A COMPARATIVE INVESTIGATION OF THE CONTROL OF PHENYLALANINE AMMONIA-LYASE ACTIVITY IN GHERKIN AND RED CABBAGE HYPOCOTYLS

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SUMMARY

In red cabbage hypocotyls the photoinduced increase in the activity of the enzyme phenylalanine ammonia-lyase (PAL) is not followed by a decline as found in gherkin hypocotyls. This indicates that a PAL inactivating system as assumed to exist in gherkin hypocotyls does not operate in red cabbage hypocotyls. For gherkin seedlings it has been postulated that at room temperature a slow synthesis of PAL is compensated by continuous inactivation and that at low temperatures the inactivating process is reversed resulting in the release of PAL from an enzyme-inactivator complex. In agreement with the assumption that red cabbage hypocotyls do not have a PAL inactivating system, it has been found that a cold dependent increase in PAL activity does not occur in these hypocotyls and that the rise in PAL activity at room temperature in darkness is much faster than in gherkin hypocotyls. It is suggested that the lack of a PAL inactivating mechanism in red cabbage hypocotyls is compensated by a better control of the photoinduction of PAL. In these seedlings the increase in PAL activity comes to a halt rather abruptly as soon as the light is turned off, whereas in gherkin seedlings in similar conditions the increase may continue for a fairly long time. This goes with a more clear-cut manifestation of phytochrome action in red cabbage hypocotyls as indicated by red/far-red reversibility and a greater effect of continuous irradiation with far-red light than with other light qualities. The effects of light on the PAL activity in red cabbage hypocotyls are parallelled to a certain degree by those on the accumulation of anthocyanin. Certain discrepancies can be resolved on the assumption that in the synthetic pathway of the latter compound there is at least one other light sensitive step.

1. INTRODUCTION

Control of enzyme levels in microorganisms can in most cases be described in terms of induction and repression. If a particular enzyme is no longer required, its synthesis is stopped and it is diluted out during subsequent cell divisions. In higher organisms this solution is usually not possible and in recent years an increasing amount of evidence has accumulated in favour of mechanisms which specifically inactivate or destroy certain enzymes (FILNER *et al.* 1969). One of the enzymes of which in certain plant tissues the activity seems to be controlled in this way is phenylalanine ammonia-lyase (PAL).

An increase in the activity of this enzyme has been obtained by irradiation (see ZUCKER 1969), wounding (*see* ENGELSMA 1968), low-temperature treatment (ENGELSMA 1969b, 1970b) and treatments with ethylene (RIOV *et al.* 1969) or gibberellic acid (REID & MARSH 1969). In several plant tissues including potato tuber slices (ZUCKER 1965, 1968), gherkin hypocotyls (ENGELSMA 1967a,b,c), excised bean axes (WALTON & SONDHEIMER 1968), *Xanthium* leaf

disks (ZUCKER 1969), mustard seedlings (DURST & MOHR 1966), buckwheat hypocotyls (SCHERF & ZENK 1967), pea buds (ATTRIDGE & SMITH 1967) and sunflower hypocotyls (ENGELSMA, unpublished results) it has been found that the initial increase in PAL activity is followed by a decline, and in the first four tissues mentioned it has been demonstrated that this decline can be blocked with inhibitors of protein synthesis. However, in more recent experiments with mustard seedlings it was found that in the case of certain light-dark treatments the induced PAL activity did not decay (WEIDNER *et al.* 1969). Preliminary experiments with red cabbage seedlings indicated that this plant was even more outspoken in this respect: under no circumstance could any decay of PAL activity be detected. In the present investigations the control of PAL activity in red cabbage hypocotyls, which apparently lack a PAL inactivating system, has been compared with that in gherkin hypocotyls, in which the occurrence of a PAL inactivating system is obvious.

