Accumulation of Lipids, Proteins, Alkaloids and Anthocyanins during Embryo Development in vivo of *Theobroma cacao* L.

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ABSTRACT

Developing embryos of *Theobroma cacao* ranging in weight from 0.01-2.2 g dry weight, equivalent to 100-180 days postpollination, were analyzed for lipids, alkaloids, proteins, and anthocyanins. Total lipid, fatty acid, triglyceride, alkaloid, and anthocyanin accumulation increased linearly after an initial lag with embryo dry weight. Palmitic, stearic, arachidic, and oleic acids had constant rates of accumulation per micromole of total fatty acid (0.29, 0.27, 0.38, and 0.01, respectively); however, linoleic and linolenic acid accumulation decreased from 0.2 and 0.02 below 0.2 g dry weight to 0.035 and 0.0035 above 0.2 g dry weight, respectively. Monounsaturated triglycerides [palmito-oleo-stearin (POS), oleo-distearin (SOS), and oleo-dipalmitin (POP)] continued to accumulate as dry weight increased but polyunsaturated triglycerides [palmito-diolein (POO), stearo-diolein (SOO), linoleo-dipalmitin (PLP), and palmito-linoleo-olein (PLO)] ceased to accumulate at about 0.4 g dry weight. Theobromine accumulation increased linearly with dry weight after an initial lag but the rate differed with cultivar. Caffeine accumulation was low until the final stages of development. The protein pattern became dominated by 4 protein species with apparent molecular weights of 43, 34, 22, and 14 kDa as embryos matured.

INTRODUCTION

Cocoa beans are one of the principal edible oil crops. Although there is extensive information on the composition of fermented mature beans (1-5), much less has been published on developmental biochemistry. Compositional changes in developing embryos from 100-180 days postpollination have been reported for lipids (6) and alkaloids (7), but proteins (8) have only been followed in the final stages of fruit ripening. The lipid study of Lehrian and Keeney (6) did not include changes in specific triglycerides. This investigation was initiated to determine the course of development and interrelationships between lipids, proteins, alkaloids, and anthocyanins in zygotic embryos developing in vivo.

METHOD AND MATERIALS

Cacao pods at different stages of development up to maturity from 2 clones identified as UF 601 and CC 67 were harvested in Turrialba, Costa Rica on January 1, 1981 and transported to the Horticulture Department, Purdue University. Pods were bisected, embryos removed and frozen at 0 C, and fresh weights determined prior to lypholization and dry weight determination. Three embryos from each pod were chosen at random and ground together for sample analysis to reduce genotypic variation among seeds within pods. Portions of each sample were used for lipid, protein, alkaloid, and anthocyanin analyses.

Lipids were extracted as described previously (9) using a procedure modified from Folch et al. (10). Half of the lipid extract was analyzed for total fatty acids using gas chromatography and the remaining half was analyzed for triglycerides using liquid chromatography.

Fatty acids were prepared by saponification of lipids using the method of Metcalfe and Schmitz (11) and methylated by heating for 3 min in a solution of 14% BF_3/methanol. Fatty acid methyl esters were separated isothermally at 180 C using a 2m x 2mm stainless steel column packed with 10% DEGS on 80/100 Supelcoport in a Hewlett Packard 5730A chromatograph equipped with a flame ionization detector. Carrier gas (nitrogen) flow rate was 30 mL/min; air was 50 mL/min; and hydrogen was 20 mL/min. Identification and quantification of fatty acid methyl esters was based on retention times and areas of known fatty acid methyl ester standards and the inclusion of 1 mg of pentadecanoic acid as an internal standard. A Hewlett Packard 3380 S integrator was used to determine fatty acid peak areas. Data reduction was accomplished by using a Control Data Corporation 6600 computing system.

Triglycerides were separated according to the liquid chromatography procedure of El-Hamdy and Perkins (12) using a Waters HPLC system consisting of a M6000 A pump, U6K injector, and a R101 differential refractometer connected with a Supelco LC-18 column (150mm x 4.6 mm). The mobile phase consisted of 64% acetone and 36% acetonitrile. Identification and quantification of triglycerides was based on retention times and areas of known standards (Supelco) and commercial cocoa butter. Trimyristin (4 mg) was included as an internal standard. Triglyceride peak areas, determined by triangulation, were used for quantification.

