

Effects of desiccation on the physiology and biochemistry of *Quercus alba* acorns[†]

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Summary Seeds that lose viability when dried to a water content of less than 12% are said to be recalcitrant. We subjected acorns of *Quercus alba* L., a species with recalcitrant seeds, to desiccation to determine the effects of drying on lipids, proteins and carbohydrates of the embryonic axis and cotyledon tissues. Samples of fresh seed and seed dried for selected intervals were analyzed for water content and germination, and for lipids, proteins and carbohydrates by Fourier transform-infrared (FT-IR) spectroscopy. Carbohydrates were further analyzed by gas chromatography (GC).

The FT-IR analysis revealed that membrane lipid structure initially exhibited reversible shifts between gel and liquid crystalline phases in response to drying and rehydration; however, reversibility declined as viability was lost. Changes in carbohydrate concentration were observed based on peak height comparisons; sucrose concentration in the embryonic axis increased dramatically after 5 days of drying. The most sensitive indicator of desiccation damage was the irreversible change in protein secondary structure in embryonic axes and cotyledon tissue. These changes were illustrated by shifts in amide absorbance near 1650 cm⁻¹. Gas chromatography indicated an abundance of sucrose in both the embryonic axes and the cotyledon tissue. Although sucrose concentrations in these tissues were initially similar, sucrose concentration in the embryonic axes became significantly greater than in the cotyledons as the acorns dried. We hypothesize that, in drying acorns, increased concentration of sucrose does not prevent loss of viability, but acts as a glycoprotectant against cell collapse and cell wall membrane damage as water stress increases.

Keywords: carbohydrates, FT-IR, GC, glycoprotectant, lipids, proteins, recalcitrance.

Introduction

Roberts (1973) defined seeds and their storage behavior on the basis of their water content, expressed on a fresh mass basis. The majority of seeds from temperate forest species are ortho-

dox, meaning they can be dried to a water content of less than 12% without damage. There are, however, temperate tree seeds that are recalcitrant (Roberts 1973), meaning they are sensitive to water loss and have short storage life spans. The critical water content below which recalcitrant seeds should not be dried varies from 12 to 31%, depending on the species (Roberts 1973, Pammenter et al. 1991, Wesley-Smith et al. 1992). Important tree species of the temperate zone with recalcitrant seeds include *Castanea* (Pritchard and Manger 1990), *Quercus*, *Aesculus* and some *Acer* species (Bonner 1990).

Several hypotheses have been proposed to explain recalcitrance. One states that it is associated with desiccation-induced changes in lipid composition or with physical disruption of the seed membranes; disruption can occur as a result of seed aging, water loss or chilling (Flood and Sinclair 1981, Seewaldt et al. 1981, Priestly and Leopold 1983). Another idea is that an increasing demand for structure-bound water elevates desiccation sensitivity (Farrant et al. 1985, 1988). Although it is believed that recalcitrance is due to metabolic aberrations that occur during hydrated storage (Pammenter et al. 1994) and during desiccation (Berjak and Pammenter 1997), the biochemical and physiological causes of recalcitrance remain unknown.

Starches and soluble sugars are prevalent in the storage reserves of many recalcitrant seeds, and they have been studied in several species. Although it is thought that sucrose and raffinose play key roles in desiccation tolerance of orthodox seeds, experimental results do not consistently support this theory (Bohicchio et al. 1997). Hoekstra et al. (1994) found high sucrose concentrations in both tolerant and intolerant states, and Ooms et al. (1994) concluded that carbohydrates were unlikely to be the sole factor determining desiccation tolerance in *Arabidopsis thaliana* (L.) Heynh. seeds. Other studies have shown that raffinose or sucrose or both are essential to desiccation tolerance of orthodox seeds. In immature *Glycine max* (L.) Merrill cv. Chippewa 64 seeds, stachyose accumulates in the axes during slow drying, resulting in desiccation tolerance (Blackman et al. 1992). Leprince et al. (1990) noted

