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Annual Variation in Sterol Levels in Leaves of *Taraxacum officinale* Weber[1]

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ABSTRACT

Sterol levels in dandelion (*Taraxacum officinale* Weber) leaves were monitored over a period of 19 months. Sitosterol was the most abundant free sterol, followed by stigmasterol, then campesterol. Cholesterol could not be detected. With the exception of stigmasterol and campesterol, esters were present in greater quantities than were free forms, with 4,4-dimethyl sterol esters being the most abundant type. Glycosides occurred only sporadically. Free 4-demethyl sterols were maximal during the winter months; levels correlated negatively with sunshine and temperature, but proportions did not alter significantly. Sitosterol ester and cycloartenol ester (but not others) showed the opposite response, with levels correlating positively with sunshine and temperature. Relative amounts of 4-demethyl sterol esters remained reasonably constant, but those of cycloartenol ester and 24-methylene cycloartanol ester varied on an annual basis and were negatively correlated with each other.

The ability of plants to respond and adapt to changes in their physical environment during the growing season is well known and extensively documented. There is growing speculation that such responses may originate, at least partly, in changes in the makeup and/or properties of cell membranes. For example, the physicochemical state of membranes, particularly their fluidity, is known to be highly temperature-dependent (20). This feature of membrane behavior has not yet been explained fully at the molecular level, but it does not appear to be wholly attributable to alterations in the phospholipid component (21). The function of the sterol component is still not entirely clear but available information suggests that it is associated mainly with the stability and integrity of membranes (8). Thus, qualitative and/or quantitative changes in sterols could bring about changes in membrane properties and, so, contribute to the responses of cells or organs to certain environmental stimuli. Sterol levels and metabolism have, in fact, been reported to vary with such factors as light intensity (5, 11), light quality (6, 13), daylength (3), and temperature (19). In most of these studies, sterols were determined over a relatively short period of time, and little information exists relating to changes over longer periods, *e.g.* a growing season. Jacobsohn and Jacobsohn (12) did find variations in the sterols of *Digitalis purpurea* seedlings over a 13-month period, but their investigation was concerned with the relationship between sterols and germination at different times of year and not with growth of plants over a long period.

In this paper, we present results of a monitoring study of sterol levels in leaves of dandelion plants grown mainly under field conditions for 19 months.

MATERIALS AND METHODS

Plant Material. Dandelion plants (*Taraxacum officinale* Weber) were collected from the University of Exeter campus. Between August and October, 1975, 30 whole plants were dug up and transferred to John Innes No. 2 compost in 13-cm-diameter plastic pots. As consistently as possible, plants were of the same size and had the same leaf form. Plants were kept in an open, north-facing cold frame for most of the time, but, in order to protect them from frost during the winter months, pots were transferred, as necessary, to an unheated glasshouse. Although growing conditions were, therefore, partially artificial, glasshouse day temperatures were of the same order as ambient temperature, and the seasonal cycle of temperature was not significantly disrupted. In this way, a continuous supply of foliage was ensured throughout the year. Plants produced flowers continuously from March until October (with the exception of June), 1976, and from March, 1977 until the end of the experiment in June, 1977. No flowers were produced prior to March, 1976, or between October, 1976, and March, 1977.

Weather Data. Rainfall, maximum day temperature, and h of sunshine were monitored daily on the University of Exeter campus, about 1 km from the growing area.

Harvesting. Leaves were harvested at monthly intervals as near as possible to the first day of the month. In each monthly sample, one juvenile and one mature leaf were removed from each of 10 to 15 plants randomly selected from the stock of 30. This was done to minimize possible differences in sterol levels between mature and immature leaves (9, 11). Following excision, leaves were immediately weighed, lyophilized, reweighed, and extracted.

Extraction and Separation of Sterols. The procedure adopted was based on that of Bae and Mercer (3) and Grunwald (8). Freeze-dried tissue (about 10-g batches) was crumbled into a Whatman parchment thimble; 0.1 μCi of [4-^{14}C]cholesterol (specific radioactivity 149 μCi/mg from the Radiochemical Center, Amersham, U. K.) was added (to measure extraction efficiency, see below); and the thimble was closed with muslin. The tissue was extracted for 16 h with ethanol in a Soxhlet extractor (Gallenkamp and Co. Ltd., London, U.K.). The ethanol extract was evaporated to dryness under reduced pressure at 40 C. Flask contents were dissolved in 150 ml 33% (v/v) ethanol in petroleum ether (b.p. 40–60 C) and partitioned with 300 ml distilled H_2O. The organic fraction (containing free and esterified sterols) was reduced to a small volume and subjected to column chromatography (see below), while the aqueous fraction (containing sterol glycosides) was hydrolyzed as follows.

