Research on the Nature of Recalcitrance in Temperate Tree Seeds: GC and FT-IR Examinations of Stored and Desiccated Seeds

K.F. Connor[1] and S. Sowa[2]

[1]U.S. Forest Service, M.S. 9681, MS State, MS 39762, USA; Ph: 662-325-2145; Fax: 662-325-3278; kconnor@fs.fed.us; [2]Department of Chemistry, Indiana University of Pennsylvania, Indiana, PA 15705, USA; Ph: 724-357-4481; Fax: 724-357-2437; ssowa@grove.iup.edu

Abstract

Quercus alba L., *Q. durandii* Buckl., and *Q. virginiana* Mill. acorns were collected, stored at +4°C and -2°C, and tested monthly to examine the physiological, biochemical, and moisture changes taking place during storage. *Aesculus pavia* L. seeds were similarly stored but tested only every three months, while those of *Q. nigra* L. and *Q. pagoda* Raf. were tested on a yearly basis. While all these seeds are classified as recalcitrant, not all deteriorate at the same rate nor does the lower storage temperature always enhance seed longevity. In addition, while gas chromatographic and Fourier transform infrared spectrometer analyses revealed that amounts of sucrose change dramatically in stored acorns, we found that there are interspecific differences observed in carbohydrate mobilization and also differences between cotyledon and embryonic axis tissue within a species. Also, any moisture loss prior to storage was fatal; and proper handling of seeds could be of greater significance than storage temperature. FT-IR studies have also found changes in membrane lipids and secondary protein structure in desiccating and stored acorns, emphasizing that low storage temperatures do not deter metabolic activity in hydrated, recalcitrant seeds.

Keywords: *Aesculus*, FT-IR, gas chromatography, *Quercus*, recalcitrance

Introduction

Roberts (1973) stated that seeds can be divided into two storage classes: 'Orthodox' or desiccation-resistant seeds that can be dried without damage to a moisture content (mc) of less than 12% and 'recalcitrant' or desiccation-sensitive seeds that cannot. The susceptibility of recalcitrant seeds to moisture loss and the necessity for hydrated storage makes any useful period of seed storage for some such seeds very short; others, such as *Quercus nigra* L. (Bonner 1973) acorns, can survive up to 3 years under proper storage conditions. Forest tree genera with recalcitrant seeds are abundant in the tropics but less common in the temperate zone. They do exist, however, and some important temperate tree genera, *Castanea* (Jaynes 1969, Prichard and Manger 1990), *Quercus* (Bonner and Vozzo 1987), *Aesculus*, and some *Acer* species (Bonner 1990) have seeds classified as recalcitrant.

Recent work has modified both Robert's initial definition of recalcitrance and our perspective of the nature of recalcitrance. Pammenter *et al.* (1994) and Berjak and Pammenter (1997) recognized the damage caused by aberrant metabolic processes while seeds are in hydrated storage and as water is lost. Other work has emphasized the changes in membrane and storage lipids and the physical disruption of seed membranes that take place as seeds deteriorate (Flood and Sinclair 1981; Priestly and Leopold 1983). Changes in seed proteins and carbohydrates, and the complexities of water properties in seeds have also been noted (Roberts 1973; Farrant *et al.* 1985, 1988; Pammenter *et al.* 1991; Wesley-Smith *et al.* 1992). Pritchard (1991) determined that damage began in recalcitrant seed embryos at a much higher mc than that proposed by Roberts (1973); embryos of *Quercus* exhibited damage when mc was still over 40%. Experiments by the authors with recalcitrant seeds from both temperate and tropical trees have yielded mixed results (Connor *et al.* 1996, 1998; Connor and Bonner 1998). While moisture and differential scanning calorimetry data exhibit a strong relationship with declining seed viability, results of lipid analyses have been conflicting.

This paper reports the results from three studies: (1) a one year storage study of Durand oak (*Quercus durandii* Buckley), live oak (*Quercus virginiana* Mill.), and red buckeye (*Aesculus pavia* L.) at 2 temperatures and a 120 day study of white oak (*Quercus alba* L.) at the same storage temperatures; (2) third year results of a water oak (*Quercus nigra* L.) and cherrybark oak (*Quercus pagoda* Raf.) acorn storage experiment at two temperatures and two mcs; and (3) a Fourier transform infrared (FT-IR) spectroscopy study of desiccating cherrybark oak acorns.

