PLANT PHYSIOLOGY

VOLUME 36 JANUARY, 1961 NUMBER 1

ANTHOCYANIN PRODUCTION IN DETACHED PETALS OF IMPATIENS BALSAMINA L.[1, 2]

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INTRODUCTION

Anthocyanin biosynthesis in plants appears to be under manifold controls. Genetic capacity for pigment production must be complemented by a sequence of rather definite developmental events and proper environmental and nutritional factors. While biochemical pathway analysis is successfully tying flavonoid synthesis to the major processes of cellular metabolism, many key physiological control mechanisms appear obscure. With the exception of intensive investigations by the Beltsville group on the light requirement for anthocyanin synthesis, there are few satisfactory accounts in the literature of the mode of action of such external influences as nutrition, temperature, age or injury. Because anthocyanin coloration takes place only under specific circumstances it is potentially an easily recognized indicator of the metabolic state of cells producing it. Anthocyanins can assume such a role only after specific controlling reactions along their biosynthetic paths have been causally related to specific cellular events. This study defines a system in which such causal connections may be detected and provides indications of some of the controlling reactions.

Petals of *Impatiens balsamina* L. were chosen as material for these investigations. Recently Alston and Hagen (1) described the genetic control of anthocyanin production by these petals and subsequently Clevenger (4) provided similar information for the flavonols present. Petals are gaining favor as suitable organs for studying several developmental phenomena, notably protein synthesis during cell expansion (11), and metabolism of senescence (19), as well as for studying specific metabolic and biosynthetic relationships (20). The immediate aims of the present work were to isolate immature petals into a suitable culture medium, to observe the development of such petals and correlate the morphogenetic events with pigment production; and to examine the physiology of both developmental and synthetic steps by attempting to alter normal development by chemical intervention.

MATERIALS & METHODS

Plants of the garden balsam (*Impatiens balsamina* L.) were grown in the greenhouse in gravel with daily watering and weekly application of a soluble commercial fertilizer. The genotypes employed were 11hhP^{r}P^r (pink), 11HHP^{r}P^r (red), and LLhhP^{r}P^r (purple) as designated by Davis et al (5). The red and purple flowers are intensely pigmented while pink is present only as a dilute color. Buds were collected, weighed, sterilized in a 1 % sodium hypochloride solution for 15 minutes, rinsed, and dissected. The four inner petals from about 20 buds were floated on sterile water. Twelve petals, picked at random, were transferred to 25 ml Erlenmeyer flasks containing 5 ml of the medium described below. This gave a petal equivalent of three flowers per flask. The culture medium was White's nutrient (22) with the modification that iron was supplied as Fe-sequestrine, and copper and cobalt were included at 0.001 and 0.01 mg/l, respectively. Unless otherwise noted the nutrient contained 20 g of sucrose per liter. Subsequent

[1] Received May 2, 1960.

[2] During part of the period in which this work was conducted, one of the authors (A.O.K.) received support from a National Science Foundation Predoctoral Fellowship. The material in this paper was included in a thesis submitted by A. O. Klein to the Graduate School of Indiana University in partial fulfilment of the requirements for the Ph.D. degree, 1959.

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culture was at 22° C under continuous illumination of 4,000 lux provided by warm white fluorescent tubes supplemented with 100 W incandescent bulbs. At the conclusion of the experiments the petals were rinsed, blotted, weighed, and quickly frozen in stoppered vials. Pigments were extracted at room temperature with 1 % hydrochloric acid in 95 % ethyl alcohol (v/v) until petals were colorless. In the tables which follow, anthocyanin concentration is usually expressed as optical density (OD) of a solution containing the pigments of one flower equivalent in 10 ml of solvent. Absorption was measured in a Beckman DU or Spectronic 20 spectrophotometer in the region of maximum absorption (510–550 mμ), the specific wave length depending on the genotype. The extracts were concentrated under reduced pressure for chromatography. Two dimensional separation was accomplished with two solvents in the following order: *tert*-butanol, acetic acid, water (3 : 1 : 1), descending; 10 % acetic acid ascending. A spray composed of 2 % aluminum chloride in 95 % ethyl alcohol was used to stabilize spots. Papers were examined by fluorescent and ultra violet light in air and in ammonia fumes. For isolating and spectrophotometrically characterizing glycosides, and preparing aglycones, extracts were streaked on Whatman no. 3 paper which was developed with the *tert*-butanol solvent. The resulting bands were eluted with Harborne's elution mixture and rechromatographed (9). Hydrolysis of glycosides was accomplished by boiling in 20 % hydrochloric acid (w/v) for 3 to 4 minutes. The aglycones were then extracted with a few drops of iso-amyl alcohol and compared chromatographically with known pigments in at least two solvents: acetic acid, hydrochloric acid, water (30 : 3 : 10) and the *tert*-butanol solvent described above. Numerical data reported are based, in general, on values obtained from three flasks containing 12 petals each, or the equivalent of 9 flowers.

