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Recalcitrant Behavior of Cherrybark Oak Seed: An FT-IR Study of Desiccation Sensitivity in *Quercus pagoda* Raf. Acorns

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ABSTRACT

The recalcitrant behavior of cherrybark oak (*Quercus pagoda* Raf.) acorns was examined in terms of effects of moisture content on seed storage longevity and (short term) seed germination. Seed samples collected over two consecutive years were fully hydrated, then subjected to drying under ambient conditions of temperature and relative humidity on the lab bench and sampled regularly for moisture determination (gravimetric analysis) and germination (greenhouse conditions). Fourier transform infrared spectroscopy (FT-IR) was used to follow changes in macromolecular structure as moisture and viability were lost. Transmission spectra were collected on dry and rehydrated samples of separate embryonic axis and cotyledon tissue. Long-term storage longevity was highly dependent on initial acorn moisture content. Germination was also highly dependent on short-term moisture content, and severely declined when seed moisture dropped below 17% (fresh weight basis). FT-IR analyses revealed significant differences in moisture and lipid profiles between embryonic axis and cotyledon tissue during short term drying. A strong absorbance near 1740 cm^{-1} in cotyledon tissue indicated a high concentration of ester carbonyl groups (storage lipids). Membrane lipid structure exhibited reversible shifts between gel and liquid crystalline phases upon drying and rehydration in both axes and cotyledons (peak frequency and bandwidth near 2850 cm^{-1}); however, reversibility declined as viability was lost. Irreversible changes in protein secondary structure, illustrated by shifts in the amide absorbance near 1650 cm^{-1}, were the most sensitive indicators of viability loss.

INTRODUCTION

Seed storage behavior has been divided into two categories (Roberts, 1973): orthodox and recalcitrant. Orthodox seeds undergo a period of desiccation prior to being shed from the plant and can be stored for long periods of time at moisture contents less than 12%. Recalcitrant seeds, however, do not go through a maturation drying phase and are extremely sensitive to moisture loss, thereby granting them poor storage longevity. Recently, recalcitrance has been redefined by the work of Pammenter et al. (1994) and Berjak and Pammenter (1997), who emphasized the importance of damage caused by aberrant metabolic processes as water is lost from seed tissues.

Some temperate forest tree genera with recalcitrant seeds are *Castanea*

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(Jaynes, 1969; Pritchard and Manger, 1990), *Quercus* (Bonner and Vozzo, 1987), *Aesculus*, and some *Acer* species (Bonner, 1990); however, the pathways and mechanisms that determine seed recalcitrance may vary from genus to genus and even between species within a genus (Tompsett, 1984; Hong and Ellis, 1990). The genus *Quercus*, for example, has two subgenera which have different seed storability traits: the acorns of the white oak subgenus, Lepidobalanus, germinate soon after seed fall and cannot be stored for more than a few months (Rink and Williams, 1984), while acorns of some red oak subgenus (Erythrobalanus) species can be stored for greater than one year although viability loss may be high (Bonner and Vozzo, 1987; Connor and Bonner, 1999).

Cherrybark oak (*Quercus pagoda* Raf.), a red oak, is an economically important bottomland hardwood species. It is among the largest of the southern red oaks, reaching heights up to 39 m and diameters of 0.9–1.5 m. While it is native to the southeastern coastal plain and Mississippi Delta, cherrybark oak favors well-drained soils in these regions and grows poorly on wet or flooded sites. The seeds have a high fat content, and, like those of most red oaks, are an excellent source of food for wildlife and birds. If properly handled and stored fully hydrated, cherrybark acorns can remain viable in cold storage for more than a year. If improperly handled and moisture is lost from the acorns, viability declines rapidly. While the characterization of recalcitrant behavior among species is highly documented, the exact biochemical mechanism of seed death by desiccation remains unknown.

