Anthocyanin Formation in Excised Segments of Buckwheat-Seedling Hypocotyls [1, 2]

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Recent studies of anthocyanin formation have involved the use of a varied assortment of plant systems: seedlings, leaves, fruits, petals, callus cultures, and Lemnaceous flowering plants (2, 9). Each of these kinds of material possesses certain advantages and certain disadvantages. In the dark-grown buckwheat seedling anthocyanin synthesis can be induced in the hypocotyl by exposure of that organ to light (6, 8); this formation of pigment is not accompanied by photosynthesis, since the hypocotyl does not contain chlorophyll. However, under many conditions which might be used in a study of anthocyanin formation, growth of the seedling, especially of the hypocotyl itself, would be a complicating factor. Furthermore, the hypocotyl is not homogeneous from top to bottom in its anthocyanin-forming capacity (1, 6).

These disadvantages might be overcome by removing the hypocotyl from the seedling before its experimental use. Accordingly, an investigation of anthocyanin formation in excised hypocotyls and hypocotyl segments was undertaken in order to ascertain the suitability of such a system.

Materials and Methods

Culture of Seedlings. Achenes of Japanese buckwheat (*Fagopyrum sagittatum* Gilib.) were soaked for 20 minutes in a 2 % sodium hypochlorite solution, rinsed thoroughly with distilled water, and placed on wet filter paper for germination. The filter paper was laid across 2 plastic dishes (10 × 10 × 1 cm) which floated on 2 cm of water in a glass tray (15 × 25 × 4 cm). The tray was covered with another like it and placed in a controlled-temperature cabinet, where germination and growth of the seedlings took place in constant darkness at 20°.

Preparation of Hypocotyl Material. Only hypocotyl material was analyzed for anthocyanin content. Sometimes entire hypocotyls were taken, but in most cases segments 20 or 25 mm long were cut from the region just below the unopened hook. This procedure was followed to eliminate variation which might be introduced by inclusion of any part of the hook, since it is different in its physiological properties from the rest of the hypocotyl (6). When studies involved a comparison of excised hypocotyl segments and hypocotyls of intact seedlings, segments comparable to those previously excised were taken from the intact plants at the end of the experimental period. When growth of hypocotyls of intact seedlings occurred, the segments were taken from the region just below a line marking the initial length of the hypocotyl as measured from the roots.

Excision. Hypocotyls or segments of hypocotyls were excised under weak illumination from a fluorescent bulb, the intact seedling being exposed for no more than 10 seconds. The excised pieces were then placed under the lamps used for light treatment after a further delay of no longer than 1 minute.

Light Treatment. Anthocyanin pigment is not synthesized in buckwheat hypocotyls in the dark. Accordingly, intact seedlings, excised hypocotyls, or excised hypocotyl segments were exposed to light by placing them in a horizontal position on wet filter paper under a light source consisting of Daylight fluorescent lamps. The illumination at the level of the plant material was 11,000 to 13,000 meter-candles, depending on position. In a given experiment the arrangement of materials under the lamps was randomized. The exposure to light always occurred at a temperature in the range of 24 to 27°.

Dark Period Treatment. After the light period the plant material was subjected to continuous darkness in controlled-temperature cabinets for various lengths of time, usually at 10° for 72 hours. In most cases the plants or hypocotyls were laid horizontally on wet filter paper during this dark period, but in some instances they were floated in solutions contained in loosely-stoppered flasks. Such solutions were not buffered because preliminary experiments revealed that anthocyanin production was almost always greater on distilled water than on various buffer solutions.

Anthocyanin Analysis. The hypocotyls or hypocotyl segments were cut transversely into small pieces which were dropped into methanol-concentrated HCl (99/1, v/v) and crushed flat with a glass rod. Usually 5 segments or small hypocotyls were extracted with 3 ml of the solvent; when the number or size of the hypocotyls was larger, a larger volume of solvent was used. Extraction occurred in the dark at

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10° for 24 hours. At this temperature no measurable conversion of leucoanthocyanin to anthocyanin occurred (12). Anthocyanin content was determined from absorbance values measured at 535 mμ in half-inch tubes in a Bausch and Lomb Spectronic 20 colorimeter. The absorbance values were multiplied by the total volume of the extract and divided by the number of hypocotyls or segments in the sample. The data thus obtained represent expressions in relative units of the amounts of anthocyanin per hypocotyl or segment.

