Effects of Desiccation on the Recalcitrant Seeds of *Carapa guianensis* Aubl. and *Carapa procera* D C.

K. F. Connor,* I. D. Kossmann Ferraz, F. T. Bonner, and J. A. Vozzo

ABSTRACT

This study was undertaken to determine if the seeds of *Carapa* guianensis Aubl. and Carapa *procera* D C. undergo physiological, biochemical, and ultrastructural changes when they are desiccated; and to find if these changes can be used to monitor viability in Carapa. Seeds were air-dried at room temperature for 7-11 days. Samples were taken at frequent intervals and germination was tested, moisture determined, lipids extracted, and samples taken for electron microscopy. The moisture content (MC) of the embryonic axes remained high throughout the experiment. The cotyledons were drier and had a higher MC variation between individual seeds during desiccation. While Karl Fisher moisture analyses indicated no relationship between axis MC and seed viability, differential scanning calorimetry (DSC) thermograms showed a strong relationship between the melting endotherm peak onset values, enthalpy (heat content) and seed germinability. Both techniques were ineffective in determining changes in seed viability when viability remained above 50%. Analyses of the bulk lipids indicated that changes were taking place, but gas chromatography (GC) results were inconsistent from year to year. Electron microscopy (EM) examinations found that cellular contents of *Carapa* showed little organization when seeds were fresh, but that spherosomes accumulated as desiccation progressed. These data and those from the moisture, DSC and GC analyses, add support to the hypothesis that storage problems of recalcitrant seeds are associated with intact seed MC and with lipid composition, metabolism, and distribution in the cells.

INTRODUCTION

The majority of seeds from temperate forest species are 'orthodox'; they can be dried without damage to a moisture content (MC) of less than 12%. There are, however, seeds from both temperate and tropical forests that are desiccation-sensitive (Roberts, 1973). This characteristic makes any useful period of seed storage extremely difficult. Important tree species of the temperate zone with seeds of known recalcitrant behavior include *Castanea* (Pritchard and Manger, 1990), *Quercus, Aesculus,* and some *Acer* (Bonner, 1990). Numerous tropical tree species have recalcitrant seeds, including some *Hopea, Shea,* and *Dipterocarpus* (Yap, 1986; Tompsett, 1987).

The physiological basis of recalcitrant behavior is not fully understood, although several hypotheses have been proposed. One proposal suggests that

K. F. Connor,* F. T. Bonner, and J. A. Vozzo, USDA Forest Service, Forestry Sciences Lab, P.O. Box 928, Starkville, MS 39760, U.S.A.; I. D. Kossmann Ferraz, Instituto Nacional de Pesquisas da Amazônia, Coordenacão de Pesquisas em Silvicultura Tropical, Manaus AM, Brasil.

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the storage problems are associated with changes in lipid composition or with physical disruption of the seed membranes which can occur as the seeds age or during drying and/or chilling (Flood and Sinclair, 1981; Seewaldt et al., 1981; Priestly and Leopold, 1983). Seed MC is also closely linked to seed decay and death; the critical MC below which recalcitrant seeds should not be dried reportedly varies from 12–31%, depending on the species being studied (Roberts, 1973; Pammenter et *al*, 1991; Wesley-Smith et al., 1992). Farrant et al. (1985, 1988) initially hypothesized that an increasing demand for structure-bound water results in increased desiccation sensitivity. It is now recognized that recalcitrant seed behavior is based on an increasingly aberrant metabolism during hydrated storage (Pammenter et *al* 1994) and as water is lost (Berjak and Pammenter, 1997).

The objective of this study was to examine the physiological, biochemical, and ultrastructural effects of desiccation on tropical recalcitrant tree seeds and relate them to loss of seed viability. Two rainforest species from the Amazon River basin of Brazil, *Carapa guianensis* Aubl. and *Carapa procera* DC., were used in these experiments. These members of the Meliaceae are widely distributed in the moist tropical forests of Central and South America.

MATERIALS AND METHODS

General

Experiments were carried out on C. *guianensis* Aubl. seeds in 1992 and 1994 and C. *procera* seeds in 1993. The seeds were collected regularly from the forest floor near Manaus, Brazil (3°06' South latitude) within 3 days of natural dissemination of fruits, cleaned by hand, and enclosed in plastic bags. The 1992 C. *guianensis* seeds were shipped by air to Starkville, Mississippi, and the 1994 C. *guianensis* seeds were hand-carried to the laboratory. The 1993 shipment of C. *procera* seeds was delayed by a strike and did not arrive at the laboratory for 5 weeks. Unlike the C. *guianensis* seed lots, these seeds were not fully hydrated upon arrival.