2. MATERIAL AND METHODS

The experiments with red cabbage seedlings (*Brassica oleracea* L., "Langedijker donkerrode") started with three-day-old seedlings that had been grown in a heat sterilized mixture of leaf mold and sand at 25° in a dark room with an air humidity of about 90%. The data on gherkin seedlings (*Cucumis sativus* L.) reported in *fig. 4* have been obtained with the variety "Venlose niet plekkers" strain Tercken VI, raised under the same conditions. The experiments with red cabbage hypocotyl segments were performed in the same way as those with gherkin hypocotyl segments (ENGELSMA 1968a) except that from each hypocotyl 5 adjoining 2-mm segments were excised immediately below the plumular hook.

The irradiations were carried out with the light and filter combinations as given before (ENGELSMA & MEIJER 1965). They took place at 25° with the following light intensities: blue, 600 $\mu W/cm^2$; red, 1000 $\mu W/cm^2$; far red, 600 $\mu W/cm^2$.

Extraction and in-vitro assay of PAL were performed as described by ENGELSMA (1967a) from acetone powders prepared from samples of 100 hypocotyls. For a quantitative determination of anthocyanin 20 hypocotyls were macerated in a mortar under liquid nitrogen. The resulting powder was dumped into 10 ml 0.1 N HCl in water. The solution was cleared by filtration and centrifugation, whereafter the optical density at 535 nm was determined.

3. RESULTS

3.1. Effect of different far-red/dark treatments on the PAL activity in red cabbage hypocotyls

If dark-grown gherkin seedlings are exposed to light the PAL activity first rises and then declines (ENGELSMA 1967a). The decline is faster in darkness than in the light. *Fig. 1* shows that a similar treatment of dark-grown red cabba-

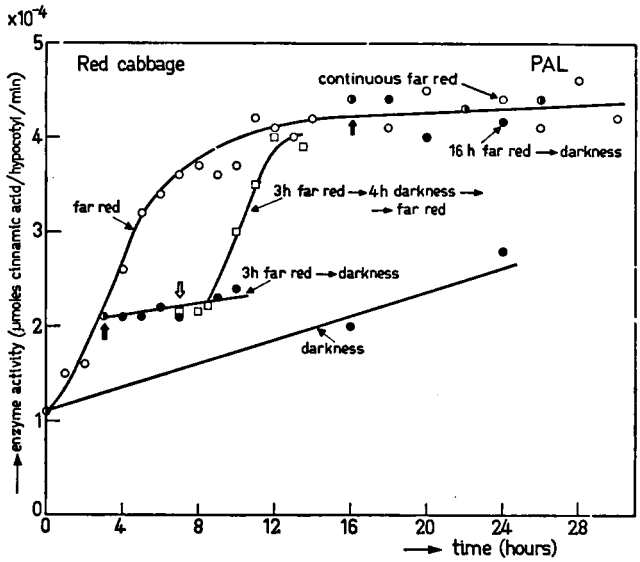

Fig. 1. Changes in the PAL activity in the hypocotyls of 3-day-old dark-grown red cabbage seedlings with different dark-light treatments with far-red light. The broad arrows indicate transfer from light to darkness, and vice versa.

ge seedlings likewise results in a temporary increase of PAL activity which thereafter, however, remains at a high and constant level irrespective of whether the seedlings remain in the light or are returned to darkness. Another point of difference between gherkin and red cabbage seedlings is that in the case of a light treatment shorter than the time needed for PAL to reach maximum activity the enzyme synthesis in the former plants continues for a fairly long time after the light has been turned off (ENGELSMA 1967a, 1968b, 1969a, 1970a), whereas in the red cabbage seedlings the increase in PAL activity then comes to a halt rather abruptly. In this respect red cabbage seedlings behave rather similarly to mustard seedlings (WEIDNER *et al.* 1969). For mustard seedlings it has been reported that on re-irradiation with far red, after a dark interval, the PAL activity increased immediately after the second irradiation (RISSLAND & MOHR *et al.* 1968). *Fig. 1* shows that, on the contrary, in a similar experiment with red cabbage seedlings the increase in PAL activity induced by the second light period is preceded by a pronounced lag phase. In gherkin seedlings, too, it has been found that the increase in PAL activity following a second irradiation is preceded by a lag phase (ENGELSMA 1967a, 1968b, 1969a). Finally it should be noted that in red cabbage seedlings the PAL activity rises quite a lot in darkness, too. In gherkin hypocotyls there is only a very slow increase in PAL activity in darkness at room temperature (ENGELSMA 1969a).

