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PHOTOPERIODIC CONTROL OF PHENOLIC METABOLISM IN *KALANCHOE BLOSSFELDIANA*

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Key Word Index—*Kalanchoe blossfeldiana*; Crassulaceae; photoperiodism; phenolic compounds; tannins; phenolic metabolism; CAM.

Abstract—When grown in conditions of long day length, the leaves of *Kalanchoe blossfeldiana* contain high levels of soluble phenolic compounds, mainly present as tannins. A decreasing concentration gradient is observed in the leaves from the apex to the base. When transferred to short day conditions, the ability of leaves of the same physiological age to accumulate phenolics decreases with time. The very low phenolic content after 25 short days indicates pronounced changes in the metabolism of the plant induced by new photoperiodic conditions. Moreover, during development in short days the amount of tannins per leaf reaches a maximum then decreases suggesting an overpolymerization or even a degradation of the substances. A similar lag time is required for the depressing effect of short days on phenolics and for their stimulating effect on CAM.

INTRODUCTION

The level of phenolic compounds in plants depends greatly on external conditions. The metabolism of phenolics can be qualitatively and quantitatively modified by different kinds of stress situations [1–3], by temperature [4–6] and especially by light conditions. Effects of light quality [7, 8] or intensity [9, 10] have been reported on the biosynthesis of these compounds, but few studies deal with the effects produced by changes in photoperiod [11].

In the short-day (SD) plant, *Kalanchoe blossfeldiana*, photoperiod has been shown to control responses both at morphogenetic (flower induction) [12, 13] and biochemical (Crassulacean acid metabolism: CAM) [14, 15] levels. Earlier work [16] established that changes in photoperiod result in modifications in the pattern of phenolic compounds in this plant. The present paper provides details on the nature of the main polyphenols in *Kalanchoe blossfeldiana* leaves and on their variations according to photoperiodic conditions.

RESULTS

Phenolic pattern of Kalanchoe blossfeldiana *leaves in long days*

Contents in different classes of phenolic compounds. The data in Table 1 show the effect of leaf age on phenolic content. It can be seen that the concentration of total phenolics in young leaves of *Kalanchoe blossfeldiana* is much higher than in other plants, either arborescent (*Quercus*) or herbaceous (*Ricinus*, *Lycopersicum*), which were examined using the same procedure. The tannins form the main phenolic fraction in *Kalanchoe blossfeldiana* and represent 70–80% of the total phenolic pool, irrespective of leaf age. This feature is quite unusual in a herbaceous plant [17]. It should be noted that the concentration of polyphenols in the different fractions decreases from younger to older leaves.

The constitution of the 'tannin' and 'non-tannin' fractions. 'Tannin' fraction. Tannins were isolated through a precipitation step with gelatin (see Experimental), and the pellet subjected to acid and alkaline hydrolyses, either directly or after dissociation of the tannin protein complex by acetone [18]. After thin layer chromatography of the hydrolysates, no gallic acid could be detected, therefore the tannins seem of the condensed type.

However, after 30 minutes heating in 2 N HCl at 100°, no red colour was produced which would indicate the formation of anthocyanin derivatives; only a light brown insoluble residue, probably a phlobaphene was formed and hence *Kalanchoe* tannins are presumably mostly composed of linked flavan-3-ol units.

Table 1. Phenolic contents of *Kalanchoe blossfeldiana* leaves (grown under long days) compared to other plants*

Kalanchoe blossfeldiana Leaf pair (from the apex)	Gallic acid equivalents % dry weight TP	T	NT
1	22.3	16.7	5.6
2	17.4	12.6	4.8
3	7.7	5.7	2.0
4	6.2	4.7	1.5
5	5.3	4.4	0.9
Other plants [40] (leaves)			
Quercus pedunculata Ehrh.	9.7	4.7	5
Ricinus communis L.	3.6	0	3.6
Lycopersicum esculentum Mill.	0.2	0	0.2

* Data for total phenolics (TP), tannins (T) and non-tannin phenolic compounds (NT). For the characteristics of the leaf material, see Fig. 1.

'Non-tannin' fraction. The supernatant obtained after selective precipitation of tannins contains simple phenolics and flavonoids. In the leaves of *Kalanchoe blossfeldiana*, the former are mainly free gallic acid on the one hand, and *p*-coumaric and *p*-hydroxybenzoic acids, identified only after alkaline and acid hydrolysis, on the other. The flavonoid fraction is composed of flavan-3-ols: chromatographic separation shows 7 spots (some of them probably corresponding to isomers) presenting the characteristics of gallocatechins.

