Formation of Anthocyanin in Leaves of Kalanchoe Blossfeldiana—a Photoperiodic Response [1, 2]

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During the course of experiments on the metabolism of Kalanchoe in short and long days (5, 8,11) it was noticed that the leaves of plants induced to flower became red, especially at the apical end and on the underside. Nonflowering plants, i.e. those kept in long days, showed only slight and delayed reddening along the leaf margins. However, it was observed that when the leaves of plants on long day were boiled in HCl, a deep red color developed in the solution. This indicates presence of a leuco-anthocyanidin (1) and suggests that the photoperiod might be causing conversion of this compound to the red anthocyanin. Since the flowers whose formation is induced by exposure to short days are also bright red, it appeared further that the photoperiodic stimulus may exert a systemic effect which would lead to the formation of anthocyanin simultaneously in several parts of the plant. A detailed study was therefore made to determine: A, if the anthocyanins in flower and leaf were the same, or related, B, if the reaction is indeed a true response to photoperiod, C, the nature of the metabolic change involved.

Although anthocyanins very commonly appear in the leaves of young growing shoots, especially of woody plants, and also in mature fully grown leaves (autumn colors) their appearance in leaves in direct association with flowering seems to have been less commonly noticed. It is not uncommon, however, for plants with red or blue flowers to develop anthocyanin in the midribs or petioles of the leaves, while those with white flowers do not.

Materials

A dwarf race of *Kalanchoe blossfeldiana* var. Tom Thumb was used. These plants have been maintained on long days in our greenhouse for 9 years in the vegetative condition. [On very rare occasions a single flower has appeared on one plant of a large lot, but it has never been followed by another. Such rare and sporadic flowering on long days has been noted before (12)]. The plants grow readily from cuttings and all our material is clonal; individuals about ten months old were used for the present experiments. They were grown for 2 months in light chambers under long day (LD, 16 hr) or short day (SD, 8 hr) at 25°. In some cases the short day was supplemented with a 1-hour light break in the middle of the night. White light (fluorescent plus incandescent) of about 18,000 lux was used throughout.

The reference sample of cyanin was kindly supplied by Sir Robert Robinson; it had originally been prepared by Willstätter. Cyanidin was prepared from it by hydrolysis with HCl. The reference sample of chrysanthemin was obtained (as picrate) from Dr. Seymour Fogel; it had been prepared in Prof. Stadler's laboratory from corn leaves. The sample of pelargonin was prepared by extracting flowers of typical scarlet *Pelargonium zonale* plants.

Identification of the Pigments. The flower petals (about 2 g) and the colored apical parts of the short-day leaves (about 10 g), were ground and extracted overnight with 200 ml of cold 0.1 N HCl. The pigments were precipitated with lead acetate, the precipitate decomposed with HCl, and the anthocyanins taken up in methanol. After evaporation the pigment was transferred as a band to Whatman No. 3 paper and developed in butanol: 2 N HCl. With extracts of both leaves and flowers, the large bulk of the color moved on the chromatogram in a single band, with a very small amount of a minor pigment which in the flowers moved ahead, in the leaves was well behind. Some of the flower preparations showed a second minor pigment (table II). However, attention was directed mainly to the major pigments.

Preliminary experiments showed that the major pigments of flower and leaf were not identical, that of the leaf behaving like chrysanthemin and that of the flower like cyanin. When the eluted bands were rechromatographed in a series of solvents, side by side with reference samples, the R_f values shown in table I were obtained. Table I also shows the positions of maximum absorption in the visible. Both pigments show a strong shift of the peak on treatment with $AlCl_3$, which, as Geissman et al. have shown (4), indicates the ortho-dihydroxy structure.

After hydrolysis with 1 N HCl for 1 hour in boiling water in an atmosphere of nitrogen, the liberated sugars were chromatographed. Each preparation

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gave only one spot due to a sugar. These spots were eluted and reacted with resorcinol (cf 7). The chromatography of the leaf pigment hydrolysate was carried out under very slightly different conditions from that of the flower, but in each case the reference sugars were run side by side at the same time. Table II summarizes the results, and leaves no reasonable doubt that the anthocyanins of both leaf and flower contain only glucose. This would be in line with the above deduction, since both chrysanthemin and cyanin are cyanidin glucosides.