3.2. Influence of light quality – Involvement of phytochrome

In gherkin seedlings no red/far-red reversibility could be observed with regard to photoinduction of PAL (ENGELSMA 1967b). Continuous irradiation with high intensity blue light was found to produce a higher peak in the PAL activity than continuous irradiation with high intensity red or far-red light. With the latter light qualities peaks of about equal heights were obtained. From these experiments the conclusion has been drawn that at least two different pigments take part in the photoinduction of PAL synthesis in gherkin seedlings and that there is no evidence for a role of phytochrome.

In *table 1* it is shown that in red cabbage hypocotyls there is a red/far-red reversible effect on the induction of PAL activity, indicating the involvement of phytochrome in these seedlings. A similar conclusion had already been reached by SIEGELMAN & HENDRICKS (1957) in respect of anthocyanin synthesis in red cabbage seedlings. Another point of difference between gherkin and red cabbage seedlings is that in the latter a continuous irradiation with high-intensity far-red light produces the highest PAL activity whereas the effects of blue and red light are now about the same.

3.3. Loss of light sensitivity

Once gherkin seedlings have been irradiated for about 6 hours (at 25°) with a high light intensity, they have become insensitive to the light as far as photoinduction of PAL is concerned (ENGELSMA 1967a). On the other hand, the response to irradiation in 5-day-old seedlings is about the same as in 3-day-old seedlings if they remain in darkness until the onset of irradiation (ENGELSMA 1969a).

Table 1. Increases in PAL activity, in the hypocotyls of 3-day-old dark-grown red cabbage seedlings, caused by different irradiation programs (D, darkness).

Program	Increase in PAL activity (μmoles cinnamic acid/hypocotyl/min) $\times 10^{-4}$
8 hours D	4
10 min red + 8 hours D	5
10 min red + 10 min far-red + 8 hours D	5
4 × (10 min red + 110 min D)	8.5
4 × (10 min red + 10 min far-red + 100 min D)	4.5
8 hours blue	11.5
8 hours red	10.5
8 hours far-red	18.5
24 hours D	13.5
24 hours blue	16.5
24 hours red	17.5
24 hours far-red	25.5

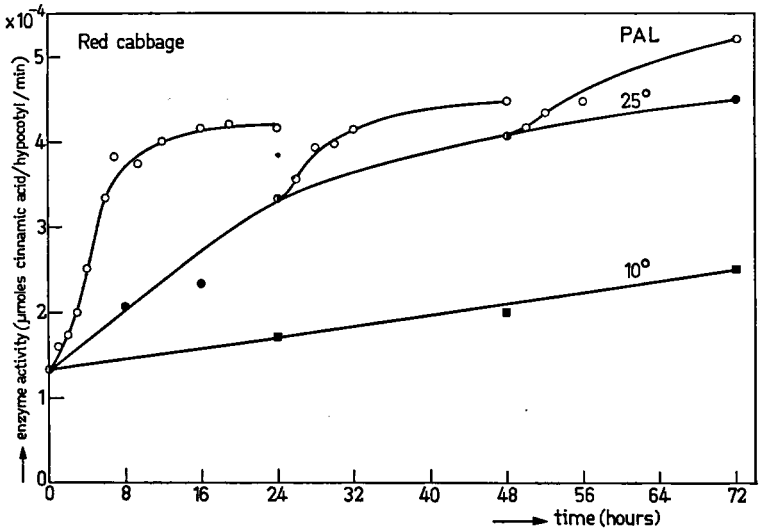

Fig. 2. Changes in the PAL activity in the hypocotyls of 3-day-old dark-grown red cabbage seedlings at 10° and 25 °C in darkness (closed symbols) and at 25 °C in far-red light, which started respectively 72, 96 and 120 hours from sowing (open circles).