Results

Young buds of *I. balsamina* are well suited for organ culture. The four showy lateral petals are tightly enclosed in an envelope consisting of the single sepal and the fifth petal. This arrangement allows for easy surface sterilization prior to dissection. Excised petals remain afloat on a liquid medium and apparently resume development with a somewhat accelerated time course. Preliminary experiments showed that White's medium (*22*) without significant additions supports the growth of petals for as long as 40 days without transfer. This medium was used throughout the investigation. The amount of sugar in the medium had a marked effect on development and anthocyanin production (table I). Although immature petals are pale green in color, carbohydrate production by photosynthesis must fall far short of the requirements for normal development. Apparently, however, no other major organic chemical contribution by the rest of the plant is required for continued growth and pigmentation. Indole acetic acid in the range of 0.005 to 0.01 mg/l did not influence the state of petals in culture. Light was found to be essential for both expansion and pigment production. Cultures which were kept in total darkness for 22 days developed only a small band of red coloration adjacent to the cut basal end. Such petals also showed arrested development but were capable of resuming growth when exposed to light.

The only reliable criterion for judging the physiological age of petals is the fresh weight of buds from which they are excised. Cultures were started as a rule with petals from buds weighing 30 to 50 mg. The fresh weight of the four petals at this stage was about ten mg. Petals of a bud on intact plants start anthocyanin production when their combined weight reaches about 40 mg. at which time total bud weight is 100 mg. When petals are fully mature they weigh ca. 400 mg per flower if left on the plant and about 150 to 300 mg if cultured. The increase in fresh weight probably represents expansion only because even the youngest petals cultured had already assumed their final shape. That weight may be directly correlated with area was demonstrated by the constancy of the ratio fresh weight/surface measured for three flasks, a total of 36 petals. The average value was 0.21 mg/mm² with a narrow range of variation.

Time Course in Culture. The time course of expansion and anthocyanin synthesis is summarized in figure 1. These curves show that expansion commences immediately and continues until about the 13th day when a sharp increase in the rate sets in. In general, anthocyanin synthesis follows the same pattern as expansion with both activities beginning to level off about 20 days after culturing.

Modification of Pigment Pattern by Culturing — Quantitative Aspect. Quantitative comparison of anthocyanins produced by detached and undisturbed petals is presented in table II. In the intensely colored flowers of 11HHP^{r}P^r and LLhhP^{r}P^r anthocyanin content of cultured petals approximates the values obtained from intact flowers. In the dilute pink line of genotype 11hhP^{r}P^r, however, cultured

Table I

Effect of Sucrose Concentration on Fresh Weight & Anthocyanin Content of Detached Petals*

Sucrose conc % (w/v)	Fresh wt/ flower mg	Anthocyanin content	
		OD/ Flower**	OD/ g Tissue***
0.00	35	0.016	0.046
0.50	115	0.235	0.204
1.00	135	0.411	0.305
2.00	174	0.575	0.330
2.50	177	0.612	0.346
3.00	182	0.575	0.317

* Genotype 11hhP^{r}P^r
** In 10 ml
*** In 100 ml

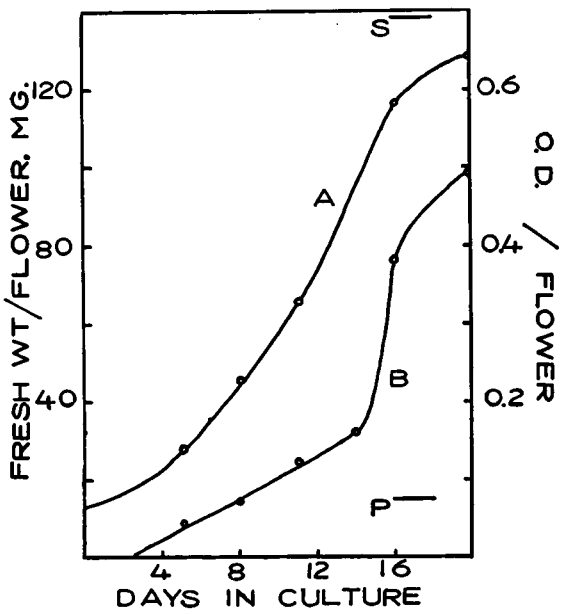

FIGURE 1.