After reextracting with 100 ml petroleum ether, the aqueous fraction was concentrated to about one-third under vacuum at 30 C and refluxed in 2 N HCl for 3 h. When cool, the hydrolysate was neutralized with 2 N NaOH. Sterols liberated by hydrolysis

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were partitioned into diethyl ether. The ether extract was then shaken with one-half its volume of 30% (w/v) methanolic KOH to remove pigments, separated, washed twice with distilled H_2O, and reduced to about 2 to 3 ml by rotary evaporation at 30 C.

Column Chromatography. The organic fraction (containing free sterols and sterol esters) was applied to an alumina column (10 g of neutral alumina activity grade III in a 10-mm-diameter glass column) and eluted with 100-ml volumes of 1%, 6%, and 20% (v/v) petroleum ether (b.p. 40–60 C) in diethyl ether to yield 4-demethyl sterol esters, free 4,4-dimethyl sterols, and free 4-demethyl sterols, respectively (3). The 6% fraction was discarded and the 1% and 20% fractions evaporated to dryness at 30 C. The 20% fraction was taken up in a small volume of diethyl ether, while the 1% fraction was refluxed in 6% (w/v) KOH in 90% (v/v) ethanol for 1.5 h. After neutralizing with 2 N HCl, the hydrolysate was partitioned with diethyl ether and the ether extract reduced to a small volume by rotary evaporation at 30 C.

TLC. Ether extracts of free, glycosylated and esterified sterols were applied as a band to 20- x 20-mm TLC plates (Kieselgel G, 0.25 mm) accompanied by a marker spot containing authentic sitosterol, stigmasterol, campesterol, and cholesterol. After developing in chloroform, plates were dried, sprayed with 0.5% (w/v) aqueous Rhodamine 6G, and viewed under UV light. The authentic sterols ran as a single spot (R_F, about 0.5), and the corresponding extract bands were scraped off and eluted with diethyl ether. The ether extracts were filtered through Whatman No. 1 paper, evaporated to dryness in an air stream, and taken up in 0.3 ml ethanol.

Extraction Efficiency. The efficiency of the extraction procedure was determined by measuring recovery of added [4-^{14}C]cholesterol. A 0.1-ml aliquot of the free sterol extract was counted in 10 ml dioxan-based scintillation fluid (NE 250; Nuclear Enterprises Ltd., Edinburgh, U. K.) in a Packard Tri Carb liquid scintillation spectrometer. Counting efficiency was about 90% and background about 28 cpm. Recovery was usually between 40 to 50% and occasionally as high as 65%. Although this correction strictly applies to free sterols only, it was used on all classes of sterols measured.

Gas-Liquid Chromatography. To each 0.3 ml of extract, 0.1 ml of an ethanol solution of 5α-cholestane (containing 400 μg) was added as internal standard. A 1-μl aliquot of the supplemented extract was then injected into a Pye series 104 gas chromatograph. The column (2.1 m × 3 mm i.d.) was packed with 3% OV-101 (80–100 mesh) on Gas-chrom Q (Phase Separations Ltd., Queensferry, Clwyd, U. K.). The column was isothermal at 250 C and the flame ionization detector temperature 300 C. Nitrogen flow rate was 40 ml/min. Sterols were quantified by referring peak areas (determined by weighing peak cutouts on precalibrated paper) to a calibration graph prepared with authentic sterols. A separate graph was constructed for each sterol studied and was checked occasionally. Graphs were replotted whenever the column was repacked or a new syringe was used.

RESULTS

Preliminary analyses of dandelion leaves indicated the presence of free, esterified and glycosylated sterols, but attempts to isolate acylated glycosides (9) were unsuccessful. Identities of individual sterols were confirmed by retention time and cochromatography with authentic sterols. The principal free sterol was sitosterol (about 60%), followed by stigmasterol (about 25%) and compesterol (about 10%). Endogenous cholesterol did not appear to be present. The principal esters were 4,4-dimethyl derivatives *viz.* cycloartenol (about 40%) and 24-methylene cycloartanol (about 30%), with 4-demethyl forms less abundant. Of the latter, sitosterol ester was present in largest amounts (about 25% of total esters) followed by stigmasterol ester at about 1.5%, with only trace quantities of campesterol ester appearing sporadically. Esters of sitosterol and 4,4-dimethyl sterols were more abundant than were free forms, but, with stigmasterol and campesterol, the situation was reversed. The only glycosides detected were those of sitosterol and stigmasterol, but, because of low concentrations and erratic occurrence, it was not possible to obtain reliable quantitative data.

