Physiology and Biochemistry of Recalcitrant *Guarea guidonia* (L.) Sleumer Seeds

K. F. Connor* and F. T. Bonner

ABSTRACT

Investigations of recalcitrant, or desiccation-sensitive, seeds have as yet failed to identify the causes of this phenomenon. Experiments with ***Guarea guidonia*** (L.) Sleumer (American muskwood) were initiated to determine the effects of desiccation on the physiology and biochemistry of the seeds of this tropical tree species. Seeds were air-dried at room temperature for 7 days. At intervals, germination was tested, moisture content determined, and lipids extracted. The bulk lipids, nonpolar lipids, monoglycerides, and phospholipids were analyzed by gas chromatography (GC), thermal characteristics of whole tissue samples were examined using differential scanning calorimetry (DSC), and moisture content was determined using the Karl Fisher analysis. DSC thermograms showed that as moisture content and germinability of seeds declined, so did enthalpy values and onset temperatures of cotyledon tissue and embryonic axes. GC analyses determined that unsaturated fatty acids accounted for approximately 70% of the bulk lipids; however, ratios of unsaturated/saturated fatty acids and amounts of individual fatty acids fluctuated between test periods. Palmitic acid was the most common saturated fatty acid, and linoleic acid was the most prevalent unsaturated fatty acid. Generally, Karl Fisher analyses of seed moisture content offered the best possibility of monitoring seed deterioration during drying.

INTRODUCTION

Seeds have traditionally been divided into two storage behavior classes (Roberts, 1973). Most 'orthodox' tree seeds undergo a period of desiccation before being shed from the tree and thus can easily be stored for long periods of time at low moisture contents and at low temperatures. On the other hand, 'recalcitrant' seeds do not undergo this final maturation drying stage and are extremely sensitive to moisture loss. This reduces their storage potential to a few months at best. Immediate causes of seed viability loss are attack by pathogens and premature germination, followed by desiccation of tender radicles and shoots.

Several hypotheses have been proposed to explain the physiological basis of recalcitrance. Because lipid breakdown was thought to play a major role in deterioration of orthodox seeds (Bewley and Black, 1994), questions about the chemistry of storage reserves of recalcitrant seeds have been raised (Tompsett, 1984). Others suggest that desiccation past a critical moisture stage leads to widespread loss of membrane integrity, a physical disruption of the seed membranes that can occur as the seed ages or during drying and/or chilling

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(Berjak et *al*, 1990; Flood and Sinclair, 1981; Seewaldt et al., 1981; Priestly and Leopold, 1983). Yet another hypothesis stresses the importance of seed moisture; Farrant *et al* (1985, 1988) theorize that an increasing demand for structure-bound water results in increased desiccation sensitivity.

Recalcitrant seeds can be found in both temperate and tropical forests. Important temperate genera with some recalcitrant-seeded species include *Quercus, Aesculus, Acer* (Bonner, 1990), and *Castanea* (Pritchard and Manger, 1990); tropical recalcitrant species are found in *Hopea, Shorea*, and *Dipterocarpus* (Tompsett, 1987; Yap, 1986). The objectives of this study were to report on the recalcitrant nature of *Guarea guidonia* (L.) Sleumer (American muskwood) seeds and to examine biochemical and physiological changes in the seeds as they dried in order to gain insight into the nature of recalcitrant behavior. G. *guidonia*, a member of the *Meliaceae*, is widely distributed in the moist tropical forests of Central America and adjoining regions.

MATERIALS AND METHODS

General

Seeds of G. *guidonia* were collected in 1992 and 1993 in Puerto Rico. The seeds were shipped by air freight to the Starkville, Mississippi laboratory soon after collection. Upon arrival, seeds were soaked overnight to insure full imbibition and desiccated for up to 7 days on a laboratory benchtop at 25 ±2°C and 40 ±10% RH. Fresh seeds and those dried for selected intervals were tested for moisture content and germinability; lipids were extracted for gas chromatographic (GC) analysis; and thermal analyses of tissue samples were performed on a differential scanning calorimeter (DSC). Seed collections were limited to one per year of 650 seeds or less. Experimental replications for each year were based on subsamples from within a single seed lot.

Moisture and germination tests

Whole seed moisture content was determined by drying two samples of 5 chopped seeds in a 105°C mechanical convection oven for 17 h (Bonner, 199 1). Samples were dried in aluminum cans and cooled for 30 min in a sealed desiccator containing Drierite prior to weighing. Moisture content was calculated as a percentage of seed fresh weight.

