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STUDIES IN THE CRUCIFERAE
CHANGES IN THE COMPOSITION OF THE STEROL FRACTION FOLLOWING GERMINATION

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Abstract—It was noted for some genera of the Cruciferae that the composition of the sterol fraction in growing plants was markedly different from that found in seed. Changes in sterol content were studied following germination of seed of *Brassica napus* L., *B. rapa* L., *B. oleracea* L., *Raphanus sativus* L., *Sinapis alba* L. and *Cheiranthus cheiri* L. In the first five cases a rise in the cholesterol proportion and a gradual disappearance of brassicasterol was noted.

INTRODUCTION

CHANGES in steroid composition consequent upon germination of seed have been studied previously for *Phaseolus vulgaris* L.,[1] *Zea Mays* L.,[2] *Haplopappus heterophyllus Bl.*,[3] *Pisum sativum* L.,[4,5] and *Avena sativa* L.[5] It is apparent from these studies that sterol biosynthesis takes place very soon after germination and that in certain organs e.g. root and shoot,[2] an increase in sterol content occurs. It also appears that in most organs[1,2] the relative proportion of each sterol remains constant. However, the sterol composition of root of *Zea Mays*[2] alters during the early stages of growth, there being a rise of stigmasterol content and a corresponding decline in the proportion of ß-sitosterol.

In the present work, part of a study of the host parasite relations of *Plasmodiophora brassicae*, changes in both the nature and relative proportions of sterols were noted. These changes showed some correspondence to stages of physiological development of the plant.

RESULTS AND DISCUSSION

Table 1 shows the variation in sterols noted for *Brassica napus f. annua* (rape) at various stages of development of the plant from early seedling through to flowering. Table 2 shows the sterol changes noted in the first 3–5 weeks of growth following germination for several examples from genera of the family Cruciferae.

[1] P. DUPERON and K. DUPERON, *Compt. Rend.* **261**, 3459 (1965).
[2] R. J. KEMP, L. J. GOAD and E. I. MERCER, *Biochem. J.* **103**, 53 P. (1967), and *Phytochem.* **6**, 1609 (1967).
[3] R. D. BENNETT, E. R. LIEBER and E. HEFTMANN, *Plant Physiol.* **42**, 973 (1967).
[4] D. J. BAISTED, *Phytochem.* **6**, 93 (1967).
[5] V. V. VIL'YAMS and N. F. KROKHINA, *Chem. Abs.* **65**, abs. no. 20,517 (1966).

TABLE 1. STEROLS IN BRASSICA NAPUS

Age or stage of development	Culture medium†	Percentage of each sterol*					
		1	2	3	4	5	6
0 days (seed)		Trace	16·1	27 8	1·1	52 3	2·7
52 hr seedlings	A	3·0	15·4	30·4	—	51·2	—
76 hr seedlings	A	7·0	12·4	29 2	—	50·9	—
118 hr cotyledons only	A	6·9	16 4	25·8	—	50 9	—
5 days cotyledons expanded	B	2·3	9·6	29 4	1·6	57·1	—
7 days cotyledons expanded	B	2·8	8·8	25·4	—	63·0	—
9 days 1st leaf expanding	B	1·8	7·0	23·6	2 1	65·5	—
11 days 2nd leaf expanding	B	2·1	4 6	20 2	Trace	73·1	—
14 days 2 leaves expanded	B	2·1	3 3	20·4	1·2	73·0	—
16 days 3rd leaf expanding ‡	B	2·6	3·9	19·5	2 5	71·5	—
19 days 3–5 leaves expanded ‡	B	2·3	1·5	21 5	2·0	72 7	—
14 days 1st leaf expanded	C	2·4	7·3	25·6	—	64·7	—
33 days 5th leaf expanding	C	3·1	—	19 1	—	77·8	—
150 days not flowered (leaf only)	C	4·4	—	14·0	—	80·3	1·3
150 days flowered, stamens not open (leaf)	C	2·3	—	14·6	2·8	73·1	7 2
150 days flowered, stamens not open (stem)	C	3·7	2·0	25·2	1 1	68·0	—
150 days flowered, seed pods forming (leaf)	C	2 1	—	14·3	2·6	75·0	6·0
150 days flowered, seed pods forming (stem)	C	1·9	3·3	20·6	4·7	69 5	—