Weather data are shown in Figure 1. Rainfall was much lower than average between December, 1975, and August, 1976, and much higher than average between September, 1976, and March, 1977. The midsummer months of 1976 also had higher than average sunshine and temperatures. Sunshine and day temperature showed a strong positive correlation (Table I), but no significant correlations existed between rainfall and any of the other weather parameters measured (Table I).

When sterol concentrations were calculated on a fresh weight basis, the pattern of seasonal variations often differed quite sig-

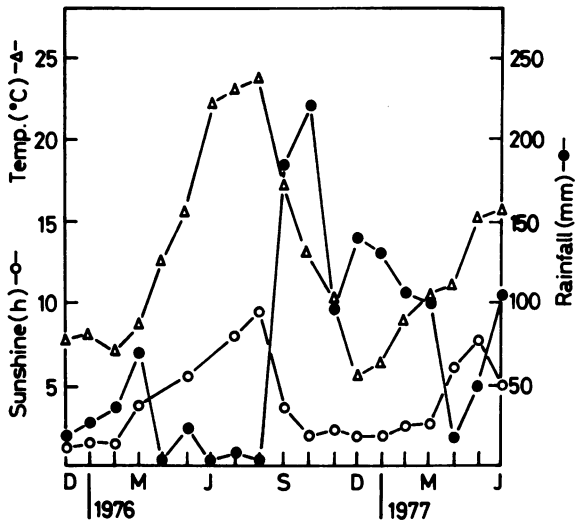

FIG. 1. Weather data for the period from December, 1975, to June, 1977, recorded about 1 km from the experimental plot. (●), Total monthly rainfall; (△), mean maximum day temperature; (○), mean daily sunshine hours. Sunshine readings for April and June, 1976, were not available.

Table I. *Correlation Data for Weather Conditions, Leaf Weights, and Sterols*

	Rainfall		Sunshine		Temperature	
	r[a]	*sig*[b]	*r*	*sig*	*r*	*sig*
Rainfall			0.29	NS	−0.29	NS
Sunshine					0.85	***
Leaf fresh wt/dry wt ratio	0.52	*	−0.70	**	−0.83	***
Free sterols						
Sitosterol	0.22	NS	−0.49	*	−0.59	**
Stigmasterol	0.16	NS	−0.57	*	−0.70	**
Campesterol	0.29	NS	−0.60	*	−0.81	***
Total 4-demethyl	0.21	NS	−0.53	*	−0.64	**
Sterol esters						
Sitosterol	−0.08	NS	0.76	***	0.79	***
Stigmasterol	0.06	NS	0.27	NS	0.21	NS
Cycloartenol	−0.07	NS	0.72	**	0.74	***
24-methylene cycloartanol	0.10	NS	0.32	NS	0.32	NS

[a] *r*, Correlation coefficient.

[b] *sig*, Significance; *, significant at 5%; **, significant at 1%; ***, significant at 0.1%.

nificantly from that based on leaf dry weight. This proved to be due to variations in the leaf fresh weight to dry weight ratio (Fig. 2). This ratio showed strong negative correlations with sunshine and with temperature but only a marginally significant positive correlation with rainfall (Table I). Variation in the fresh weight to dry weight ratio could have been brought about by changes in water content, dry matter accumulation, or both; but it seems probable that water content was the major variable and, so, results have been expressed on a dry-weight basis.

Variations in total free 4-demethyl sterols are shown in Figure 3. Levels tended to be at a maximum between October and March and minimal in the summer months; significant negative correlations were found with sunshine and (particularly) temperature but not with rainfall (Table I). Individual sterols showed similar variations (both to each other and to total free sterols), and negative correlations again occurred with sunshine and temperature (Table I). The proportion of campesterol remained reasonably constant over the 19-month period (Table II). Relative amounts of sitosterol and stigmasterol, as well as the sitosterol to stigmasterol ratio, tended to be more variable but apparently were not correlated with weather conditions (Table II).

