitors of an enzyme, IAA oxidase, which is responsible for the inactivation of IAA (HILLMAN and GALSTON, 1957). This hypothesis is based on investigations by TANG and BONNER (1947, 1948) who found that peas grown in the light contain more inhibitors than etiolated peas. The inhibitors were later identified as kaempferol-3-triglucoside, kaempferol-3-(triglucosyl-p-coumarate) (FURUYA, GALSTON *et al.*, 1962; MUMFORD *et al.*, 1962) and related compounds with quercetin instead of kaempferol (FURUYA, GALSTON *et al.*, 1962). However, so far a relation between the effects of light on the synthesis of the kaempferol derivatives and the development of the pea seedling could not be found (FURUYA and THOMAS, 1964). For a large number of other plants as well it has been found that light induces or stimulates the synthesis of structurally related phenolic compounds like hydroxycinnamic acids, flavonols and anthocyanins (SIEGELMAN, 1964). Whether this demonstrates the widespread significance of a phenol-dependent IAA regulating mechanism remains to be established.

The inhibitory effect of light of different spectral regions on the elongation of gherkin seedlings has been the subject of a number of investigations (MEIJER, 1958a, 1958b, 1959, 1965). In preliminary experiments it appeared that the hypocotyls of these seedlings are particularly well suited to a study of the IAA oxidase-growth relationship since they contain only one IAA oxidase cofactor and one inhibitor. Moreover the tissue does not contain appreciable amounts of other compounds absorbing in the region where these phenolic modifiers have their main light absorbance, which permits a direct spectrophotometric determination of the phenols (hydroxycinnamic acids) in an aqueous extract.

A second reason why we are particularly interested in the light dependent synthesis of these hydroxycinnamic acids is that it offers a starting point for investigating how and where light acts on the metabolism.

The influence of the light quality on the synthesis of phenolic compounds will be described in this paper. In a second paper an analysis will be given of the IAA oxidase system of gherkin seedlings and it will be discussed whether the light dependent changes in this system can be related to the concomitant developmental changes induced in the seedling.

METHODS AND MATERIALS

Plants

All the experiments were carried out with gherkin seedlings (*Cucumis sativus* "Venlose niet plekkers", strain Tercken VI). The seeds were germinated in a mixture of leaf mould and sand in a dark room with an air humidity of about 90 % at 25° C. The light treatments started 60 hours after sowing, the seedlings having been picked for a uniform length of the hypocotyl of about 25 mm. During the

next 48 hours the elongation of the dark-grown seedling is linear with time (MEIJER and ENGELSMA, 1965).

Irradiation

Irradiations were carried out in light cabinets. The characteristic of the red and blue light were as given before (MEIJER, 1957). Far-red irradiation ($\lambda > 7000$ Å) was obtained from incandescent light filtered with a combination of a water filter, two sheets of blue and one sheet of red "Plexiglas" (Röhm and Haas, Darmstadt, nos 27 and 1 respectively, thickness 3 mm). Light intensities were measured with a thermopile or with a calibrated photocell. The light intensity of the far-red radiation is given for the 7000–8000 Å region.

Separation and identification of phenolic compounds

The hypocotyls of gherkin seedlings contain the sugar esters of two hydroxycinnamic acids, p-coumaric acid and ferulic acid, of which the former always predominates. They were extracted as follows. Immediately after harvesting the hypocotyls were macerated in a mortar under liquid nitrogen. The resulting powder was added slowly to boiling water, 10 ml per gram fresh weight, to destroy any enzyme action as rapidly as possible. After continued boiling for 5 min the mixture was cooled and centrifuged at 20000 g. The precipitate was washed with water and the combined supernatant and washing liquids were concentrated in vacuo at about 35° C. From time to time methanol was added to the solution to lower the boiling-point. The concentrated solution was chromatographed on paper (Macherey, Nagel & Co, MN 2261 A) using the descending method with isopropanol-1N-ammonia-water (10:1:1, by vol.) as solvent mixture. The phenolic bands are easily detected and characterized by examining the chromatograms under a UV source before and after impregnation with ammonia vapour. A disadvantage of this method is that under the influence of the UV light the hydroxycinnamic acids are easily converted from the naturally occurring trans-form into the cis-form.

From the cotyledons three p-coumaric acid sugar derivatives, one ferulic acid sugar derivative and four other compounds of flavonoid nature have been isolated by a similar procedure. The compounds were provisionally separated by paper chromatography of a concentrated aqueous extract with isopropanol-ammonia-water as a solvent system. A further separation could be achieved by rechromatography of the different bands with water or n-butanol-acetic acid-water (5:1:4, by vol.) as a solvent system. The characteristics of the compounds isolated are given in table 1.

Chromatography of alcoholic extracts gives the same bands of phenolic compounds. This method, however, has the disadvantage that a number of non-phenolic compounds, probably carotenoids which interfere with the chromatographic separation, are extracted as well.

The identity of p-coumaric and ferulic acid was confirmed by comparison with original samples of the ultraviolet spectra and the

TABLE 1

Properties of phenolic compounds isolated from gherkin seedlings.
Solvent systems:
A Water.
B Isopropanol – 1N ammonia – water (10:1:1, by vol.).
C n–Butanol – acetic acid – water (5:1:4, by vol.).
IAA oxidase modifiers: + Cofactor.
— Inhibitor.