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that the appearance of stachyose and an increase in sucrose concentration in *Brassica campestris* L. embryos were coincident with the onset of desiccation tolerance. Others have found that the raffinose to sucrose mass ratio is important in conferring desiccation tolerance in maize seeds. Chen and Burris (1990) concluded that the presence of raffinose at certain concentrations may be the key factor in protecting maturing seeds from damage during high-temperature drying, and Koster and Leopold (1988) reported that loss of raffinose may correspond to the loss of desiccation tolerance in axes of *Zea mays* L. cv. Merit seeds. Greggains et al. (2000) determined that there were higher concentrations of oligosaccharides, sucrose and dehydrins in the orthodox seeds of Norway maple (*Acer platanoides* L.) than in the recalcitrant seeds of sycamore maple (*Acer pseudoplatanus* L.).

Because lipid breakdown is thought to play a major role in deterioration of orthodox seed (Bewley and Black 1994), studies have focused on the chemistry of membrane lipids and storage reserves of recalcitrant seeds (Tompsett 1984). Many researchers have suggested that increasing lipid peroxidation is linked with reduced viability during seed drying (Chaitanya and Naithani 1994, Finch-Savage et al. 1996, Li and Sun 1999). Finch-Savage et al. (1993) found decreased antioxidant activity and increased generation of free radicals in drying *Quercus robur* L. acorns. Similar results have been reported for other species (Chaitanya and Naithani 1994, Finch-Savage et al. 1994a).

Seed proteins may be adversely affected during hydrated storage and desiccation. Loss of viability has been associated with loss of cellular constituents and with processes such as protein degradation and changes in membrane phospholipids leading to loss of integrity (Ching and Ching 1976, Jain and Shivanna 1989). Additionally, late-embryogenesis-abundant proteins and seed maturation proteins have been examined to determine their role in seed longevity (Finch-Savage et al. 1994b). Although their part in conferring protection against the effects of desiccation is unclear (Han et al. 1997), it is possible that these proteins interact with other cellular features to determine the degree of seed recalcitrance (Berjak and Pammenter 2002).

Among seeds of the Fagaceae, white oak (*Quercus alba* L.) acorns are among the most recalcitrant. Maturing in the fall, they can be stored at 4 °C for no more than 4 to 6 weeks before root emergence. In this study, we investigated the effects of desiccation on the structure and composition of lipids, proteins and carbohydrates of *Q. alba* acorns. We hypothesized that, because of the short life span of these seeds under optimal storage conditions, biochemical changes in the lipids, carbohydrates and proteins of desiccating acorns will be detectable within days of seed maturation.

Materials and methods

Plant material

Acorns were collected near Starkville, MS, within 2 days of natural dissemination in Year 1 and purchased from a local

seed supplier in Year 2. Seeds from the supplier were stored fully hydrated at 4 °C before purchase. In the Year 1 studies, half of the acorns were shipped from Mississippi to Pennsylvania for Fourier transform-infrared (FT-IR) spectrometry, and Ralf were used locally for gas chromatography (GC) studies. In Year 2, all acorns were used for the FT-IR experiments. Before the start of the experiments, acorns were soaked for 15 h in water and spread on blotter paper in a single layer on a laboratory bench to dry. Water content and acorn viability were determined for each FT-IR and GC experiment performed. Laboratory conditions of temperature and relative humidity were monitored with a hygromograph. Samples of fresh seeds were used for each analysis.

Moisture content

Moisture content (MC) of whole acorns was determined for four to five replicates of three acorns each by the procedures recommended for large seeds with high MCs (Bonner 1981, International Seed Testing Association 1993). Briefly, randomly selected acorns were cut into quarters and dried in aluminum cans at 103 ± 2 °C for 24 h in a mechanical convection oven. Seed MC was calculated on a fresh mass basis (Roberts 1973).

Germination

At each sampling period, 100 acorns were randomly selected and germinated as two replications of 50 seeds each. After imbibition in tap water for 1.5 h, acorns were cut in half. The half with the cup scar was discarded (Bonner and Vozzo 1987), and the pericarp was removed from the remaining half. Acorns were placed cut side down on moist Kimpak and germinated either under greenhouse conditions (IT-IR studies) or in a Stultz germinator (GC studies) with an 8-h photoperiod and day/night temperatures of 30/20 °C (Bonner and Vozzo 1987). Germination was tallied each week for up to 4 weeks. An acorn was scored as germinated if both the radicle and shoot had emerged and exhibited normal morphology and growth.

