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CHANGES IN LIPIDS OF MATURING *CEIBA PENTANDRA* SEEDS

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Abstract—Lipids were extracted with chloroform-methanol from *Ceiba pentandra* seeds at different stages of maturity, separated by TLC and tested for the Halphen response. Cyclopropene fatty acids (CFA) were absent from mono- and diglycerides and phospholipids. Free fatty acids, diglycerides and phospholipids were maximum on the 23rd, 65th and 65th day respectively and minimum on the 96th day (mature) after flowering. The fatty acid compositions were determined by GLC. Dihydromalvalic acid reached a maximum value on the 81st day, while malvalic acid remained almost constant from the 38th day and sterculic acid steadily increased. Dihydrosterculic acid was noticed only in the free fatty acids and phospholipids. The positional distribution in the triglycerides was determined by lipolysis. Since CFA inhibitied lipolysis partially the triglycerides were dissolved in chloroform and treated with methanolic silver nitrate prior to lipolysis. CFA were found in the 2-position, though to a lesser extent than in the primary positions, indicating rearrangement of glycerides during maturation.

INTRODUCTION

CHANGES in composition of lipids in oilseeds during maturation have been studied in several species.[1–6] Development of unusual fatty acids during maturation has been followed in a few species; epoxyoleic in *Vernonia anthelmintica*,[7] ximenynic in *Santalum acuminatum*,[8] crepenynic in *Crepis rubra*,[9] and malvalic and sterculic in some members of the Malvaceae and Sterculiaceae.[10] This report describes the changes in compositions of lipid classes and fatty acids during maturation of seeds of *Ceiba pentandra* Linn. (var. Kapok, Bombacaceae), which contain cyclopropene fatty acids.[11]

RESULTS AND DISCUSSION

Changes in Lipid Classes

Contents of total lipids, triglycerides, free fatty acids, diglycerides and phospholipids at various stages of seed maturity are shown in Table 1. The lipid contents doubled between the 51st and 65th day after flowering and remained constant thereafter. The free fatty acids decreased from the 23rd to 51st day, remained fairly constant between the 51st and 81st day and decreased thereafter. The phospholipids also decreased after the 65th day. The monoglycerides were present in traces only in the initial 15- and 23-day samples. The

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[9] W. G. HAIGH, L. J. MORRIS and A. T. JAMES, *Lipids* **3**, 307 (1968).
[10] A. R. JOHNSON, J. A. PEARSON, F. S. SHENSTONE, A. C. FOGERTY and J. GIOVANELLI, *Lipids* **2**, 308 (1967).
[11] F. L. CARTER and V. L. FRAMPTON, *Chem. Rev.* **64**, 497 (1964).

TABLE 1. CHANGES IN LIPID CLASSES* DURING MATURATION OF *C. pentandra* SEEDS

	Days after flowering					
	23	38	51	65	81	96
	Moisture content (%)					
	87·7	76·8	71·9	63·7	52·3	9·9
	mg/100 g dry wt. of seeds					
Lipid content	2100	12 800	11 800	22 700	23 100	23 600
Triglycerides	1251	10 020	—	—	20 420	21 880
Free fatty acids (as oleic)	340	269	115	116	127	71
Diglycerides	—	614	—	1839	1016	0
Phospholipids	—	908	897	1180	416	307

* Triglycerides and diglycerides were estimated by gravimetry after preparative TLC, free fatty acids from acid values and phospholipids from phosphorus contents.

presence of partial glycerides and free fatty acids indicates that Kartha's 'quantum' mechanism of triglyceride biosynthesis[1] may not be operating in this plant. Negative response of the mono- and diglycerides and phospholipids to the Halphen test, which is specific for CFA and sensitive up to 0·1%,[12] indicated that these lipid classes did not contain CFA.

Changes in Fatty Acid Compositions

Total lipids. The contents of different fatty acids of the total lipids at various stages of seed maturity are shown in Table 2. Linoleic acid increased till the 65th day after flowering. Sterculic and oleic acids steadily increased. Malvalic acid remained almost constant from the 38th day with a maximum value on the 65th day. Palmitic and dihydromalvalic acids reached a maximum value on the 81st day. Traces of dihydrosterculic acid were present at all stages.