To determine moisture distribution in the seeds, embryonic axis and cotyledon tissues were dissected, weighed to the nearest 0.1 mg, and immersed in 20 ml of methanol (MEOH) to extract the water. Tissue sample weights ranged from 10 to 60 mg, but most were in the 15 to 25 mg range. Mean values were based on individual measurements of tissue samples from 10 seeds. After 48 h, aliquots of the MEOH were injected into an Aquastar® V1B titrator for Karl Fischer analysis of moisture content (AOAC, 1965).

Thirty-four to fifty G. *guidonia* seeds were randomly selected at each sampling time for the germination test. They were germinated as two replications of 17-25 seeds each on trays lined with Kimpak®/ blotter paper. Trays were placed in a Stults® wet box set on a 20–30°C temperature cycle with 8 h of light at 30°C and 16 h of dark at 20°C. Germination was considered complete

when the cotyledons emerged. Because fungi that were apparently associated with the seed coats were harmful to the germination of the 1992 seeds, coats were removed from seeds in the 1993 germination tests.

Lipid analyses

Seed coats were removed from 17 randomly selected seeds at each sampling time. The selected seeds were then individually chopped, the pieces immediately immersed in liquid nitrogen (LN_2), and the entire sample ground by a LN_2-cooled Wiley mill equipped with a 20-mesh screen. Lipids were extracted in 2: 1 chloroform ($CHCl_3$)/MEOH and purified as described in Connor *et al.* (1996). The resulting bulk lipid sample was dried under a stream of N_2, weighed, and redissolved in 1 ml of $CHCl_3$. A portion of the bulk lipid sample was loaded onto a 10g column of chloroform-washed 100-200 mesh silica gel; nonpolar lipids (NPL) were eluted with $CHCl_3$, monoglycerides (MGL) with 5:1 $CHCl_3$/MEOH, and phospholipids (PL) with MEOH. Samples were esterified using premixed 14% boron trifluoride in MEOH (Metcalfe and Schmitz, 1961) and analysed on a 5880 Hewlett-Packard@ GC equipped with a 4 mm x 2.44 m glass column packed with GP 3% SP2310/2% SP2300 on 100/120 Chromosorb W AW (Supelco®, Inc., Bellefonte, PA). The initial oven temperature of 150°C was held for 8 min and then increased to 190°C at 3°/min, next to 214°C at 4°/min, and then to 240°C at 5°/min where temperature was held for 12 min. Injector and detector temperatures were set at 250°C. Retention factors were calculated from injections of oil reference factor AOCS No. 3 (Sigma Chemical Co., St. Louis, MO). All solvents used in the extractions contained 5 mg/L of butylated hydroxytoluene (Pearce and Abdel Samad, 1980). A petroleum ether Soxhlet extraction was also performed to determine the amount of lipid in a gram of ground, dried seed. Analyses were based on the four GC injections made for each lipid sample.

Thermal analyses

The Perkin Elmer@ differential scanning calorimeter (DSC-7) was calibrated using indium (melting point = 156.6°C) and hexane (melting point = -95.3°C). In 1993, at each sampling time, two seeds were selected for DSC analysis. The embryonic axis and a portion of the cotyledon tissue were dissected from the seeds and individually sealed in aluminum pans. Samples were cooled from 30°C to -150°C at 10°C/min, held at -150°C for 5 min, and then warmed at 10°/min to 35°C. Melting endotherm peak onset temperature and enthalpy (heat content) values were determined using instrument software.

RESULTS

Moisture and germination tests

The 1992 seeds had high initial germination (57%), but viability fell to 3% by day 3 of the desiccation test (Fig. 1). We repeated the experiment in 1993 and are primarily showing data from that year. Even though the 1992 seeds had been surface-sterilized by a 30 second wash of a 10% solution of commercial bleach, fungus killed many of the seeds before germination was com-