FT-IR Spectroscopy

In Year 1, transmission spectra were recorded on thin slices of cotyledon and embryonic axes squashes (three to five per sample) that were placed between CaF₂ windows of a removable transmission cell. Duplicate samples were analyzed on each sampling day. For each spectrum, 512 scans at 2 cm⁻¹ resolution were collected on a Nicolet 20 DXB spectrometer equipped with an MCT-A detector. Single beam spectra were ratioed against an open beam background to yield transmission spectra. In Year 2, attenuated total reflectance spectra were recorded on finely divided embryonic axes and cotyledon tissues that were layered onto a 45° ZnSe crystal in an ARK sampling device (SpectraTech, Shelton, CT). Instrument collection parameters were the same as for the transmission experiment; however, "absorbance" is equivalent to $\log(1/R)$, where R equals reflectance.

The sampling schedule for even-numbered days (0, 2, 4, 6, 8, 12) involved fresh mass determination, FT-IR sampling and soaking acorns for germination. The FT-IR analysis was conducted on the next odd-numbered day (3, 5, 7, 9, 11) so that spectra were recorded for both dry and rehydrated samples. Sampling continued until seed MC decreased below 15%.

Gas chromatography

At each sampling time, embryonic axes with immediately adjacent (within 1 mm) cotyledon tissue were dissected from acorns; tissues were frozen in liquid nitrogen and lyophilized. The freeze-dried cotyledons were finely ground in a Wiley mill to pass a 20-mesh screen; lyophilized embryonic axis tissue was ground by hand with a mortar and pestle. We used a 0.3–0.5 g dry tissue sample for each carbohydrate extraction. Three to five extractions were made from each cotyledon sample and one to two from each embryonic axis sample, depending on the amount of tissue available. The tissue sample was placed in 10 ml of 80% ethanol, incubated at 75 °C for 1 h and filtered. The acorn tissue was extracted twice more with ethanol solution and the ethanol extracts combined and roto-evaporated until dry. The evaporation flask was rinsed with 10 ml of distilled water, and the water plus contents stirred with 1 g of Amberlite MB-3 resin for 1 h. The sample was then filtered, rinsed and freeze-dried overnight. Phenyl- β -D-glucopyranoside was added as an internal standard. The dried sample was dissolved in 1 ml of trimethylsilylimidazole, incubated at 75 °C for 30 min, blown to dryness, and redissolved in 1 ml of chloroform and stored at -20 °C. Analyses were performed within 2 days on a Hewlett Packard 5890 GC equipped with a Supelco SPB-5 capillary column (30 m x 0.25 mm ID x 0.25 film thickness). Carbohydrates were identified by comparison with standards of pure sugars prepared and chromatographed in the same manner as the experimental samples. Differences between means were determined by t-tests, where $P < 0.05$ indicated significance. Samples were tested on days 0, 1, 3, 5, 7 and 9 for Year 1 acorns only.

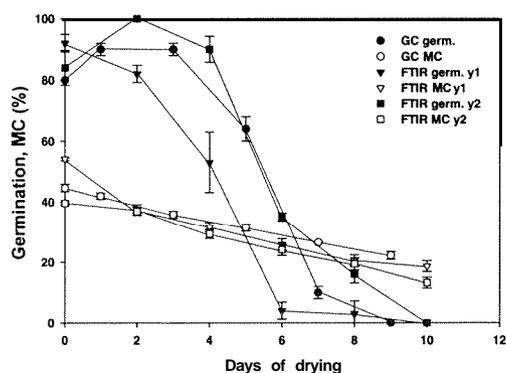


Figure 1. Germination (%) and water content (MC; % fresh mass) of desiccating *Quercus alba* acorns used for gas chromatography (GC) and Fourier transform-infrared spectroscopy (FT-IR) analyses. Abbreviations: y 1 = Year 1 acorns and y2 = Year 2 acorns.

