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FLAVAN PRODUCTION IN STRAWBERRY LEAVES

L. L. CREASY* and T. SWAIN

Low Temperature Research Station, Downing Street, Cambridge

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Abstract—The physiology of flavan formation has been studied in strawberry leaves using a method developed for measuring specific simple flavans utilizing thin-layer chromatography. It was found that flavans were synthesized in darkened areas of leaves from light-induced translocatable precursors. The synthesis of flavans, unlike that of anthocyanins, shows no light-induction period, but is stimulated by both light and sucrose feeding. The synthesis of both flavans and anthocyanins in mature leaves show a phytochrome response, but this is affected by the concentration of sucrose fed.

INTRODUCTION

STUDIES on the physiology of flavonoid compounds have been mainly carried out with the anthocyanins[1] and information on the physiology of other flavonoids, particularly flavans, is comparatively scanty. The chemistry and distribution of these latter compounds have been extensively studied because of their importance as precursors of condensed tannins,[2,3] and their suggested central role in flavonoid biosynthesis.[2,4,5]

Studies on the physiology of flavans have previously been concerned in determining the total amounts of the various classes of flavans which can be extracted under different condition or which react to specific reagents.[6] For example, the production of leucoanthocyanins (measured by conversion to anthocyanidins) has been studied in several plants.[7–10] The amount of leucoanthocyanin produced by plants was found to be increased by exposure to light,[9–13] but it was shown that the synthesis of these substances is not fully light dependent.[7,8,11] Separation of leucoanthocyanins into classes roughly related to their molecular size has been utilized to show that changes in the proportion of polymeric leucoanthocyanins (flavolans[14]) are important in the astringency of ripening fruits.[3] Studies of the physiology

* Present address: Department of Pomology, Cornell University, Ithaca, New York, U.S.A.

[1] H. W. SIEGELMAN, *Biochemistry of Phenolic Compounds* (Edited by J. B. HARBORNE) pp. 437–456. Academic Press London (1964).

[2] See chapters in *The Chemistry of Flavonoid Compounds* (Edited by T. A. Geissman). Pergamon Press, Oxford (1962).

[3] J. L. GOLDSTEIN and T. SWAIN, *Phytochem.* **2**, 371 (1963).

[4] T. ROBINSON, *The Organic Constituents of Higher Plants*. Burgess, Minneapolis 1963.

[5] T. SWAIN, *Wood Extractives* (Edited by W. E. HILLIS) pp. 277–313. Academic Press, New York (1962).

[6] T. SWAIN and J. L. GOLDSTEIN, *Methods in Polyphenol Chemistry* (Edited by J. B. PRIDHAM) pp. 131–146. Pergamon Press, Oxford (1964).

[7] J. R. TROYER, *Phytochem.* **3**, 535 (1964).

[8] M. BOPP, *Naturwissenschaften* **47**, 158 (1960).

[9] W. E. HILLIS and T. SWAIN, *J. Sci. Food Agr.* **10**, 135 (1959).

[10] R. E. ALSTON, *Am. J. Botany* **45**, 289 (1958).

[11] M. BOPP, *Z. Botan.* **48**, 153 (1960).

[12] W. E. HILLIS and T. SWAIN, *Nature* **179**, 586 (1957).

[13] R. E. ALSTON, *Botan. Gaz.* **120**, 99 (1958).

[14] T. SWAIN *The Chemistry of Flavonoid Compounds* (Edited by T. A. GEISSMAN) pp. 513–552. Pergamon Press, Oxford. (1962).

of the more simple flavans (catechins and leucoanthocyanins) have been limited by the lack of specific reagents to measure them individually in plant extracts.[6]

Anthocyanin synthesis has been previously studied in strawberry leaves[15] which were found to be a suitable material for the investigation of flavonoids. The leaves are a mature non-growing tissue which contain initially small amounts of a variety of flavonoid compounds[16] under normal greenhouse conditions, but possess the ability to produce them rapidly when treated under suitable conditions. We have therefore examined the physiology of flavans in the strawberry leaf, using a method developed for measuring specific simple flavans utilizing thin-layer chromatography. In addition to examining changes in the concentration of the simple flavans, (+)-catechin and strawberry biflavan,[17] we included measurements of the complex flavolans[2,6] and of the total anthocyanin.