Designation Cotyledons	Rf values in solvent			Colour		Spectral maxima and shoulders(s)		Activity as IAA oxidase modifier	Hydrolysis product
	A	B	C	Ultraviolet	Ultraviolet/ ammonia	95 % ethanol	+ KOH		
PC 1	0.80	0.65	0.80	Colourless	Blue	225(s), 275, 313	—, 300, 310, 368	+	p–Coumaric acid
PC 2	0.75			Colourless	Blue	223(s), 276(s), 315	245(s), 300, 310(s), 368	+	p–Coumaric acid
PC 3	0.70			Colourless	Blue	223(s), 301(s), 316	245(s), 300(s), 310, 370	+	p–Coumaric acid
FE 1	0.65	0.50	0.70	Blue	Green	223, 286(s), 313	245(s), 300, 310, 364	—	Ferulic acid
FL 1	0.60	0.60			Yellow green	274, —, 316	279, —, 370	—	
FL 2	0.40	0.50			Yellow green	271, 287(s), 329	279, 332, 398	—	
FL 3	0.25	0.30			Yellow green	264, —, 332	265, —, 407	—	
FL 4	0.15	0.20			Yellow green	273, —, 315	280, 296, 363	—	
Hypocotyls									
PC 4	0.66	0.55	0.69	Colourless	Blue	225(s), 279, 315	—, 308(s), 351	+	p–Coumaric acid
FE 2	0.57	0.46	0.62	Blue	Green	—, 295, 317	—, 305, 353	—	Ferulic acid

Rf values in two other solvent systems in addition to the systems mentioned above: chloroform-methanol-formic acid-water (10 parts of chloroform saturated with a mixture of 1 part methanol and 1 part 4 % formic acid) and benzene-ethyl methyl ketone-formic acid-water (a mixture of 9 parts benzene and 1 part ketone saturated with 1 part 2 % formic acid) (REIO, 1959; 1961).

The spectra were obtained with a Cary Model 14 recording spectrophotometer. For the p-coumaric and ferulic acid derivatives of the hypocotyls the molar absorbances in aqueous solutions of pH > 10.0 are 3.08×10^4 cm²/mmole (351 mμ) and 2.85×10^4 cm²/mmole (353 mμ) respectively. The calculation of these values is based on the amounts of hydroxycinnamic acids obtained on hydrolysis with KOH. Hydroxycinnamic acid contents in the hypocotyls given in this paper have been calculated by using a molar absorbance of 3×10^4 cm²/mmole at 350 mμ in solution of water at pH > 10.0.

Quantitative determination of phenolic compounds

For a quantitative determination of the hydroxycinnamic acids in the hypocotyls two methods will be described. The first method is a modification of a procedure used by ELEMA (1960).

I. Three hundred hypocotyls were frozen in liquid nitrogen and powdered in a mortar. The resulting powder was stirred into one and a half times its weight of a 0.2 N solution of sodium hydroxide in water, and the suspension left at room temperature for 4 hours to complete hydrolysis of the sugar esters. The precipitate was centrifuged off and washed twice with 0.2 N sodium hydroxide. The combined sodium hydroxide solutions were acidified with 0.1 N sulphuric acid until pH 2.5. The precipitate was centrifuged off and washed twice with water. The acidic solution was extracted overnight in a shaking machine with 150 ml ether. After separation the water phase was extracted two more times with ether. The combined ether extracts were extracted three times with 70 ml portions of a saturated sodium hydrogen carbonate solution. The hydrogen carbonate solution was acidified with 0.1 N sulphuric acid to pH 2.5 and extracted six times with ether. The ether extracts were evaporated in vacuo and the residue dissolved in 100 ml of ethanol. This solution can be used for a spectrophotometric estimation of the total amount of hydroxycinnamic acids. The calculations are based on the absorbance at 320 mμ. At this wavelength p-coumaric acid and ferulic acid have the same molar absorbance. By means of paper chromatography it was checked that no other compounds absorbing at 320 mμ are present in the alcoholic solution.

II. The second method consists of a direct spectrophotometric determination of the unhydrolysed hydroxycinnamic acid derivatives in an aqueous solution. Thirty hypocotyls were frozen in liquid nitrogen and macerated in a mortar. The resulting powder was added to 25 ml boiling water. After continued boiling for 5 min, followed by cooling, the precipitate was filtered off and washed with a small volume of water. The combined filtrate and washing liquid were

filled up with water to 22.5 ml. From this solution 4.5 ml was taken which, after addition of 0.5 ml phosphate buffer, 0.2 M, pH 6.0, was centrifuged at 20000 g for 40 min. The supernatant is perfectly clear and does not show any light absorption at 400 mμ. Fig. 1 gives an example of such a UV spectrum with peaks at 310 mμ, due to the hydroxycinnamic acids, and at 260 mμ, due to soluble proteins and nucleic acids.

The 310 mμ band shifts to 350 mμ on addition of KOH. As mentioned before, the absorbance at this wavelength has been used for calculations.

To compare both methods over a range of different phenol concentrations and for different parts of the hypocotyl, the following experiment was performed. From a box with three-days-old dark-grown seedlings 330 plants were harvested, and the remaining seedlings were exposed to blue light (700 μW/cm²). From these plants portions of 330 seedlings were harvested after 8 and 24 hours. The hypocotyls were cut into three sections, the apical part (5 mm), the upper half of the remaining part of the hypocotyl and the lower half. 300 sections were used to determine the amount of hydroxycinnamic

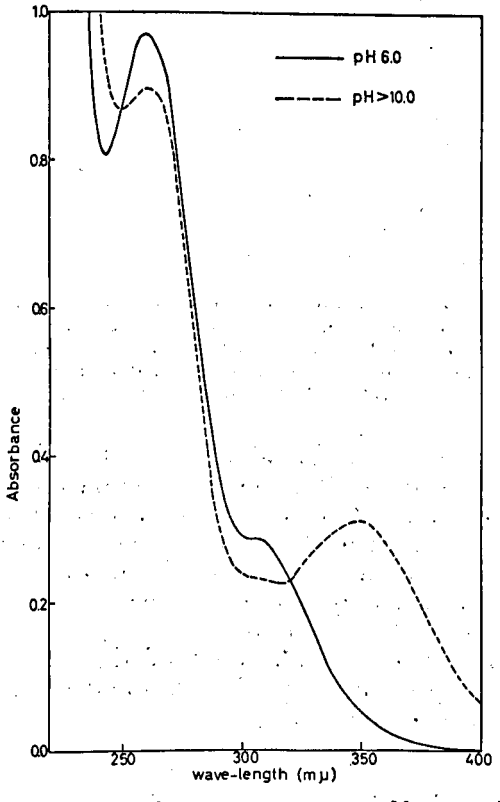

Fig. 1. Ultraviolet spectrum of an aqueous extract of hypocotyls from with blue light irradiated gherkin seedlings prepared according method II (see text).

acids in the different parts by method I, whereas for method II samples of 30 sections were taken. As can be seen in fig. 2, the first method always gives slightly higher values than the second method. This may be due to:

1) Adsorption of phenolic compounds to the precipitate. The values could not, however, be increased by further washing of the precipitate.