Results and discussion

FT-IR Experiments

Viability of white oak acorns was highly dependent on MC and started to decline significantly after 4 days of drying, when MC reached about 30% (Figure 1). Seed water content declined to about 20% within 8 days of drying; by this time, viability was nearly lost.

Seed lipids and proteins were examined by FT-IR spectroscopy. Storage lipids, or triacylglycerols, have three ester bonds per molecule that absorb near 1740 cm^{-1} . The amide bonds of proteins exhibit IR absorbances between 1700 and 1500 cm^{-1} that are indicative of types of secondary structure (Golovina et al. 1997). Many other dipoles can be used to identify macromolecular structure with IR absorbances in the "fingerprint" region of frequencies (800–1800 cm^{-1}). Both frequency and bandwidth of IR absorbance spectra provide useful information about biomolecular structure. Transmission FT-IR spectra of the embryonic axes showed peak shifts of the symmetric and asymmetric $-\text{CH}_2$ vibrations near 2850 and 2910 cm^{-1} to lower frequencies in response to drying, accompanied by decreased bandwidth (Figure 2a). Such changes have been associated with loss of membrane fluidity, or transition to gel phase (Casal and Mantsch 1984, Sowa et al. 1991). After rehydration, the changes were even more visible, and irrevers-

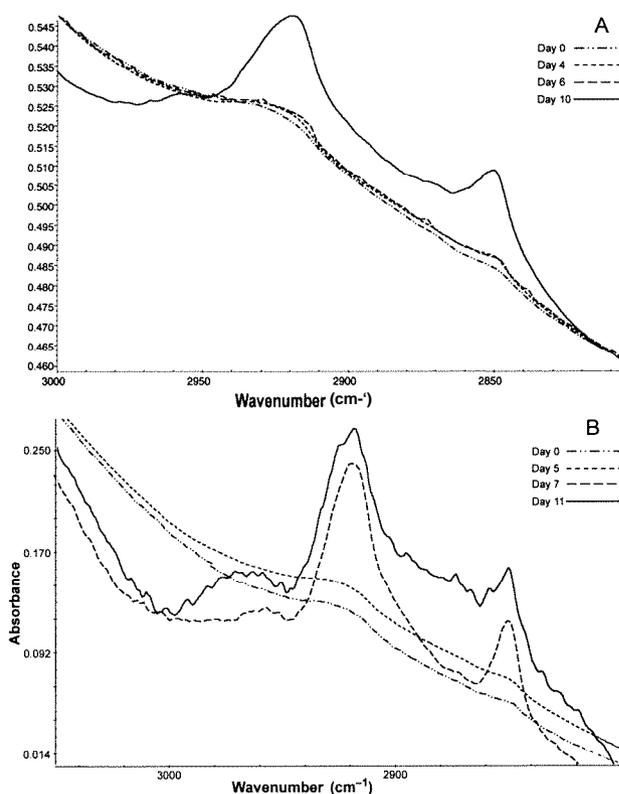


Figure 2. Transmission infrared (IR) spectra of the membrane lipids of (A) dried and (B) rehydrated *Quercus alba* embryonic axes (Year 1).

ible, in embryonic axes and in cotyledons after the tissues had been dried below the critical MC (Figure 2b).

Changes in protein secondary structure were indicated by both frequency and bandwidth of the amide I and II vibrations near 1650 and 1550 cm^{-1} , respectively (Sowa and Connor 1995, Golovina et al. 1997). It is known that alpha-helix structures absorb near 1650 cm^{-1} , whereas beta-sheets absorb near 1630 cm^{-1} . Denatured proteins typically take on extended beta-sheet conformations that absorb at frequencies of less than 1630 cm^{-1} . Changes in peak width also indicate denaturation or a change in secondary structure. Transmission spectra of *Q. alba* embryonic axes showed irreversible loss of protein secondary structure as viability was lost (Figure 3), even after rehydration. A similar pattern was found in cotyledon tissue.