RESULTS AND DISCUSSION

Initial studies were carried out using the whole strawberry leaves, areas of which were covered with aluminium foil. Sucrose solution (0·1 M) was fed through the petioles and the leaves were supported so that the leaf blades were perpendicular to the light. Figure 1 shows

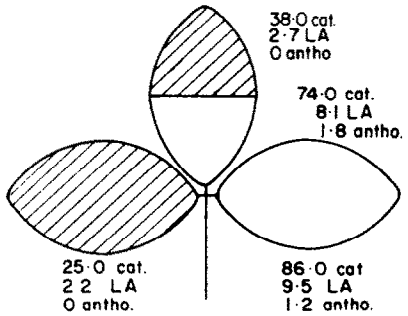

FIG. 1. PRODUCTION OF FLAVANS AND ANTHOCYANIN IN INTACT LEAVES.

The shaded areas were enclosed in aluminium foil to exclude light. The numbers given are the content in $\mu g/cm^2$ after 96 hr light (6000 lx) at 20° with 0·1 M sucrose.

Cat. = (+)-catechin; LA = amount of cyanidin produced from extracted flavolans; antho. = cyanidin-3-glucoside. A fully enclosed leaf = 10·0 cat.; 0·9 LA; antho.

the amounts of (+)-catechin, extractable flavolan and anthocyanin present in the various parts of the leaf after four days under one set of the conditions used. Under these conditions a fully covered leaf produced no increase in any of the compounds examined. However, as can be seen, in the covered areas of partially shaded leaves flavans but not anthocyanins were produced, whereas in the exposed areas, as in a completely uncovered leaf, considerable quantities of all classes of flavonoids were formed (Fig. 1). Since anthocyanins were only produced in the areas which were directly exposed to light, their synthesis must initially be completely light-dependant, although once started it can continue at a slow rate in darkness. There is no apparent light requirement, however, for flavan synthesis but since their formation does not take place in a completely darkened leaf, it must be presumed that a precursor is formed in the areas exposed to light and translocated to the covered area of the leaf.

[15] L. L. CREASY, E. C. MAXIE and C. O. CHICHESTER, *Phytochem.* **4**, 517 (1965).
[16] L. L. CREASY, E. C. MAXIE and V. L. SINGLETON, *Proc. Am. Soc. Hort. Sci.* **85**, 325 (1964).
[17] L. L. CREASY and T. SWAIN, *Nature* **207**, 150 (1965).

Strawberry leaf disks were capable of producing large amounts of flavonoids for long periods of time when floated on sucrose solutions and treated with continuous light (Fig. 2). During nine days of constant light, the chlorophyll content decreased by 44 per cent, the dry weight after extraction increased by 33 per cent, but the rate of production of the flavans based on leaf area remained reasonably constant. The initiation of synthesis did not occur simultaneously for all the flavonoid compounds studied. (+)-Catechin synthesis appeared to start before production of strawberry biflavan, anthocyanin and polymeric flavolan. The apparent lag in the start of simple flavan synthesis is probably due to the time involved in sugar uptake rather than to a light specific induction stage as occurs for anthocyanin synthesis.[15,18]

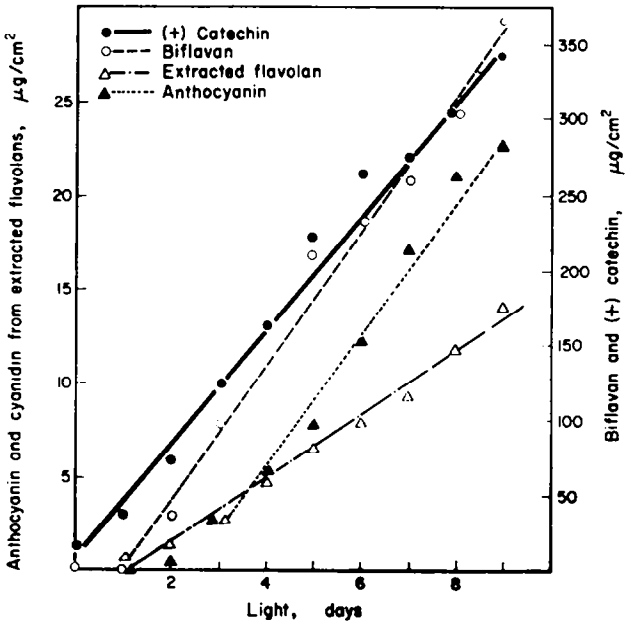

FIG. 2. FLAVAN AND ANTHOCYANIN PRODUCTION IN STRAWBERRY LEAF DISKS FLOATED ON 0·1 M SUCROSE WITH CONSTANT LIGHT (6000 lx) AT 20°.