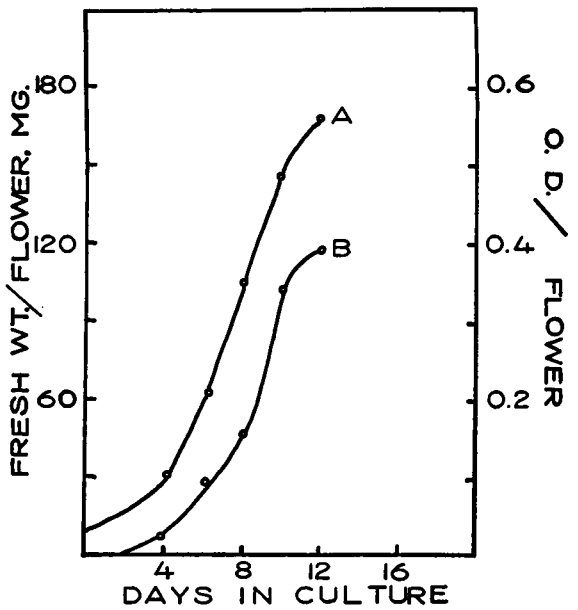

FIGURE 2.

FIG. 1. Time course of anthocyanin production and expansion of petals in culture. Expansion measured as fresh weight of four petals (curve A). Anthocyanin content expressed as OD (at λ max) of an extract containing the pigments of four petals in 10 ml of 95 % ethanol containing 1 % HCl (curve B). Pigment content of intact petals (P) and sepals (S). Genotype llhhPrPr.

FIG. 2. Time course of anthocyanin production and expansion of injured petals in culture. For description of intentional injury see text. Genotype llhhPrPr.

petals approximate intact sepals in their anthocyanin content. It thus appears that in this genotype culturing allows the production of larger quantities of pigments than are produced in situ.

MODIFICATION OF PIGMENT PATTERN BY CULTURING — AGLYCONES & GLYCOSIDES. The flavonoid pigments produced by detached petals are compared with those produced by intact flowers in table III. Undisturbed petals contain no anthocyanin or flavonol with a 3'4'-dihydroxylated B ring, whereas in culture all three genotypes produced cyanidin, two produced quercetin and one produced peonidin, all of these compounds having a basic 3',4'-dihydroxy pattern on the B ring of the flavonoid nucleus. In this respect cultured petals took on some of the characteristics of sepals in the pink and red flowers. Also sepaloid in nature was the distribution of pigment in cultured petals, for veins became strongly delineated while in undisturbed petals the coloration is more evenly distributed.

To test the possibility that these departures from normal quantitative and qualitative patterns of pigment production resulted from mechanical injury or

TABLE II

COMPARISON OF ANTHOCYANIN CONTENT OF INTACT FLOWER PARTS & CULTURED PETALS

GENOTYPE	COLOR	FLOWER PART	ANTHOCYANIN CONTENT			
			INTACT FLOWERS		CULTURED PETALS	
			OD/FLOWER	OD/g TISSUE	OD/FLOWER	OD/g TISSUE
llhhPrPr	Pink	Petal	0.075	0.015	0.633	0.345
		Sepal	0.690*	0.262	...	...
llHHPrPr	Red	Petal	1.420	0.230	1.018	0.750
		Sepal	0.490*	0.130	...	...
LLhhPrPr	Purple	Petal	0.470	0.090	0.425	0.278
		Sepal	0.440*	0.139	...	...

* Values given are for four sepals.

premature exposure to white light which cultured petals received, the following simple experiments were performed. A large number of 11hhP^{r}P^r buds were pierced with a needle at the stage usually collected for culturing. The injured buds were allowed to develop in situ and the petals were examined at anthesis. In another experiment approximately 20 buds were divested of their outer envelope formed by the sepal and fifth petal and were then enclosed in a transparent film of "Saran Wrap" to reduce water loss. In this way white light was admitted to immature petals without detaching them. At the conclusion of these experiments the treated petals showed no departure from the usual, natural patterns in either the quantity, distribution, or quality of coloration. The dilute pink color of the 11hhP^{r}P^r genotype would have allowed easy visual detection of any alteration usually associated with cultured petals.