Experiments with the aglycones, the anthocya-

Table I

Chromatographic & Optical Properties of Anthocyanins

	BuOH: HOAc:H_2O (4: 1: 5)	BuOH: 2 N HCl (4: 6)	1 % HCl	BuOH: 27 % HOAc (1: 1)	HOAc: HCl:H_2O (30: 3: 10)	Wavelength of max. absorption in 0.01 % HCl in methanol	
						Direct	+ $AlCl_3$
	R_f	R_f	R_f	R_f	R_f	mμ	mμ
Kalanchoe (SD) leaf	0.39	0.21	0.05	0.54	0.26	525	565
Chrysanthemin chloride	0.38	0.21	0.07[7]	...	0.26[7]	525[6]	...
Kalanchoe (SD) flower	0.28	0.08	0.13	0.43	0.43	524	560
Cyanin chloride	0.28	0.08	0.13	0.45	0.43	523	560

Table II

Identification of the Sugar in the Two Major Anthocyanins of Kalanchoe blossfeldiana.

	BuOH:HOAc:H_2O (4: 1: 5) R_f	EtOAc:Pyridine:H_2O (2: 1: 2) R_f	EtOAc:HOAc:H_2O (3: 1: 3) R_f	Absorption maxima of resorcinol complexes	
				Peak mμ	Inflection mμ
Sugar from SD leaf pigment	0.28	0.23	0.05	494	430
Glucose	0.28	0.23	0.05		
Galactose	0.25	0.19	0.05		
Sugar from SD flower pigment	0.25	0.22	0.05	494	430
Glucose	0.25	0.22	0.05	494	430
Galactose	0.22	0.19	0.05	432, 502	556
Rhamnose	0.43	0.38	0.22	424, 490	548

Table III

Chromatographic & Optical Properties of Anthocyanidins Liberated on Hydrolysis

	HOAc:HCl:H_2O (30: 3: 10)	HCOOH:HCl:H_2O (5: 2: 3)	Wavelength of max. absorption in 0.01 % HCl in methanol	
			Direct	+ $AlCl_3$
	R_f	R_f	mμ	mμ
Kalanchoe (SD) leaf anthocyanidin	0.49	0.28	535	565
Kalanchoe (SD) flower anthocyanidin	0.49	0.28	535	570
Kalanchoe (LD) leuco-compound	0.49	0.28	535	565
Cyanidin	0.49	0.28	536	560
Delphinidin (4, 7)	0.32	0.13	546	569
Petunidin (9)	0.44	0.26	543	566
Pelargonidin (4, 7)	0.68	0.33	520	520

nidins, from both leaves and flowers, are summarized in table III, which includes also data for the anthocyanidin formed on boiling the extract from the long-day leaves with acid (see below). It is evident that none of the pigments is identical with delphinidin, pelargonidin, or petunidin, and less complete figures in the literature for hirsutidin and peonidin exclude identity with these pigments also. The accumulated data, together with the fact that all three Kalanchoe pigments show the $AlCl_3$ shift, confirm the conclusion that the aglucone of all three is cyanidin.

That the flower pigment is cyanin and the leaf pigment chrysanthemin agrees with the observed colors, both of which were bluish pink in the visible; in the ultraviolet the flower pigment and cyanin were both bright orange, turning red in NH_3 vapor, while the leaf pigment and chrysanthemin were pink.

For the leuco-anthocyanidin of the leaves of plants grown in long days, about five grams of the leaves were extracted with methanol, and the crude leuco-compound precipitated with lead acetate; the precipitate was decomposed with a few drops of cold 2 N HCl and redissolved in methanol. The lead acetate precipitation procedure was repeated and the lead complex decomposed with 10 ml of cold 2 N HCl. The solution was decanted off from the lead chloride after centrifugation and then heated on a boiling water bath for 20 minutes (1). The deep red anthocyanidin thus obtained was readily extractable with isoamyl alcohol. The aqueous layer, after freeing from acid by washing three times with equal volumes of a 10 % solution of di-*n*-octylmethylamine in chloroform (10) and once with pure chloroform, was evaporated to a small volume. When this was spotted on paper and chromatographed, only a very faint spot of glucose was found. The amount of red pigment extracted into the isoamyl alcohol was far greater than the amount which would have been extracted after hydrolysis of enough anthocyanin to yield a comparatively dark glucose spot. It is therefore believed that the leuco-compound exists as leuco-anthocyanidin, the trace of glucose coming only from glucosidic impurities.