Chromatography. Paper-chromatographic analysis of extracts of anthocyanin-containing hypocotyls was carried out by 1- and 2-dimensional descending techniques on Whatman No. 3MM paper. The 3 solvent systems employed were acetic acid-concentrated HCl-H_2O (30: 3: 10, v/v/v), the lower phase of *n*-butanol-2 N HCl (1/1, v/v), and formic acid-2 N HCl (1/1, v/v). The sample of cyanidin used for comparative chromatography was purchased from California Corporation for Biochemical Research.

Results

Anthocyanin Pigments. In an earlier chromatographic study of anthocyanins of buckwheat-seedling hypocotyls (11) 3 pigments were found, an anthocyanidin and 2 of its glycosides. Repetition of this work indicated the presence of the same 3 substances only when certain solvents were used. A possible third glycoside was revealed by 2 of the solvents (table I). It was found that 2 of these substances actually appeared or increased in quantity in the course of hydrolysis of buckwheat extracts. Therefore, some extracts were prepared in such a manner that their temperature never rose above 10° and chromatograms of these were run at 5°. On all such low-temperature chromatograms only 1 anthocyanin spot was evident (table I). Comparison of the chromatographic behavior of the anthocyanidin present in hydrolyzed hypocotyl extracts with that of authentic cyanidin (table I) indicates, as previously suggested (6, 11, 12), that the buckwheat anthocyanin is probably a glycoside of cyanidin.

Duration of Light Period. The amount of anthocyanin formed in hypocotyl segments excised from 5-day-old seedlings increased with increasing duration of illumination when the light treatment was followed by a period of darkness at 10° (fig 1). At the particular illuminance used, the amount of pigment formed varied linearly with duration of exposure only for light periods longer than 4 hours. The existence of a lag period or induction period in this plant under these conditions is thus evident.

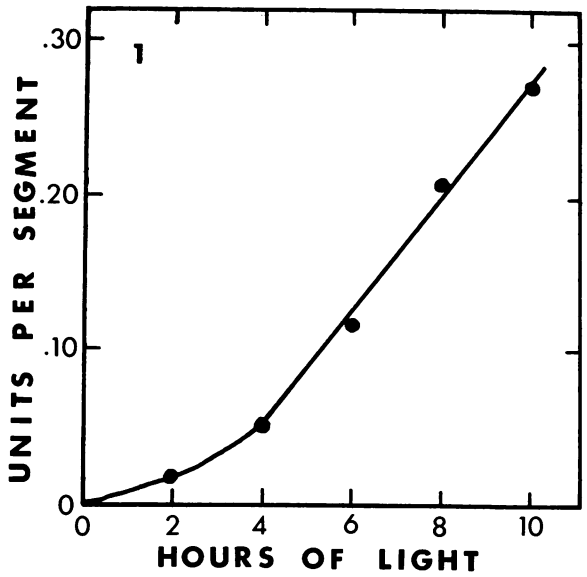
Temperature during Dark Period. Anthocyanin synthesis in hypocotyls of 5-day-old seedlings was affected by the temperature to which the hypocotyls were subjected during the dark period following a 5-hour light exposure (fig 2). The largest amount of pigment was formed in intact seedlings at 10°, and the smallest amount in excised segments at the same temperature. At both 10° and 25° less anthocyanin was produced in excised segments than in comparable portions of intact plants, but this difference was much larger at 10° than at 25°. The behavior of intact and excised materials was similar in that at 10° the amount of anthocyanin leveled off eventually to a plateau value, but at 25° a maximum value was reached at about 48 hours, after which a decline occurred.