Neyland *et al.* [16] have reported the presence of flavan-3,4-diols in *Kalanchoe blossfeldiana*; we could not confirm this result and it is possible that, if present, these substances are in very small amounts as compared to flavan-3-ol derivatives.

Quantitative variations of phenolics after a change in photoperiod

Two different experimental approaches were designed in order to study the effect of a change in photoperiodic conditions on leaf polyphenols:

(a) analysis of leaves of the same physiological age (Fig. 1A), sampled according to strict morphological criteria after an increasing number of short days; in this case the results show the effects of the change in photoperiod, and the fact that the leaves, sampled when they reach the 2nd node, have previously received a decreasing number of long days;

(b) analysis of leaves which at time 0 had the same physiological age (1st pair from the apex) and hence have received the same number of long days, and which are sampled at different stages of their development under long days (Fig. 1B) or short days (Fig. 1C).

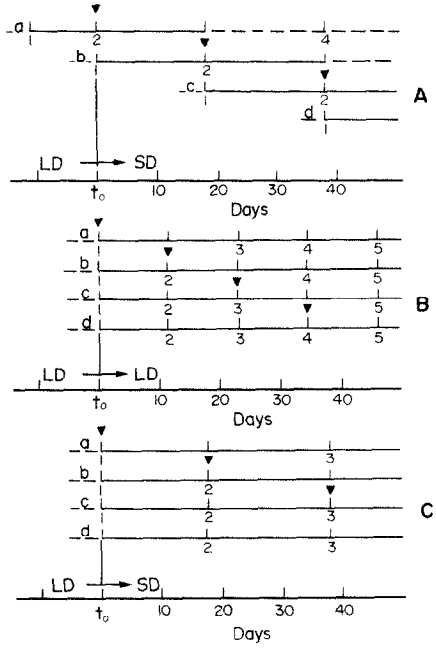


Fig. 1. Plant material: sampling method for the study of the effect of an increasing number of short days (SD) or long days (LD) on the content of phenols in *Kalanchoe* leaves. A—Leaves of identical physiological age (2nd pair from the apex); B—leaves of different ages sampled as a function of time in LD on different plants (at time 0 the first pair from the apex is sampled; C—leaves of different ages sampled as a function of time after transfer of the plants from LD to SD (at time 0 the first pair from the apex is sampled). The first leaf pair refers to the large primordia of *ca* 5 mm length; the 2nd leaf pair refers to expanded leaves (about 20 mm length).
▼ Leaf sampled from different plants (a, b, c, d . . .); 1 . . . 2 . . . leaf range; ⌊—⌋—plastochrone.

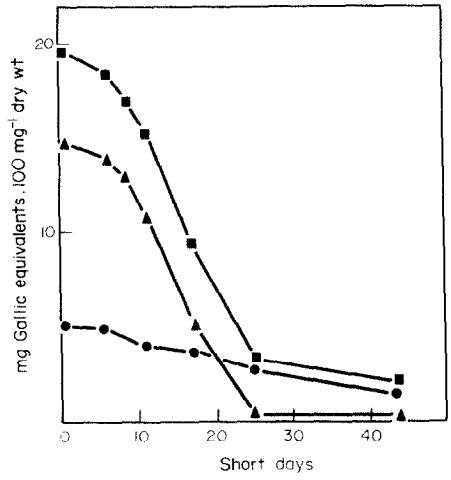


Fig. 2. Variation in total phenolics, ■—■; tannins, ▲—▲; non-tannins, ●—●; in leaves of identical physiological age (2nd pair from the apex) after the transfer of *Kalanchoe blossfeldiana* from LD to SD.

The analysis of leaves of the 2nd pair (from the apex) sampled after an increasing number of short days shows a decrease in the total phenolics (Fig. 2). After 44 SD, leaves of the 2nd pair, which were almost entirely grown under short days (see Fig. 1A), have a phenolic content *ca* 8 times lower than leaves under long days. The tannin fraction is particularly affected by short days and drops to *ca* 4% of its initial content in long days; in contrast, the non-tannin fraction decreases by *ca* 50%. Hence, changing from LD to SD results in a modification of the ability of synthesis, degradation and (or) accumulation of phenolics by the leaves.