Fourier transform infrared spectroscopy, FT-IR, has proved to be a particularly useful technique to study qualitative and quantitative changes in macromolecular structure in intact biological tissues, including plant propagules such as seed and pollen (Golovina et al., 1997; Sowa et al., 1991; Wolkers et al., 1999). Advances in sampling techniques and data handling facilitate study *in vivo* and *in situ*, to allow the examination of intact tissue and to avoid spectral artifacts that might arise from sample manipulation. Many macromolecules contain infrared-active functional groups, so that the full-frequency spectrum collected by an FT-IR can provide a wealth of structural information. Studies have been published that describe changes in membrane lipid structure in seeds and pollen (Crowe et al., 1989) as well as changes in proteins and metabolism (Sowa and Connor, 1995). Frequencies of FT-IR vibrations between 3100 and 2800 cm^{-1} are used to study C-H stretching of membrane lipids, specifically the symmetric and asymmetric $-CH_2-$ (2850 and 2920 cm^{-1}) and $-CH_3$ (2955 and 2865 cm^{-1}) groups, as well as the -C=C- bond stretch near 3010 cm^{-1}. Shifts to lower frequency, accompanied by narrowing of the absorbance bands, are associated with a loss in membrane fluidity, i.e. a phase change from liquid crystalline to gel (Casal and Mantsch, 1984). Ester carbonyl groups absorb infrared radiation near 1740 cm^{-1}.

Storage lipids, or triacylglycerols, have three such ester bonds per molecule. The amide bonds of proteins exhibit IR absobances between 1700–1500 cm^{-1} that can be used to identify types of secondary structure (Golovina et al., 1997). Many other dipoles can be used to identify macromolecular structure

with infrared absorbances in the 'fingerprint' region of frequencies (400–1800 cm^{-1}). We have also used the FT-IR measurement of metabolic CO_2 production as a viability indicator in cell suspension cultures (Sowa and Towill, 1991).

In this study we document the sensitivity of cherrybark oak seed viability to moisture content on both long-term and short-term bases. We provide evidence of the extreme sensitivity of acorn storability to initial seed moisture content and also examine changes associated with loss of viability upon desiccation and rehydration of both cotyledon and embryonic axis tissue using FT-IR spectroscopy.

MATERIALS AND METHODS

Seed materials

Acorns were collected locally in Starkville, MS (Year 1) and purchased from a commercial seed company the following year (Year 2). Upon receipt in the laboratory they were fully hydrated by soaking overnight in tap water, and then stored at +4 °C until the start of the experiments.

Determination of seed moisture content

Moisture contents of whole acorns were determined gravimetrically on five samples of 3–5 seeds each as recommended by the ISTA (International Seed Testing Association, 1993). Acorns were chopped into small pieces (4–8 each) and the tissue was placed in aluminum cans, weighed, then dried overnight (18 h) in a mechanical convection oven at 103 $\pm$ 2 °C. Moisture contents were expressed on a fresh weight basis.

Germination

One hundred randomly selected acorns were germinated in two replications of 50 seeds each. Seeds were imbibed overnight in tap water, then the lower half of each seed was cut off horizontally, and the cup scar portion discarded. The pericarp was removed from the remaining half, which was then placed cut side down (embryonic axis side up) on moist Kimpak and incubated either under a diurnal cycle of 20 °C for 16 h in the dark and 30 °C for 8 h with light in a germination cabinet or under greenhouse conditions for up to 4 weeks, with germination tallied weekly. An acorn was scored as germinated if both the radicle and shoot had emerged and exhibited normal morphology and growth.

Long-term storage

Before storage, acorns from Year 2 of the experiment were imbibed in tap water overnight and visibly damaged acorns and floaters were discarded. Acorns were then spread on a laboratory counter and allowed to surface-dry. Half of the acorns was counted into 150-acorn lots and stored in plastic bags at +4 °C and –2 °C. The other half was allowed to dry on the lab bench for two days. Moisture content and germination were recorded prior to storage. Moisture contents of whole acorns were determined on three subsamples of 3 each, while germination was determined from 2 replications of 50 seeds each. Acorns were retested for viability and moisture content at yearly intervals.