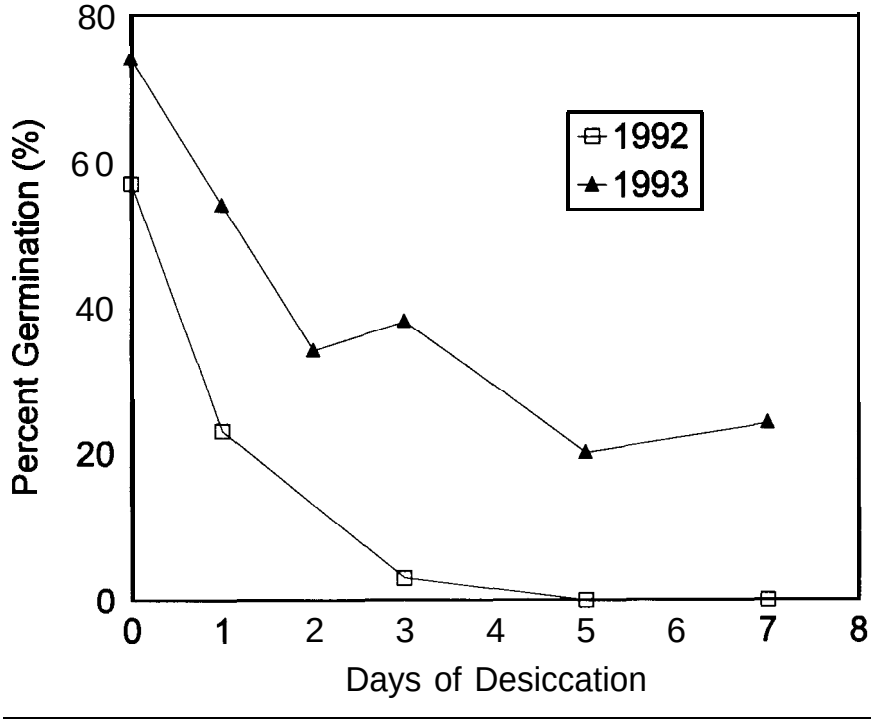
plete. In 1993, in addition to surface sterilization, seed coats were removed prior to testing for germination. As a result, the 1993 G. *guidonia* seed germination was greater than that of 1992 (Fig. 1). The tissue moisture values in 1993 (Fig. 2) were lower prior to the start of desiccation than in 1992 (data not shown). As the 1993 seeds dried, axis moisture content fell below 25% at day 5, and germination fell from 74% to 25% (Fig. 1). At this point there was little difference between axis and whole seed moisture content. Higher moisture contents were recorded on day 7, probably due to a difference in seed size.

Lipid analyses

The petroleum ether Soxhlet extraction yielded 0.1901 g lipid/g dry weight of ground seed material. Nonpolar lipids comprised 90.3% of the bulk lipids, while MGLs and PLs accounted for 6.1% and 3.6% of the total, respectively. In all lipid fractions, palmitic acid was the most common saturated fatty acid, and linoleic acid was the most common unsaturated fatty acid (Tables 1,2). GC analyses were complicated by the presence of some 15 early-eluting peaks. Using GC-mass spectrometry, these compounds were identified as sesquiterpenes.

Since GC analyses of the lipids from 1992/1993 G. *guidonia* seeds were fairly

FIGURE 1. Germination of 1992 (❑) and 1993 (A) *Guarea guidonea* seeds in relation to days of desiccation.



similar, only data from the 1993 analyses are shown (Tables 1,2). As is evident, no clear patterns of change in the fatty acid contents were found in the deteriorating seeds. Fluctuations in amounts of individual fatty acids occurred as the seeds dried, but in an apparently random fashion, resulting in low coefficient of determination (r^2) values when lipid percentage figures were regressed on seed germination.

The bulk and NPL fractions of G. *guidonia* seed had a high linolenic acid content (Table 1) and also contained small quantities of arachidic acid (0.14–0.35 mg/g). The ratio of unsaturated/saturated fatty acids in these lipid extractions was always >2.00, but dropped as low as 1.17 in the PL fraction (Table 2) due to a high palmitic acid and a low oleic acid content.

FIGURE 2. Dynamics of seed moisture for 1993 ***Guarea*** *guidonia* intact seeds (○), embryonic axes (●), and cotyledons (▽) in relation to days of desiccation. Vertical bars represent ± se of the mean of measurements from 10 seeds.