Fig. 1 shows that, similarly to gherkin seedlings, irradiation of red cabbage seedlings that continues beyond 6 hours does not contribute any longer to the induction of PAL activity. However, in the red cabbage seedlings the light sensitivity declines in darkness rather rapidly as well (*fig. 2*). The dark level of PAL activity continues to rise from the 3rd through the 5th day, whereas the extra increase due to irradiation declines.

Fig. 3 shows that the light sensitivity with respect to the photoinduction of anthocyanin synthesis declines to a similar extent as the seedlings grow older. Similar observations have been made for the photoinduction of anthocyanin synthesis in turnip (GRILL & VINCE 1964, GRILL 1969) and mustard seedlings (WAGNER & MOHR 1966b).

3.4. Development of PAL activity in hypocotyl segments

The loss of light sensitivity with respect to induction of the PAL synthesis in gherkin hypocotyls has been attributed to repression of PAL synthesis by the end products cinnamic acid and its derivatives (ENGELSMA 1967a). This hypothesis was tested by using hypocotyl segments, a method in which advantage was taken of the fact that PAL synthesis became derepressed if the excised tissue was floated on water (ENGELSMA 1968a). This phenomenon is mainly restricted to the cell layers adjacent to the cut surfaces. After 10-mm segments from gherkin hypocotyls had floated for 24 hours on water the PAL activity appeared to be 5 times higher in the terminal 2-mm parts than in the inner part (ENGELSMA, unpublished results).

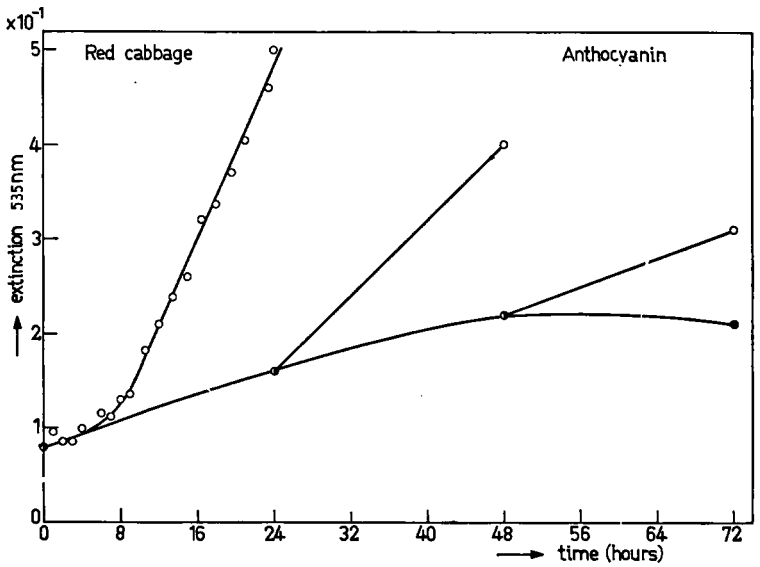

Fig. 3. Accumulation of anthocyanin in the hypocotyls of 3-day-old dark-grown red cabbage seedlings in the conditions as mentioned for *fig.* 2.