The glycosidic pattern of intact and cultured petals was found to be quite similar upon chromatographic comparison. It was of interest to observe in several developmental stages whether or not the various pigments appear in a specific order. As will be shown below, injury has the effect of accelerating development. The anthocyanin patterns of two groups of 11HHP^{r}P^r petal cultures were therefore compared. The first group of petals was allowed to develop in culture without any additional treatment while the second group received mechanical injury in the form of two needle tears per petal to increase pigmentation. When harvested after 14 days of incubation the injured petals showed a much higher pigment content than the control. In the control petals one of the two major pelargonidin glycosides (designated as Spot no. 3) was far more intense on the two dimensional chromatogram than the other (designated as Spot no. 4). In the more intensely colored, injured petals, Spot no. 4 seemed to predominate slightly over Spot no. 3. Densitometric measurements of chromatogram strips confirmed this observation. A comparison of the absorption spectra of the two eluted pigments showed that the pigment of Spot no. 3 possessed a shoulder at 440 mμ in addition to the major pelargonidin peak, while the glycoside of Spot no. 4 had no such shoulder. According to results published by Harborne (9) an E_{440}/E_{max} ratio of 0.39 is indicative of a free hydroxyl group in position 5 of the pelargonidin molecule, while a ratio of around 0.20 characterizes glycosides with a sugar occupying position 5. The E_{440}/E_{max} ratios of Spots no. 3 and no. 4 were 0.39 and 0.22, respectively. Full chemical and chromatographic identification of these spots as a pelargonidin-3-monoglucoside and a pelargonidin-3,5-diglucoside will be published elsewhere. Thus, it appears that, as has been suggested by Harborne and Sherratt (8), glycosidation proceeds in a stepwise fashion, in this case first the 3 and then the 5 hydroxyl groups reacting. Judging by the intensity and area of spots representing the pelargonidin glycosides of llhhP^{r}P^r petals, the sequence of events in that genotype is similar. Similar observations have been made in developmental studies on intact plants by Hagen (unpubl).

Experimental Control of Development & Pigmentation — Natural Sequence of Events. Further work with this petal system was designed to shed light on the relation between developmental events and synthetic processes leading to pigmentation. We shall first describe the course of pigment development in culture and then present the results of attempts to interfere with the observed pattern. The excised petals are initially pale green, tightly furled organs. Within 2 days after culture they flatten out and the portion of the claw adjacent to the cut end begins the production of anthocyanin. The red area slowly enlarges as its boundary moves toward the blade. At this time the cells in the blade adjacent to the claw begin to lose their green appearance and simultaneously start to expand. Expansion causes a slight hump to form at the border between the still-green and the white portions of the blade. The hump slowly travels across the surface acropetally until the entire petal has lost its chlorophyll and reached

Table III

Anthocyanidins & Flavonols Found in Cultured Petals & Intact Flower Parts

Genotype	Color	Part	Anthocyanidins				Flavonols		
			Pelargonidin	Cyanidin	Peonidin	Malvidin	Kaempferol	Quercetin	Myricetin
llhhP^{r}P^r	Pink	Intact Petal	+	...	...	...	+	...	...
		Cultured Petal	+	+	+	...	+	+	...
		Sepal	+	+	+	...	+	+	...
llHHP^{r}P^r	Red	Intact Petal	+	...	...	...	+	...	...
		Cultured Petal	+	+	...	...	+	+	...
		Sepal	+	+	...	...	+	+	...
LLhhP^{r}P^r	Purple	Intact Petal	...	...	...	+	+	...	+ (?)
		Cultured Petal	...	+	...	+	+	...	+ (?)
		Sepal	...	...	...	+	+	+	+ (?)

TABLE IV

EFFECT OF INJURY ON FRESH WEIGHT & ANTHOCYANIN CONTENT OF CULTURED PETALS

EXP.	GENOTYPE	DAYS IN CULTURE	FRESH WT/FLOWER mg			ANTHOCYANIN CONTENT OD/FLOWER		
			CONTROL	INJURED	% INCREASE	CONTROL	INJURED	% INCREASE
I–80	LLhhP^{r}P^r	7	29.4	61.0	107	0.069	0.221	220
I–83	llhhP^{r}P^r	12	41.3	95.3	131	0.041	0.100	144
II–3	llhhP^{r}P^r	14	69.0	127	85	0.121	0.426	252

maximum expansion. At the same time the zone showing anthocyanin also enlarges acropetally closely following behind the hump. Anthocyanin and chlorophyll were never observed to coexist over the same area nor was anthocyanin found in an area which had not yet expanded. The consistent sequential occurrence of bleaching, expansion, and the anthocyanin formation suggested that perhaps the mechanism responsible for anthocyanin synthesis is set in motion by conditions generated in the cell as a consequence of its rapid expansion. These conditions may also be incompatible with the maintenance of chlorophyll in the cells. Curves A and B of figure 1 show the relation of maximum rates of expansion and anthocyanin production.