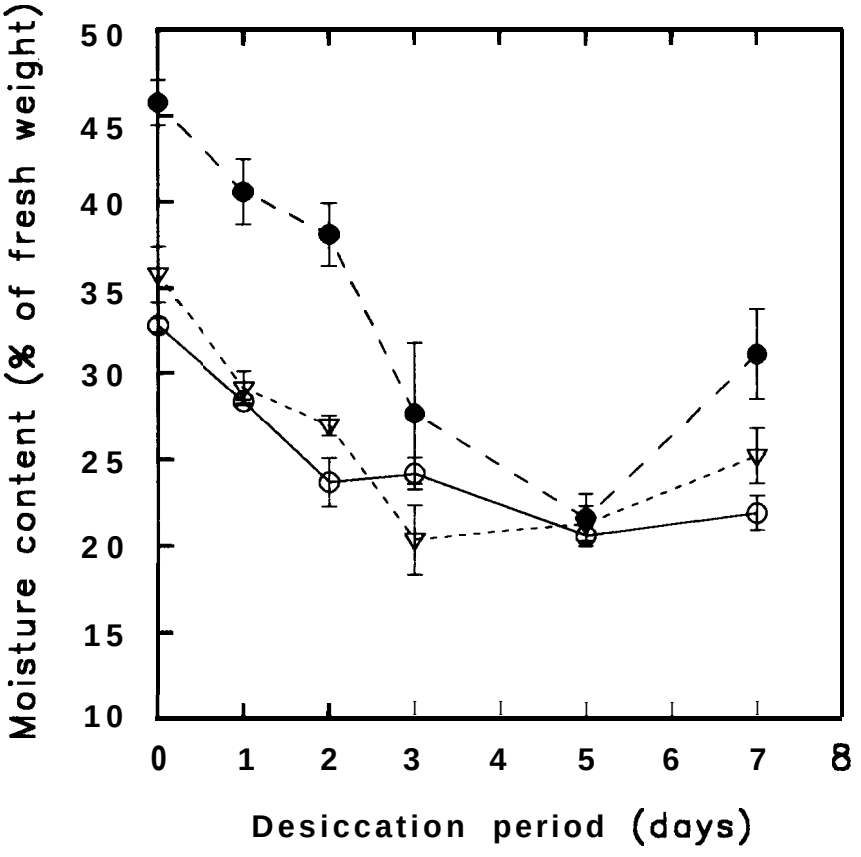


TABLE 1. Percentages of individual fatty acids in bulk and nonpolar lipid (NPL) fractions of 1993 *Guarea guidonia* seeds as affected by days of desiccation.

Fraction	Day	Palmitic	Stearic	Oleic	Linoleic	Linolenic	U/S†
Bulk	0	17.1	10.0	15.7	41.0	15.3	2.68
	1	17.2	9.1	13.1*	42.5*	17.7*	2.79
	2	19.4*	10.3	14.8	42.8*	12.7*	2.52
	3	18.5*	8.7	12.2*	45.1*	15.6	2.80
	5	15.8*	12.5*	11.2*	43.6*	16.0*	2.50
	7	16.5*	11.9*	13.9*	40.5*	16.0*	2.52
NPL	0	16.7	11.3	16.8	38.4	14.5	2.41
	1	16.9	10.7	13.6*	39.6	17.2*	2.55
	2	15.8	10.5	13.7*	39.9	16.8*	2.67
	3	16.7	12.0	13.0*	39.3	16.6*	2.37
	5	15.9	13.8	10.9*	41.6	15.6*	2.29
	7	16.8	12.1	14.7*	39.6	15.6*	2.42

† U/S = ratio of unsaturated/saturated fatty acids.

* = significantly different from the fresh (day 0) amount ($p = 0.01$); significance based on ± se of the mean of 4 injections per sample.

TABLE 2. Percentages of individual fatty acids in the monoglycerides (MGL) and polar lipids (PL) of 1993 *Guarea guidonia* seeds as affected by days of desiccation.

Fraction	Day	Palmitic	Stearic	Oleic	Linoleic	Linolenic	U/S†
MGL	0	17.4	12.2	10.4	47.6	12.1	2.38
	1	16.1*	12.0	9.4*	48.7	13.8	2.54
	2	18.6	12.4	8.2*	47.2	13.4	2.23
	3	15.2*	20.5*	8.8*	43.2*	11.1	1.77
	5	17.6	10.0*	14.3*	38.9*	13.6	2.40
	7	19.9*	13.3*	10.2	36.4*	9.9	1.69
PL	0	30.0	8.3	30.2	25.4	5.1	1.57
	1	36.3*	9.9*	6.4*	38.3*	9.1*	1.17
	2	32.4	9.0	5.7*	42.3*	10.6*	1.42
	3	32.2	10.1*	6.2*	40.7*	10.8*	1.36
	5	21.9*	9.7	7.2*	44.4*	16.6*	2.17
	7	24.6	9.2	22.4*	30.3*	5.6*	1.38

†U/S = ratio of unsaturated/saturated fatty acids.