Short-term desiccation/infrared spectroscopy

Short-term desiccation experiments were conducted on acorns harvested for the two consecutive years. On Day 0 of the desiccation experiment, seeds (presoaked overnight) were spread in a single layer onto blotter paper on the lab bench to dry. Laboratory conditions of temperature and relative humidity were monitored using a hydrothermograph. Subsamples of fresh seed were taken for the following analyses: moisture content, germination under greenhouse conditions, and FT-IR spectroscopy. Moisture content was determined on 5 subsamples of 3–5 seeds each. Germination was determined on two replications of 50 seeds each, with greenhouse counts made for up to 4 weeks.

FT-IR spectra were recorded of thin slices of cotyledon tissue and of embryonic axis squashes (3–5 axes) that were placed between CaF_2 windows of a demountable transmission cell. A minimum of duplicate samples was analyzed. For each spectrum, 512 scans at 2 cm^{-1} resolution were collected and averaged on a Nicolet 20 DXB spectrometer using a liquid nitrogen-cooled MCT-A detector. Single beam spectra were ratioed against an open beam background to yield transmission spectra.

The sampling schedule for 'even' days (2, 4, 6, 8) involved fresh weight determination for moisture analysis, FT-IR analysis, and soaking acorns (150) for germination on the next 'odd' day (3, 5, 7, 9) so that spectra were recorded for both dry and rehydrated samples. Sampling continued until seed moisture content dropped below 15%.

RESULTS

Effect of seed moisture content on cherrybark oak germination after long-term storage

Cherrybark storage longevity proved to be extremely sensitive to seed moisture content. Viability dropped significantly in the 'dry' acorns (sample 2) after one year of storage at both temperatures (Table 1). In fact, it was almost completely lost (down to 5%) compared to a slight loss to 88% germination in the 'wet' seed when stored at +4 °C. Storage temperature had a much smaller effect

TABLE 1. Cherrybark oak seed germination after long term storage.

	Storage Temperature +4 °C			Storage Temperature −2 °C	
	day 0	1 Y	2 Y	1Y	2Y
Sample 1 "Wet"					
mc (%)	29.6	25.2	28.37	26.01	29.80
± sd	1.7850	1.1423	3.3755	1.3548	1.1581
germination (%)	100	88	67	97	76
Sample 2 "Dry"					
mc (%)	19.9	19.89	21.43	17.84	19.84
± sd	0.667	1.4451	1.1753	0.8579	0.1879
germination (%)	98	5	0	22	0

on viability, but –2 °C did have a slight advantage, with practically no loss after one year in the 'wet' seed, yet significant loss in the 'dry' seed to 22%.

Short-term desiccation

Seed moisture content. Seed moisture content declined from an average of 31.6% to 12.1% within six days of drying on the lab bench (Table 2), where

TABLE 2. Short-term desiccation of cherrybark oak acorns.

	Seed Moisture Content (%)				
	Day 0	Day 2	Day 4	Day 6	Day 8
Year 1	29.70±0.9390	20.77±0.9526	16.69±1.695	10.63±0.7659	n.d.
Year 2	33.52±0.7663	23.79±1.386	17.49±0.5510	13.48±1.024	11.03±0.1685

FIGURE 1. Cherrybark oak seed moisture loss after days of drying under ambient laboratory conditions. Moisture is expressed on a fresh weight basis.