Seeds were inspected for damage and imbibed in tapwater overnight at room temperature. Seed size varied greatly (typical of recalcitrant seeds), and it was impossible to use a completely uniform size, but extremely large and extremely small seeds were discarded. Uniform desiccation of the seeds was carried out on a laboratory benchtop at a room temperature of 27±2°C and an ambient RH of 40±10%. Randomly selected seeds were periodically removed to provide subsamples between full imbibition and death of the seeds due to desiccation. Depending on the seed lot, this period ranged from 7 to 11 days. Each seed subsample was divided randomly prior to testing.

Germination

Germination was tested on samples ranging in number from 5 to 20 seeds. The only pretreatment was a 30-second submersion in a 10% solution of sodium hypochlorite (commercial bleach). After a rinse in running tapwater, seeds were placed on moist Kimpak® and incubated under a diurnal cycle of 20°C for 16 h in the dark and 30°C for 8 h with light. A seed was scored as germinated when both radicle and plumule appeared without obvious abnormalities.

Moisture analyses

MC of whole seeds was determined on five or six subsamples of one or two seeds each. The seeds were cut into halves or quarters, and MC was determined by procedures recommended for large seeds with high MCs (Bonner, 1991; Connor et al., 1996; International Seed Testing Association, 1993). MC was expressed as a percentage of fresh weight. To determine distribution of moisture within the seeds, the embryonic axis with surrounding tissue, cotyledon proximal to the axis (PCOT -within 10 mm), cotyledon distal to the axis (DCOT -- opposite side of the seed), and seedcoat material (1992 and 1993 only) were dissected from a randomly selected seed sample, rapidly weighed to the nearest 0.1 mg on an electronic balance, and immersed in 20 ml of anhydrous methanol (MEOH). Sample weights ranged from 10 to 100 mg, but most were in the 15 to 25 mg range. After dehydration in the MEOH for 48 h, MCs were measured on an aliquot of the MEOH by Karl Fisher analysis with an Aquastar® V1B automatic titrator (Association of Official Agricultural Chemists, 1965). Five to ten seeds were used for these measurements each sample day.

Thermal analysis

A Perkin-Elmer@ DSC-7 was calibrated using indium (melting point = 156.6°C) and hexane (melting point = -95.3°C). Fresh embryonic axes with surrounding tissue, PCOT, and DCOT samples were sealed in aluminum pans and cooled from 30°C to - 150°C at 10°C/min. Samples were held at - 150°C for 5 min and then warmed at 10°C/min to 35°C. Melting endotherm onset and enthalpy (heat content) values were determined using instrument software.

Lipid extraction and analyses

C. *guianensis* seed coats were either peeled or cut away from the cotyledon. In 1992, embryonic axes were dissected from all but a small core of cotyledon tissue and analyzed separately; the small number of seeds available in 1994 precluded separate analyses. Samples were extracted and purified in the manner reported by Connor et *a l* (1996) and analyzed on a Hewlett Packard@ gas chromatograph (GC). A 4 mm x 2.44 m glass column packed with GP 3% SP2310/2% SP2300 on 100/200 Chromosorb W AW (Supelco®, Inc., Bellefonte, PA) was used for the 1992 analyses. A J & W Scientific@ DB-23 30 m x 0.53 mm x 0.5µ megabore column was used to analyze the 1994 samples. Response factors were calculated from injections of AOCS oil reference mix no. 3 (Sigma Chemical Co., St. Louis, MO). GC analyses of the lipids of C. *procera* were not done because of the low initial germination values of the seeds.

Electron microscopy

Seeds of 1992 and 1994 C. *guianensis* and 1993 C. *procera* were taken at random at 0, 1, 3, 5, 7, and 11 days from the bulk collection of drying seeds. The embryonic axis was extracted as were 3-mm^3 plugs of cotyledon tissue. All sections were immediately placed in a 2.5% glutaraldehyde solution of pH 7.2 phosphate buffer. Tissues were fixed in osmium tetroxide, dehydrated in ace-

tone, embedded in Epon®, sliced at 0.5 to 0.75 μm, and stained with uranyl acetate as reported in Vozzo and Song (1989). Thick sections were observed at 1.2 MeV using high-voltage transmission electron microscopy (HVEM) to determine the effects of desiccation on cell ultrastructure and membrane integrity.