Reflectance spectra, collected in Year 2, resolved the carbohydrate peaks in the IR frequency range near 1000 cm^{-1} . The peak at 1045 cm^{-1} was assigned to sucrose based on reference values (library spectra). The identity of the peak was confirmed by GC analysis of seed extracts. Analyses confirmed that cell membrane content remained constant during seed drying. Therefore, based on peak height comparisons of absorbance at 1045/2850 cm^{-1} , the FT-IR spectra indicated sucrose mobilization; sucrose accumulated in the embryonic axes and decreased in the cotyledons during the period of greatest loss of viability and water content (Figure 4). These results were verified by the more detailed GC analyses examining sucrose concentration of desiccating embryonic axes and cotyledon tissue (Figure 5). Although GC analyses revealed no significant reduction in sucrose concentration in the cotyledon tissue, differences in sucrose utilization and accumulation between the Year 2 FT-IR and the Year 1 GC experiments were not surprising as they were performed with acorns collected in different years.

Membrane lipid structure initially exhibited reversible shifts between gel and liquid crystalline phases in response to drying and rehydration; however, reversibility declined as viability was lost. Changes in carbohydrate concentration were observed based on peak height comparisons; sucrose concentration in the embryonic axis increased dramatically after 5 days of drying. The most sensitive indicator of desiccation damage as revealed by FT-IR was the irreversible changes in

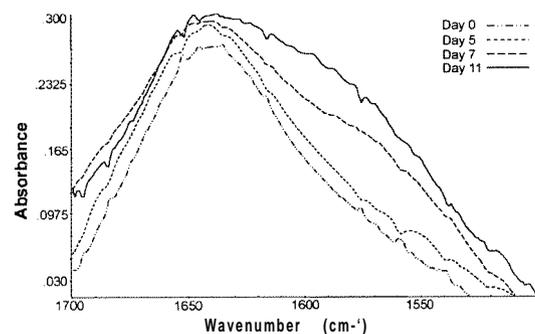


Figure 3. Transmission infrared (IR) spectra of proteins of rehydrated *Quercus alba* embryonic axes (Year 1).

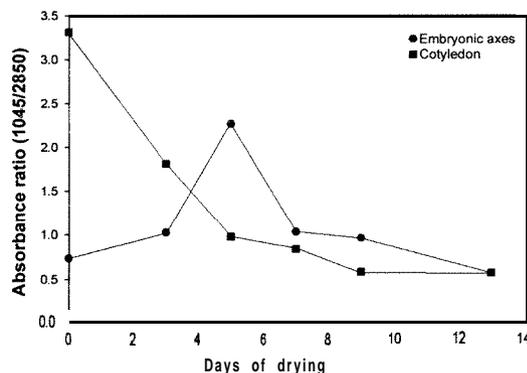


Figure 4. Relative sucrose concentration versus days of drying. Relative sucrose concentration was inferred from the ratio of absorbance in the carbohydrate region at 1045 cm^{-1} and in the lipid region at 2850 cm^{-1} . Data from Year 2 Fourier transform-infrared experiments.

protein secondary structure in embryonic axes and cotyledon tissue. These changes were illustrated by shifts in the amide absorbance near 1650 cm^{-1} .

Gas chromatography experiments

Initial germination and MCs of the acorns used for the GC analyses paralleled those used for the FT-IR experiments, although viability was lost at a slightly higher MC (22.2% versus 18.6 and 13.2%). Unlike in many orthodox seeds and some recalcitrant seeds, raffinose and stachyose were not detected in *Q. alba* acorns. There was, however, an abundance of sucrose in both embryonic axis and cotyledon tissues. Although sucrose concentrations in the cotyledon and axis tissues were initially similar, sucrose concentration in the embryonic axis tissue became significantly greater than in the cotyledon as the acorns dried (Figure 5). Changes in sucrose concentration of cotyledons were not significant until Day 5 of drying. Sucrose concentration dropped by 0.39 \times from Day 3 to Day 5, and increased by 3.3 \times from Day 5 to Day 7. There was no significant