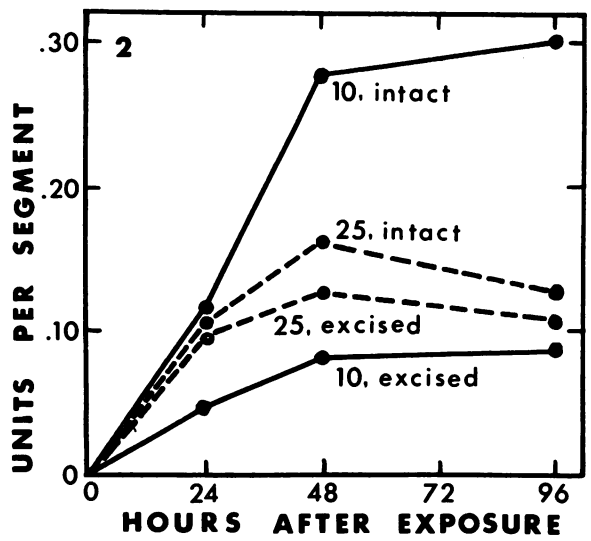
Age of Plant and Time of Excision. The ability of buckwheat seedlings of different ages to form anthocyanin was studied by treating intact seedlings with a 5-hour light period followed by a 72-hour dark period at 10°. Entire hypocotyls were taken for analysis. The amount of anthocyanin formed in the hypocotyl in response to the conditions used increased as the age of the seedling increased up to 7 days, but decreased thereafter (fig 3A). Thus, in terms of total amount, the anthocyanin-forming capacity of the seedlings was greatest at an age of 6 to 7 days. During the 10-day experimental period the seedlings grew steadily; between the ages of 3 days and 10 days the hypocotyl increased in mean length from 1 cm to 13 cm, and in mean wet weight from 8 mg to 138 mg. The amount of anthocyanin formed per g of wet weight of the hypocotyl decreased steadily (fig 3B). On this basis the anthocyanin-forming capacity was greatest at a seedling age of 3 days.

Table I. *R_F Values of Anthocyanin Pigments in Extracts of Buckwheat-Seedling Hypocotyls*

Each R_F value is the mean of at least 5 determinations. Solvent composition is designated in parts by volume.

Solvent	Temperature	Unhydrolyzed extract		Anthocyanidin in hydrolyzed extract	Authentic cyanidin
		Principal spot	Other spots		
Formic acid-2 N HCl (1/1)	25°	0.82	0.68, 0.51, 0.30	0.30	0.30
	5°	0.71	None present	...	...
n-Butanol-2 N HCl (1/1) lower phase	25°	0.46	0.28, 0.22, 0.12	0.15	0.14
	5°	0.26	None present	...	...
Acetic acid-conc HCl-H_2O (30:3:10)	25°	0.74	0.52	0.50	0.51