RESULTS AND DISCUSSION

The purpose of this study was to determine if a pattern existed in MC, GC, EM, and DSC data collected from desiccating tropical *Carapa* seeds. Analyses of seed tissues using the above techniques indicate that all may offer possibilities for monitoring seed desiccation and deterioration in ***Carapa*** spp.

Germination

In 1992 and 1994 C. ***guianensis*** had high initial germination (80% and 69% respectively; Table 1). After 10 days of desiccation in 1992, germination had dropped to 10%, while in 1994 it was 0% after 11 days. In 1993 C. ***proceru*** seeds were subjected to a 5 week delay in shipping and had a low initial germination (33%), which dropped to 8% by the third day. While it is noted that recalcitrant seeds can be shed at different stages of maturity (Berjak and Pammenter, 1997) and that moisture loss during shipping can adversely affect germination, the good initial germination of the *Carapa* ***guianensis*** seed lots is indicative of successful collection and handling techniques.

Moisture content

Initial MCs of whole seeds were very high. Seeds of both C. ***guianensis*** seed lots had initial MCs of about 50%, while the comparable value for whole seeds of C. ***procera*** was near 40%. Variation among individual seeds was high, as expected for large seeds with high moisture levels (Bonner, 1984).

Moisture analyses of whole seeds and individual tissues during desiccation are illustrated by the 1994 measurements on C. ***guianensis*** (Figure 1). Results of MC for the other year and species (not shown) were similar. Karl Fisher analyses indicated that the highest MCs throughout the experiment were in

TABLE 1. Germination (%) of *Carapa guianensis* (1992/1994) and *Carupa procera* (1993) seeds.

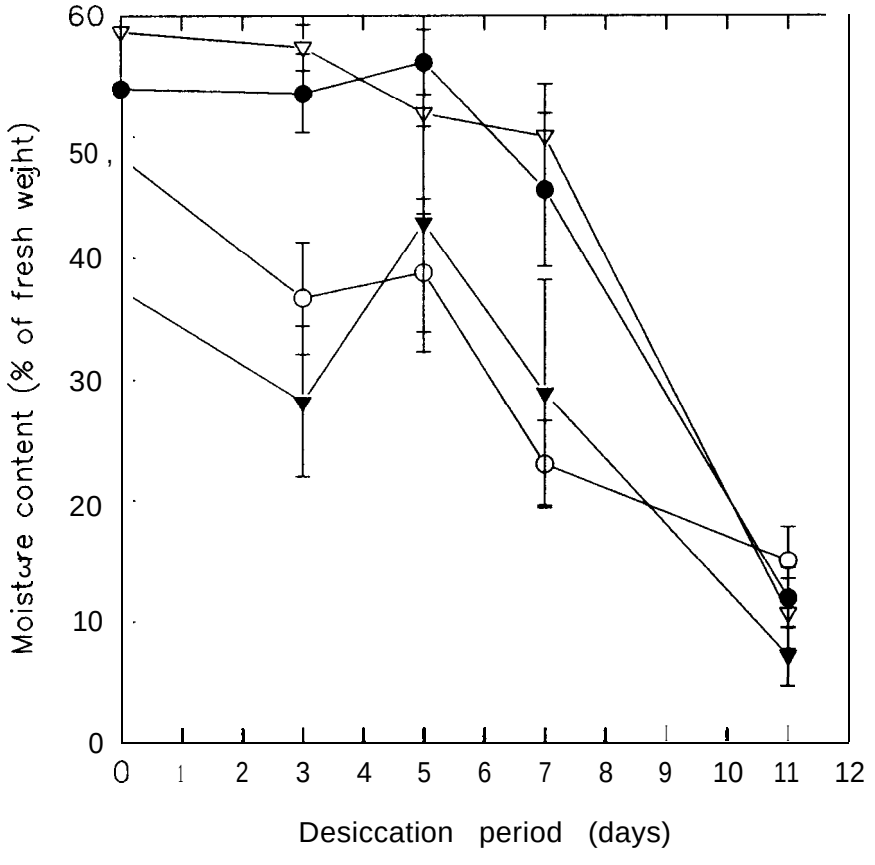
Drying Period (days)	Germination (%)		
	1992	1993	1994
0	80	33	69
3	70	8	42
5	60	0	15
7	40	0	5
10	10	0	–
11	–	–	0

the axes and **PCOT** tissue **(55** to **60%).** MC of the DCOT tissues was initially less than 40%, and except for an unexplained high mean value on day 5, it decreased at about the same rate as the other tissues. The seed coats' MCs were initially 28 to 30%, but declined to a minimum of 10% after only one day of desiccation (data not shown).