Alkaloids were extracted using the method of Kreiser and Martin (13). Theobromine and caffeine were separated using a Spectra Physics 8700 HPLC system connected with a Whatman Partisil PXS 5/25 ODS stainless steel column (150 mm x 4.5 mm). Alkaloid peaks were detected using a SP8300 UV absorbance (254 nm) detector. The mobile phase used was 20% methanol, 79% water, and 1% acetic acid. Theobromine and caffeine were identified using known standards, and quantified with the inclusion of 0.5 mg theophylline as an internal standard and measuring peak heights.

Proteins were extracted with 0.031 M Tris-HCl, 1.5% SDS, 2.5% 2-mercaptoethanol, pH 6.8 buffer. Samples were heated at 100 C for 3 minutes. Protein extracts were separated electrophoretically on 12.5% SDS polyacrylamide slab gels containing 0.4% SDS. The stacking gel contained 3% polyacrylamide. Proteins were stained with Coomasie Brillant Blue-R. MW of major protein bands were estimated by distance travelled compared with SDS low standards (Bio-Rad). Total protein was determined by the method of Lowry et al. (14).

Total anthocyanins were determined spectrophotometrically according to previously published methods (15).

RESULTS AND DISCUSSION

Dry weight accumulation of developing cacao embryos is extremely slow during the first 100 days postpollination, but then increases with time at a rate of about 20-40 mg/day, depending on cultivar, until maturity at about 170-180 days (6). The results presented here use dry weight (DW) for purposes of comparison to embryo development, since the embryos analyzed for this investigation were all harvested from different stages on the same day. Dry weight has been used previously to sort embryos into different stages of development (6,7,8).

Fresh weight and protein accumulation were high initially but decreased to constant rates of 0.8 and 0.027 g/g DW,

respectively, above 0.2 g DW (Fig. 1). In contrast, lipid, fatty acid, triglyceride, and alkaloid accumulation was low initially and increased to constant rates of 570 mg, 520 mg, 400 μmoles, and 30 mg per g DW, respectively (Fig. 2 and 3). These results indicate that fresh weight and protein accumulation precedes lipid and alkaloid accumulation during embryo development. The fresh weight, protein, and lipid accumulation in soybean and castor seeds follows this same pattern of development (16,17).

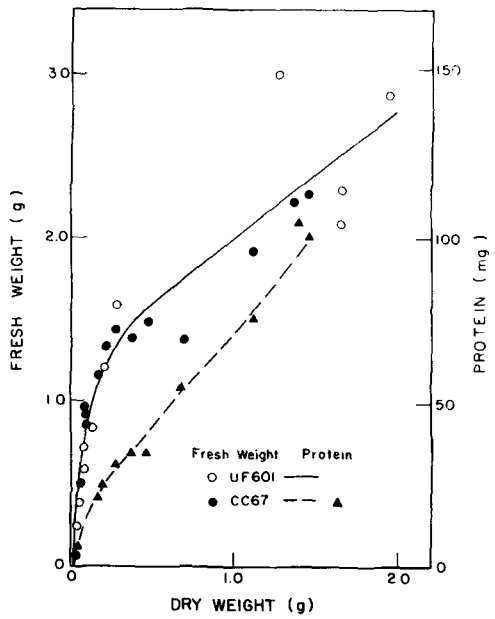


FIG. 1. Change in total fresh weight and protein content as dry weight increases in developing embryos of cacao.

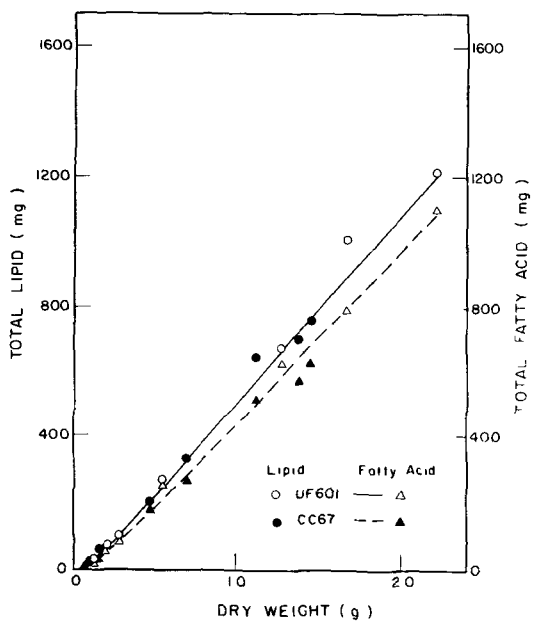


FIG. 2. Change in total lipids and fatty acids as dry weight increases in developing embryos of cacao.