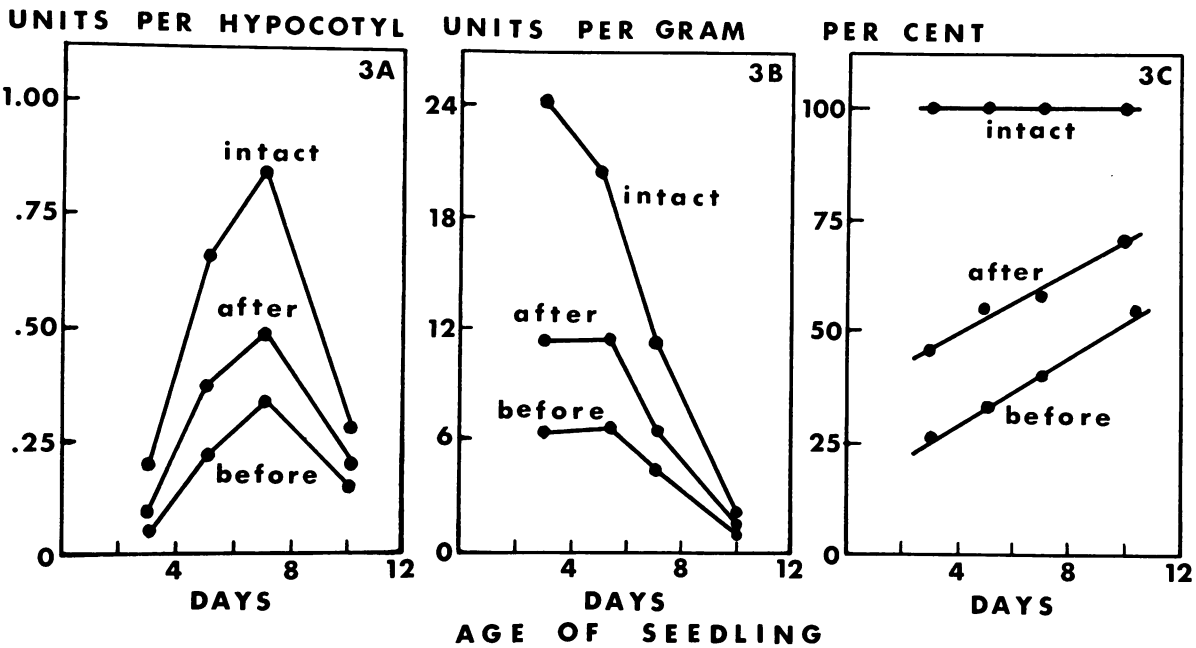


FIG. 1 (*upper left*). Amounts of anthocyanin formed in excised 20-mm hypocotyl segments in response to various durations of exposure to light at 25° followed by darkness at 10°. Measurement was made 72 hours after the start of the light period. Seedlings 5 days old were used. Each point represents the mean of 5 replications.

FIG. 2 (*upper right*). Amounts of anthocyanin formed in the dark at 2 temperatures in excised 20-mm hypocotyl segments and in comparable portions of intact seedlings. The anthocyanin formation was induced by exposure of the plant material to light for 5 hours at 25°. Seedlings were 5 days old at the start of the experiment. Each point represents the mean of 4 replications.

FIG. 3 (*lower*). Anthocyanin formation in entire hypocotyls of buckwheat seedlings of different ages following exposure to light at 25° for 5 hours and subsequent darkness at 10° for 72 hours. Hypocotyls were either excised before or after the light period, or were parts of intact plants throughout the experiment. Each point represents the mean of 10 replicates. A) Anthocyanin content expressed as relative units per hypocotyl. B) Anthocyanin content expressed as relative units per g wet weight. C) Anthocyanin content expressed as per cent of the content of hypocotyls of intact seedlings for each age. The straight lines shown for excised hypocotyls are fitted least-squares lines.

Excision of hypocotyl segments from seedlings resulted in a reduction in the amount of anthocyanin formed. The variation of this excision effect with seedling age was studied by cutting off the entire hypocotyl from the remainder of the seedling either immediately before or immediately after the exposure to light. These excised hypocotyls were otherwise treated in the same manner as the intact seedlings. In all cases removal of the hypocotyl from the seedling decreased the amount of anthocyanin formed, and the reduction was always greater when the excision occurred before the light period than when it occurred afterward (fig 3A, B). When the anthocyanin formed in the hypocotyls of intact seedlings was taken as 100% for each seedling age and the amounts formed in the excised hypocotyls were expressed on that basis, then the latter increased linearly with age (fig 3C). Statistical tests of the fit of the least-squares lines indicated no significant deviation from linearity in either case. Also, comparison of the slopes of the 2 lines supported the hypothesis of no significant difference between them.