Both species exhibited a general gradient of moisture from axis/PCOT→ DCOT→ seed coat, and this relationship essentially stayed the same throughout desiccation. However, complete distinction between axis and PCOT moisture was impossible because of the way the axes are embedded in the fused cotyledons.

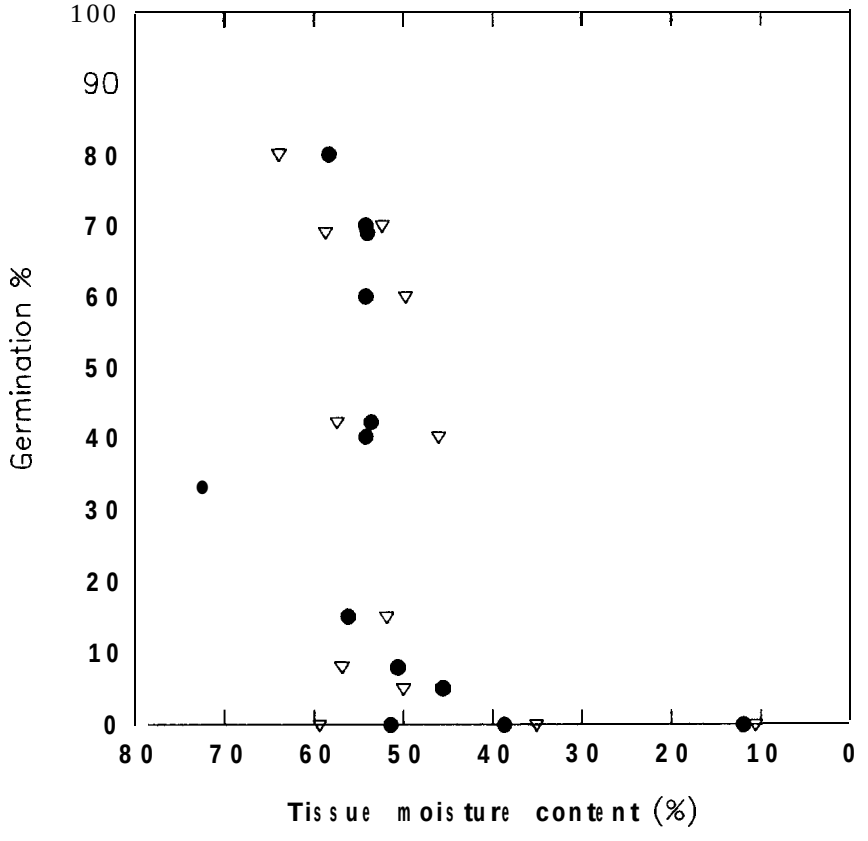
The relationship between axis MC and loss of germination was of special interest. In 1994, C. *guianensis* germination fell to 15% at day 5 (Table 1), but axis moisture was still above 50% (Figure 1). On day 7, when germination was

FIGURE 1. Seed moisture for 1994 ***Carapa guianensis*** intact seeds (○), axes (●), proximal (PCOT) (▽) and distal (DCOT) (▼) cotyledon tissue during desiccation period.



only 5%, axis moisture was still 45%. Axis and PCOT moisture contents for both species in all years were plotted against the respective germination values at all stages of desiccation. Surprisingly, the results strongly suggest that loss of viability was not related to axis or PCOT MC (Figure 2). Germination declined from around 80 to below 10% with axis MCs still averaging above 50%. The two outlying points (33% germination) in Figure 2 are from the C. ***procera*** sample that was delayed in shipment. These low germination values could have been caused by something other than desiccation. This is contrary to most information available on recalcitrant seeds, which stress critical MCs for maintaining seed viability. In the case of ***Carapa,*** it may be that the critical moisture content is very high, and it is also advisable to use intact seed or DCOT MCs, rather than axis or PCOT moisture, as more reliable indicators of seed viability. DCOT and intact MCs changed over the course of the exper-

FIGURE 2. Relationship between C. *guianensis* and C. *procera* moisture contents in axis (●) and proximal cotyledon (▽) and seed germination during desiccation.



iment; it may be that the axis and PCOT tissues are such strong moisture sinks that they are rendered useless as viability indicators.