EFFECTS OF INJURY. Observations on the effects of accidental injury to petals led us to an experiment in which petals were deliberately injured by two needle tears at the time of culturing. Both fresh weight gain and anthocyanin synthesis are stimulated by such treatment (table IV). A time course study on injured petals (fig 2) clearly shows that the periods of most active expansion and coloration occur some 6 to 8 days earlier than in uninjured cultures. To determine the upper limit of stimulation by injury, petals in three parallel sets of cultures were left uninjured, cut in halves, and cut in quarters, respectively. While halving resulted in a doubling of weight and tripling of pigment production further injury by quartering the petals resulted in no further expansion and only a very slight further stimulation of anthocyanin synthesis (table V). In all cases, however, injury stimulated both expansion and coloration.

TABLE V

DEGREE OF INJURY CORRELATED WITH FRESH WEIGHT & ANTHOCYANIN CONTENT OF CULTURED PETALS*

TREATMENT	FRESH WT/ FLOWER mg	% INCREASE	OD/ FLOWER	% INCREASE	OD/g TISSUE	% INCREASE
Petals whole (control)	66.6	...	0.143	...	0.214	...
Petals halved	141.0	112	0.432	198	0.306	43
Petals quartered	138.7	108	0.460	222	0.332	55

* Genotype llhhP^{r}P^r

EFFECTS OF KINETIN. In an attempt to achieve chemical stimulation of expansion, kinetin, reported to have such action on leaf discs by Miller (12), was added to the medium. At the optimum concentration of 0.5 mg/l, kinetin indeed increased the fresh weight of petals (table VI) but its effects on pigmentation were even more pronounced. In general, kinetin greatly increased anthocyanin production. These results are in contrast with Thimann's observations (15) in which he found no stimulation of anthocyanin synthesis by kinetin in his Spirodela system.

TABLE VI

EFFECTS OF KINETIN ON FRESH WEIGHT & ANTHOCYANIN PRODUCTION OF CULTURED PETALS*

KINETIN CONC mg/l	FRESH WT/ FLOWER mg	ANTHOCYANIN CONTENT	
		OD/FLOWER	OD/g TISSUE
0.0	83	0.214	0.258
0.5	135	0.571	0.422
1.0	112	0.342	0.306

* Genotype llhhP^{r}P^r

EFFECTS OF MANNITOL & TRIIODOBENZOIC ACID. As it had been found that both chemical and mechanical stimulation of expansion produced similar stimulation of anthocyanin synthesis, an attempt was made to restrict expansion and observe whether or not pigment production would also be inhibited. For this purpose petals were grown in graded concentrations of mannitol to interfere osmotically with water uptake and also in the presence of triiodobenzoic acid (TIBA), a potent antagonist of auxins (7), to suppress expansion by disturbing hormonal controls. The results obtained from the mannitol experiments (table VII) indicate that anthocyanin production is inhibited to a greater degree by higher, osmotically active, concentrations than is petal expansion itself. That no specific toxicity to the pigment producing system is involved may be surmised from the slightly stimulatory effects of the lowest mannitol concentra-

TABLE VII

EFFECTS OF MANNITOL CONCENTRATION ON FRESH WEIGHT & ANTHOCYANIN CONTENT OF CULTURED PETALS*

MANNITOL CONC M	FRESH WT/ FLOWER mg	% CHANGE	OD/FLOWER	% CHANGE	OD/g TISSUE	% CHANGE
0.000 (control)	267	...	0.344	...	0.125	...
0.005	277	+4	0.399	+16	0.154	+19
0.010	303	+13	0.384	+12	0.126	0
0.050	211	−21	0.299	−29	0.110	−12
0.100	138	−48	0.084	−76	0.061	−51
0.150	110	−59	0.056	−84	0.050	−60
0.200	105	−61	0.057	−84	0.057	−54
0.250	80	−70	0.034	−90	0.044	−65

* Genotype llhhPrPr

tions. Results obtained with TIBA are summarized in table VIII. The most significant information derived from TIBA treatment is not quantitative, however. When cultures were observed after 10 days of incubation the following alteration in the normal sequence of developmental events was noted. Petals in the presence of 5 mg/l TIBA lost their chlorophyll and had expanded considerably without any trace of anthocyanin appearing. Two days later, however, rapid anthocyanin synthesis set in and, as table VIII shows, at the conclusion of the experiment pigment content reached 65 % of the control value. We thus succeeded in creating a time gap between bleaching and expansion on one hand and the appearance of anthocyanins on the other.