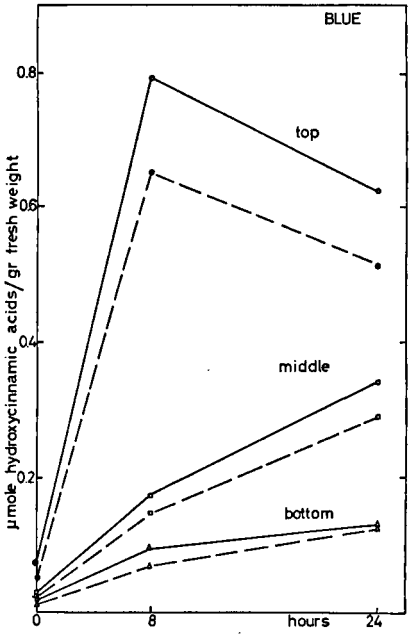

Fig. 2. Time dependent distribution of hydroxycinnamic acids in the top of the hypocotyl (5 mm) and the upper and lower half of the remaining part of the hypocotyl of gherkin seedlings in blue light of 700 μW/cm^2. The cinnamic acid derivatives have been determined by both methods mentioned in the text. Unbroken line: method I. Broken line: method II.

2) The possibility that in addition to the sugar esters of the hydroxycinnamic acids small amounts of the phenols occur as glycosides (RUNECKLES and WOOLRICH, 1963). Hydroxycinnamic acids thus bound are included in the measurements by method I but not by method II. Since we are concerned with modifiers of IAA oxidase the method by which only compounds with free hydroxyl groups are determined should be preferred.

In each experiment the phenolics of duplicate samples were determined. The results presented in fig. 5 and table 3 are averages of three different experiments.

RESULTS

The hypocotyls of gherkin seedlings contain two phenolic compounds, p-coumaric acid and ferulic acid. They occur estrified to sugars. A

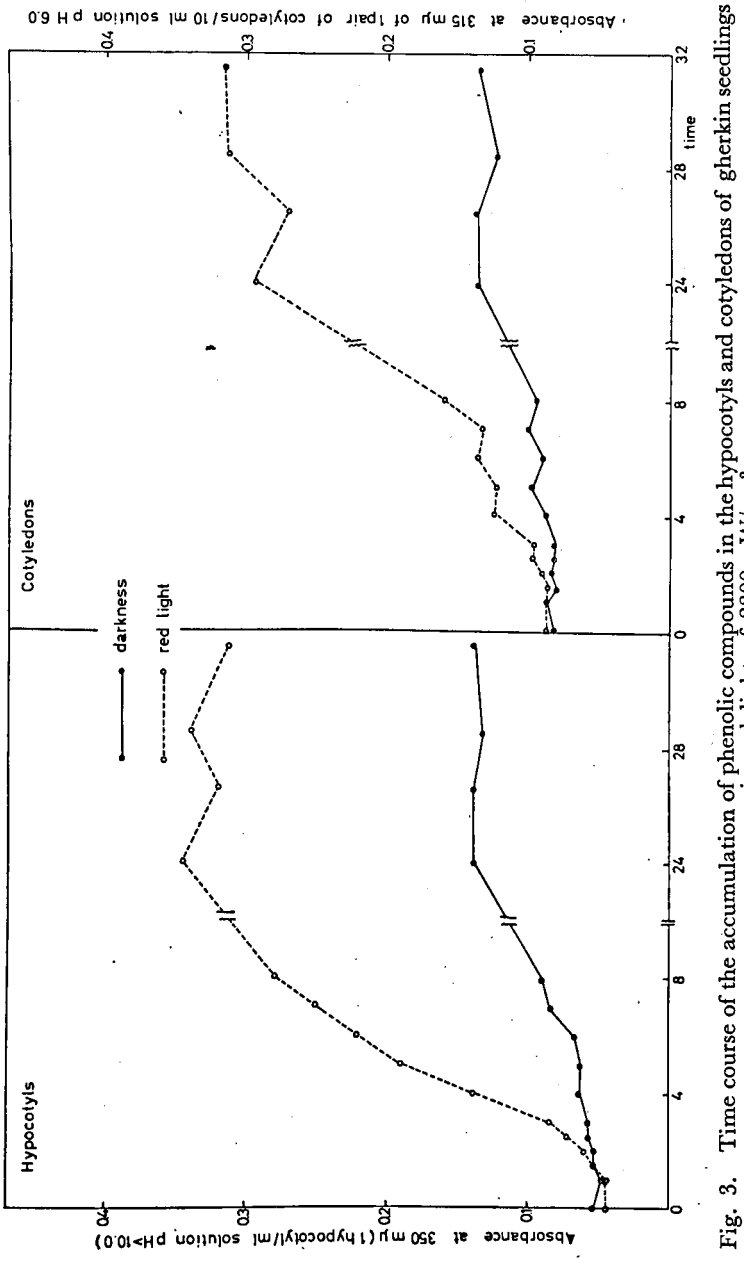

Fig. 3. Time course of the accumulation of phenolic compounds in the hypocotyls and cotyledons of gherkin seedlings in red light of 2300 $\mu W/cm^2$.

watery extract of the hypocotyl does not contain measurable amounts of other compounds absorbing above 300 mμ. This part of the seedling is therefore particularly well suited for a quantitative spectrophotometric determination of the hydroxycinnamic acids. The cotyledons contain, besides sugar derivatives of the two hydroxycinnamic acids mentioned, a number of flavonoid compounds all absorbing in the same wavelength region (Table 1). A detailed study of the accumulation of phenolic compounds in this part of the plant is therefore much more difficult.