The isoamyl alcohol extract of anthocyanidin was applied to a sheet of Whatman No. 3 filter paper and purified by chromatographing once in Forestal solvent. The band was cut out, eluted with methanol containing 0.5 % HCl and identified by its R_f values and maximum absorption in the visible spectrum. The results in table III show that the anthocyanidin is cyanidin. The leuco-compound in the long-day leaves is therefore leuco-cyanidin.

Table IV presents R_f values of the minor pigment of the short-day leaves and the two minor pigments of the flowers. In the absence of a more detailed study the minor pigment is tentatively considered to be cyanin and the minor flower pigments pelargonin and chrysanthemin; this agrees in each case with their visible colors.

Table IV

R_f Values of the Minor Pigments of Kalanchoe Leaves & Flowers

The values underlined are the presumed identities.

	BuOH : 2 N HCl (4 : 6)			BuOH : HOAc : H_2O (4 : 1 : 5)		
Short-day leaves (red spot)		0.08			0.26	
Flowers (orange-red and red spots resp.)	0.14		0.21	0.31		0.38
Cyanin		0.08			0.25	
Pelargonin	0.14			0.30		
Mecocyanin		0.18			0.33	
Chrysanthemin			0.21			0.38

Quantitative Determination of the Leaf Pigments. If the anthocyanin that appears on exposure to short days is formed from the leuco-anthocyanidin present in leaves kept on long days, it should be possible to show a quantitative relationship between the two. For this purpose the amount of leuco-pigment must be determined, and this has to be done by converting it stoichiometrically to anthocyanidin. The normal procedure of heating with HCl (1), as used above, gives only a poor yield (cf 3) and much of the material is converted to a red-brown polymer. For this reason, the amount of leuco-anthocyanidin was determined by measuring the optical density at 500 mμ of the red compound formed with vanillin and HCl (2). Leaves (1 g) were ground in a mortar with methanol, made up to 25 ml in volumetric flask and allowed to stand at 4° for 3 days. The extract was filtered. To 1 ml of the extract, 1 ml of a saturated solution of vanillin in ethanol (30 %) and 1 ml of 3 N HCl were added, and the optical density measured at 500 mμ in the spectrophotometer.

For determination of visible pigment, the amount of which was much less than that of leuco-anthocyanidin, 1 g of leaves were ground with 0.1 N HCl and made up to 10 ml. The solution was centrifuged to remove fine suspended particles before measuring the optical density. Leaves of plants on long days, short days, and short days with interrupted night were used. The results are shown in table V.

Just as in the photoperiodic induction of flowering, short day with interrupted night produces the same physiological effect on the anthocyanin pigment as does long day. Under either condition, young leaves synthesize a large amount of leuco-compound and the synthetic activity drops off with age. When induced by exposure to short days with 16-hour nights, however, there is a drastic decrease of leuco-anthocyanidin in the young leaves, and a smaller decrease in the older leaves. Simultaneously the visible anthocyanin appears. If we set the

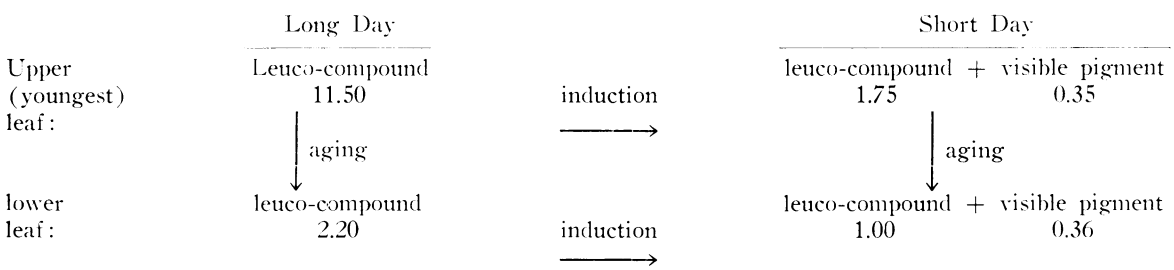
Table V

Quantitative Determination of Leuco-anthocyanidin & Anthocyanin in Kalanchoe Leaves, after 44 Days in Light Room under Three Photoperiodic Conditions