Thermal Analyses

The moisture data on the insensitivity of *Carapa* axis tissue is not supported by the DSC data. In DSC whole-tissue analyses, the decline in enthalpy values as desiccation progressed was consistent and reproducible (Table 2). Unlike the moisture analyses, the axis tissue proved to be the most sensitive to moisture loss. While DCOT and PCOT tissue produced high enthalpy values until germination was below 15%, axis measurements showed a significant drop between days 3 and 5, when seed germinability fell from 42 to **15%.**

DSC peak onset values also proved sensitive to moisture loss and germinability decline in both axis and cotyledon tissues. Onset values for all tissues were close to zero or above zero until day 5, when they sharply declined (Table 2). This corresponded to the seed viability drop between days 3 and 5. Thus, while only axis enthalpy values accurately indicated a decline in seed viability, the onset values of all tissues of C. *guianensis* corresponded to declining germinability. When onset values dropped well below zero, the ability of the seeds to germinate also sharply declined. This is an expected result since onset values reflect the water content in the seeds. However, like the moisture tests of axis and PCOT, the DSC proved ineffective in pinpointing changes in viability when viability remained above 50%.

A sharp, secondary peak in DSC thermograms of *Carapa* tissues occurred at 0, 5, and 7 days. This peak (Figure 3) was also found by Pammenter *et al.* (1991), Vertucci *et al.* (1991), and Berjak *et al.* (1992) in *Landolphia kirkii* Dyer seeds. Pammenter *et al.* (1991) ascribed it to the presence of almost-pure water, and Vertucci *et al.* (1991) noted that, if the peak was present in the melting endotherm, the axis which produced it failed to survive in tissue culture. These sharp peaks were found in both cotyledon and axis tissues in all *Carapa* samples through day 7 of the experiment. Similar to Pammenter *et al.* (1991),

TABLE 2. Melting endotherm onset and enthalpy values for 1994 *Carapa guianensis* embryonic axes and cotyledons.

Drying Period (days)	Axes		PCOT†		DCOT‡	
	Onset	Enthalpy	Onset	Enthalpy	Onset	Enthalpy
	(°C)	(J/g)	(°C)	(J/g)	(°C)	(J/g)
0	3.4	179.1	1.4	197.6	3.2	142.0
3	1.9	177.4	-0.2	204.2	0.4	148.5
5	-6.6	84.6	-5.4	114.6	-4.6	131.7
7	-10.7	51.9	-10.7	91.7	-2.5	103.4
11	–	–	-23.7	14.4	-7.4	91.0

† PCOT = cotyledon tissue proximal to the embryonic axes.
‡ DCOT = cotyledon tissue distal to the embryonic axes.

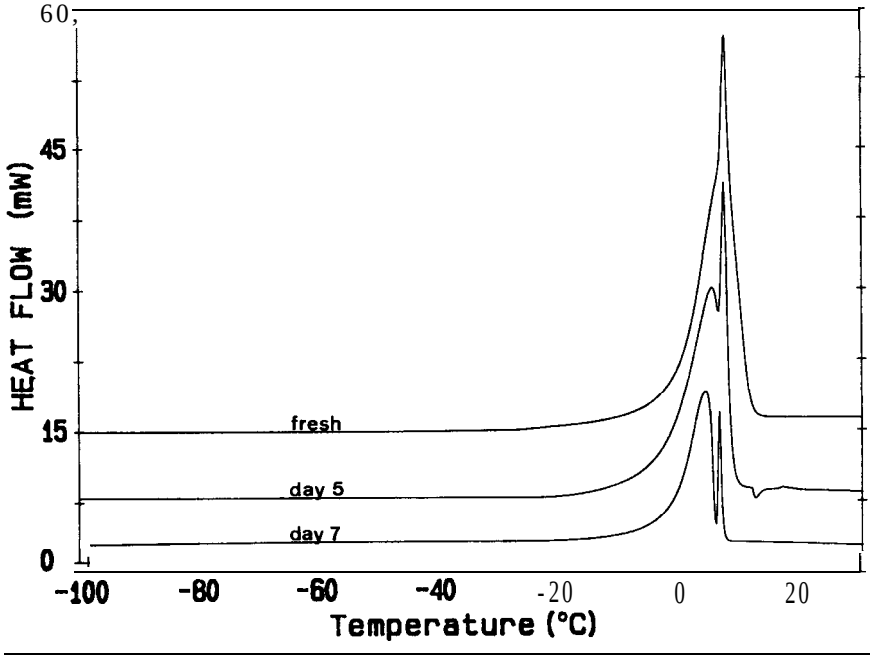
it was found that enthalpy declined with moisture content and that drying resulted in damage to the seed.