Lag period of the photoresponse

No phenolics have been found in light-treated seedlings that were not already present in the dark-grown seedlings, but light stimulates the synthesis of these compounds. There is a lag of 1½ to 2 hours between the beginning of the irradiation and a detectable increase of the hydroxycinnamic acids in the hypocotyls. In fig. 3 this is shown for three-days-old seedlings transferred from darkness to a cabinet with red light of 2300 μW/cm² at the time zero of the graph.

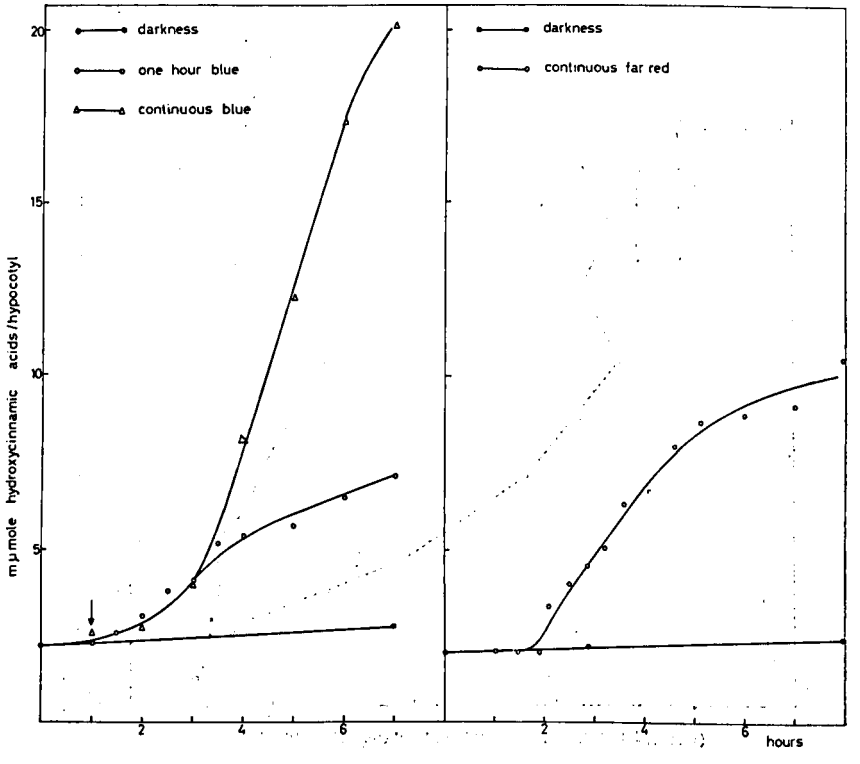

Fig. 4. Time course of the accumulation of hydroxycinnamic acids in the hypocotyls of gherkin seedlings, continuously irradiated with blue light, one hour of blue light and then returned to darkness, and continuously in far-red radiation. Light intensities: blue 800 μW/cm², far red 600 μW/cm².

Lags of about the same length were obtained in blue light (800 μW/cm^2) and far-red radiation (600 μW/cm^2) (fig. 4).

In the cotyledons the increase in synthesis of phenolic compounds as measured from the increase of absorbance at 315 mμ is less drastic during the first 8 hours of irradiation. This makes a determination of the lag less accurate but the length seems to be of the same order as for the hypocotyls (fig. 3).

Effects of light intensity, light quality, and duration of irradiation on the phenol synthesis

When the seedlings are continuously irradiated the lag period is followed by a stage in which the rate of accumulation of hydroxycinnamic acids in the hypocotyl is roughly linear with time. In a third stage the rate has much declined. For seedlings returned to darkness after a light treatment with blue or far-red this decline in phenol synthesis occurs at an earlier point (fig. 4). The next figure (5)

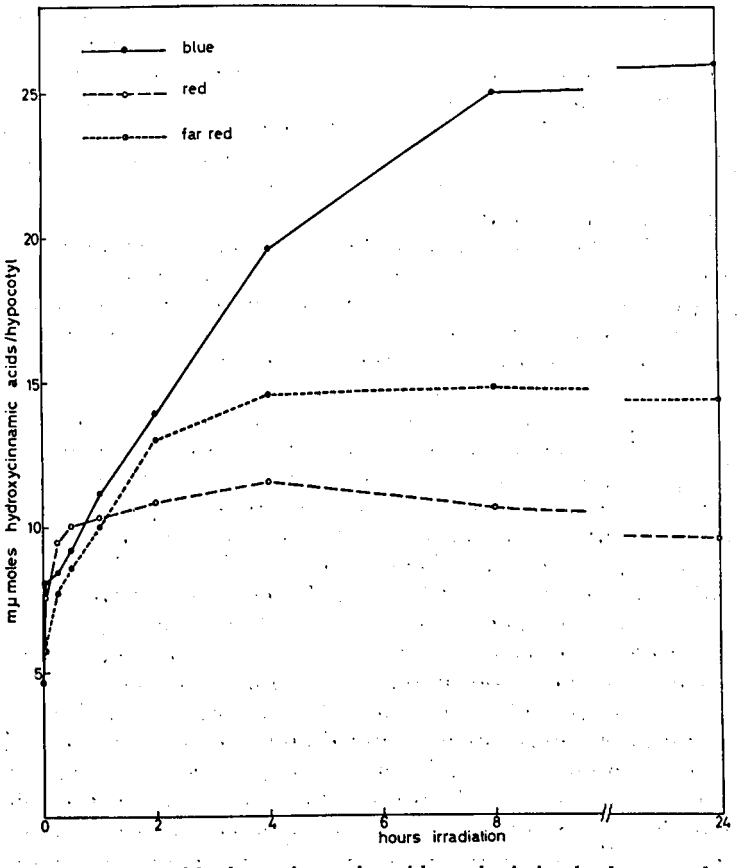

Fig. 5. Dependence of hydroxycinnamic acid synthesis in the hypocotyls on the time of light exposure in the blue (800 μW/cm^2), red (400 μW/cm^2), and far-red (600 μW/cm^2) wavelength regions.