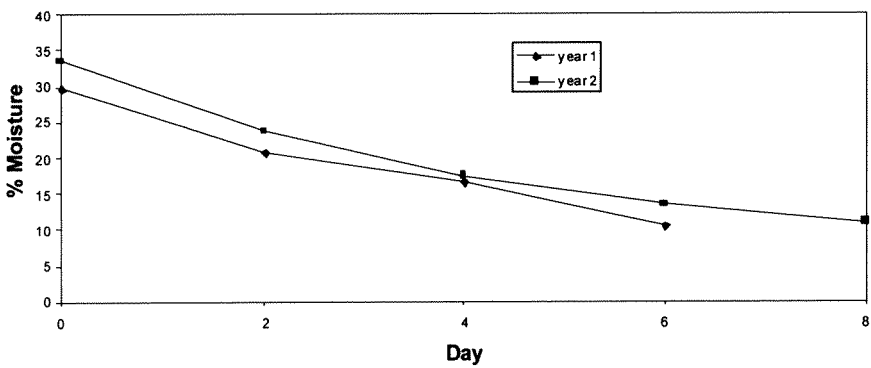
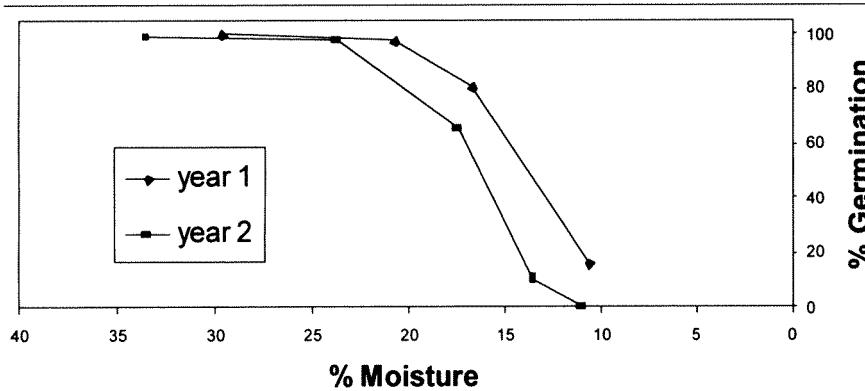


FIGURE 2. Effect of short-term seed moisture loss on viability of cherrybark oak acorns.



ambient conditions of temperature and relative humidity ranged from 20–26 °C and 40–60%, respectively. The drying trend was repeated in both experiments (Fig. 1).

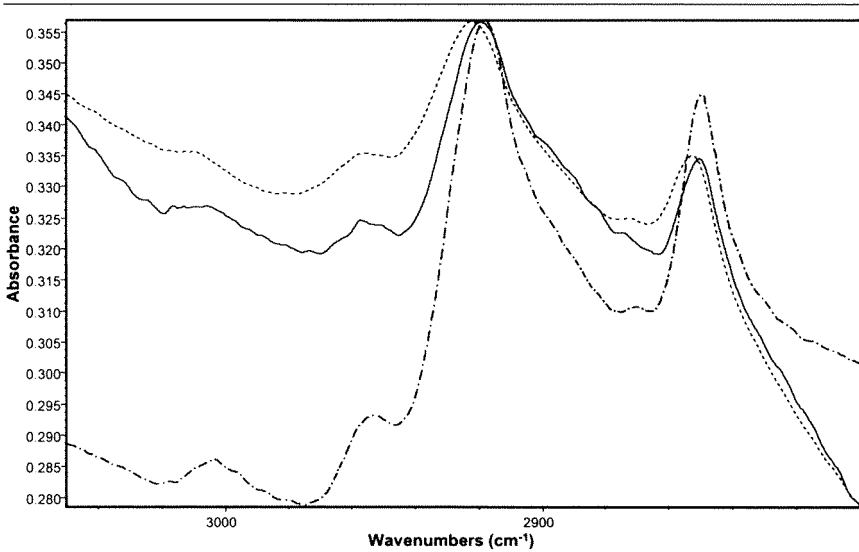
Germination. A rapid loss of seed viability accompanied loss of seed moisture content (Fig. 2). The seemingly critical level of moisture content varied between experiments, and is slightly higher in Year 1. However, once seed moisture content dropped below 17%, viability declined rapidly. This occurred in both experiments after 4 days of drying on the bench, so that following Day 6, the samples were effectively dead.