Influence of Sucrose on Excision Effects. An experiment was performed in which sucrose was supplied artificially to excised hypocotyls. Entire hypocotyls or intact seedlings of 3 ages were exposed to light for 5 hours, then floated on distilled water or on sucrose solutions during the subsequent 72-hour dark period at 10° (table II). In 7-day-old seedlings treatment with 0.06 M sucrose increased the amount of anthocyanin formed by about 35% regardless of the time of excision. This effect was sufficient to raise the anthocyanin content of hypocotyls excised after exposure to a value equivalent to that of intact seedlings. With hypocotyls excised before the light period, supplying sucrose became more effective with advancing age of the seedlings. In 3-day plants 0.03 M sucrose was without effect. In 7-day plants sucrose levels up to 0.12 M increased the amount of anthocyanin formed, but in every case this amount was still considerably smaller than that formed in intact seedlings. In 10-day plants 0.03 M sucrose not only overcame the effect of excision, but even increased the amount of anthocyanin formed to a value much higher than that in intact seedlings.

Excised Epidermis. In the buckwheat hypocotyl the anthocyanin pigment occurs in most of the cells of the epidermis and in some of the subepidermal cortex cells, but not elsewhere (6). Attempts were therefore made to induce the synthesis of the pigment in isolated strips of epidermis. In no case, however, was a detectable amount of anthocyanin ever formed in such a piece of epidermis, whether removal occurred before or after the period of exposure to light. Also, anthocyanin never appeared in any cells of hypocotyl segments the epidermis of which had been completely stripped off. Such results were consistently obtained even when the strips or segments were floated on sucrose solutions before, during, or after the light period. Careful visual inspection revealed no obvious evidence of injury or drying-out of any of the materials.

Discussion

The Anthocyanin Pigment. The pigment synthesized in excised hypocotyls was identical with that formed in hypocotyls of intact seedlings; thus, excision apparently does not qualitatively alter the anthocyanin-forming capability of the hypocotyl. This situation is simpler than that found in *Impatiens* petals (7). The results described are consistent with the conclusion that only 1 anthocyanin, a cyanidin glycoside, occurs in hypocotyls of buckwheat seedlings. Previously 3 pigment spots were observed on some chromatograms of buckwheat extracts (11). However, these chromatograms were developed with strongly acid aqueous solvents at room temperature. Since in the present study only 1 pigment appeared on chromatograms run at a low temperature, it seems likely that the other substances are actually artifacts resulting from hydrolysis during the chromatographic development. This idea is supported by correspondence of the spots in question with pigments which increase in quantity during hydrolysis of anthocyanin-containing extracts. Factors contributing to this effect would be the high acid concentration in the solvents in question, and the high temperatures (25°–30°) and long times (12–17 hr) associated with the development process.

Table II. *Anthocyanin Formation in Excised Hypocotyls and Hypocotyls of Intact Buckwheat Seedlings upon Treatment with Various Levels of Sucrose*

All materials were exposed to light for 5 hours, then floated on sucrose solutions in the dark for 72 hours at 10°. Each value is the mean of 5 replications.

Age of seedling	Treatment	Molarity of sucrose				
		0	0.015	0.03	0.06	0.12
Days		Units of anthocyanin per hypocotyl				
3	Intact seedlings	0.19	...	...	...	...
	Excised before exposure to light	0.06	...	0.06	...	...
7	Intact seedlings	0.54	...	...	...	...
	Excised before exposure to light	0.26	0.32	0.31	0.35	0.36
	Excised after exposure to light	0.44	...	...	0.59	...
10	Intact seedlings	0.28	...	...	...	...
	Excised before exposure to light	0.19	...	0.42	...	...