Materials and Methods

Durand oak, white oak, and red buckeye seeds were collected locally in Oktibbeha County, Mississippi (MS), USA. The water oak and cherrybark oak acorns were purchased from a local supplier, while the live oak acorns were collected in Washington County, MS, USA. All seeds were cleaned by floatation, soaked overnight, and then stored at 4°C until the start of the experiment. Original mc for each drying regime was determined by drying 2-4 samples of seeds at 105°C for 16-17 h. In preparation for germination tests, acorns were cut in half horizontally. The seed coat was removed from the half containing the embryo, and the half with the cup scar was discarded. Buckeye seeds were germinated intact. Germinations were conducted on moist Kimpak at an alternating temperature regime of 20°C for 16 h in the dark and 30°C for 8 h with light. Since sprouting in storage can be a common problem, counts were made of the number of seeds in a sample, which had sprouted during storage. Experiments were conducted as follows:

Experiment 1

General: This experiment examined temperate tree species with highly recalcitrant seeds. Samples of 250 fully hydrated acorns of Durand oak and live oak were stored in plastic bags at either 4°C in a Lab-Line Ambi-Hi-Low Chamber or at -2°C in a modified chest freezer. Percent germination and mc were determined for the fresh acorns and every 30 days (d) thereafter for one year, as acorn supplies and deterioration permitted. A subsample of acorns was dissected, and the embryo and cotyledon cryostored for carbohydrate analyses. Acorns were germinated as two replications of 50 seeds each per sampling period and were rehydrated overnight in tap water prior to germination testing. White oak acorns and red buckeye seeds were stored as above; however, the white oak acorns were stored in batches of 185 per bag and, since they rapidly deteriorate when stored, tested only through 120 days. Germination tests were conducted on 2 replications of 25 seeds per sampling period. Red buckeye seeds were tested only at fresh, 90-, 180-, and 360-d intervals and were stored in batches of 59 seeds per bag. Germination tests consisted of 2 replications of 15 seeds each per sampling period.

Carbohydrate analyses: At each sampling time, *Quercus* embryoic axes with immediately adjacent cotyledon tissue were dissected from surrounding tissue. Samples were immediately frozen in LN_2 and lyophillized. The cotyledons were finely-ground in a Wiley mill using a 20-mesh screen; embryonic axis tissue was ground by hand with a mortar and pestle. A 0.3-0.5g dry tissue sample was used for each carbohydrate extraction. The tissue sample was placed in 10 ml of an 80% ethanol solution and heated in a 75° C water bath for 1 h. The sample was then filtered, rinsed with more of the ethanol solution and rotoevaporated to dryness. The evaporation flask was rinsed with 10 ml of distilled water, and the sample was then filtered, rinsed, and freeze-dried overnight. The dried sample was dissolved in 1 ml of trimethylsilylimidazole, heated in a 75° C water bath for 30 min, blown to dryness and then redissolved in 1 ml chloroform and stored until analysis. Analyses were performed on a HP® 5890 gas chromatograph (gc) using a Supelco® SPB-5 capillary column (30m x 0.25 mm ID x 0.25 film thickness).

Experiment 2

High and low moisture levels for water and cherrybark oak acorns were imposed by either soaking in tap water for 16 h or by drying on a lab bench for 48 h. Lots consisting of 110-120 acorns were stored in 4-mil polyethylene bags at either 4°C or at -2°C as described above. Original percent germinations and mcs were determined for fresh acorns and thereafter at yearly intervals. Acorns were germinated as two replications of 50 seeds per sampling period and were soaked overnight in tap water prior to germination testing.

Experiment 3

Cherrybark oak acorns collected in 1999 were spread on blotter paper in a single layer on the lab bench. Cotyledon samples of fresh seeds and those that had been dried for 2,4,6, and 8d were analyzed by FT-IR spectroscopy as follows: thin slices of cotyledon tissue were placed between CaF_2 windows of a demountable transmission cell. For each spectrum, 512 scans at 2/cm resolution were collected on a Nicolet 20 DXB spectrometer using an MCT-A detector. Single beam spectra were ratioed against an open beam background to yield transmission spectra. Sampling continued until seed mc dropped below 15%, and the samples were analyzed for changes in macromolecular structure that might occur during drying and during rehydration. The experiment was replicated on acorns collected in 2000.

Results

Experiment 1

Durand oak acorns stored at -2°C had significantly higher viability than those stored at 4°C in as little as 30d (Table 1). After 210d, acorns stored at -2°C averaged 83% viability, while only 6% of those stored at 4°C survived. Red buckeye seeds also remained viable longer if stored at -2°C. The differences in viability did not occur, however, until after 90d in storage. Acorns of live oak were the only ones tested that survive longer if stored at 4°C. Storage at -2°C resulted in significant damage to the acorns. Fresh mcs were 38.1, 60.6, and 56.6% for Durand oak, red buckeye, and live oak, respectively, and did not change greatly during storage. White oak acorns began sprouting in as little as 60d when stored at 4°C and by the 90d test, 96% of all the 4°C acorns had sprouted (Table 2). Only 3% of acorns stored at -2°C had sprouted by the 120d test and viability was still 90%. Mcs remained high in the acorns.

Table 1. Germination and moisture content of Durand oak, red buckeye, and live oak seeds stored for up to one year at +4C and -2C.