The analysis of leaves at different stages of their development under short days as compared to leaves growing under long days shows that under LD (Fig. 3A)

Table 2. Variations of flavonoids and simple phenolic compounds in leaves of *Kalanchoe blossfeldiana* during growth under short days (at time 0 the first pair from the apex is sampled)

No. of SD	Gallic acid equivalents (mg leaf) Flavonoids	Simple phenolics
0	0.016	0.01
6	0.08	0.04
27	0.10	0.13
48	0.08	0.97

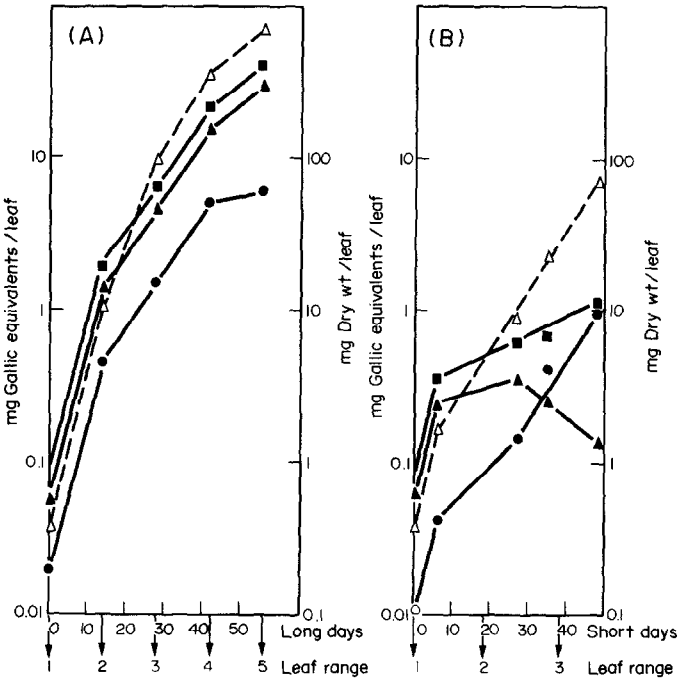


Fig. 3. Semi-logarithmic plots of the changes in dry weight, △—△; total phenolics, ■—■; tannins, ▲—▲; non-tannins, ●—●; in *Kalanchoe blossfeldiana* leaves. A—Under LD conditions; B—after the transfer from LD to SD as a function of increasing number or SD (at time 0 the first pair of leaves from the apex is sampled).

the variation per leaf of the different phenolic compounds parallels the variation of dry weight during about 2 plastochrones (the plastochrone is defined as the time interval between the formation of two successive leaves), and then slows down. It can also be seen that after transfer to SD (Fig. 3B), the amount of phenolics rises in a similar way as the dry weight during the first 6 SD; then the rate of accumulation decreases concomitantly with a decrease in leaf growth and a lengthening of the plastochrone. After 20 SD, the amount of the tannin fraction per leaf shows a net decrease. Data in Table 2 show that the net accumulation of flavonoids per leaf stops after 6 SD whereas the 'simple phenols' continue to accumulate through the experiment. Thus, tannins and flavonoids (hence more generally the C_{15}-substances) are more affected by short days than simple phenolics.

DISCUSSION

A promoting effect of light on polyphenol synthesis in plants has been reported by a number of authors. In *Kalanchoe blossfeldiana*, phenolics accumulate very substantially in young leaves under long days: in leaves of the 1st pair from the apex, the amount of soluble phenols (expressed on gallic acid basis) appears to be *ca* 20% of the tissue's dry weight. In contrast, culture under short days depresses polyphenolic content, and the very large amplitude of variation in total phenolics after a transfer from long to short days may be emphasized. These results on total soluble phenolics agree with those reported by different authors for particular phenols in plants grown under varied photoperiodic conditions [19–23].

Under long days, a decrease can be observed in the concentration of phenols according to the age of the leaves. Similar variations have been reported for some phenolic compounds in tobacco (which is also a day-length controlled plant [24, 25]) and in coffee [26]; an opposite effect was found in sunflower [27]. Moreover, variations in phenylalanine ammonia-lyase activity were also shown in different plants [28, 29].

In a leaf growing under long days (Fig. 3A), the accumulation of phenolic compounds proceeds at a constant rate until the leaf reaches range 3 (40th LD); later it slows down. In contrast, after transfer from long to short days, the accumulation rate drops from the 6th SD onwards (Fig. 3B): this result clearly demonstrates a definite effect of photoperiod on phenolic metabolism, as distinct from ageing.

The results reported in Fig. 3B also show that the amount of soluble tannins per leaf decreases after 27 short days. This decrease is a remarkable feature: it can hardly be ascribed to a transfer to other organs on account of the size and the reactivity of these molecules, and also because no correlated increase is found in the other leaves (unpublished results).