The extracted dry weight was 1·99 mg/cm² at the start and 2·64 mg/cm² at the end of the experiment.

Due to the possibility of contamination with micro-organisms, most of the subsequent experiments were completed within three days of the preparation of leaf disks.

The effect of sucrose and light on the production of anthocyanin and flavans in disks (Table 1) reveals that light, although stimulatory, is not essential for the production of flavans as it is for anthocyanins. Sucrose is stimulatory to the synthesis of both flavans and anthocyanin in the light and to the synthesis of flavans in the dark. The additional light requirement for anthocyanin production must therefore act in a manner separate from any system common to the synthesis of these other flavonoids. Leucoanthocyanins production in plants has been shown to be distinct in its light requirements from anthocyanin synthesis.[8]

The effect of concentrations of sucrose (0·05 to 0·3 M) on the rate of production of flavans in light or in darkness was investigated for anthocyanin, (+)-catechin, strawberry biflavan,

[18] H. W. SIEGELMAN and S. B. HENDRICKS, *Plant Physiol.* 33, 409 (1958).

TABLE 1. EFFECT OF LIGHT AND 0·1 M SUCROSE ON THE PRODUCTION OF FLAVANS AND ANTHOCYANIN DURING 72 HR

	μg/cm²/72 hr*				
				Flavolan	
Treatment	Anthocyanin	(±)-Catechin	Biflavan	Extractable	Non-extractable
Water-darkness	0	14	15	0·4	0·4
Sucrose-darkness	0	38	24	1·0	0·7
Water-light	3·7	123	76	2·3	1·3
Sucrose-light	7·8	132	127	2·0	2·5

* The production of flavolans is expressed as the amount of cyanidin produced from hydrolysis.

extracted flavolans and non-extracted flavolans. All substances showed similar responses to sugar with an optimum concentration of 0·2 M sucrose for light production, while the optimum concentration for dark production was not reached at 0·3 M. The response for the production of non-extracted flavolans is shown in Fig. 3. These results are similar to those

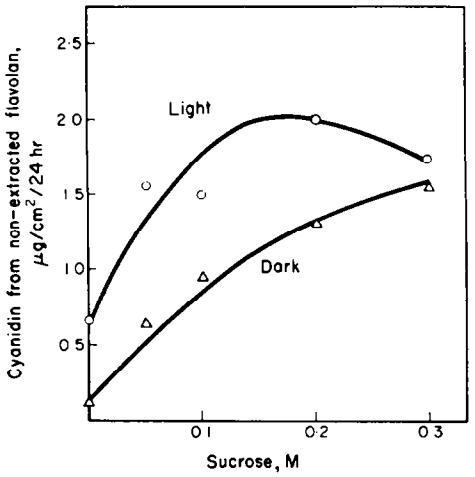

FIG. 3. NON-EXTRACTED FLAVOLAN PRODUCED AT 20° IN LEAF DISKS ON DIFFERENT CONCENTRATIONS OF SUCROSE.

Production is either during light (6000 lx) or during a dark period following a pre-illumination of 24 hr.

previously reported for anthocyanin production in the same tissue.[15] Since the stimulation of light production by sucrose decreases at the higher concentrations, it is possible that some substance whose production is also stimulated by light and sucrose uses a precursor of the flavonoids.

Alternatively, the suggestion that the high energy reaction (HER) necessary for anthocyanin production (and presumed for other flavonoids) is associated with photosynthesis[19]

[19] R. J. DOWNS, H. W. SIEGELMAN, W. L. BUTLER and S. B. HENDRICKS, *Nature* **205**, 909 (1965).

could explain the finding of an optimum sugar concentration in light but not in darkness. Photosynthesis might be inhibited at the higher sucrose concentrations and therefore reduce the contribution of the HER system to flavonoid synthesis, forcing it to rely primarily on the system which drives it in the dark. The production by the dark system would show no such diminishing effect of higher sugar concentrations and would be expected to increase because of the greater amounts of precursor and energy available. The difference between the light requirements of anthocyanin synthesis and flavan synthesis appears to be confined to the lack of an induction stage for the initiation of flavan synthesis. The relative stimulation of production of flavans and anthocyanin by light is similar once production is initiated, and it is assumed that the HER is acting on both. More work needs to be done on the requirements of the induction stage for anthocyanin synthesis to see how it is separated from the synthesis of other flavonoid compounds.