* 1. Cholesterol. 2. Brassicasterol. 3. 24-methylcholesterol (campesterol). 4 Stigmasterol. 5. 24-ethylcholesterol (β-sitosterol). 6. Others, including 24-ethylidenecholesterol.

† See experimental for details.

‡ Leaf + stem material only.

It is apparent from Table 1 that following germination of *B. napus* a significant rise in cholesterol present in the sterol fraction occurred within about two days and that the rise was much less marked afterwards. The relatively high level of cholesterol noted in seedlings grown in sand (A) may possibly be attributable to a higher level of application of nutrient solution relative to the other cases (B, C).

A less rapid initial change in the relative proportion of brassicasterol was noted but the level began to fall after 2–3 days and by the twentieth day had fallen by a factor of ten. It is not clear whether this represented conversion of brassicasterol into another compound or whether synthesis of other sterols occurred sufficiently rapidly to reduce the relative concentration of this compound until it was no longer detectable (usually less than 5 weeks).

The data in Table 1 also show that synthesis of ß-sitosterol in the growing plant represented a greater proportion of the total sterol synthesis than occurred during development of the ripe seed. Thus the relative level of ß-sitosterol showed a marked rise during the first 2 weeks of growth followed by a nearly constant relative level of synthesis over the next 3–4 months.

After flowering it was possible to divide the plant into leaf and flowering stem (including developing fruits) for analysis. As might be expected, flowering stem showed a change in sterol content to include brassicasterol and there was also an appreciable rise in synthesis of stigmasterol, the C_{24} homologue of brassicasterol. Leaf material showed no brassicasterol

TABLE 2. STEROLS IN CRUCIFERAE

Species and age of seedling		Sterol*						
		1	2	3	4	5	6	Others
Brassica rapa (Golden Ball)	0 days	2·7	13·4	22·4	Trace	61·5	Trace	
	8 days	3·8	16·7	26·4	1·7	49·7	1·7	
	14 days	5·2	5·6	18·8	1·9	64·1	4·4	
Brassica rapa (Wallace)	0 days	0·3	19·6	25·2	—	53·4	1·5	
	14 days	5·4	16·4	26·6	—	49·0	2·6	
	33 days	4·5	5·8	21·4	1·7	58·7	5·6	2·3
B. oleracea f. capitata (Primo)	0 days	Trace	12·5	26·4	—	61·1	—	
	5 days	1·9	8·6	31·6	2·6	55·3	—	
	19 days	7·6	4·8	26·4	4·0	55·4	1·8	
Raphanus sativus (French Breakfast)	0 days	2·3	8·5	24·2	—	62·0	3·0	
	5 days	4·3	9·4	24·6	2·2	59·5	Trace	
	19 days	6·1	5·5	22·6	4·7	61·1	Trace	
Sinapis alba	0 days	3·2	5·2	34·6	—	43·8	13·2	
	5 days	4·5	5·5	32·5	—	57·5	Trace	
	19 days	2·5	2·2	22·2	Trace	69·0	4·1	
	‡22 days	3·9	1·1	20·8	1·5	69·6	3·1	
Cheiranthus cheri (Blood Red)	0 days	15·1	—	19·5	—	51·5	3·8	10·1†
	19 days	17·4	—	22·7	—	53·4	4·0	2·5
	33 days	5·0	—	22·7	—	70·4	1·9	—