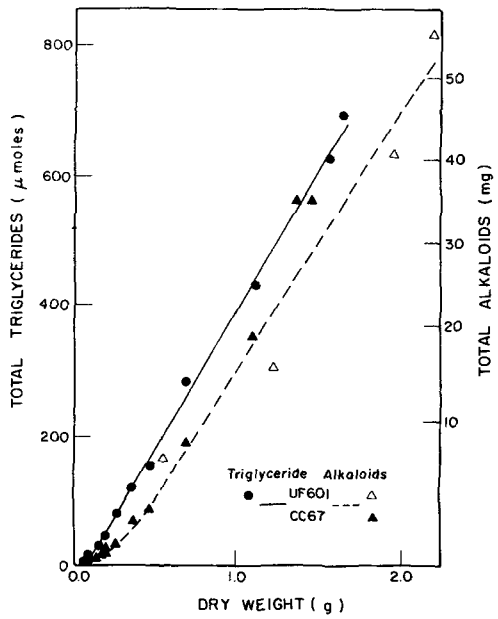


FIG. 3. Change in total triglycerides and alkaloids as dry weight increases in developing embryos of cacao.

The molecular percentage (mole %) of palmitic and arachidic acids based on total fatty acid composition decreased slightly from 33.0 and 1.2 to 29.0 and 1.0, respectively, as DW increased during embryo development (Fig. 4). The mole % of stearic and oleic acids increased from 4.0 and 24.0 to 27.0 and 38.0, respectively, but linoleic and linolenic acid decreased from 33.0 and 3.0 to 4.0 and 0.5, respectively. These results are similar to those of Lehrian and Keeney (6).

Linoleic acid and linolenic acid accumulation decreased from 0.2 and 0.02 μmoles/μmole of total fatty acid below 0.2 g DW to 0.035 and 0.0035 μmoles/μmole of total fatty acid above 0.2 g DW (Fig. 5). Palmitic, stearic, oleic, and arachidic acid accumulation did not change appreciably during embryo development (Fig. 5). The accumulation rate for palmitic acid was 0.285; for stearic acid was 0.270; for oleic acid was 0.390; and for arachidic acid was 0.008 μmoles/μmole of total fatty acid. Thus, the decrease in the rates of accumulation of linoleic and linolenic acid account for the changes observed in the mole % fatty acid composition for cacao embryos (Fig. 4), and suggest that oleic acid desaturation decreases during development. This is in contrast to soybean and castor beans in which polyunsaturated fatty acid accumulation increases during development (16, 17,18).

Monounsaturated triglycerides (POS, POP, and SOS) after a short lag accumulated at constant rates of 0.38, 0.23, and 0.20 μmoles/μmole of total triglyceride, respectively (Fig. 6). Accumulation of polyunsaturated triglyceride (PLO, PLP, POO, and SOO) was about 0.10, 0.05, 0.25, and 0.35 μmoles/μmole of total triglyceride but accumulation ceased before maturation (Fig. 7). Saturated triglycerides such as tripalmitin (PPP) or tristearin (SSS) which represent about 1% of the total triglycerides were not detected using these methods. These results indicate that the dominant monounsaturated triglycerides of mature embryos accumulate during the entire course of development but not polyunsaturated triglycerides.