Anthocyanin Formation in Excised Hypocotyls. Most investigations of anthocyanin formation in seedlings have involved culture and treatment of the entire plant. This was the approach used by Kuilman (8) and Karstens (6) in their studies of buckwheat. However, Arnold and Alston (1) have described the use of small pieces of *Impatiens* hypocotyls in experiments on growth and anthocyanin synthesis, and Eberhardt (4) briefly mentioned an investigation of the effect of 2 respiratory inhibitors on anthocyanin formation in pieces of buckwheat hypocotyls. The fact that anthocyanin is formed in excised hypocotyls or small pieces of hypocotyls following exposure to light indicates that this organ of the dark-grown buckwheat seedling contains in itself a raw-material supply and metabolic apparatus sufficient for anthocyanin synthesis. The other portions of the seedling are thus not essential to the process, although the excision effects discussed later show that such parts may exert a quantitative influence. Various previous observations (6) indicate that there is no apparent translocation of the effect of light from 1 part of the hypocotyl to another. The individual cells in which the anthocyanin ultimately appears may thus be self-sufficient for pigment formation. It is difficult to reconcile this likelihood with the fact that no anthocyanin synthesis took place in strips of epidermis or in hypocotyl pieces from which the epidermis had been removed, unless one assumes an inhibitory effect of injury resulting from the drastic treatment involved. Such injury might not be evident under the visual examination conducted.

Temperature during Dark Period. Recorded observations on the influence of temperature on anthocyanin synthesis are numerous. Most of the early workers reported that greater amounts of anthocyanin are formed at low temperatures (2). Recently maximum synthesis at 15° to 20° has been found in apple skin (10) and in *Sorghum* seedlings (4). Most temperature studies have not involved examination of the time-course of anthocyanin formation; exceptions to this statement are works with buckwheat (8) and with red cabbage (5). The observation reported here that the peak amount of pigment formed in intact seedlings at 10° was greater than that at 25° is consistent with the results of Kuilman (8), but unlike those of Frey-Wyssling and Blank (5). The disappearance of anthocyanin at a higher temperature, observed here after about 48 hours at 25°, is similar to the situations described in both of the reports just mentioned.

On the basis of this study, incubation in the dark at 10° for 72 hours following exposure to light was adopted as a standard practice. This low-temperature treatment was chosen for 4 reasons: the pigment content remains the same over a long period of time after 48 hours, the difference between excised and intact materials is maximized, no growth and no adventitious root formation occur, and treatment of the hypocotyls with solutions of various reagents would involve fewer problems than at a higher temperature.

Duration of Illumination. The observed variation of anthocyanin formation in excised segments under different periods of illumination is similar to that previously recorded for intact seedlings by Kuilman (8), who used sunlight of unspecified intensity and exposure periods of no more than 5 hours. The lag period suggested by a plot of Kuilman's data is shorter than that observed in the present instance. However, this difference may be attributed to the different exposure conditions used, since it has been demonstrated that the length of the lag or induction period may vary with light intensity (3) as well as with other factors (10). A light period of 5 hours was chosen for routine use.

Age of Seedling. Karstens (6) observed that buckwheat seedlings 6 or 7 days old were capable of forming more anthocyanin than either younger or older seedlings in response to a brief light period followed by several days in darkness. The present study confirms this finding, and indicates in addition that excised hypocotyls show the same general pattern as hypocotyls of intact plants. Since growth of the hypocotyl occurred continuously throughout the 10-day period, while the amount of anthocyanin formed per g of wet weight declined steadily, the increase in anthocyanin-forming capacity during the first 7 days apparently does not keep pace with growth processes. In further experimentation the use of seedlings 6 or 7 days old would clearly be of advantage in maximizing the amount of anthocyanin which potentially could be formed.

Effects of Excision. Karstens (6) concluded that the supply of readily available carbohydrate in the hypocotyl itself was the principal factor determining the anthocyanin-forming capacity of the organ. However, it is possible that some other factor is involved which is associated with the other parts of the seedling, since excision of the hypocotyl results in formation of a smaller amount of pigment. A similar effect of excision of the first internode in *Sorghum* has been mentioned (3). The possibility that excision may decrease anthocyanin formation by removing the hypocotyl from a source of additional carbohydrate is consistent with those cases in which supplying sucrose to excised hypocotyls resulted in as large an amount of anthocyanin as in intact plants. However, availability of carbohydrate is probably not the only factor, since sucrose did not overcome the effect of excision in all cases. The situation is further complicated by the fact that hypocotyls from seedlings of different age differ in these respects.