Leaf sample		Leuco-anthocyanidin OD**/g of leaf	Relative amount***	Anthocyanin: OD†/g of leaf	Relative amount††
SD	upper*	0.193	1.75	0.038	0.35
	middle	0.151	1.35	0.038	0.35
	lower	0.110	1.00	0.040	0.36
SD interrupted night	upper	1.215	11.05	0.003	0.027
	middle	0.408	3.70	0.003	0.027
	lower	0.262	2.40	0.004	0.036
LD	upper	1.265	11.50	0.007	0.064
	middle	0.567	5.15	0.008	0.073
	lower	0.245	2.20	0.003	0.027

* Upper leaves were shorter than 1.5 cm, middle leaves were 2.3 to 2.7 cm long, and lower leaves 3.5 to 4.0 cm long.
** Measured as OD at 500 mμ of the red compound formed with vanillin and HCl.
*** Since the oldest SD leaves contain least leuco-compound their content has been taken as 1 for convenience.
† Unit weight of leaf was extracted with 2/5 the volume of liquid used for extraction in **. For comparison, the OD given has been multiplied by 2/5.
†† Ratio relative to the same value, 0.110, which was taken as unity for the leuco-anthocyanidin.

amount of leuco-anthocyanidin in the oldest short-day leaves as equal to 1.0, then the relative values are as follows:

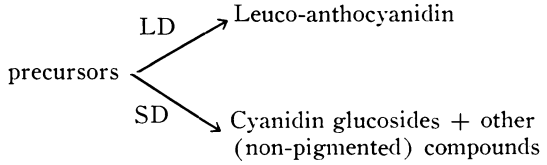


Unfortunately the optical density of the vanillin-phloroglucinol complex used for measuring the leuco-pigment is not necessarily the same as that of the visible anthocyanin. Hence the concentrations of the two substances cannot be compared exactly. Nevertheless it is quite clear that photoperiodic induction causes a far greater decrease in the amount of leuco-anthocyanidin than increase in anthocyanin. That there are other changes too is shown by the observation that when the extract from long-day leaves is boiled with HCl the polymeric precipitate resulting is red-brown, while that from short-day leaves is nearly black. The effect of induction, therefore, is not to cause a simple conversion of leuco-anthocyanidin to visible pigment; the change in metabolism is more profound.

Discussion

The aglycones of the various pigments show a very satisfactory unity. The major pigment of the leaves is chrysanthemin (i.e. cyanidin-3-glucoside) and that of the flowers cyanin (i.e. cyanidin-3-5 diglucoside); in addition the leaves contain a little cyanin and the flowers a little chrysanthemin. The leuco-pigment in the long-day leaves is leuco-anthocyanidin. The only exception to this unity is the apparent occurrence of a little pelargonin in the flowers, but the identification of this pigment is only tentative. The sugar is glucose. Evidently the metabolism of Kalanchoe tends strongly to the formation of cyanidin glucosides.

Notwithstanding the close chemical relationship between the pigments, the data show clearly that leuco-cyanidin is not simply converted to cyanidin glucosides when flowering is induced. The relatively small amount of cyanidin monoglucoside which appears in the leaves, as well as the diglucoside which appears in the flowers, may or may not be directly formed from the leuco-pigment, but most of this substance evidently undergoes some other fate. It is suggested that photoperiodic induction causes, along with the morphological change from leaf to flower, a change of metabolism:



Interrupted long nights behave like long days in preventing the induction both of flowering and of anthocyanin formation.

Summary

The development of significant amounts of anthocyanin in the leaves of *Kalanchoe blossfeldiana* is a photoperiodic response and occurs only under conditions which also lead to flowering, i.e. short days. Exposure to long day, or to short day with interrupted nights, prevents both flowering and formation of any more than a trace of anthocyanin.

The major anthocyanin of the flower petals was identified as cyanin (cyanidin-3,5-diglucoside), with minor amounts both of chrysanthemin (cyanidin-3-monoglucoside) and probably of pelargonin (pelargonidin-3,5-diglucoside). In the leaves of the flowering plants the major pigment is chrysanthemin with minor amounts of cyanin. The pigments are thus dominated by cyanidin derivatives.

A leuco pigment identified as leuco-cyanidin is abundant in leaves of the vegetative plants, and the amount present decreases greatly after exposure to short days. However, such photoperiodic induction causes a greater decrease in the amount of leuco-cyanidin than could be accounted for by the increase in anthocyanin. It is concluded that the effect of short day induction is not simply to convert leuco-cyanidin to anthocyanin, but rather to change the metabolic route of a common precursor.

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