Theobromine accumulation per g DW increased from 0 to 30.0 mg/g DW for CC 67 and from 0 to 15.0 mg/g DW

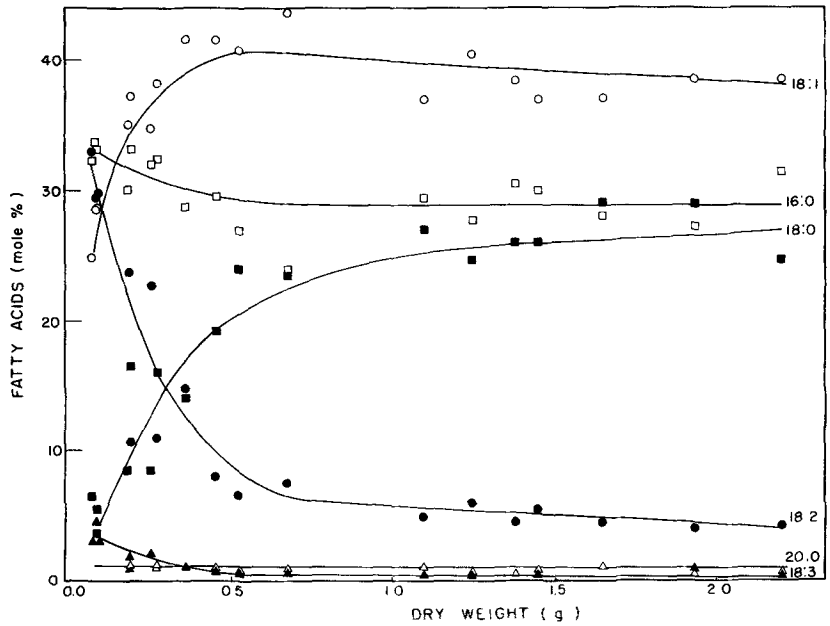


FIG. 4. Change in fatty acid content (mole %) as dry weight increases in developing embryos of cacao.

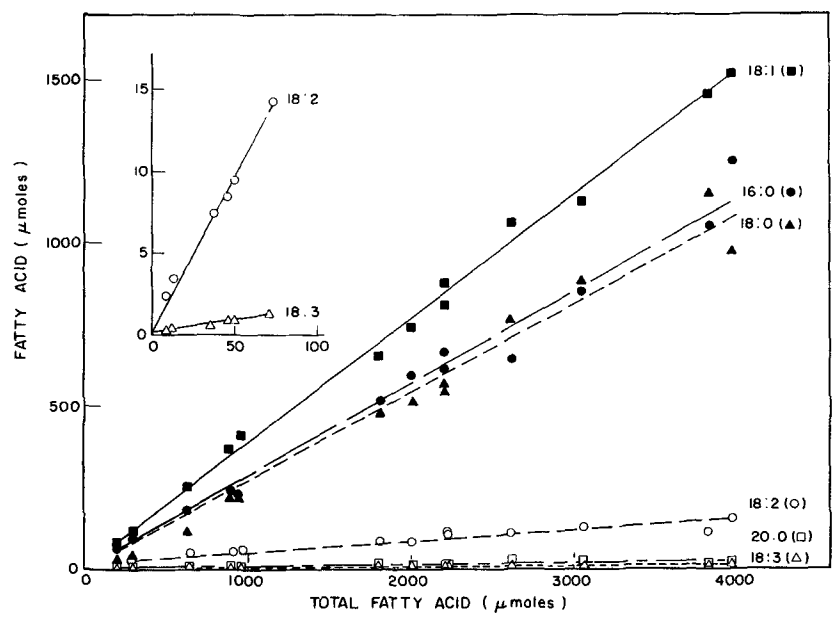


FIG. 5. Relative rate of accumulation of palmitic, stearic, oleic, linoleic, arachidic and linolenic fatty acids as total fatty acids increase in developing embryos of cacao.

for UF 601 (Fig. 8). Caffeine accumulation in both cultivars was identical, although UF 601 accumulated more caffeine because of its higher embryo dry weight. These results are consistent with the observation that the theobromine and caffeine content of different cacao cultivars is variable (19). Caffeine accumulation increased rapidly late in embryo development and some of the genotypic variability in caffeine cotent might represent differences in maturity.

The MW distribution of proteins were similar for both cultivars (Fig. 9). Young embryos (< 100 mg DW) contain a very heterogeneous mixture of proteins with widely diffeent MW, but in older embryos (> 100 mg DW) the protein pattern becomes dominated by 4 major protein species with apparent MW of 43, 34, 22, and 14 kDa. The 22 kDa protein appeared in embryos greater than 100 mg DW, with the 14 and 43 kDa proteins appearing slightly later in embryos greater than 200 mg DW. The 34-kDa protein did not appear until embryos had gained at least 600 mg DW. The sequence of appearance of these 4 protein species can be considered markers for embryo development.