* 1. cholesterol. 2. brassicasterol. 3. 24-methylcholesterol (campesterol). 4. stigmasterol. 5. 24-ethylcholesterol (β-sitosterol). 6. 24-ethylidenecholesterol.
† Various Δ^7-sterols. ‡ Flowered.

at these stages but stigmasterol was evident, although it was absent from leaf of plants of the same age which had not flowered. The data in Table 1, part C, were obtained on SE-30, and this stationary phase does not resolve 24-ethylidenecholesterol from 24-ethylcholesterol. Separate analyses in a different experiment showed that the former compound is only measurable in cases corresponding to late stages of flower development and only to a maximum of 2–3 per cent. Bennett *et al.*[3] record a change in phenolic compounds, presumed to be sterols, following flowering in *Haplopappus heterophyllus*.

In other species of Brassica (Table 2) and for *Raphanus sativus* (radish) the relative rise in cholesterol and fall in brassicasterol proportions were similar to those described above. However, it seemed that some reduction in the ß-sitosterol level occurred initially. The relative level of this sterol then increased in *B. rapa* (turnip), whereas the corresponding level was not markedly affected during the first 3 weeks of growth of *B. oleracea f. capitata* (cabbage) and radish.

With respect to *Sinapis alba* (white mustard) the level of cholesterol remained approximately constant but, as in the above cases, the brassicasterol level fell following germination. There was also a rise in the ß-sitosterol level and a fall in the level of 24-ethylidene cholesterol in mustard.

Cheiranthus cheiri (wallflower) represented a special case in that the seed was found to contain an exceptionally high relative proportion of cholesterol. This proportion persisted for a while following germination and was then followed by a decline. The ß-sitosterol

proportion correspondingly showed a rise to a higher level. The Δ^7-sterols (including Δ^7-cholestenol and 4α-methyl-Δ^7-cholestenol) were suppressed to the point of non-detectability in less than 5 weeks.

In marked contrast to previous work with other species, considerable variation in the sterol fraction of members of the Cruciferae occurred following germination. Thus, except where the content of cholesterol was high in the seed, the proportion of cholesterol in the sterol fraction rose following germination and the level of brassicasterol fell to a minimum. The latter compound reappeared in *B. napus* following flowering. The ß-sitosterol proportion often rose appreciably in the first 2 or 3 weeks following germination. Where it showed any tendency to variation (e.g. *B. napus* and *S. alba*), campesterol was found to decrease in proportion to the other sterols.

The isomer of 24-ethylidenecholesterol present in various samples analysed was shown by gas chromatography on OV-17 to be always the same isomer as occurs in seed of *Avena sativa* (oat).

It has been shown that cholesterol is a major component of a tightly bound sterol fraction of chloroplasts,[6] and it was considered possible, therefore, to look upon the early biosynthesis of cholesterol as a photomorphogenetic change in the plant. No correlation between cholesterol synthesis and chlorophyll content was demonstrable and, in fact, seed of *B. napus* germinated in Peralite in darkness produced etiolated seedlings containing a proportion of about 2 per cent cholesterol in the sterol fraction.

Sterol synthesis has been shown to take place in the dark in *Phaseolus vulgaris*[1] and in *Pisum sativum* and *Avena sativa*.[5] In the latter cases an inverse correlation between total sterol content of seedlings and synthesis of chlorophyll and carotene was noted. Presumably, therefore, sterol synthesis is more active in plants grown in a dark regime. This is in general agreement with other results.[1]

In the present case plants were grown under alternating periods of light and dark and it is possible that most of the sterol changes occurred during dark periods. However, to determine the effects of light v. dark in this context over the periods used would require much more elaborate growth cabinets than are currently available in our laboratories.

EXPERIMENTAL

Cultivation of Plants

A. Seeds were sown in sand in trays and, after watering with nutrient solution,* were germinated at 20° in a growth cabinet. This cabinet was operated on alternating 16 hr light and 8 hr dark periods and was illuminated with fluorescent tubes (alternate warm white and daylight tubes to give a total energy of $2{\cdot}08 \times 10^{-3}$ cal/cm^2/min). Watering was carried out daily with the nutrient solution.