* = significantly different from the fresh (day 0) amount (p = 0.01); significance based on ± se of the mean of 4 injections per sample.

Thermal analyses

Thermograms of all 1993 ***Guarea guidonia*** tissues, throughout the course of the experiment, had a small, sharp peak that formed on the broad endothermic peak of the main moisture melt (Fig. 3). The small peak had an average enthalpy value of 1.63 J/g, and, in the drying seeds, it became more distinct as the onset of the larger peak shifted to cooler temperatures.

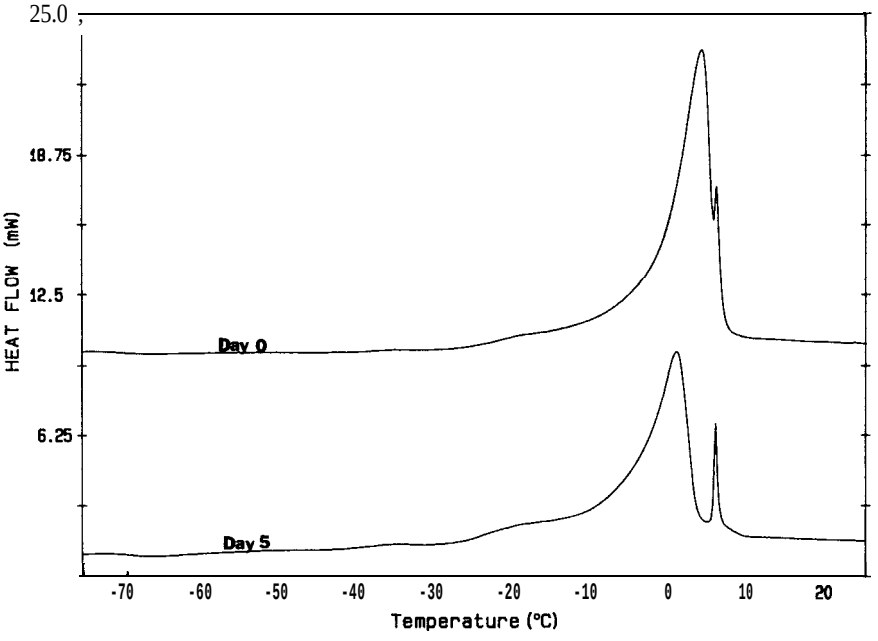
The onset temperature for the broad peak dropped from - 1.23 to - 10.46 in the cotyledon tissue and from -2.85 to -9.23 in the embryonic axes (Table 3). Enthalpy values for the axes were always higher than the corresponding cotyledon values and remained mostly above 100 J/g until germination dropped below 25%.

DISCUSSION

These, experiments were initiated to examine changes in the moisture and lipids of desiccating G. ***guidonia*** seeds and to determine if a biochemical or physiological pattern of change could be detected in the drying seeds. Experiments were designed to discover if relationships existed between seed germinability and any changes so detected.

The lack of consistent shifts in the fatty acid makeup of the G. ***guidonia*** lipid fractions does not lead to conclusions regarding the importance of the

FIGURE 3. DSC warming thermograms of 1993 ***Guarea guidonia*** cotyledon tissue at experiment days 0 and 5. The sharp peak becomes more distinct as the onset temperature of the broad endotherm became more negative.



lipid components in the desiccation process. While slight changes are clearly taking place, they are not consistent from year to year and often fluctuate in a cyclic manner. For instance, GC analyses show that shifts in the unsaturated/saturated ratio obviously are occurring (Tables 1,2); but the random nature of these changes does not permit any firm conclusions on their importance to the germination and desiccation process.

However, GC separations indicate a much higher saturated fatty acid content in the G. *guidonia* PL fraction than in the bulk, NPL, and MGL fractions. It was also found that between-year comparisons of seed lipid data can be a difficult undertaking if there is no way to filter out the probable effects of environmental fluctuations (Dornbos and Mullen, 1992; Rikin *et al*, 1993) and handling procedures on seed chemical composition.