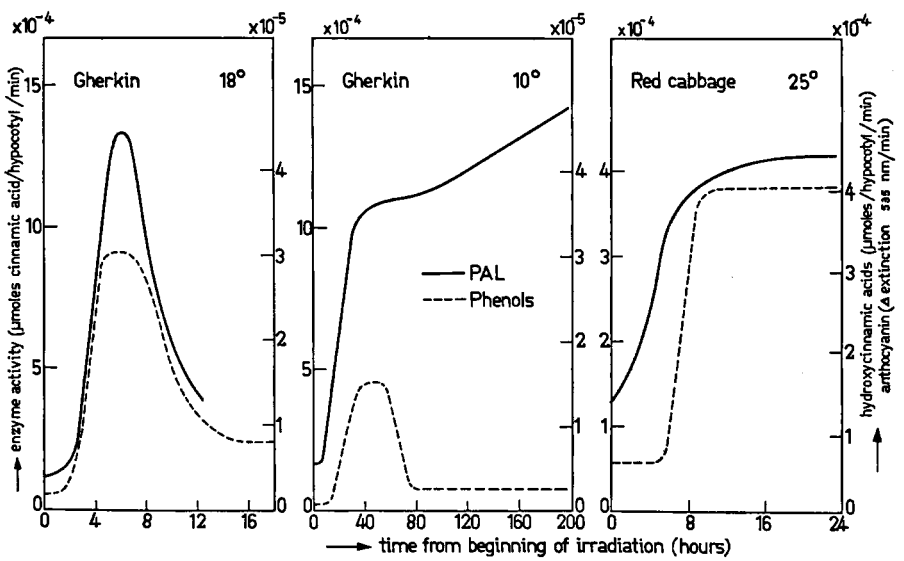

Fig. 4. Comparison between the light-induced changes in PAL activity in the hypocotyls of dark-grown gherkin and red cabbage seedlings and the accumulation of hydroxycinnamic acids and anthocyanin, respectively. The data on gherkin seedlings are derived from *figs. 1* and *2* of ENGELSMA 1968c and *figs. 1* and *3* of ENGELSMA 1970b, and those on red cabbage seedlings from *figs. 2* and *3* of this paper.

In order to test whether the products of the reaction catalyzed by PAL cause repression of this enzyme in red cabbage hypocotyls similar experiments have been performed with segments from these hypocotyls. *Table 2* shows that in the segments excised from dark-grown seedlings the PAL activity increases if they are floated on water and that the increase is much greater in 2-mm than in 10-mm segments. 10^{-3} M concentrations of cinnamic acid and p-coumaric acid in the incubation medium were found to inhibit the development of PAL activity in red cabbage hypocotyl segments but unlike in gherkin hypocotyl segments (ENGELSMA 1968a) these concentrations appeared to affect also the development of peroxidase activity in red cabbage hypocotyl segments. The effect may therefore be an unspecific one due to damage of the segments, which in some experiments could actually be observed. Therefore no conclusions will be drawn form these results.

3.5. Lack of low-temperature dependent development of PAL activity in red cabbage hypocotyls

In gherkin hypocotyls a treatment at 10 °C or lower temperatures leads to a rise in the PAL activity (ENGELSMA 1969b, 1970b). This has been attributed to the combined effect of de-novo PAL synthesis, which below 10° is no longer compensated by inactivation, and of reversal of the inactivation process, which causes the release of PAL from an enzyme-inactivator complex. Since in red cabbage hypocotyls apparently no inactivation of PAL occurs, no such low-temperature effects need to be expected in these seedlings. Accordingly we find that in darkness the development of PAL activity is much slower at 10° than at 25 °C (*fig. 2*). It was found further that a treatment of red cabbage seedlings at 4 °C for 24 hours did not result in a rise of PAL activity after transfer back to the higher temperature, as occurs in gherkin hypocotyls (ENGELSMA 1969b, 1970b).

Table 2. Development of PAL activity in hypocotyl segments of gherkin and red cabbage seedlings. Influence of the length of the segments and of the pre-irradiation of the seedlings (D, darkness; FR, far-red light)

Pre-treatment seedlings (hours)	Lenth of the segments	Incubation time (hours)	PAL activity (μmoles cinnamic acid/10-mm segment or 5 × 2-mm segments/min) × 10^{-4}	
			Red cabbage	Gherkin
72 D	10 mm	0	1.0	0.5
72 D	10 mm	24	1.8	3.5
72 D	2 mm	24	3.1	5.9
72 D 24 FR	10 mm	0	2.3	
72 D 24 FR	2 mm	24	2.3	