TABLE VIII

EFFECT OF TIBA ON FRESH WEIGHT & ANTHOCYANIN CONTENT OF CULTURED PETALS*

TIBA CONC mg/l	FRESH WT/ FLOWER mg	ANTHOCYANIN CONTENT	
		OD/ FLOWER	OD/g TISSUE
0.0	154	0.517	0.335
1.0	176	0.500	0.284
5.0	144	0.326	0.226
10.0	PETALS DEAD		
20.0	PETALS DEAD		

* Genotype llhhPrPr

EFFECTS OF ETHIONINE. Protein synthesis has frequently been associated with cell expansion (17) but the independence of these two events in some petals has also been demonstrated (11). We therefore grew petals in the presence of ethionine, an amino acid analogue, in an attempt to interfere with protein synthesis and in order to determine whether anthocyanin production and expansion depend on this process. Both anthocyanin production and expansion were sharply reduced (table IX). In an attempt to shed some light on the mechanisms of action of kinetin and ethionine their combined effects on expansion and pigmentation were examined. As table X shows, ethionine completely erases any stimulation by kinetin indicating that both agents may act on the same metabolic sequence. That this sequence involves the synthesis of materials at a specific stage of development is demonstrated by the results of an experiment in which ethionine inhibition was tested on very young as well as more mature petals. Petals from 70 mg buds, although devoid of visible pigment, have apparently partially completed the ethionine sensitive step in the assembly of their anthocyanin producing

TABLE IX

EFFECTS OF ETHIONINE ON FRESH WEIGHT & ANTHOCYANIN CONTENT OF CULTURED PETALS*

ETHIONINE CONC M	FRESH WT/ FLOWER mg	% CHANGE	ANTHOCYANIN CONTENT			
			OD/FLOWER	% CHANGE	OD/g TISSUE	% CHANGE
0.00	81	...	0.22	...	0.272	...
10^{-5}	67	−17	0.24	+09	0.358	+31
6×10^{-5}	34	−38	0.08	−64	0.235	−14
10^{-4}	22	−78	0.06	−73	0.272	...
2×10^{-4}			MOST PETALS DEAD			
4×10^{-4}			MOST PETALS DEAD			

* Genotype llhhPrPr

TABLE X

COMBINED EFFECTS OF ETHIONINE & KINETIN ON CULTURED PETALS*

ADDITIONS TO MEDIUM	FRESH WT/ FLOWER	% CHANGE	OD/FLOWER	% CHANGE	OD/g TISSUE	% CHANGE
None	93.7	...	0.238	...	0.254	...
Ethionine 6×10^{-5} M	42.6	−55	0.074	−69	0.174	−31
Kinetin 2.5×10^{-5} M	114.6	+22	0.288	+21	0.252	0
Ethionine + Kinetin	47.4	−49	0.103	−57	0.218	−14

* Genotype llhhP^{r}P^r

mechanism and were less effected by the inhibition. Petals from 30 mg buds, on the other hand, are much more susceptible to such inhibition. In all these experiments we were unable to divorce expansion from anthocyanin production although it is now clear that a time gap may be introduced between the two and that the quantity of pigment produced is not in direct proportion to the degree of expansion.

DISCUSSION

Our results indicate that petals at a bud stage of 25 mg fresh weight and higher possess the complete machinery for the production of anthocyanins. No exogenous substrates are needed beyond inorganic salts, vitamins, and sugar. From their experiments on buckwheat, Underhill et al (18) concluded that quercetin, a flavonol related to cyanidin, may be synthesized in the stem and stored in the leaves. Although a similar relationship between balsam petals and the rest of the plant is not ruled out, isolated petals appear fully competent to produce at least the usual complement and quantity of anthocyanins and flavonols.