Lipid analyses

A petroleum ether Soxhlet extraction performed on lyophilized C. *guianensis* cotyledon tissue yielded 0.486 g of lipid/g dry weight. In both axes and cotyledons, palmitic acid was the most common saturated fatty acid ($\bar{x}$ = 26.4%), and oleic acid was the most prevalent unsaturated fatty acid, accounting for about 50% of the total fatty acid content in the cotyledons and 40% in the axes. GC analyses of the fatty acids of 1992 C. *guianensis* cotyledons showed no clear pattern of change in the percentages of fatty acids in the deteriorating seeds (Table 3). Fluctuations of individual fatty acids occurred as the 1992 seeds dried, but in an apparently random fashion, resulting in low coefficient of determination (r^2) values when the percent fatty acid was regressed on seed germination. However, the data for 1994 cotyledons showed a decline in the major saturated fatty acids and an increase in oleic acid (Table 3) as viability declined. Thus, the r^2 values for the saturated fatty acids, palmitic and stearic, were 0.60 and 0.84 respectively, while oleic acid was 0.98.

This inconsistency in GC data makes interpretation of the results difficult and lessens the value of the data when it is related to seed viability. It is not the

FIGURE 3. DSC warming thermograms of *Carapa guianensis* distal cotyledon tissue at day 0 (fresh), 5, and 7 of desiccation. The small secondary peak was present through day 7.



first time this difficulty has been encountered. While Harman and Mattick (1976) reported reductions in unsaturated lipids in aged pea seeds, Priestly and Leopold (1979) found no changes in soybean fatty acids with seed deterioration. Connor et al. (1996) also reported inconsistent changes in naturally aged *Quercus* spp. acorns.

Electron microscopy

There were no major ultrastructural distinctions seen between C. *guianensis* and C. *procera* during these trials. The micrographs showed differences for both embryos and cotyledons before and during the drying treatments, but both species had similar cellular responses.

A micrograph of a parenchymatous axis cell from a control treatment having loosely distributed cytoplasm, intact cell walls, and small spherosome

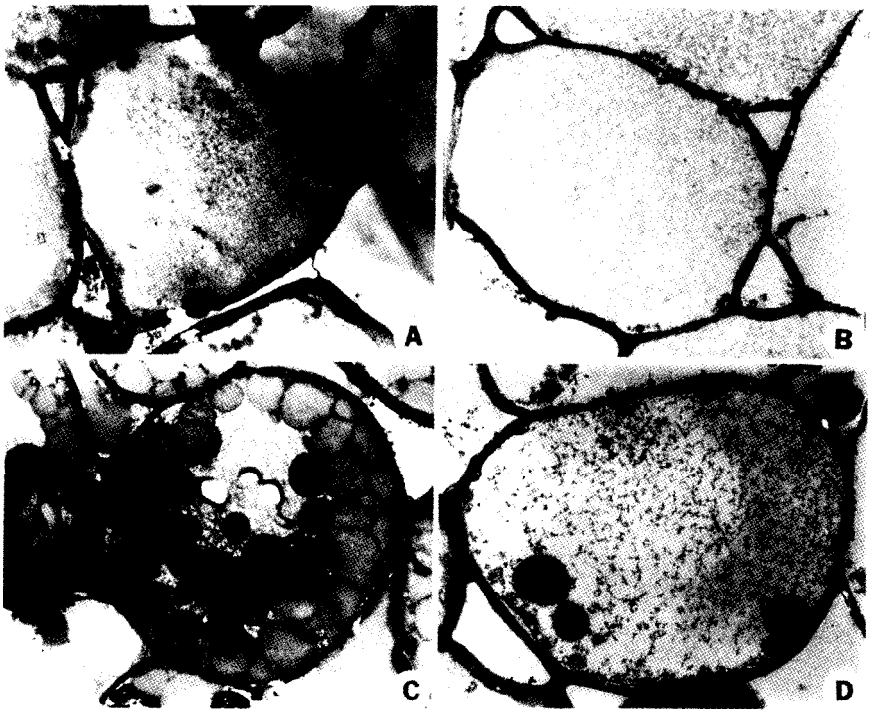
TABLE 3. Fatty acid composition in bulk lipid samples from 1992 *Carapa guianensis* cotyledons and axes and 1994 cotyledons. Standard deviations are in parentheses.