3.6. PAL activity and phenol synthesis

In gherkin hypocotyls the pattern of phenolic compounds is very simple compared with that in most other plant tissues, only hydroxycinnamic acids being produced (ENGELSMA & MEIJER 1965). *Fig. 4* shows that at 18 °C the changes in the rate of synthesis of these hydroxycinnamic acids if gherkin seedlings are exposed to the light, correlate closely with the changes in PAL activity, which indicates that the step catalyzed by this enzyme is rate limiting. At 10 °C the PAL activity rises to high levels both in the light and in darkness, but only in the light is there a considerable accumulation of hydroxycinnamic acids (ENGELSMA 1970b). This indicates that another step in the synthetic pathway of these compounds can become rate limiting and that this step is somehow affected by irradiation. At 10 °C in the light there is initially a good correlation. But this disappears as time progresses: the PAL activity remains high whereas the rate of hydroxycinnamic acid synthesis eventually declines.

The pattern of phenolic compounds in red cabbage hypocotyls is more complicated and consists of hydroxycinnamic acids, flavonoids, and anthocyanin. The accumulation of the latter compound only has been determined. It has been stated already that the light sensitivity with respect to photoinduction of anthocyanin synthesis declines in parallel with that of the induction of PAL activity. Comparison of the results presented in *figs. 2* and *3* shows that the accumulation of anthocyanin in the light correlates better with the extra increase in PAL activity due to the irradiation than with the total PAL activity. Further it should be noted that the lag phase in respect of the induction of anthocyanin synthesis is much longer than that of the increase of PAL activity (*fig. 4*). A likely interpretation of these results is that a fairly high PAL activity is a prerequisite for anthocyanin synthesis but that the rate is determined by another enzyme which, rather like PAL, is affected by irradiation but which drops to a low level in darkness.

Similar conclusions have been reached for anthocyanin synthesis in buckwheat (SCHERF & ZENK 1967) and mustard seedlings (RISSLAND & MOHR 1967).

4. DISCUSSION

In gherkin hypocotyls increases in PAL activity have been obtained by irradiation (ENGELSMA 1967a), excision and ageing (ENGELSMA 1968a), and application of low temperatures (ENGELSMA 1969b, 1970b). All these phenomena can be explained on the basis of a mechanism which involves: induction of de-novo synthesis of PAL, reversible inactivation of PAL by a proteinaceous factor, and repression of PAL synthesis. A schematic representation of this mechanism has been given in *fig. 6* in ENGELSMA 1967a. Similar mechanisms have been advanced for the induced changes in PAL activity in potato-tuber slices (ZUCKER 1965, 1968), *Xanthium* leaf disks (ZUCKER 1969) and mustard seedlings (WEIDNER *et al.* 1969). Certain differences from this mechanism have been observed in red cabbage hypocotyls; these are presented in this paper.

4.1. Photoinduction of PAL

From the red/far-red reversibility it may be concluded that phytochrome is involved in the photoinduction of PAL in red cabbage hypocotyls. Continuous irradiation with high-intensity far-red light was found to produce a bigger effect than with red light. Similar observations have been made with respect to photoinduction of anthocyanin synthesis in mustard (WAGNER & MOHR 1966a) and turnip seedlings (GRILL 1967). The explanation given by these authors is that red light destroys phytochrome.

Whether in addition to phytochrome other pigments, with a role similar to those in gherkin seedlings (ENGELSMA 1968b), are involved in the photoinduction of PAL in red cabbage hypocotyls does not appear from these experiments. Recently CREASY (1968b) and ZUCKER (1969) have reported that the photosynthetic system is involved in the photoinduction of PAL in strawberry and *Xanthium* leaf disks, respectively. This clearly demonstrates that the light-dependent increase of PAL in plant tissues is not necessarily tied to phytochrome action.

After termination of the inducing light period the PAL synthesis comes to a halt much more quickly in red cabbage than in gherkin hypocotyls. It might be that a more sensitive control of the photoinduction of PAL in red cabbage seedlings makes up for the lack of a system that removes this enzyme. Possibly this is related to a clearer manifestation of the involvement of phytochrome in these plants.