shows that particularly in these two spectral regions the amount of phenolic compounds synthesized during 24 hours is an increasing function of the duration of the irradiation up to a certain length of time. It is noteworthy that on the basis of total amount of hydroxycinnamic acids per hypocotyl the process becomes more or less saturated though the capacity of the seedling to synthesize phenols has not been fully exhausted. In red light the total amount that accumulates during 24 hours in the hypocotyls of continuously irradiated plants is even smaller than when the seedlings are irradiated for ½ to 4 hours and then are returned to darkness. On a fresh weight basis, however, the concentration in continuously irradiated plants is higher, due to a stronger growth inhibition of these plants compared with the seedlings transferred back to darkness. Results of the experiments to be described next (fig. 6) suggest that the height of the saturation level for a particular light quality as shown in fig. 5 increases as the light intensity increases.

A very short irradiation (10 sec) with low intensity red light (50 μW/cm^2) already induces a net increase of 3.0 mμ mole hydroxycinnamic acids per hypocotyl (measured after 24 hours), which is more than half the value obtained for plants continuously irradiated with red light during 24 hours. A far-red irradiation (600 μW/cm^2) of 1 min immediately after the red light treatment results in almost complete reversal. A short irradiation with blue light (1 min) has a comparatively large effect as well, but no reversal by a subsequent far-red irradiation could be obtained.

Also for the phenol synthesis in the cotyledons a (partial) red/far-red reversibility was observed. The results indicate that Phytochrome ("photoresponse II") is involved in these processes. The response in blue and far-red radiation shows the characteristics of the light involved process indicated as "photoresponse I" (Siegelman 1964), also known as "high energy reaction".

In order to study whether there is a correlation between the effects of light on the phenol synthesis and the developmental phenomena, we set up a number of large-scale experiments. All the seedlings of one experiment were raised at the same time under the same conditions to avoid any influence of temperature or humidity on the results. For the three wavelength regions, blue, red and far-red, intensities of 400, 40 and 12 μW/cm^2 were used. Three days after sowing, boxes with each at least 120 seedlings were, except for the dark controls, transferred to the different light cabinets. For these experiments we chose continuous irradiations in order that the plant would have to go only once through a change in environment in the course of the experiment. Immediately before transfer and after 8, 24 and 48 hours the lengths of the hypocotyls were measured and 30 plants were harvested from each box. Because the phenolic compounds are inhomogeneously distributed in the plant, each seedling was divided into four parts: the cotyledon, the upper part of the hypocotyl of about 5 mm (plumular hook), the upper half of the remaining part of the hypocotyl and the lower half. The different