TABLE 2. CHANGES IN FATTY ACID COMPOSITION OF TOTAL LIPIDS DURING MATURATION OF *C. pentandra* SEEDS

Fatty acid	Days after flowering 38	65	81	96
	mg/100 g dry wt. of seeds			
Myristic	13	159	23	24
Palmitic	2560	4770	5410	5380
Dihydromalvalic	128	136	370	165
Stearic	64	295	231	189
Oleic	2940	5470	6670	7080
Linoleic	4500	8830	7670	7760
Malvalic	2040	2250	1980	2200
Sterculic	269	681	739	802

Free fatty acids. The changes in fatty acid composition at the initial stages of seed maturity (15 and 23 days after flowering) are given in Table 3. Malvalic, sterculic and dihydrosterculic acids were present in the 23rd day sample, while the 15th day sample did not contain malvalic acid. In both cases sterculic acid was present in larger proportions than malvalic.

[12] F. C. MAGNE, *J. Am. Oil Chem. Soc.* **42**, 332 (1965).

TABLE 3. CHANGES IN FATTY ACID COMPOSITION OF FREE FATTY ACIDS IN MATURING *C. pentandra* SEEDS

Fatty acid	Days after flowering 15	23
	mg/100 g dry wt. of seeds	
Palmitic	34	47
Oleic	24	41
Linoleic	40	50
Dihydrosterculic	10	7
Malvalic	0	5
Sterulic	7	11

Diglycerides. The changes in fatty acid composition of diglycerides at two stages of seed maturity (38 and 65 days after flowering) are shown in Table 4. CFA and their dihydro-derivatives were not detected at either stage.

Phospholipids. The variations in fatty acid compositions of phospholipids at different stages of seed maturity are shown in Table 5. Dihydrosterculic and oleic acids were present in maximum amounts on the 65th day.

TABLE 4. CHANGES IN FATTY ACID COMPOSITION OF DIGLYCERIDES IN MATURING *C. pentandra* SEEDS

Fatty acid	Days after flowering 38	65
	mg/100 g dry wt. of seeds	
Palmitic	173	586
Steraic	13	40
Oleic	147	586
Linoleic	280	623

TLC on Silica Gel G of the phospholipids from immature seeds showed, in the initial stages only, small amounts of phosphatidyl choline and phosphatidyl ethanolamine. A large part of these phospholipids was made up of components with R_f values higher than that of phosphatidyl ethanolamine. Their amounts decreased as the seeds matured, and phosphatidic acid was identified as one of the components.

TABLE 5. CHANGES IN FATTY ACID COMPOSITION OF PHOSPHOLIPIDS IN MATURING *C. pentandra* SEEDS

Fatty acids	Days after flowering 38	51	65	96
	mg/100 g dry wt. of seeds			
Palmitic	375	280	446	119
Stearic	24	22	27	8
Oleic	143	157	267	68
Linoleic	333	400	390	83
Dihydrosterculic	33	38	45	28

Distribution of Fatty Acids in the Triglycerides

A modified procedure was used for pancreatic lipase hydrolysis since CFA were found to react with lipase causing partial inhibition of activity. When *C. pentandra* oil was subjected to lipolysis, the liberated monoglycerides and fatty acids as well as the unhydrolysed triglycerides failed to respond positively to the Halphen test. To study the nature of the reaction, a sample of freshly extracted *Sterculia foetida* oil, which contains a high concentration CFA,[11] was subjected to lipolysis. The monoglycerides did contain CFA as shown by GLC of the methyl esters after treatment with methanolic silver nitrate.[13] GLC also showed an unidentified peak emerging between linoleic and malvalic acids. The methyl esters of the unhydrolysed triglycerides isolated from the lipolysis products also gave the same unidentified GLC peak. These triglycerides responded only faintly to the Helphen test. The IR spectrum showed a peak for cyclopropane group (1028 cm^{-1}) but not for cyclopropene group (1008 cm^{-1}). The data indicate that lipase reacted with CFA by addition to the ring. But the NMR spectrum did not show a peak at 9·4 τ characteristic of the cyclopropane ring. CFA are known to react with —SH compounds[14] and there is evidence to show that —SH groups are present in pancreatic lipase[15,16] though they do not form part of the active site.[17] Combination with —SH groups may cause steric blocking which interferes with the activity of the enzyme. CFA have been shown to inhibit the activity of other —SH enzymes.[18,19]