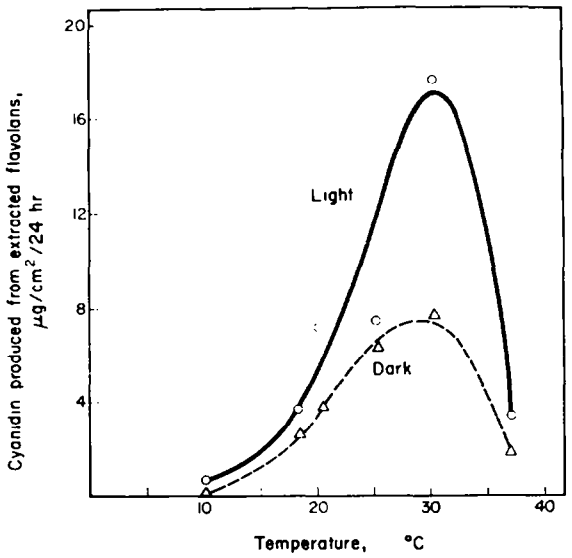

FIG. 4. EXTRACTED FLAVOLAN PRODUCTION IN LEAF DISKS ON 0·1 M SUCROSE DURING THE 24 HR AFTER A 24 HR PRE-ILLUMINATION PERIOD.

Attempts were made to feed (+)-catechin, (−)-epicatechin and cocoa biflavan[20] at 10^{-3}, 10^{-4}, and 10^{-5} M to strawberry leaf disks. At these concentrations, the compounds had only a slight effect on the levels of flavans in leaf disks. Measurements of the amounts of the compounds remaining in solution after the three days revealed that strawberry leaf disks apparently do not readily take up these simple flavans and this fact should be considered when carrying out radioactive feeding studies.

The effect of different temperatures on the production rates of flavans and anthocyanin were determined during light and during a dark period following a light period. The anthocyanin response was similar to that already described[16] with the maximum production at 30° in light and at 25° in darkness. All the flavans had similar curves with the maximum production at 30° in both light and in darkness. The results for extractable flavolans are shown in Fig. 4.

[20] W. G. C. FORSYTH and J. B. ROBERTS, *Biochem. J.* 74, 374 (1960).

In order to confirm that the light requiring induction stage was necessary for anthocyanin synthesis but not for flavan synthesis we gave disks variable lengths of time in light at 10° or 30° and measured the production of flavans and anthocyanin during a dark period at 30°. Before the disks were given any light they were kept in the dark at 20° for 20 hr to allow them to take up sugar (0·1 M sucrose). Dark synthesis rates at 30° are plotted for anthocyanin, strawberry biflavans, extracted flavolans and non-extracted flavolans according to the preceding light treatment (Fig. 5). The induction period for anthocyanin synthesis was 12 hr at 30°, but was not completed in 24 hr at 10°, suggesting that the light reactions associated with the induction stage are coupled to dark reactions occurring too slowly at 10° to allow its

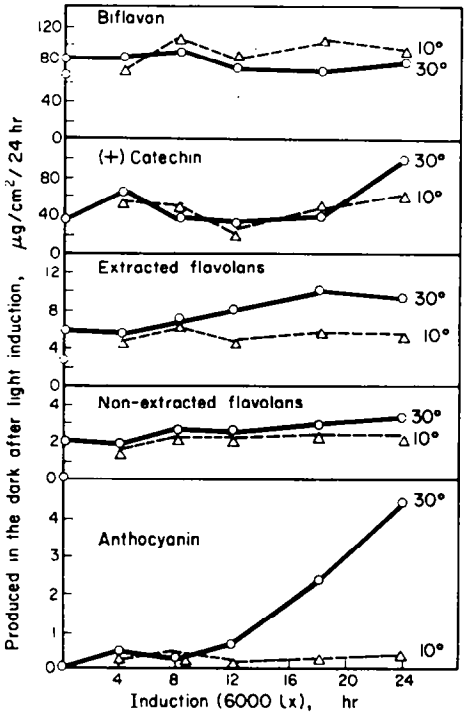

FIG. 5. DARK PRODUCTION OF FLAVONOIDS BY STRAWBERRY LEAF DISKS FLOATED ON 0·1 M SUCROSE AT 30°.