Changes in macromolecular structure measured by FT-IR

Membrane lipids. Membrane lipids in both axes and cotyledons exhibited a phase change from liquid crystalline to gel as drying occurred. The frequency of the symmetric -CH_2- stretch decreased as days of drying increased, with the biggest shift occurring between Day 2 and Day 4. When the acorns were rehydrated, the peak shifted back to higher frequency and regained some of its original bandwidth, indicating reversal to a more fluid phase. By Day 8 (Year 2), however, when viability was completely lost, the peaks did not return to their original frequency/bandwidth when rehydrated on Day 9, indicating that the membrane lipid phase remained less fluid than in the fresh/viable state in embryos (Fig. 3) and cotyledons (Fig. 4).

Membrane phase transition from fluid to gel can be visualized by a plot of peak frequency of the symmetric -CH_2- stretch vs. seed moisture content (Fig.

FIGURE 3. Membrane lipid vibrations in fresh, or Day 0 (– – –), Day 8 (dry; – · – ·) and Day 9 (rehydrated; ——) cherrybark embryos. Peak frequencies of the symmetric -CH_2- stretch occur at 2852.54, 2849.71, and 2850.30 cm^{-1}, respectively.



5). Represented graphically, this shift can be considered a diagram of phase behavior and likened to a phase transition diagram with acorn moisture content (days of drying) the independent variable. A distinct inflection point can be seen on the curve for Year 1, which would be analogous to a "Tm" but in

FIGURE 4. Membrane lipid vibrations in fresh, or Day 0 (– – –), Day 8 (dry; –·–·) and Day 9 (rehydrated; ——) cherrybark cotyledons. Peak frequencies of the symmetric -CH_2- stretch occur at 2851.69, 2847.22, and 2848.78 cm^{-1}, respectively.

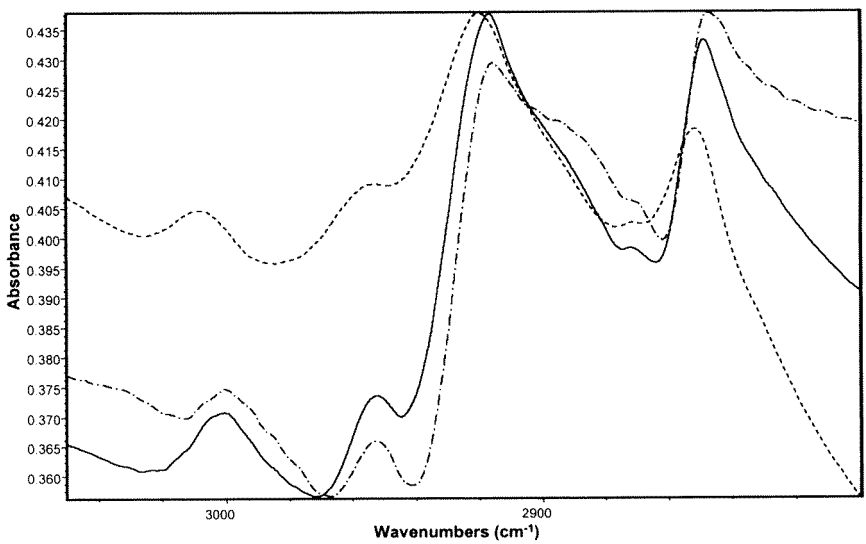


FIGURE 5. Membrane phase transition from liquid to gel as a function of seed moisture content; traditional "Tm" is more accurately described as an isothermal gel point.