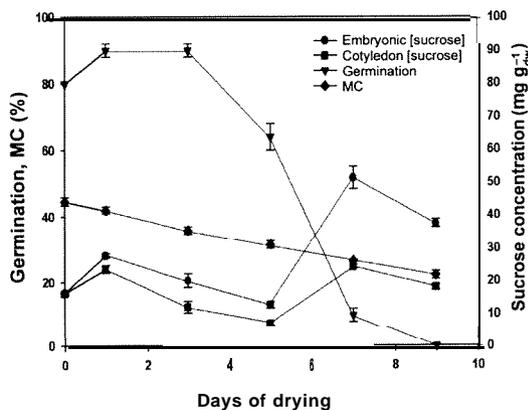


Figure 5. Germination (%), moisture content (MC; %), and sucrose concentration ($\text{mg g}_{\text{dw}}^{-1}$) in desiccating *Quercus alba* embryonic axes and cotyledons. Data from Year 1 gas chromatography experiment.

difference in sucrose concentration in cotyledons between Day 0 and Day 9. A similar pattern occurred in desiccating embryos when sucrose concentration decreased 0.36 \times from Day 3 to Day 5 (Figure 5). Although this decrease was not statistically significant, the following 3.9 \times increase in sucrose concentration at Day 7 was statistically significant, and the sucrose concentration recorded on Day 9 was significantly higher (2.2 \times) than the fresh embryo value.

The increase in sucrose concentration in the embryonic axis, coupled with declining viability, suggested that sucrose was no longer being used for growth and development or that starch was being broken down by enzymatic activity in the deteriorating seeds, or both. An increased concentration of sucrose in response to drying was also observed in

cacao L. seeds (Li and Sun 1999). These authors noted that desiccation sensitivity of embryonic axes did not appear to be associated with a lack of sugar-related protective mechanisms during desiccation and suggested that desiccation-sensitivity and decline in axis viability were related to a decrease in the activities of free-radical-scavenging enzymes, superoxide dismutase, ascorbate peroxidase and peroxidase. Lin and Chen (1995) also reported an increase in sucrose concentration when developing seeds of *Machilus thunbergii* Sie. & Zucc. were slowly desiccated and suggested a protective role for sucrose. Circumstantial evidence for a glycoprotective effect was found in electron micrographs of desiccating tissues of *Q. alba* and *Quercus nigra* L. acorns (Connor et al. 1996) showing that, despite the stress imposed by decreasing MCs, the cell membranes of sucrose-enriched embryonic axis and cotyledon tissues of *Q. alba* remained intact. Those of *Q. nigra*, a species with acorns that have high lipid contents, were damaged after as few as 3 days of drying.

We note, however, that this explanation does not account for the increase in sucrose concentration in acorns that had dropped below the critical MC and were losing viability. For example, the sucrose concentration in embryonic axes on Day 7 of drying was much higher than in fresh acorns (3.1 \times), but viability had dropped from 92 to 10%. The high sucrose concentrations in the drying embryos and the continued accumulation of sucrose in the cotyledons, although obviously not being used for growth and development in the deteriorating acorns of *Q. alba*, may have acted secondarily as a glycoprotectant, preventing both desiccation damage of cell membranes and cell collapse.

Bruni and Leopold (1991) mentioned the importance of disaccharide glass formation as a protective mechanism in seeds and suggested that leakage or hydrolysis of disaccharides would hinder glass formation. But as Pammenter and Berjak (1999) pointed out, glass formation would occur only at moisture contents well below the lethal limit in recalcitrant seeds, because tissues in desiccation-sensitive seeds suffered fatal damage at relatively high MCs. Although the combination of high sucrose concentration and high axis MC may have protected membranes in *Q. alba* tissues, the mechanism did not function to preserve viability, which dropped rapidly after Day 5. Thus, although the sugar-related protective mechanisms mentioned by Li and Sun (1999) were in place in these

acorns, they did not preserve viability, indicating that an unidentified metabolic failure is responsible for the desiccation sensitivity of *Q. alba* acorns.

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