The quantitative aspects of pigment production in culture must be tested separately in the dilute pink line and the more intense red and purple lines. In the latter anthocyanin production in culture and on the plant were nearly the same. In the pink line of genotype llhhP^{r}P^r, however, culturing of petals resulted in an eightfold increase in anthocyanin content and thus brought petals to the level of pigmentation normally found in the deep sepals of this line. Anthocyanin production in petals thus appears to operate under a quantitative control mechanism which depends on the continued association of these parts with the rest of the plant. Once petals are removed from such control they may produce higher amounts of pigment. Although both the upper limit of anthocyanin synthesis and the relative pigment content of sepals and petals seems to be governed by the same gene, it follows from our observations that only the former action of this gene is the governing influence residing in the petal cells, while the latter action is exerted somewhere else in the plant. Culturing has the effect of removing this second influence.

The invariable appearance of the 3′,4′-dihydroxy pattern on the B ring of flavonoids found in cultured petals is open to several interpretations two of which will be considered here. It is conceivable that both the higher pigment yield and the presence of additional pigments in cultured petals may be ascribed to the effects of injury incurred in the process of their removal. Indeed, Bopp has recently shown (2) that deliberate injury may induce the appearance of anthocyanins in normally unpigmented structures. The kind of anthocyanin produced was usually the one found in some other portion of the plant. In balsam cyanidin is normally present in the stems of all three and the sepals of two of the three genotypes here investigated. This might lead to the conclusion that, as in Bopp's findings, the appearance of cyanidin in cultured petals is a consequence of injury. Yet when petals are injured in situ no additional anthocyanin is developed and no change in the kind normally present is noted by visual observation.

Developing sepals bear closer resemblance to vegetative leaves than do petals. Sepals remain green and firm longer, expand later and are exposed to the atmosphere and direct sunlight much sooner than petals. In all the genotypes studied, sepals produce the 3′,4′-dihydroxy flavonol quercetin; in two genotypes they produce the corresponding anthocyanin, cyanidin, which is the same pigment found in vegetative structures. Cyanidin is the most widely distributed and also frequently considered to be the primitive and, perhaps, biosynthetically key anthocyanidin (eg. 17). Thus, sepals present, in general, a less specialized morphological and biochemical appearance than petals. Yet, when petals are placed in culture they also produce cyanidin. We suggest that this phenomenon may be ascribed to the partial inhibition of biosynthetic steps which normally convert one of the precursors of this pigment to the other anthocyanins. It is presumably easier to interfere with a highly specialized process than with a more primitive one. Accordingly the interpretation we give to the appearance of cyanidin and quercetin in cultured petals is that the more specialized steps, production of 4′- mono- and 3′,4′,5′-trihydroxy derivatives have been disturbed. Currently available data suggests that the 3′,4′-dihydroxy pattern is introduced

at the level of phenylpropanoid precursors to the B ring. From the order of appearance of pigments in the grape, Ribereau-Gayon (13) considers that cyanidin serves as a precursor of delphinidin and malvidin. Since both anthocyanins and flavonols are similarly affected by culture conditions this would indicate that the hydroxylation pattern is modified in a compound which serves as a precursor to both.

With respect to the developmental physiology of petals as it relates to anthocyanin synthesis, we are still far from a coherent picture. The stimulatory effects of kinetin confirm the view (14, 15) that anthocyanin synthesis is dependent on purine and pyrimidine metabolism. It is gratifying that we were able to enhance rather than restrict pigment production, thus escaping the general criticism leveled against conclusions based on inhibitor studies alone. Nevertheless, the involvement of nitrogen bases in flavonoid synthesis still cannot be tied to a definite mechanism. Thimann and Radner (16) showed that in *Spirodela pyrimidine* effects are mediated by riboflavine metabolism. Strauss, in a recent paper (14) noted the stimulation of anthocyanin synthesis in corn endosperm by nucleotides but could not demonstrate the mediation of riboflavine. Growing petals are neither wholly autotrophic like Spirodela, nor are their cells specialized for the storage of massive amounts of reserves as in corn endosperm. Anthocyanin synthesis in these three experimental objects is superimposed on three markedly different metabolic patterns, and may be most sensitive to experimental intervention at different points along a common course.

Our ethionine results suggest that kinetin may effect expansion and pigmentation by influencing protein synthesis. The close correlation of morphological expansion with biochemical events and the disturbance of this relationship by TIBA may mean that both are triggered by a hormonal mechanism. Anthocyanin production and the disappearance of chlorophyll may be indications of a major shift in metabolism. It is tempting to draw a parallel between this possible shift and the observations of Hotta et al (10) who found that renewed protein synthesis and a change in the kind of ribose nucleic acid formed was closely linked with the onset of a specific morphogenetic stage. The advantage of using a simple pigment as an indicator for a specific morphogenetic step lies in the hope that eventually this may greatly aid in the metabolic characterization of a developmental event.