In order to explain the observed decrease in tannins, the hypothesis of further polymerization resulting in the formation of insoluble polyphenols [30, 31] could be considered (as it has been suggested in the case of fruit ripening); but as proposed in earlier publications [32–34], tannin degradation cannot be ruled out. In *Kalanchoe blossfeldiana*, C_{15}-monomers arising from the breakdown of polymers, could be degraded and enter the general metabolism [36, 37] since there is no increase in free flavonoids in the soluble phenolic fraction

(Table 2). These different hypotheses require further research.

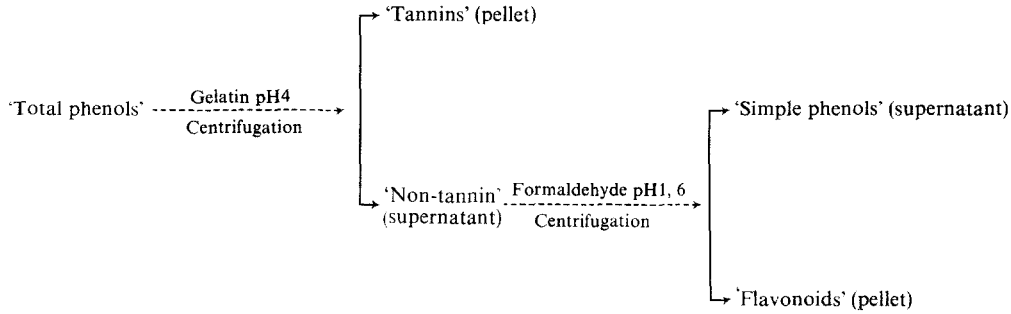
After transfer from long days to short days, phenolic accumulation in *Kalanchoe blossfeldiana* leaves proceeds during the first 6 or 7 short days at a rate similar to that observed in long days. It is noteworthy that this lag time, required for achieving a different level of phenolic metabolism, is of the same order than the time required for the photoperiodic induction of CAM [38]. Hence, the same stimulus (change of photoperiod) acts simultaneously on the metabolism of malate and the metabolism of phenolic compounds. Time relationships between these two metabolic pathways under photoperiodic control are under current investigation.

EXPERIMENTAL

Plant material. Plants of *Kalanchoe blossfeldiana* Tom Thumb were grown in 'Le Phytotron' (Gif sur Yvette) under conditions described previously [14]. After 8 weeks of long days (16 hr) the plants were kept either under the same conditions or transferred to short days (9 hr). Leaves sampled at 15.00 hr were frozen in liquid N_2, lyophilized, ground to powder and stored at $-20°$ in sealed flasks.

Analysis of phenolic compounds. Extraction. 50 mg lyophilized powder were extracted by 80% ethanol (5 × 50 ml) as previously described [39]. The extract was dried, lipid soluble pigments removed by petrol, and the residue dissolved in H_2O.

Fractionation. Selective precipitations, conducted as described by Marigo [40], were used to fractionate 'total phenols' (TP) into 'tannins' (T) and 'non-tannin' (NT) fractions composed of flavonoids (F) and simple phenolic compounds (SP).



Identification. TLC was performed on Merck cellulose plates in the following solvents: A, $CHCl_3$–HOAc–H_2O (2:1:1, lower phase); B, 1% HOAc; C, *n*-BuOH–HOAc–H_2O (4:1:5, upper phase); D, 15% HOAc; E, 5% MeOH.

Simple phenolic compounds. These phenols were obtained from fraction SP by extraction with Et_2O at pH 2 before and after alkaline or acid hydrolysis, followed by 2-D chromatography in solvents A and B. Identification was obtained by spraying with *p*-nitraniline reagent [39] and comparison with standards.

Flavonoids. These compounds were identified in fraction NT by direct chromatography: (a) in solvents C (1st dimension) and D (2nd dimension); (b) in solvent E. The compounds were detected with: *p*-nitraniline reagent [39]; $AlCl_3$ [41]; vanillin chloride [42]; and *p*-toluene sulfonic acid (affording distinction between flavan-3-ol and flavan-3,4-diols [43].

Tannins. The 'tannin fraction' was previously hydrolysed by NaOH or HCl and the free compounds extracted by Et_2O at pH 2. Identification of the compounds released by hydrolyses was conducted as in the case of simple phenols and flavonoids.

Quantitative analysis. The Swain and Hillis method for Folin–Denis reacting phenols [44] as modified by Marigo [40] was used for measuring the level of 'total phenols' and the level of the different phenolic fractions. The 'tannin fraction' was evaluated by difference using the values obtained for 'total phenols' and 'non-tannin fraction'. In the same way, the 'flavonoid fraction' corresponds to the difference between 'non-tannin fraction' and 'simple phenol fraction'. Results are expressed as 'gallic acid equivalents'.

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