A general drop in DSC enthalpy values and onset temperatures as seeds dry and germination declines has proven a constant feature in desiccating seeds of both temperate-recalcitrant Quercus species (Connor *et al*, 1996) and tropical-recalcitrant *Carapa* species (Connor *et al*, 1998). While G. *guidonia* seeds are recalcitrant, regression analyses reveal that the connection between seed germination and DSC moisture endotherm data is not as strong in G. *guidonia* as it is in these other species. The relationship between germination and the onset temperature and enthalpy values of the embryonic axes yielded r^2 values of 0.51 and 0.25, respectively, while the values for the cotyledons were 0.54 and 0.63, respectively. The r^2 figures for similar analyses of *Quercus* tissues were as high as 0.99, while those from *Carapa* went up to 0.90. These results imply that seed moisture content, while still important to seed viability, may not have as commanding an influence on seed germination in G. *guidonia* as it does in *Quercus* and *Carapa*. However, the importance of moisture to the nature of the recalcitrant seed cannot be denied; like *Carapa* and *Quercw*, *G. guidonia* clearly exhibited a large drop in germination before axis moisture fell below 38%. Although seed did not reach zero viability in 1993, the critical seed moisture content can be estimated at 15-20% .

While DSC moisture content analyses are generally sensitive to declining

TABLE 3. Onset and enthalpy values for 1993 *Guarea guidonia* embryonic axes and cotyledons.

Drying Period (days)	Axes		Cotyledon	
	Onset	Enthalpy	Onset	Enthalpy
	°C	J/g	°C	J/g
0	-2.85	90.27	-1.23	57.17
1	-2.45	116.82	1.88	60.50
2	-4.07	105.72	-4.19	46.69
3	-3.77	103.12	-3.82	51.65
5	-6.52	71.40	-5.42	35.08
7	-10.62	44.69	-10.46	21.42

seed viability, they do not function well as an accurate predictor of seed germinability. For example, high enthalpy values in the embryonic axes would seem good indicators for high seed viability; however, in G. ***guidonia,*** the enthalpy values remain over 100 J/g until germination has dropped to less than 25%. Similarly, in ***Carapa,*** there is not a significant decline in enthalpy values until germination drops below 50% (Connor et ***al,*** 1998).

The DSC data is somewhat contradicted by the Karl Fisher moisture analyses. The relationship between germination and intact seed moisture content is strong (r^2 = 0.99); and embryonic axis moisture content also appears fairly important to seed germination (r^2 = 0.73), although not to the extent it is in ***Quercus*** *spp.* and ***Carapa*** *spp.* Intact seed moisture content may be the fastest, easiest way of estimating G. ***guidonia*** seed viability.

Interestingly, as reported by Pammenter et ***al*** (1991) and Berjak et ***al*** (1992) for ***Landolphia kirkii,*** the presence of a sharp peak whose onset temperature does not change in both embryonic axis and cotyledon tissue (Fig. 3) was noted. However, while Pammenter et ***al*** (1991) reported that the peak disappears at water contents of < 0.5g/g, the G. ***guidonia*** peak is still observed at water contents of 0.2g/g. Similar to Pammemter et ***al*** (1991), the enthalpy generally declines with moisture content (Table 3), and drying results in damage to the seed (Fig. 1).

Despite the DSC moisture analyses, the difference between the germinability of the 1992 and the 1993 seeds might tempt conclusions about the significance of high moisture content to enhanced seed viability. While this might indeed be the case, removing the seed coat prior to testing G. ***guidonia*** seed germination may be more important. Many of the 1992 seeds which initiated germination were killed by fungal attack before cotyledon emergence. Removing the seed coats greatly reduced this problem and led to the survival of a greater number of the seeds. In this instance, this latter action was primarily responsible for the enhanced 1993 germination.

In conclusion, desiccating G. ***guidonia*** seeds lacked systematic changes in the lipid composition and did not exhibit the strong relationships between percent germination and DSC moisture endotherm data found in other species. However, G. ***guidonia*** does have features in common with other recalcitrant-seeded species; viability was reduced by more than 50% only 2-3 days into the experiment, while intact seed moisture content was still at least 24% and embryonic axis moisture was still above 38%. A high seed and axis moisture content does not appear uncommon even after seed viability has fallen below 20%. Both DSC and GC studies demonstrate that changes are occurring in the moisture content and lipid fraction as germinability decline, but the random nature of the lipid changes make it impossible to draw firm conclusions concerning their importance in these recalcitrant seeds.

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