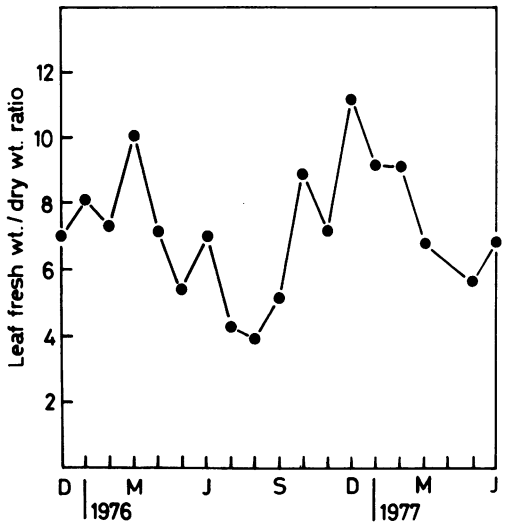

FIG. 2. Variation in dandelion leaf fresh weight to dry weight ratio over a 19-month period. Values for April, 1977, were not obtained.

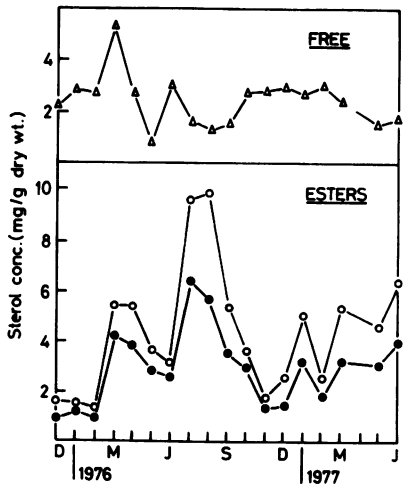

FIG. 3. Variation in free sterols and sterol esters in dandelion leaves over a 19-month period. Free sterol data represent the total of sitosterol, stigmasterol, and campesterol, all of which showed similar variations which correlated with weather conditions. Data for sterol esters relate only to those derivatives which correlated with weather conditions. (△), Total free sterols; (●), sitosterol ester; (○) cycloartenol ester. Values for April, 1977, were not obtained.

Table II. *Relative Amounts of Free 4-Demethyl Sterols in Dandelion Leaves over a 19-Month Period*

Year	Month	Sitosterol	Stigmasterol	Campesterol	Sitosterol/ Stigmasterol
		%	%	%	*ratio*
1975	Dec	58.9	30.9	10.2	1.9
1976	Jan	60.4	30.1	9.5	2.0
	Feb	65.4	24.1	10.5	2.7
	Mar	62.7	27.0	10.3	2.3
	Apr	66.0	22.7	11.3	2.9
	May	69.7	20.4	9.9	3.4
	Jne	67.0	23.8	9.2	2.8
	Jly	58.9	31.4	9.7	1.9
	Aug	76.6	11.6	11.8	6.6
	Sep	64.5	25.3	10.2	2.6
	Oct	68.0	21.2	10.8	3.2
	Nov	69.3	20.9	9.8	3.3
	Dec	61.3	27.1	11.6	2.3
1977	Jan	56.5	34.6	8.9	1.6
	Feb	60.7	27.2	12.1	2.3
	Mar	59.9	27.9	12.2	2.2
	Apr	NO[a]	NO	NO	NO
	May	65.1	27.2	7.7	2.4
	Jne	59.9	28.6	11.5	2.1

[a] NO, Data not obtained.

Sitosterol was the only esterified 4-demethyl sterol which correlated with weather conditions, but, in contrast to the free form, levels peaked mainly in the summer months with minimum values occurring between November and February (Fig. 3). This pattern of changes correlated strongly and positively with sunshine and temperature but not with rainfall (Table I). Campesterol ester was detected infrequently while stigmasterol ester, although more abundant (about 6% of 4-demethyl sterol esters), was not correlated with any of the weather parameters (Table I). Changes in cycloartenol ester (Fig. 3) were very similar to those in sitosterol ester, also being positively correlated with temperature and with sunshine (Table I); variations in 24-methylene cycloartanol ester levels resembled those of stigmasterol ester and, likewise, were not correlated with any of the weather parameters (Table I).