The existence of at least 2 excision effects is also suggested by the fact that removal of the hypocotyl before exposure to light always resulted in less anthocyanin being formed than removal after the light period. In 7-day plants sucrose overcame the latter effect completely, but the former only partially. Furthermore, although the anthocyanin-forming ability of excised hypocotyls relative to that of hypocotyls of intact seedlings increased steadily with age, there was a constant difference for segments excised before and after the light period. It is possible that

this constant difference, which amounted to about 22% of the value for intact plants (fig 3C), represents an effect of excision on the photosensitive systems, while the remainder of the effect represents an influence of excision directly on the anthocyanin-forming system. In this connection the assumption is made that in buckwheat the role of light in anthocyanin synthesis is indirect (9).

Summary

The suitability of hypocotyl material excised from dark-grown buckwheat seedlings (*Fagopyrum sagittatum*) for use in the study of anthocyanin synthesis has been investigated. Under the conditions used, anthocyanin formation in response to light and a subsequent dark period occurs in much the same manner in excised hypocotyls or hypocotyl segments as in hypocotyls of intact plants. The 1 pigment formed is evidently a glycoside of cyanidin. After an initial lag period the amount of pigment formed varies linearly with the duration of the light period at the particular constant illumination used, and is greater if the temperature during the dark period is low (10°). Hypocotyls from seedlings 6 or 7 days old can form more anthocyanin than those from older or younger plants.

The quantity of pigment formed in excised hypocotyls is smaller than that synthesized in comparable organs of intact plants. The magnitude of this decrease caused by excision becomes smaller as the age of the seedling increases. Excision of the hypocotyl before exposure to light results in an even greater reduction in the amount of anthocyanin synthesized than does excision after the light period. Under some conditions supplying sucrose increases the pigment formed in the excised material to a value equal to that formed in intact plants.

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Literature Cited

1. Arnold, A. and R. E. Alston. 1961. Certain properties of hypocotyl of *Impatiens balsamina* reflecting physiological complexity. Plant Physiol. 36: 650–56.
2. Blank, F. 1958. Anthocyanins, flavones, xanthones. In: Encyclopedia of Plant Physiology, X. W. Ruhland, ed. Springer-Verlag, Berlin, p. 300–53.
3. Downs, R. J. and H. W. Siegelman. 1963. Photocontrol of anthocyanin synthesis in milo seedlings. Plant Physiol. 38: 25–30.
4. Eberhardt, F. 1954. Über die Beziehungen zwischen Atmung und Anthocyansynthese. Planta 43: 253–87.
5. Frey-Wyssling, A. and F. Blank. 1943. Untersuchungen über die Physiologie des Anthocyans in Keimlingen von *Brassica oleracea* L. var. *capitata* L. f. *rubra* (L.) Ber. Schweiz. Botan. Ges. 53A: 550–78.
6. Karstens, W. K. H. 1939. Anthocyanin and anthocyanin formation in seedlings of *Fagopyrum esculentum* Moench. Rec. Trav. Bot. Néerl. 36: 85–179.
7. Klein, A. O. and C. W. Hagen, Jr. 1961. Anthocyanin production in detached petals of *Impatiens balsamina* L. Plant Physiol. 36: 1–9.
8. Kuilman, L. W. 1930. Physiologische Untersuchungen über die Anthocyane. Rec. Trav. Bot. Néerl. 26: 287–416.
9. Mohr, H. 1962. Primary effects of light on growth. Ann. Rev. Plant Physiol. 13: 465–88.
10. Siegelman, H. W. and S. B. Hendricks. 1958. Photocontrol of anthocyanin synthesis in apple skin. Plant Physiol. 33: 185–90.
11. Troyer, J. R. 1958. Anthocyanin pigments of buckwheat hypocotyls. Ohio J. Sci. 58: 187–88.
12. Troyer, J. R. 1961. Occurrence of a leucoanthocyanin in hypocotyls of etiolated buckwheat seedlings. J. Elisha Mitchell Sci. Soc. 77: 137–40.