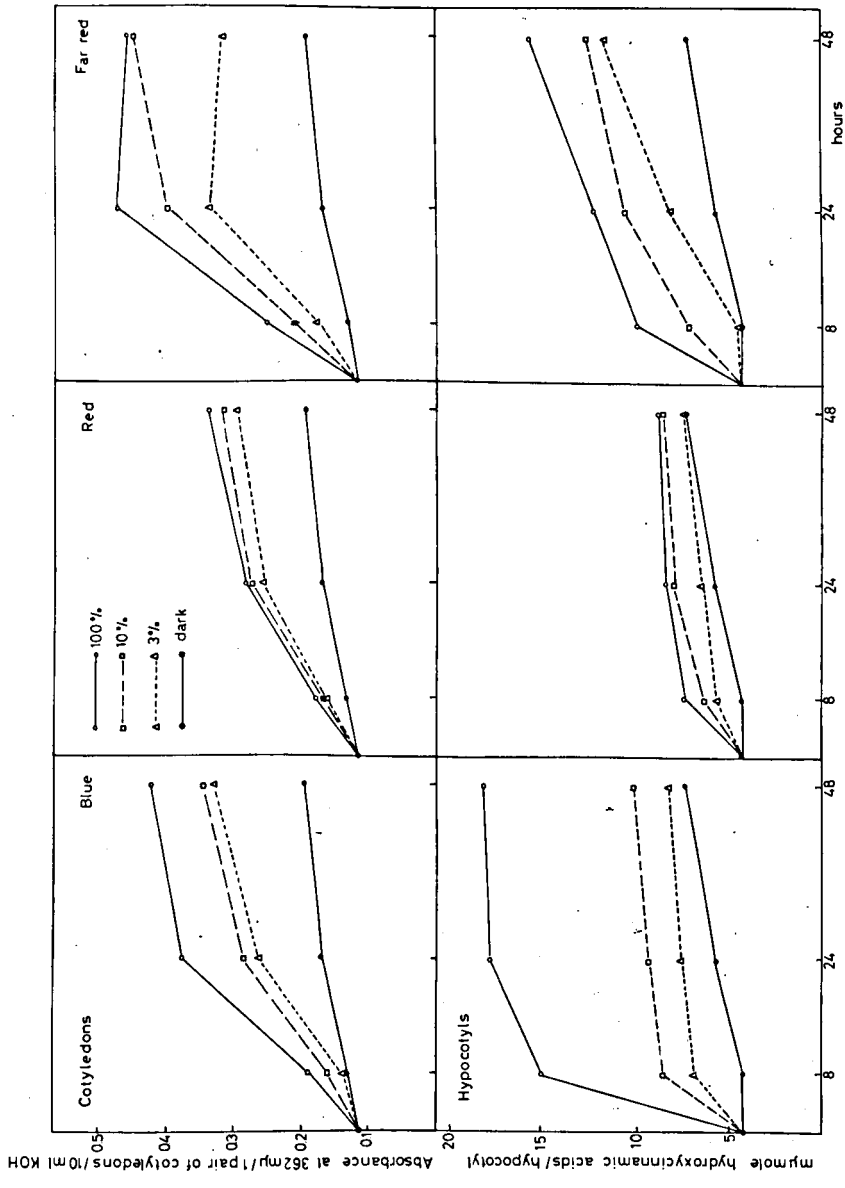

Fig. 6. Accumulation of phenolic compounds in gherkin seedlings under different light conditions (continuous irradiation). Highest light intensity (100 %) for the three wavelength regions 400 μW/cm^2.

parts were weighed and the amounts of phenolics determined spectrophotometrically. Fig. 6 gives the total amounts of phenolic compounds synthesized under the different conditions and fig. 7 shows the light and time dependent changes in the distribution pattern of the hydroxycinnamic acids in the hypocotyl. The data for the concomitant inhibition of the hypocotyl elongation and the expansion of the cotyledons will be published in the next paper.

As to the phenolic compounds, from this and four duplicate experiments, which gave very similar results, the following conclusions can be drawn: In blue and far red the amounts of phenolic compounds per cotyledon and per hypocotyl increase much more strongly with increasing light intensity than in red light. In blue light, particularly at higher intensities, the growth inhibition is very strong and the rate of phenol synthesis high during the first 8 hours of irradiation. This reults in short plants with very high concentrations of hydroxycinnamic acids in the hypocotyls. In far-red, on the other hand, the inhibition of elongation and the rate of accumulation of phenolics are less than in blue light of comparable intensity. Accumulation of hydroxycinnamic acids, however, continues for a longer period and eventually the amount per hypocotyl may be higher than in the plants treated with blue light whereas the amount on a fresh weight basis is much lower.

Light-dependent changes in concentration and distribution of the phenolic compounds in the seedling

In dark-grown seedlings the concentration of hydroxycinnamic acids is always higher in the apical part of the hypocotyl than in the remaining part. This can be made visible by viewing the seedling under an ultraviolet source, after soaking in concentrated ammonia. Only in the part immediately below the cotyledon can the blue fluorescence due to p-coumaric acid be clearly seen. On irradiation of the seedling the increase of phenolic compounds is first of all in this section (fig. 2 and fig. 7). Particularly in blue light high concentrations can be obtained here, up to 8.10^{-4} M, for the highest intensity we had available (800 $\mu W/cm^2$). Later there is always a decrease in this part of the hypocotyl, whereas in the lower part a further accumulation may occur. These hypocotyls show a blue fluorescence under a UV source, which becomes bluish green when they are soaked in concentrated ammonia. This is due to the presence of ferulic acid, particularly in the lower parts of the hypocotyl, as shown in table 2.

To determine the ferulic acid percentages, which are of importance for the IAA oxidase inhibitor-cofactor balance, we separated the hydroxycinnamic acid esters from the hypocotyls by paper chromatography (IAW). The concentrations of both compounds were determined spectrophotometrically in the alcoholic solution obtained by exhaustive extraction of the bands from the paper. The data of table 2 show that in blue light the ratio of ferulic acid to p-coumaric acid increases as a function of the irradiation time. Although no exact