Flavolan production is expressed as the amount of cyanidin produced on hydrolysis. The two temperatures are those during the light induction period.

completion. Extrapolation of the previous temperature experiment indicated that production started at about the same time for all the temperatures above 10° (where there was no production at all), suggesting that above 10° the light reactions become limiting and production starts when these have been satisfied. As can be seen flavans do not show a light dependent *induction* stage although their synthesis is stimulated by exposure to light. Nor is their synthesis affected by prior exposure to low temperatures (10°) (Fig. 5).

The time course of the production of some flavans and anthocyanin in leaf disks at 30° in the light is given in Fig. 6 and again indicates the lack of an induction stage for flavan synthesis. In addition, the synthesis of non-extractable flavolans shows a slight lag compared to the simple biflavan and this may be due to the necessity of building up sufficient quantities

of simple flavans which then can be utilized in the production of the more complex substances.[21]

The phytochrome system is known to influence the anthocyanin synthesizing ability of many plants,[22, 23] but difficulty has been experienced in demonstrating phytochrome responses in mature green tissues.[1,9] A phytochrome system, not necessarily associated with anthocyanin formation, has been shown to exist in strawberry leaves.[24] To determine whether this system can control the synthesis of flavonoids in strawberry leaves, we pre-illuminated leaf disks for 24 hr with fluorescent tubes, gave them a final 5 min period of either red or far-red light, and then placed them in the dark. We found that the concentration of sucrose used had a great effect on the response of strawberry leaf disks to far-red light. No inhibition of dark synthesis of flavans or anthocyanin by far-red light was found when 0·3 M sucrose was used. If lower concentrations were used (0·05 M), the dark production was basically lower

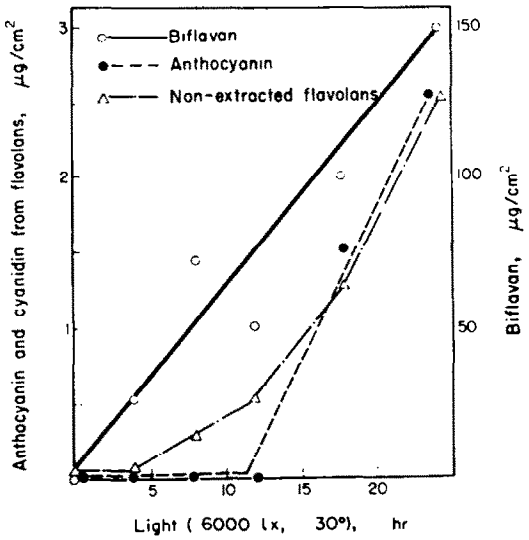

FIG. 6. TIME COURSE OF THE PRODUCTION OF FLAVONOIDS IN LEAF DISKS ON 0·1 M SUCROSE AT 30° IN LIGHT.

and far-red treatments were effective in inhibiting both flavan and anthocyanin synthesis. The dark synthesis of each flavan was inhibited about 30 per cent by a far-red treatment while anthocyanin synthesis was inhibited 47 per cent. Further work must be done to explain the effect of high sucrose concentrations in masking the phytochrome response but it may be related to the phenomena described earlier (cf. Fig. 3).

EXPERIMENTAL

Plant Material

Strawberry plants (*Fragaria vesca* var. alpine)* were grown in the greenhouse. Fully expanded young leaves were used immediately after picking.

* Kindly supplied by the John Innes Institute, Hertford, Herts.

[21] D. G. ROUX, *Nature* **181**, 1454 (1958).
[22] R. J. DOWNS *J. Wash. Acad. Sci.* **54**, 112 (1964).
[23] R. J. DOWNS and H. W. SIEGELMAN, *Plant Physiol.* **38**, 25 (1963).
[24] L. W. MAPSON and T. SWAIN, *Nature* **204**, 758 (1964).