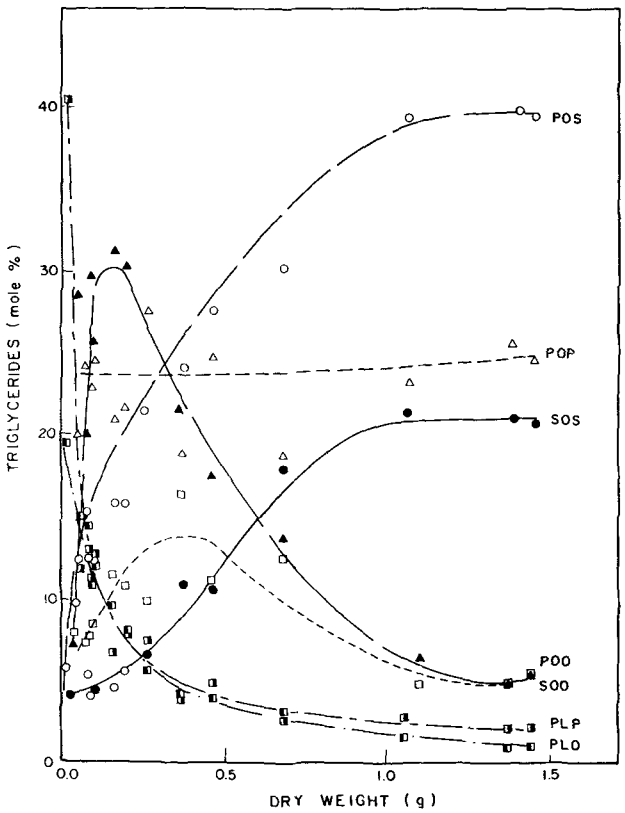


FIG. 6. Change in triglyceride content (mole %) as dry weight increases in developing embryos of cacao.

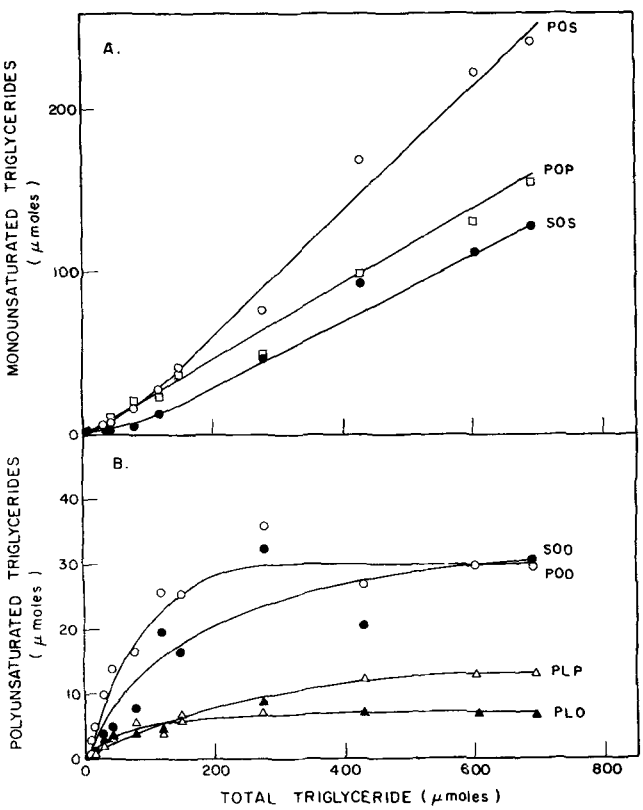


FIG. 7. Relative rate of accumulation of monounsaturated triglycerides (A) and polyunsaturated triglycerides (B) as total triglycerides increase in developing embryos of cacao.