TABLE 6. DISTRIBUTION OF FATTY ACIDS IN THE 2-POSITION OF TRIGLYCERIDES OF MATURING *C. pentandra* SEEDS

	Fatty acids (% mol)														
	Palmitic			Oleic			Linoleic			Malvalic			Sterculic		
Days after flowering	Total	2-position	% in 2-position*	Total	2-position	% in 2-position	Total	2-position	% in 2-position	Total	2-position	% in 2-position	Total	2-position	% in 2-position
81	25·1	1·2	1·5	28·2	42·6	50·3	32·6	51·1	51·9	8·4	1·8	6·9	3·0	3·4	37·7
96	24·4	1·2	1·7	29·3	35·5	40·4	32·4	56·1	57·8	9·1	1·2	4·4	3·2	2·4	25·2

* % proportion in 2-position = (2-position × 100)/(Total × 3).

Table 6 gives the percentage proportion of any particular fatty acid in the 2-position of the triglycerides at two stages of seed maturity, i.e. 81 and 96 days after flowering. The total lipids as such without purification were subjected to lipolysis because free fatty acids, diglycerides and phospholipids together were present in small amounts. The percentage proportion of palmitic acid in the 2-position was small and remained almost constant. Oleic acid decreased from the 81st to the 96th day after flowering; the reverse change was noted with linoleic. The percentage proportions of both malvalic and sterculic acids in the 2-position decreased during ripening. Sterculic acid was present in larger percentage than malvalic at the 2-position.

The absence of CFA in diglycerides, monoglycerides and phospholipids at any stage of seed maturation shows that CFA are introduced into the trilyceride molecule only at the

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final stage of triglyceride biosynthesis. Since the diglycerides formed from phosphatidic acids are 1,2-diglycerides, the CFA would be expected to be present only in the 3-position of the triglyceride, if α-glycerophosphate pathway is involved. But the lipase hydrolysis data (Table 6) showed their presence also in the 2-position. This may indicate that either the diglycerides or triglycerides rearrange or a pathway other than α-glycerophosphate pathway is operative. Rearrangement of fatty acids in the glycerides was also indicated in other studies on maturation of sunflower seeds[4] and soybeans.[5]

EXPERIMENTAL

Seeds were obtained from a single *C. pentandra* tree on the 15th, 23rd, 38th, 51st, 65th, 81st and 96th day after flowering. Moisture content of the seeds was determined by heating a known amount of the crushed sample at 110° to a constant weight.

Extraction of lipids. Soon after collection, the seeds were weighed, crushed quickly, placed in a flask and soaked immediately with $CHCl_3$–MeOH (2:1, v/v) for 18 hr at room temp. in the dark. The extract was filtered and the material was ground again and reextracted. Five extracts thus obtained were combined and concentrated below 40° *in vacuo*. The concentrated extract was transferred to a separating funnel, diluted with H_2O and extracted thrice with light petroleum (60–80°). The extracts were combined, washed with 10% Na_2SO_4 and dried (Na_2SO_4). The combined extract was concentrated as before and transferred to a 50-ml volumetric flask and made up with petrol. An aliquot of the extract was evaporated to determine the lipid content.

Analysis of lipids. Acid values[20a] for an estimate of free fatty acids and the Halphen response[20b] for the presence of CFA were determined according to the Official and Tentative Methods of the American Oil Chemists' Society on a semi-micro scale. Phosphorus content of the total lipids was determined according to the procedure of Harris and Popat.[21] Approximate percentages of phospholipids were obtained by multiplying the phosphorus contents by 25.