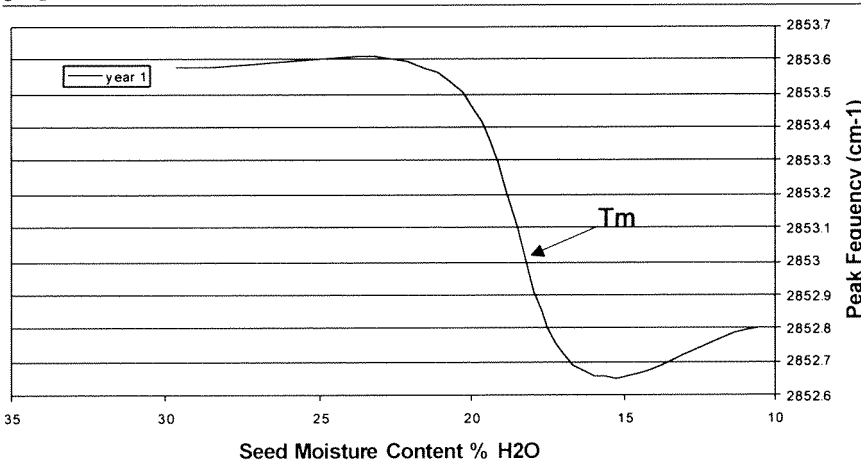


FIGURE 8. Storage lipid and protein vibrations in Day 0 (fresh; – – –) and Day 8 (dry; –·–·), cherrybark cotyledons. Peak height of the ester carbonyl vibration near 1740 cm^{-1}, representing triacylglycerols, remains the same in intensity, while the amide I vibration representing protein secondary structure is practically eliminated after 8 days of desiccation.

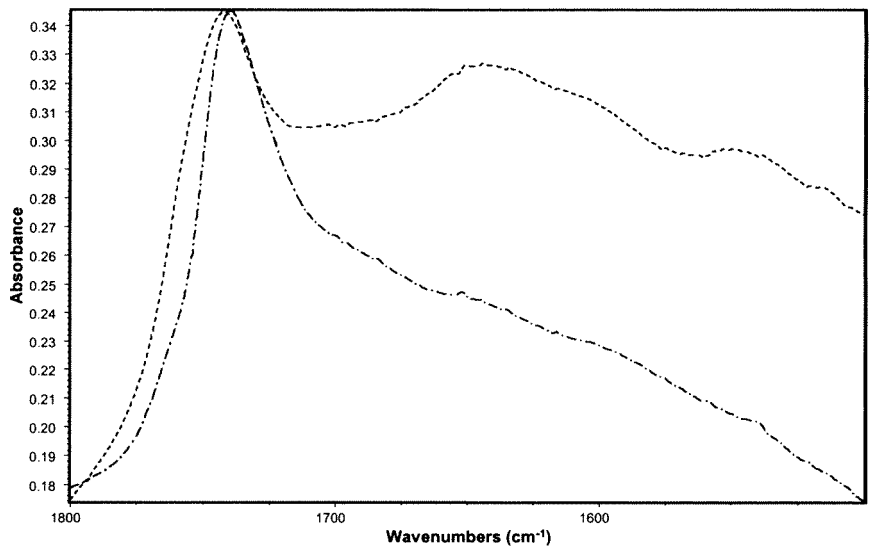
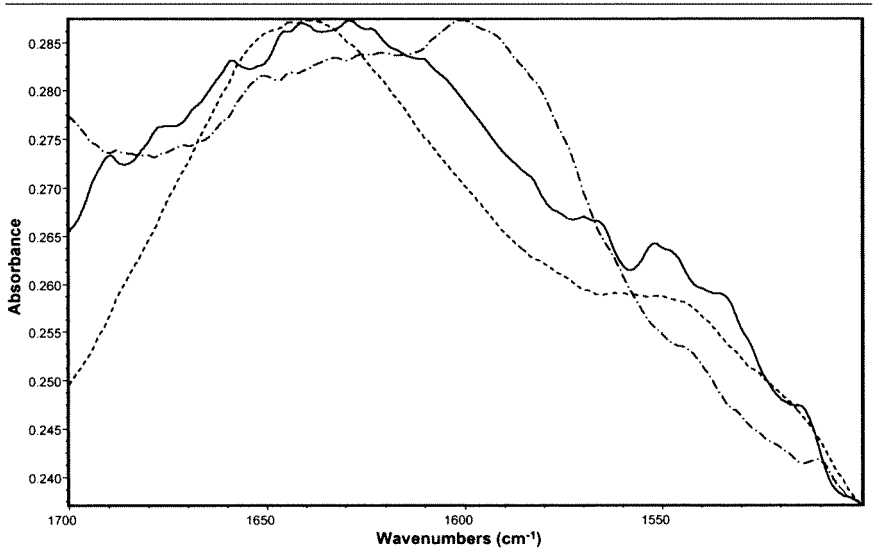