	Day	Palmitic 16:0	Stearic 18:0	Oleic 18:1	Linoleic 18:2	Linolenic 18:3	Arachidic 20:0
		(%)					
1992 Cotyledon	0	**23.0**	7.0	**53.6**	**15.0**	0.3	1.0
		(.2)	(–)	(.0)	(.2)	(.0)	(.0)
	5	**20.6**	5.0	**50.0**	**24.1**	0.3	0.0
		(.7)	(.1)	(.1)	(.2)	(.3)	(–)
	7	**26.1**	16.2	**54.6**	**11.5**	0.3	1.0
		(.0)	(.0)	(.1)	(.0)	(–)	(.1)
1992 Axes	0	**23.7**	9.1	**34.1**	**26.6**	1.4	1.3
		(.1)	(.5)	(.2)	(.1)	(.0)	(.1)
	7	**21.7**	4.8	**46.6**	**22.0**	0.6	0.6
		(.3)	(.1)	(.2)	(.1)	(–)	(.0)
1994 Cotyledon	0	**31.4**	7.9	**46.5**	**10.6**	0.0	0.0
		(2.3)	(.4)	(4.0)	(.7)	(–)	(–)
	3	**32.8**	6.6	**49.1**	9.5	0.0	0.0
		(.1)	(.0)	(.1)	(.0)	(–)	(–)
	5	**30.4**	6.0	**51.2**	9.9	0.3	1.6
		(.8)	(.2)	(.9)	(.1)	(.0)	(.4)
	7	**28.4**	6.4	**51.8**	**11.7**	0.2	0.8
		(.2)	(.1)	(.3)	(.0)	(.0)	(.0)
	11	**26.1**	5.4	**53.3**	**12.7**	0.3	0.9
		(.1)	(.4)	(.3)	(.0)	(.0)	(.1)

bodies is shown in Figure 4A. The cell appeared flaccid and perhaps under turgor stress. This was typical of many tissue sections and may have been due to shipping conditions of the samples. Germination tests, however, indicated that this is no serious challenge, at least during a short transit period. (Note that cotyledon cells withstood shipping conditions better than axis cells.) Typically, the cotyledon cells appeared similar to cells of the axis before beginning a drying regime.

A typical cotyledon cell of the control treatment is shown in Figure 4B. After drying for 7 days, both embryonic axis cells (Figure 4C) and cotyledon cells (Figure 4D) exhibited differences from the control seeds. There was still a generally unorganized cytoplasm, but spherosomes began to enlarge and accumulate during drying. Cotyledon cells became more rounded and full as the lipid bodies became prominent. Cotyledon cells (Figure 4D) show the enhanced spherosomes, but not the accumulation as seen in axis cells (Figure 4C). As the lipid bodies became evident, respiration rates probably followed that of temperate recalcitrant *Quercus nigru* L., which relies on similar liposomes as its primary respiration substrate (Bonner and Vozzo, 1987).

FIGURE 4. Parenchyma cells of *Carapa* seeds for the control and after 7 days ambient drying treatments. (A) control, embryonic axis cells; (B) control, cotyledon cells; (C) embryonic axis cells after 7 days; and (D) cotyledon cells after 7 days. All x2500 magnification.



Would results have been different if other desiccation rates had been used? Pritchard (1991) suggested that loss of viability during desiccation of recalcitrant seeds is related to desiccation rate. Bonner (1996) found that at 27°C, rate of desiccation made no difference in the lethal moisture level for intact seeds of Quercus nigra L. Pammenter et *al* (1984) reported that rapid drying favored viability retention in excised embryos of *Avicennia marina* Firsk.. However, excised embryos do not always react the same as intact seeds during desiccation (Bonner 1996), so comparisons should be made with caution. Ultrastructural images reported here did not support either argument. They did, however, fundamentally agree with suggestions that storage problems of recalcitrant seeds are indeed associated with lipid metabolism, composition, and distribution in the cells.

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