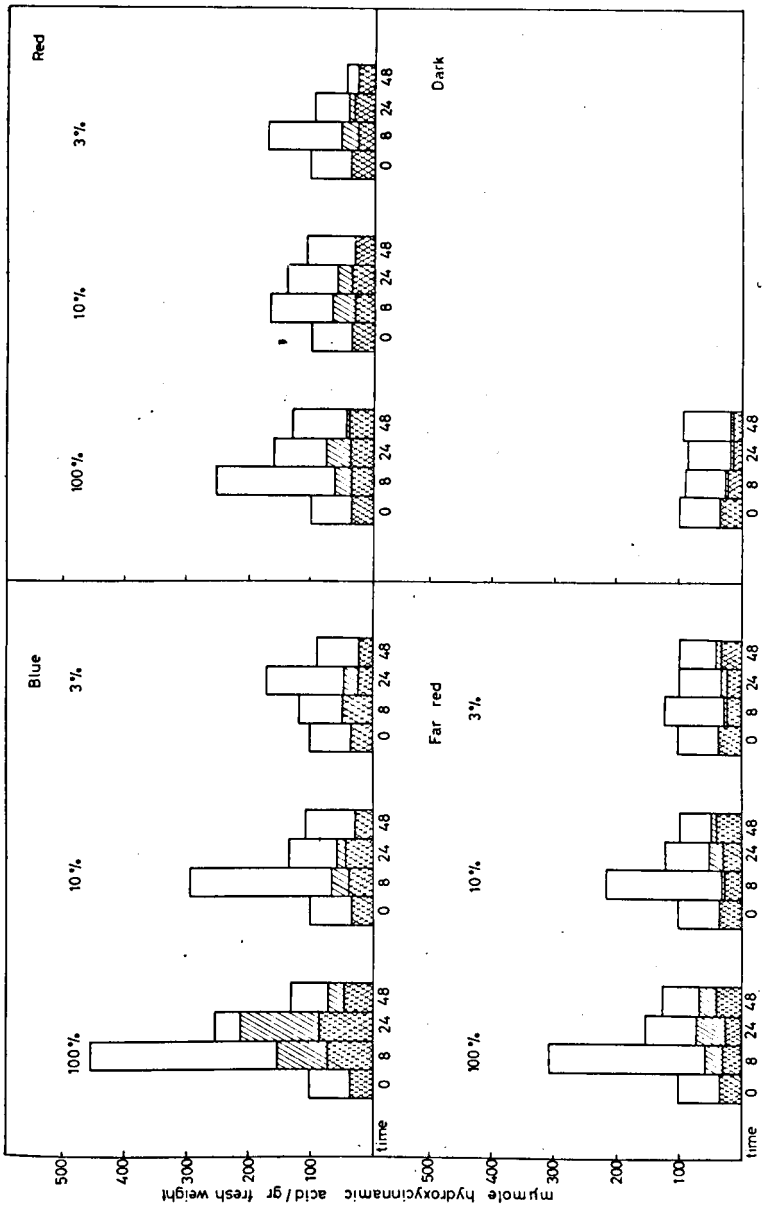

Fig. 7. Time dependent distribution of hydroxycinnamic acids in the hypocotyls of gherkin seedlings under different light conditions. Numbers above the graphs: Light intensities in percentages of the highest intensity (400 μW/cm^2). Complete bar: apical part of the hypocotyl (5 mm). Lightly shaded plus double shaded bar: upper half of the remaining part of the hypocotyl. Double shaded bar: lower half of the remaining part of the hypocotyl.

TABLE 2

Percentages of ferulic acid of the total amount of hydroxycinnamic acids synthesized in the hypocotyls in blue light of 700 μW/cm^2.

Part of the hypocotyl	After 8 hours blue light		After 24 hours blue light	
	Exp. I	Exp. II	Exp. I	Exp. II
Top	7	8	13	16
Middle	11	15	14	15
Bottom	17	19	25	28
Total	9	13	16	19

data were determined, we know from viewing chromatograms that this is the case in red and far-red too.

As already stated, the cotyledons contain, in addition to hydroxycinnamic acid esters, a number of flavonoid compounds which make it impossible to determine the former compounds in a watery extract. But here we can combine both methods to make an estimation of the accumulation of these flavonoids. By method II the absorbance of flavonoids plus hydroxycinnamic acids at 362 mμ is measured in a solution of pH $>$ 10.0. From the amount of p-coumaric acid plus ferulic acid obtained by method I we can estimate how much the hydroxycinnamic acid esters contribute to this absorbance. For this purpose we made the assumption that the hydroxycinnamic acid esters from the cotyledons have the same molar absorbances as those from the hypocotyls. The results presented in fig. 8 indicate that there is an increase in the ratio of flavonoids to hydroxycinnamic acid derivatives in the cotyledons of plants continuously irradiated with blue light (400 μW/cm^2). The conclusion is only valid, however, if the composition of the two groups remains the same during the experiment, or, if this is not the case, the molar absorbances of the components of each group do not differ very much.

Light steps in the pathway of hydroxycinnamic acid biosynthesis

A number of preliminary experiments have been undertaken to study the light steps in the biosynthetic pathway of the phenolic compounds. In table 3 the accumulation of hydroxycinnamic acids

TABLE 3

Effect of the excision of the cotyledons on the synthesis of hydroxycinnamic acids in the hypocotyls of gherkin seedlings during 24 hours. The seedlings were irradiated continuously or during 3 hours and then returned to darkness. Light intensities: 400 μW/cm^2 for the three wavelength regions. The numbers are mμ moles hydroxycinnamic acids/hypocotyl.

	Darkness	Blue		Red		Far red	
		3B21D	24B	3R21D	24R	3FR21D	24FR
Cotyledons excised	3.2	8.0	11.3	4.9	4.8	4.1	5.4
Intact plants	5.4	16.6	19.0	12.6	9.5	13.7	14.3

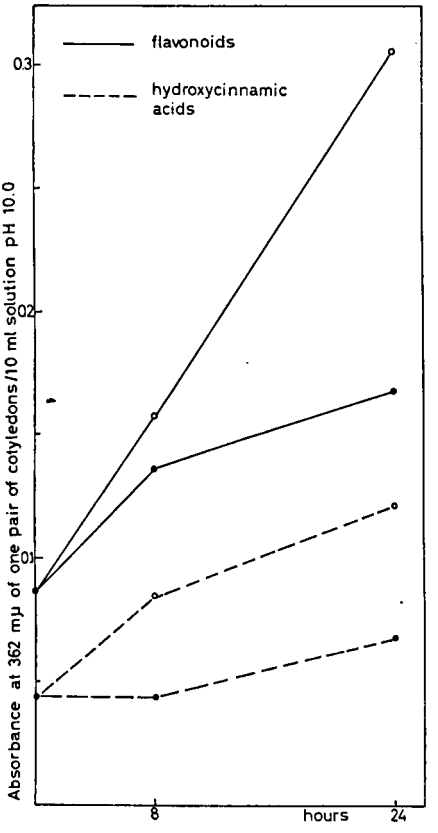

Fig. 8. Increase in flavonoids and hydroxycinnamic acids in the cotyledons of gherkin seedlings in darkness (closed circles) and in blue light of 400 μW/cm^2 (open circles).

in the hypocotyls of seedlings from which the cotyledons have been removed has been compared with the synthesis in intact seedlings. It appears that in red and far-red light the phenol synthesis in the hypocotyls is largely dependent on the cotyledons. In blue light there is also a considerable increase when the cotyledons have been removed although the value is 40 % to 50 % lower compared with the intact plant. The accumulation of phenolics in the hypocotyls is also much lower when the cotyledons alone are irradiated. The results might indicate that a precursor moves from the cotyledons to the hypocotyls and that in this part of the plant a light-influenced step is involved in the further pathway to hydroxycinnamic acids. No explanation for the deviating results in blue light has been found so far. It is not likely that photosynthesis (carbon fixation) plays an essential role. Table 3 shows that the blue light effect is equally pronounced when the seedlings are only irradiated for three hours and are then returned to darkness. During the light treatment no detectable greening of

the hypocotyls has occurred. Similar results as with growing hypocotyls were obtained with 5-mm sections from dark-grown seedlings cut immediately below the plumular hook, which were irradiated while floating on distilled water. Only in blue light (400 μW/cm^2) is there a considerable increase of hydroxycinnamic acids in these sections (net increase during 24 hours 0.25 mmole/gr fresh weight). The results are the same when carbon dioxide has been carefully excluded from the system, offering more evidence against an involvement of photosynthesis.