FIGURE 9. Amide I and II vibrations representing protein secondary structure in fresh, or Day 0 (– – –), Day 8 (dry; –·–·) and Day 9 (rehydrated; ——) cherrybark embryos. Peak frequencies of the amide I vibrations occur at 1638.49, 1635.00 and 1629.20 cm^{-1}, respectively.



occurs somewhere near a seed moisture content of 17%. At moisture levels below the 15% value, viability was essentially lost.

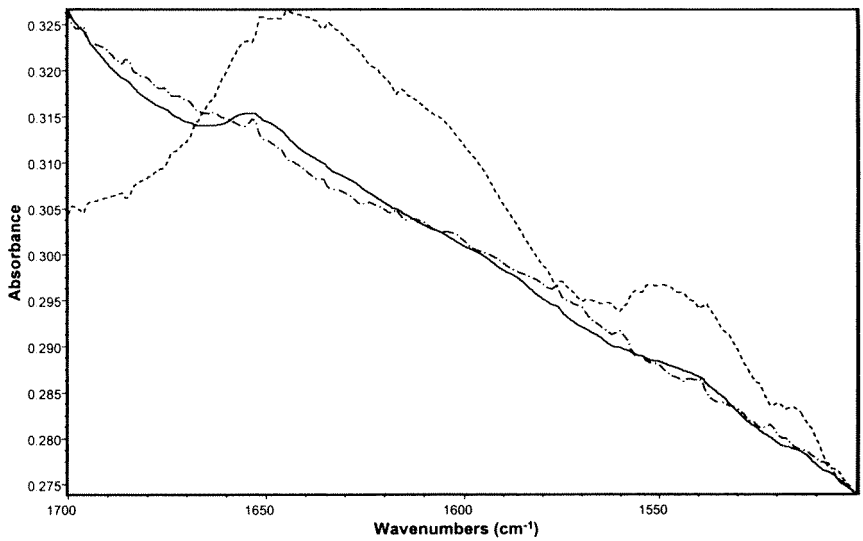
FT-IR analyses of macromolecular structures during these times of moisture/viability loss provided some biochemical insight into the processes that were occurring. We verified the high oil content of the cotyledons by a prominent peak at 1742 cm^{-1} which would indicate a high concentration of ester carbonyl bonds in triesters of fatty acids and glycerol that comprise oil molecules. Relative peak intensity did not change, i.e. it did not go down in the cotyledons and up in the embryonic axes (Fig. 8); so we conclude that lipid mobilization did not occur between the tissues. This is contrary to spectral changes we have seen in carbohydrate-storing acorns such as white oak, *Quercus alba* L. (Connor and Sowa, 2003). The membrane lipid profiles of embryonic axis vs. cotyledon at the same stage of drying, Day 4, indicated more membrane fluidity, i.e. broader absorbance peaks at higher frequencies, in the axes compared to (and perhaps at the expense of) the cotyledons (Fig. 6). This is direct evidence of a different survival mechanism, maintaining higher moisture content in the embryonic axis to maintain viability.