The proportions of sitosterol and stigmasterol esters fluctuated over the sampling period but were not correlated with weather conditions (Table III). There was, however, a strong negative association between the percentage of cycloartenol and that of 24-methylene cycloartanol ($r = -0.94$, significant at 0.1% level). In the summer months, cycloartenol was relatively more abundant and constituted 40 to 50% of total esters, whereas 24-methylene cycloartanol was at a minimum around 25% (Table III). In the winter months, the situation reversed, with cycloartenol reducing to about 33% and 24-methylene cycloartanol rising to about 40% of total esters (Table III).

DISCUSSION

The sterol complement of dandelion leaves is broadly similar to that reported earlier for dandelion inflorescence stalks (1) and flowers (14).

Our data suggest that dandelion leaf sterols fluctuate with the season and that temperature may be the most important causal factor. Because of the membrane-stabilizing properties of free sterols (8), the greater abundance of these compounds in leaves during the winter months raises the question of their contributing to adaptive responses by the plant to low temperatures. This finding would appear to contrast with other reports of either little change in free sterols with temperature (7, 22) or a positive

Table III. *Relative Amounts of Sterol Esters in Dandelion Leaves over a 19-Month Period*

Year	Month	Sitosterol	Stigmasterol	Campesterol	Cycloartenol	24-Methylene Cycloartanol
		%	%	%	%	%
1975	Dec	26.6	0.9	ND[a]	26.9	45.6
1976	Jan	25.3	0.8	ND	31.6	42.3
	Feb	24.8	1.0	ND	34.7	39.5
	Mar	28.6	0.5	ND	36.7	34.2
	Apr	27.8	1.8	ND	39.9	30.5
	May	32.8	1.1	ND	41.7	24.4
	Jne	33.7	1.2	ND	41.8	23.3
	Jly	27.7	1.5	0.8	41.4	28.5
	Aug	27.0	1.2	0.8	46.9	24.1
	Sep	29.0	ND	ND	44.9	26.1
	Oct	27.0	1.6	ND	33.0	38.4
	Nov	27.0	ND	ND	33.8	39.2
	Dec	21.8	2.1	ND	37.7	38.4
1977	Jan	20.8	3.1	ND	33.2	42.9
	Feb	24.0	2.3	ND	34.4	39.3
	Mar	19.6	2.6	ND	32.6	45.2
	Apr	NO[b]	NO	NO	NO	NO
	May	28.8	1.4	ND	44.6	25.2
	Jne	28.6	2.0	0.3	45.7	23.4

[a] ND, Not detectable.
[b] NO, Data not obtained.

correlation between leaf sterols and temperature (19), but it should be noted that the correlations with temperature reported here derive from a study conducted over 19 months, whereas, in other investigations (7, 19, 22), plants were grown under different temperature regimes for a maximum of only 35, 7, and 28 days, respectively. Of interest, also, is the report by Jacobsohn and Jacobsohn (12) of an annual variation in free sterols in *D. purpurea* seedlings over a 13-month period and their suggestion that this might relate to low temperature adaptation in this species. Our findings stress the need for yet further investigation in this area.

Explanation of the physiological significance of the greater accumulation of sterol esters in leaves in the summer months is hindered by the dearth of information on the function(s) of this group of compounds. Esters of 4-demethyl sterols are thought not to contribute to membrane stability (8), and the suggestion that they represent intercellular translocatable forms (15) has not attracted experimental support (2). However, the inverse relationship observed between esters and free sterols possibly suggests some interconversion between these forms. Such a relationship has also been reported in tobacco in relation to leaf age (9, 11). It seems possible, therefore, that 4-demethyl sterol esters might act as 'reservoirs' of free sterols and/or translocatable forms at a subcellular or transmembrane level. The inverse relationship found between cycloartenol ester and free sterols is of interest in view of the proposal (10) that 4,4-dimethyl sterol esters may be involved in the regulation of 4-demethyl sterol biosynthesis.

Other physical factors which could have contributed to the fluctuations in dandelion sterols but which were not measured (although they would probably correlate positively with temperature) are light intensity, light quality (particularly red/far red content), and daylength. All of these are known to be capable of influencing sterol levels in plants (3, 5, 6, 11, 13).

At present, the apparent correlations between flowering in dandelion and in leaf sterols are difficult to assess. Of the few studies which have been made of sterol metabolism in relation to flowering (*e.g.* 4, 16–18) none supports a central role for sterols in this process. It seems unlikely, therefore, that the relationship observed here is a causal one.

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