The amount of hydroxycinnamic acids which accumulates in the sections during 24 hours in red or far-red light is very small compared with the increase in blue light, and is hardly higher than the level reached in sections kept in the dark. When the sections are supplied with glucose and cinnamic acid or phenylalanine there is a considerable enhancement in phenol synthesis in all the three wavelength regions. A similar result is obtained when the sections are irradiated while floating on distilled water, and subsequently are transferred to the precursor solution in darkness. Thus a direct action of light on an intermediate in the biosynthetic pathway of hydroxycinnamic acids is unlikely. The effect of light can be blocked by pre-incubation in darkness with chloramphenicol (2 mg/ml) or with actidione (5 μg/ml), inhibitors of protein synthesis (ENGELSMA and MEIJER, to be published).

DISCUSSION

Dark-grown gherkin seedlings synthesize hydroxycinnamic acids and the related flavonoids. The rate of accumulation is highly increased on exposure of the seedling to light. The results suggest that in this light-dependent synthesis of phenolic compounds different light-influenced processes occurring either in the same or in different parts of the plant play a role.

1) To obtain synthesis of hydroxycinnamic acids in the hypocotyl this part of the plant has to be exposed to the light. Inhibition of the light-induced phenol synthesis caused by cloramphenicol and by actidione offers an indication that enzyme synthesis is involved. For the synthesis of chlorogenic acid in potato tuber slices similar results have been obtained (LEVY and ZUCKER, 1960; ZUCKER, 1963). More direct evidence in support of light-induced protein synthesis has recently been given by WILLIAMS and NOVELLI (1964) who found that ribosomes from light-treated plants show a markedly enhanced capability for C^{14}-leucine incorporation into protein.

2) The rate of accumulation of hydroxycinnamic acids in the hypocotyl depends on the availability of precursors. Phenol synthesis in excised hypocotyls is much smaller than in the hypocotyls of intact seedlings. In the former case the rate of accumulation can be much enhanced by adding hydroxycinnamic acid precursors such as phenylalanine and cinnamic acid. It is likely that the supply of precursors from the cotyledons is influenced by light. Cytochemical investigations carried out in this laboratory (PELETIER-BRIDGEWATER,

1964) have shown that light accelerates the mobilization of the storage products in the gherkin cotyledons.

Evidence for different light reactions involved in a particular photoresponse comes also from investigations by GRILL and VINCE (1964) who found that in the formation of anthocyanin in the hypocotyls of turnip seedlings two photoreactions are involved, one occurring in the cotyledons, the other in the hypocotyls.

3) Light-dependent processes consuming hydroxycinnamic acids or their precursors, for instance lignification, have also to be considered. Saturation of the accumulation of hydroxycinnamic acids at different levels in the different wavelength regions (fig. 5) may be partly due to the latter process.

Particularly in blue and far-red light the accumulation of phenolic compounds is an increasing function of the light intensity and the length of the irradiation period, large light doses being required. This type of photoresponse, for which the term "high energy reaction" has been used for some time, was recently designated less ambiguously by SIEGELMAN (1964) as photoresponse I. Small doses of red light have a comparatively pronounced effect but above a certain limit the intensity dependence is much smaller than in the other wavelength regions. From the red/far-red reversibility it may be concluded that phytochrome plays a role in the phenol synthesis in the gherkin (photoresponse II). Obviously there is an analogy with the light-dependent synthesis of anthocyanin and flavonols in buckwheat seedlings, where both types of photoresponses were also found to be involved (MOHR and VAN NES, 1963; HARRASCHAIN and MOHR, 1963).

As to the nature of the photoreceptors, the existence of the photoreceptor responsible for photoresponse II, the phytochrome, is well established (HENDRICKS and BORTHWICK, 1963), but a photoreceptor corresponding to photoresponse I has not been found so far. The fact that more than one light-effected process may be involved might explain why the action spectra for different light-dependent phenomena in the same plant, the buckwheat seedling (HARRASCHAIN and MOHR, 1963), are not the same, and why the action spectra for the same reaction, like anthocyanin synthesis, differ widely from plant to plant (SIEGELMAN, 1964; NG, THIMANN and GORDON, 1964). The parallelism of the dose-response curves in the blue and far-red wavelength regions for a number of light-dependent phenomena in buckwheat seedlings has been seen as an indication for the existence of one photoreceptor, with peaks in the blue and far-red, for the "high energy reaction" (HARRASCHAIN and MOHR, 1963). More recent experiments with lettuce seedlings, however, suggest that the "high energy reaction" in the far red is mediated by phytochrome (HARTMANN, personal communication). Our experiments give indications for a separate light action in the blue wavelength region.

1) In excised hypocotyls an appreciable stimulation of phenol synthesis is found only in blue light.

2) The lag between the beginning of irradiation and the measurable inhibition of hypocotyl elongation for dark-grown gherkin seedlings

is much shorter in blue light than in the other wavelength regions (ENGELSMA and MEIJER, 1965b).

Further investigations into the nature of the photoreceptor(s) involved in the light-dependent formation of p-coumaric acid, and into the manner in which the photoreactions are linked to the synthetic pathway, are at present being undertaken with hypocotyl sections. The synthesis of phenolic compounds in the intact seedling will be related to the growth phenomena in the next paper (ENGELSMA and MEIJER, 1965b).

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