Preparation of Extracts

Disks (1 cm in dia.) cut from the lamina of the leaves were floated with the lower epidermis upward on appropriate solutions in petri dishes. After the desired treatment the disks were removed, blotted dry and extracted first three times with hot absolute methanol (5 ml/g) and then four times with hot 50% aqueous methanol (5 ml/g).[9] The extracted residue was used directly for the estimation of non-extractable flavolans.[9]

Analysis of Extracts

(*a*) *Leucoanthocyanins.* The analysis of both non-extractable and aqueous-methanol soluble leucoanthocyanins was carried out with suitable aliquots using 5% HCl in *n*-butanol heated at 95° for 1½ hr.[25] After cooling the absorbance was measured at 550 mμ. The results are expressed as the amount of cyanidin produced from this treatment. The production of cyanidin from flavolans is not strictly quantitative[26] but was found to be reproducible.[25] Synthetic leucocyanidin polymers yielded 17% cyanidin under these conditions[15] and this conversion may be similar for natural flavolans.

(*b*) *Anthocyanins.* Anthocyanin in the absolute methanol extracts was measured spectrophotometrically by determining the visible absorption spectrum of each extract after acidification (final concentration 1% HCl), and measuring the absorbance at 550 mμ after adjustment for interfering substances.

(*c*) *Simple flavans.* (i) *By TLC chromatography.* The simple flavans ((+)-catechin and strawberry biflavan) were fully extracted by the absolute methanol. This fraction was concentrated under vacuum, separated by TLC (250 μ cellulose, MN 300, developed in butanol–aectic acid–water, 4:1:2·2). After drying, the chromatograms were lightly sprayed with 1% vanillin in 70% sulfuric acid[25] to reveal the flavans. The cellulose in the relevant portions of the chromatograms was carefully scraped off into small flasks containing 1 ml water. With constant shaking and cooling, 5 ml of the vanillin reagent was added and, after 15 min, when the cellulose powder had dissolved in the reagent leaving a clear solution, the absorbance was measured at 500 mμ. A standard curve determined from amounts up to 20·0 μg of (+)-catechin gave a straight line, although the scatter was large below 2·0 μg. A similar method was utilized for strawberry biflavan based on material purified from strawberry leaves by repeated paper chromatography.[15] This method can be directly applied to any flavan or, without the use of vanillin, to any other substance which has a suitable spectrum[27] or reacts with 70% sulfuric acid. The method is particularly useful since the cellulose does not interfere with the spectrophotometric measurement in the visible range, and the size of the spot and therefore the amount of cellulose does not affect the technique.

(ii) *Direct method.* After repeated chromatography of absolute methanol extracts of strawberry disks, it was observed that only two components ((+)-catechin and strawberry biflavan) reacted to vanillin-sulfuric acid, and only one of these to the leucoanthocyanin reagent (strawberry biflavan). This permitted a more rapid method to be used for the estimation of these compounds in strawberry leaf extracts; both the leucoanthocyanin and the vanillin-sulfuric estimation were applied directly to samples of the methanol extract, and by appropriate calculation, the amounts of (+)-catechin and strawberry biflavan could be calculated.

[25] T. SWAIN and W. E. HILLIS, *J. Sci. Food Agri.* **10**, 63 (1959).
[26] D. G. ROUX and E. PAULUS, *Biochem. J.* **82**, 320 (1962).
[27] T. SWAIN and L. L. CREASY, In preparation.
[28] H. MOHR, *Planta* **49**, 389 (1957).

Light Sources

All illuminated samples received light from fluorescent tubes which provided 6000 lx at the disk level. Red light for the phytochrome experiments was obtained with fluorescent lamps and red cellophane which provided light at wavelengths above 585 mμ. The red light did not include appreciable far-red because of the low production of far-red light by fluorescent lamps. Far-red light was provided by a 275-W tungsten projector lamp with a filter made from red and green cellophane and 5 cm of water. This filter transmitted light only at wave-lengths above 695 mμ. The light sources were tested for their ability to control the phytochrome system by treating pre-illuminated three day-old white mustard seedlings (which have been reported to be phytochrome responding[28]) with red (4 min) or far-red (4 min) light. Final flashes of far-red light resulted in 75 per cent inhibition of anthocyanin synthesis (compared to red treated) during a subsequent dark period.

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