Separation of lipids into classes The total lipids were separated into classes by preparative TLC on Silica Gel G (E. Merck, 500 μ-thick). The lipids were dissolved in $CHCl_3$, and developed in a mixture of light petroleum–Et_2O–HCOOH (60:40:1, v/v). The separated bands were located using I_2 vapour. These were identified with the help of reference compounds as triglycerides, free fatty acids, diglycerides and a mixture of monoglycerides and phospholipids. The components were isolated by extraction with Et_2O or MeOH. The mixture of monoglycerides and phospholipids was further separated by the same method using the solvent system Et_2O–petrol (9:1, v/v). The different lipid classes thus isolated were examined for the presence of CFA by the Halphen test. The isolated triglycerides and diglycerides were weighed. Monoglycerides were present in very small amounts and not estimated. The phospholipids were further resolved, for qualitative identification, by TLC on Silica Gel G using the solvent system[22] $CHCl_3$–MeOH–H_2O (65:25:4, v/v).

Isolation of free fatty acids. The total lipids were esterified with CH_2N_2 and the methyl esters were separated by column chromatography on silica Gel (National Chemical Laboratory, Poona, India; 20 g in a column 23 × 16 mm). The lipids (350 mg) were adsorbed. Elution was carried out first with light petroleum (60–80°, 50 ml) and then with 2% Et_2O in petrol (175 ml) which eluted all the methyl esters free from contamination as checked by TLC on Silica Gel G using the light petroleum ether–Et_2O–HOAc (97:3:1, v/v).

Determination of fatty acid composition. Methyl esters were prepared from total lipids, diglycerides and phospholipids using 1% NaOMe in MeOH. The esters from total lipids containing CFA were treated with a saturated solution of $AgNO_3$ in MeOH (15 ml/100 mg of sample) as described by Schneider *et al.*[13] The methyl esters were analysed by GLC with a thermal conductivity detector. The column (2·4 m × 6 mm) was packed with 15% DEGS on Chromosorb W (60–80 mesh) and maintained at 210°. H_2 (60 ml/min) was used as the carrier gas. Peak areas were obtained by triangulation. From the area percentages, the fatty acid composition (wt. %) was obtained. The peaks were identified using reference esters.

Pancreatic lipase hydrolysis. Lipids isolated from seeds collected on the 81st and 96th day after flowering contained only small amounts of free fatty acids, diglycerides and phospholipids (0·5, 4·4, 1·8 and 0·3, 0·0, 1·3%, respectively). Hence the total lipids without further purification were hydrolysed with pancreatic lipase (E.C. 3.1.1.3). Preliminary experiments on lipolysis of *C. pentandra* and *S. foetida* oils and analysis of products including the unhydrolysed triglycerides by the Halphen test, GLC, IR spectrophotometry and NMR spectroscopy indicated that CFA reacted with pancreatic lipase causing partial loss of activity. Hence

[20] *Official and Tentative methods of the American Oil Chemists' Society*, Revised edition (1947–1960), (a) *Ca* 5a-40; (b) *Cb* 1-25.

[21] W. D. Harris and P. Popat, *J. Am. Oil Chem. Soc.* **31**, 124 (1954).

[22] H. Wagner, C. Horhammer and P. Wolff, *Biochem. J.* **334**, 175 (1961).

the procedure was modified as follows: To the fat (*ca.* 100 mg) dissolved in dry $CHCl_3$ (5 ml) was added a saturated solution (20 ml) of $AgNO_3$ in MeOH. The mixture was left at room temp. in the dark for 20 hr. The triglycerides were isolated by extraction with light petroleum. The extract was washed free of $AgNO_3$ and dried (Na_2SO_4). After evaporation of the solvent, the triglycerides were subjected to pancreatic lipase hydrolysis according to Luddy *et al.*[23] The 2-monoglycerides were isolated by preparative TLC on Silica Gel G using the solvent system light petroleum–Et_2O–HCOOH (60:40:1, v/v). These as well as the triglycerides treated with $AgNO_3$ were converted to methyl esters as described above and analysed by GLC.

[23] F. E. LUDDY, R. A. BARFORD, S. F. HERB, P. MAGIDMAN and R. W. RIEMENSCHNEIDER, *J. Am. Oil Chem. Soc.* **41**, 693 (1964).

Key Word Index—*Ceiba pentandra*; Bombaceae; lipids; maturation of seeds; cyclopropene fatty acids; glycerides.