Summary

I. Immature petals of *Impatiens balsamina* L. continue to expand when they are excised and floated on a simple nutrient medium. Anthocyanin synthesis by such petal tissue was studied.

II. In two intensely colored genotypes (llhhP^{r}P^r & LLhhP^{r}P^r) pigment production per petal was nearly as high in culture as in intact flowers. Anthocyanin content was three times the normal on a fresh weight basis. In a lightly colored genotype (llhhP^{r}P^r) cultured petals produced more than eight times the normal amount of anthocyanin. Pigment content per unit weight was 23 times that of undisturbed petals.

III. Cyanidin appeared as an added anthocyanidin in the petals of all three investigated genotypes when these were placed in culture. Quercetin was found as an added flavonol in two of the genotypes. Peonidin appeared as an added anthocyanidin in one genotype. As a contrast no 3′,4′-dihydroxy flavonoids have been identified in intact petals although they are generally present in the sepals. This may be interpreted as evidence for a return to a more primitive 3′,4′-hydroxy pattern under the influence of culture conditions.

IV. Evidence was found for the sequential appearance of the two major pelargonidin glycosides in two of the genotypes. The order of appearance was: 3-glycoside followed by the 3,5-diglycoside.

V. Morphogenetic steps leading to the expansion and coloration of petals in culture are described. Injury was found to accelerate both expansion and anthocyanin production. Kinetin at 0.5 mg/l had similar effects. When expansion was limited by concentrations of mannitol greater than 0.1 M anthocyanin synthesis was also curtailed. Triiodobenzoic acid at 5.0 mg/l introduced a time gap between expansion and anthocyanin synthesis. Ethionine at 6×10^{-5} M/l reduced both expansion and anthocyanin content. Ethionine completely erased any stimulation by kinetin.

VI. These results are interpreted to point to a close relationship between specific morphogenetic events, such as cellular expansion and the loss of chlorophyll, and the appearance of anthocyanins in petals. Anthocyanins are seen as useful indicators of metabolic conditions accompanying differentiation.

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PYRIMIDINE METABOLISM IN GERMINATING SEEDLINGS[1, 2, 3]

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In the course of an inquiry into the biochemical events associated with germination and dormancy in seeds an attempt was made to assess the turnover or new production of ribonucleic acid and deoxyribonucleic acid in germinating rape seeds. The use of uracil and thymine, both labelled in the 2-position with C^{14} has disclosed that at least in the early stages of germination, these two pyrimidines, the first of which is found in ribonucleic acid and the second in deoxyribonucleic acid, are only sparingly incorporated into polynucleotides although the bases are rapidly decomposed by a sequence of events suggestive of that found in higher animals.

MATERIALS AND METHODS

CHEMICALS USED: Uracil and thymine were obtained from the Nutritional Biochemical Corp., dihydrouracil (DHU)[4] and dihydrothymine (DHT) from the California Corp. for Biochemical Research and were used without further purification. β-Ureidopropionic acid (BUP) and β-ureidoisobutyric acid (BUIB) were made from DHU and DHT, respectively, by alkaline hydrolysis (10). (mp of BUP found, 170–171° C; reported 169–170° C; mp of BUIB, found, 114–116° C, reported, 116–119° C (10). Uracil-2-C^{14} (0.0263 mc/mg) and thymine-2-C^{14} (0.011 mc/mg) were purchased from the New England Nuclear Corp.

GERMINATION OF SEED: The rape seeds (*Brassica napus* L., var. Dwarf Essex) were obtained from the Crawfordsville Seed Co., Crawfordsville, Ind. Germination was carried out in small cylindrical dishes (1 cm high × 1 cm dia.). A disc of Whatman No. 1 filter paper was inserted into each dish. Fifty μliters of a 0.01 % solution of either uracil-2-C^{14} or thymine-2-C^{14} were pipetted into each dish. Five

[1] Received May 9, 1960.
[2] This work was supported in part as a collaborative contributing project under North Central Regional Research Project NCM–23, entitled "Improving the Marketing of Seed Through Improved Testing and Handling Procedures".
[3] Journal Paper No. 1611 of the Purdue University Agricultural Experiment Station.

[4] The following abbreviations are used throughout: BUIP, β-ureidoisobutyric acid; BUP, β-ureidopropionic acid; DHT, dihydrothymine; DHU, dihydrouracil.