4.2. Inactivation of PAL

For gherkin seedlings it has been assumed that at temperatures above 10 °C both the slow synthesis of PAL in darkness and the extra increase of PAL caused by irradiation are compensated by inactivation of PAL (ENGELSMA 1970b). The consequences of the fact that below 10 °C the inactivation mechanism no longer functions are that below this temperature the PAL activity increases in darkness and that the PAL activity induced by irradiation does not decay. This corresponds exactly to what has been found for red cabbage hypocotyls for the whole temperature range of 4 to 32 °C, indicating the total absence of a PAL inactivating system in this tissue.

In gherkin hypocotyls the increase in PAL activity following a cold treatment has been attributed to a reversal of the PAL inactivating reaction. In agreement with the assumption that this inactivating process does not occur in red cabbage hypocotyls no cold-dependent increase in PAL activity could be observed in these seedlings. The increase in PAL activity in red cabbage seedlings in darkness, in contrast to that in gherkin seedlings, is faster at 25° than at 10 °C. This correlates with the finding of FRY-WYSSLING & BLANK (1943) that in red cabbage seedlings in darkness more anthocyanin accumulates at 20° than at 10 °C.

4.3. Repression of PAL synthesis

It has been found previously (ENGELSMA 1968a) that in gherkin hypocotyl

tissue in which, through preceding irradiation, the PAL activity was no longer affected by irradiation, the light sensitivity reappeared after segmentation and floating the segments on water. In line with this there are the publications in respect of potato tubers (ZUCKER 1965), *Helianthus* tubers (NITSCH & NITSCH 1966), strawberry leaves (CREASY 1968a) and *Xanthium* leaves (ZUCKER 1969), in which it is reported that the light-dependent increase of PAL activity is stimulated by, or depends on, excision. In the case of gherkin seedlings, though, it could be proved that excision (ageing) and irradiation did not cause the production of the same factor, or of different factors that act synergystically (ENGELSMA 1968a). The interaction of irradiation and excision can therefore best be explained by the hypothesis that in the PAL controlling mechanism at least two opposing factors are involved: an inducing factor, the formation of which is induced or increased by irradiation (induction), and a repressing factor, which disappears as a consequence of excision and ageing (derepression).

For potato tuber slices (ZUCKER 1965), excised bean axes (WALTON 1968) and excised gherkin hypocotyl segments (ENGELSMA 1968a) it has been reported that cinnamic acid and hydroxycinnamic acids inhibit the development of PAL. The loss of light sensitivity which occurs in the course of the irradiation in gherkin seedlings has been attributed to a repression of PAL synthesis by the accumulation of these compounds (ENGELSMA 1967a). In red cabbage seedlings, in contrast to gherkin seedlings, it appears that the light sensitivity for the induction of PAL decreases fairly rapidly in darkness as well. This correlates with a much faster increase of the PAL activity in darkness in the former seedlings. From this a relatively faster synthesis of cinnamic and/or hydroxycinnamic acid(s) might be expected in red cabbage seedlings, and thus the hypothesis that these compounds are involved in the repression of PAL synthesis might account for the different behaviour of red cabbage and gherkin seedlings.

4.4. Variety in the control of PAL

For most plant tissues, including gherkin and red cabbage hypocotyls, it is true that, once PAL synthesis has become completely repressed, the light sensitivity does not reappear unless, as stated above, recourse is taken to excision. This has been a difficulty in relating photoinduction of PAL to photoperiodic phenomena (ENGELSMA 1969a). In this respect it is interesting that ZUCKER (1969) recently reported that in *Xanthium* leaf disks the PAL activity repeatedly increased and decreased during alternating cycles of light and darkness.

It can be stated that with respect to the control of photoinduced PAL synthesis three variations can be detected:

1. In potato tuber slices and gherkin hypocotyls a surplus of PAL is removed by an inactivating or degradating process and further enzyme synthesis is blocked by irreversible repression.
2. In *Xanthium* leaf disks PAL is subject to inactivation or degradation, but its synthesis does not become irreversibly repressed.
3. In red cabbage hypocotyls PAL synthesis becomes eventually irreversibly repressed, but enzyme already formed is not readily removed.

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