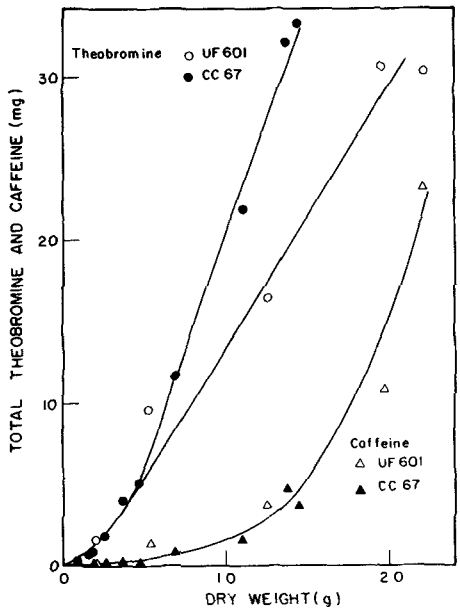
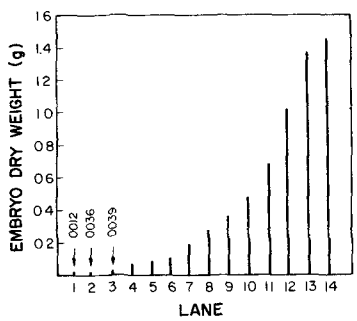


FIG. 8. Change in total theobromine and caffeine as dry weight increases in developing embryos of cacao.



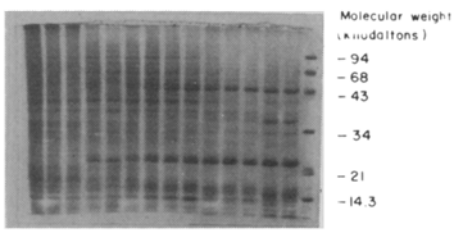


FIG. 9. Change in protein composition in developing embryos of cacao clone CC 67 as dry weight increases.

Total anthocyanins increased linearly with dry weight (Fig. 10). Anthocyanin accumulation cannot be considered a critical marker of embryo development because various cultivars are known which have little or no pigmentation.

Growth of cacao embryos during the first 100 days post-pollination is slow (6); the first cell division occurs at 50 days (20). After this initial lag lipids, alkaloids, proteins, and anthocyanins accumulate almost linearly with increasing dry weight such that the biochemical development of cacao embryos appears indeterminant with respect to dry weight accumulation. Embryo maturity appears to be associated with a cessation of dry weight accumulation which occurs at various times depending on cultivar. If the data was plot-

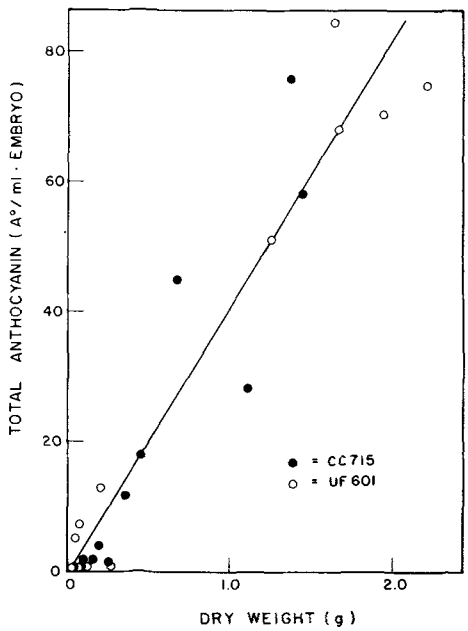


FIG. 10. Change in total anthocyanins as dry weight increases in developing embryos of cacao.

ted against days of development, the accumulation of lipids, alkaloids, proteins and anthocyanins would not be linear but should parallel the dry weight accumulation curve which increases sigmoidally with time (6). Cacao embryo development based on dry weight accumulation with time can be divided into three phases, an initial lag phase (0 to ≅ 100 days), an accumulation phase (100 to ≅ 160 days), and a maturation phase (160 to ≅ 180 days). Our data indicates that the rate of lipid, alkaloid, protein, and anthocyanin accumulation is constant with respect to dry weight in the last 2 phases. These results are similar to that of other oil seed crops (16-18), and suggest that seed maturation is characterized by a decrease in overall dry weight accumulation and not by a qualitative change in dry weight partitioning into seed components such as lipids and proteins. It also suggests that there is a single program for the accumulation of embryo constituents. Studies are underway in this laboratory to determine factors which influence the accumulation of these constituents.

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