B. Seeds were sown in John Innes potting compost in trays and placed in the growth cabinet. Watering was performed daily and nutrient solution was applied after 14 days.

C. Seeds were sown outside in April following application of fertilizer to the soil. Seedlings were thinned and then allowed to grow. A proportion of plants flowered in September and October and were harvested.

D. Seeds of various Cruciferae were sown thickly in boxes of John Innes potting compost and placed in an unheated greenhouse during August. Watering was performed daily.

* Modified Hoagland's: Calcium nitrate, 81 per cent solution (1 ml/l. nutrient solution); potassium nitrate 20 per cent (2·5 ml/l.); magnesium sulphate $7H_2O$, 49 per cent (1 ml/l.); potassium dihydrogen phosphate, 14 per cent (1 ml/l.); sequestrene 138Fe, 2·5 per cent (1 ml/l.), and a micro nutrient solution [copper sulphate (35·4 mg/l.); manganese sulphate (609 mg/l.); zinc sulphate (97·4 mg/l); boric acid 126·9 mg/l. and molybdic acid $4H_2O$ (39 8 mg/l.)] (0·1 ml/l.).

[6] E. I. MERCER and K. J. TREHARNE, in *Biochemistry of Chloroplasts* (edited by T. W. GOODWIN), p. 181. Academic Press, London (1966).

Harvesting

In series A, B and C plants were pulled or uprooted and washed in water to remove most of the adhering soil or sand. Material was air-dried at 80° for extraction, whole plants being used except where indicated in Table 1. In series D plants were cut off at soil level and air-dried at 80°.

Isolation of Sterols

Seed or air-dried material was broken up in a coffee grinder and extracted with light petroleum in a Soxhlet extractor. Total sterol was isolated by evaporation of the light petroleum followed by the successive steps of saponification of the residue, ether extraction and digitonin precipitation of sterols from the nonsaponifiable fraction.[7]

Acetates were prepared by treating the sterol fraction with acetic anhydride in pyridine overnight.[8] Reagents were removed in a stream of N_2 and were dissolved in ether or $CHCl_3$ for gas chromatography. Trimethylsilyl ethers were prepared by dissolving the sterol fraction in bis-trimethylsilylacetamide (100 μl) or by treating a $CHCl_3$ solution of the sterol fraction with hexamethyldisilazine and trimethylchlorosilane (5:1 ratio of reagents) at room temperature overnight.[8] The resultant solutions were used directly for gas chromatography.

Gas Chromatography

A Pye 104 model 14 chromatograph was used with N_2 carrier gas. Support material (Gas Chrom P) and stationary phase packings (1 per cent SE-30) were prepared as described elsewhere[9] or were purchased from Applied Science Laboratories (3 per cent OV-17 on Gas Chrom Q). Two columns were used, a 9 ft 1 per cent SE-30 column at 250° and a 9 ft 3 per cent OV-17 column at 256°. Sterols were recognized by comparison of retention data with authentic compounds and by combined gas chromatography–mass spectrometry (for sterols from seed of *Brassica oleracea*, *Raphanus sativus*, *Sinapis alba* and *Cheiranthus cheiri*).

Relative proportions of sterols were determined by triangulation of peaks on chromatograms, making the assumption that all sterols were both eluted from the column, and detected, with equal efficiency.

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[7] W. BERGMANN, *J. Biol. Chem.* **132**, 471 (1940).
[8] B. A. KNIGHTS, *Memoirs of the Society for Endocrinology, No.* 16 (edited by J. K. GRANT), p. 211 (1967).
[9] E. C. HORNING, W. J. A. VANDEN HEUVEL and B. G. CREECH, *Meth. Biochem. Anal.* **11**, (1963).