Membrane lipid phase change was observed in axes as moisture content decreased (Fig. 3). Data for the Year 1 experiment showed a phase change from fluid to gel between Day 2 and Day 4, where seed moisture content dropped from 20.77 to 16.69% (Table 2). This phase change was reversible when seeds were rehydrated so that the peak frequencies and bandwidths recovered when samples were tested on Day 3 and Day 5. However, when viability was completely lost, as on Day 8 of Year 2, the reversibility was only partial (Fig. 3); although the rehydrated axes on Day 9 increased bandwidth and frequency, they did not return to the fresh state values from Day 0. The same was true in the cotyledon samples (Fig. 4).

The determination of an "isothermal gel point", i.e. the seed moisture content at which the membrane lipids become immobile, is a novel approach to the analysis of desiccation sensitivity. Although we have seen determinations of membrane Tm in the literature, and the shift in that parameter with temperature and moisture content (Crowe et al., 1989), we have not seen FT-IR analyses used to directly determine the moisture content of the phase change (Fig. 5). A 3-dimensional plot of lipid peak frequency vs. seed moisture content vs. temperature would provide a surface for the physical analysis of phase behavior, perhaps with an intersection point. Comparisons of this parameter among different recalcitrant species, among seeds exhibiting different degrees of recalcitrant behavior, and between recalcitrant and orthodox seeds could prove to be a key element in understanding the phenomenon of desiccation sensitivity.

Changes in protein secondary structure were also observed during desiccation of both embryonic axes and cotyledons. The amide I vibration, from the carbonyl stretch of the amide bond in polypeptides, absorbs infrared radiation near 1650 cm^{-1} when proteins are in alpha-helix conformation. Beta-sheets have an absorbance maximum closer to 1640 cm^{-1}. The amide II vibration, from the C-N stretch and N-H bend of an amide bond, absorbs infrared

FIGURE 10. Protein vibrations in fresh, or Day 0 (– – –), Day 8 (dry; –·–·) and Day 9 (rehydrated; ——) cherrybark cotyledons. The amide I and II vibrations near 1650 and 1550 cm^{-1} that are clearly present in the fresh sample are practically absent in the dry and rehydrated samples.



radiation near 1550 cm^{-1}. These absorbances are clearly seen in fresh cherrybark embryos (Fig. 9). However, by Day 8, the amide I peak has shifted considerably to the right, and rehydration brings it back—but only to 1630 cm^{-1}. Extended beta-sheet conformation, associated with protein denaturation, exhibits infrared absorbance frequencies less than 1630 cm^{-1} (Golovina et al., 1997). We therefore conclude that the proteins in the axes have undergone irreversible denaturation as viability is lost. The protein changes in the cotyledons are similar, and more pronounced (Fig. 10). This is contrary to observations by Golovina et al. (1997) who observed no changes in protein secondary structure using FT-IR techniques in a variety of naturally aged embryos from orthodox seeds. This behavior might be an indication of significant protein structural changes that occur during dehydration of recalcitrant seeds that are absent in orthodox, desiccation tolerant seeds.

The results presented here confirm the recalcitrant seed behavior of cherrybark oak, and emphasize the care that should be taken in the collection and preservation of temperate recalcitrant species. A small change in initial moisture content can have a large effect on storability. A seed moisture content just below 20% can be lethal. This correlates to the seed moisture content at the isothermal gel point, which can be determined using FT-IR spectroscopy.

FT-IR spectroscopy can also provide more detailed structural information about the biochemical changes occurring during loss of viability. The data collected are straightforward and can provide a wealth of structural information in a very brief sampling time (minutes). They are collected with no sample

manipulation except for slicing cotyledon tissue and squashing whole embryos. We made no mathematical manipulation to the data whatsoever; the spectra presented are as they were collected, therefore we are comfortable that no artifacts are included. In fact, baseline corrections for variable water content would only exaggerate the peak shifts. Our results showed that although membrane phase behavior exhibits some reversibility upon drying, complete reversibility to the membrane fluidity of a fresh or high viability sample does not occur. There is an even more sensitive indicator of viability, the denaturation of protein structure, which loses all reversibility on dehydration with loss of viability in these recalcitrant seeds.

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