Species	Days stored	Germination (%) +4C	Germination (%) -2C	Moisture Content (%) +4C	Moisture Content (%) -2C
Durand oak	0	98	98	38.1	38.1
	30	88	98	37.3	37.7
	60	87	97	39.7	39.9
	90	75	93	39.7	37.1
	120	69	93	40.3	39.1
	150	10	50	40.6	38.4
	180	--	--	---	---
	210	7	83	41.0	38.7
	360	0	13	*	39.8
Red buckeye	0	93	93	60.6	60.6
	90	100	100	63.4	61.4
	180	83	100	59.1	62.4
	360	0	44	---	*
Live oak	0	92	92	56.6	56.6
	30	84	79	52.3	57.6
	60	91	46	54.6	50.4
	90	68	20	58.9	53.4
	120	45	10	53.4	57.4
	150	30	7	61.5	59.6
	180	14	2	54.8	53.3
	210	4	6	61.3	52.6
	240	2	7	56.2	53.6

*Seeds selected for the moisture test were all dead.

Table 2. Germination, sprouting, and moisture content of white oak acorns stored for 120 days at +4C and -2C.

Days stored	Germination (%) +4C	Germination (%) -2C	Moisture Content (%) +4C	Moisture Content (%) -2C	Sprouting(%) +4C	Sprouting(%) -2C
0	96	96	51.9	51.9	0	0
30	100	100	50.1	52.1	0	0
60	42	94	52.2	50.5	4.9	0
90	0	98	51.2	50.8	95.7	1.1
120	---	90	---	50.2	---	3.2

It was obvious from the carbohydrate analyses that, even when stored at temperatures of -2°C, seed metabolism was still very active (Fig. 1). Sucrose was still being mobilized and transported despite the cool temperatures, a difficulty commonly encountered in recalcitrant seeds, which must be stored fully hydrated. In several of the species studied in this experiment, there were no oligosaccharides present. In others, quantities were very small (< 3mg/g). It is difficult to tie specific physiological events in stored seeds to fluctuations in sucrose content; however, another experiment with *Q. alba* determined that sucrose was significantly reduced in the embryo and cotyledon tissue when the radicle emerged.

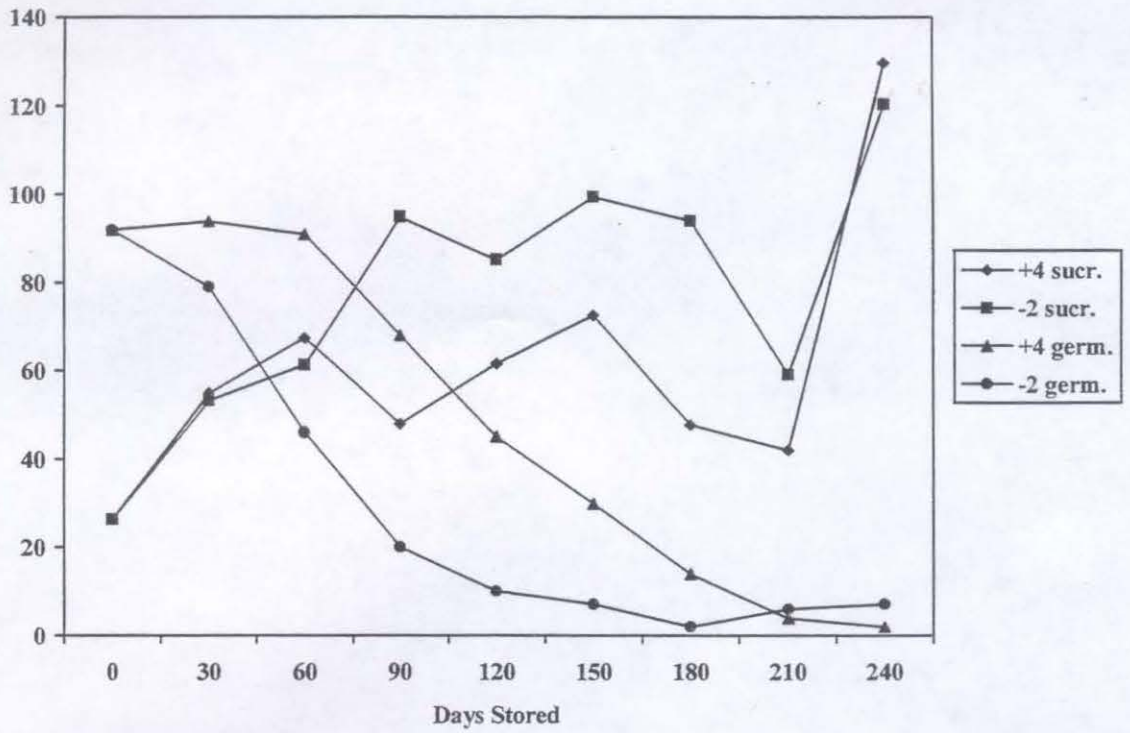

Figure 1. Germination (%) and sucrose content (mg/g dry wt.) of live oak acorn cotyledons stored at +4°C and –2°C for 240 days.

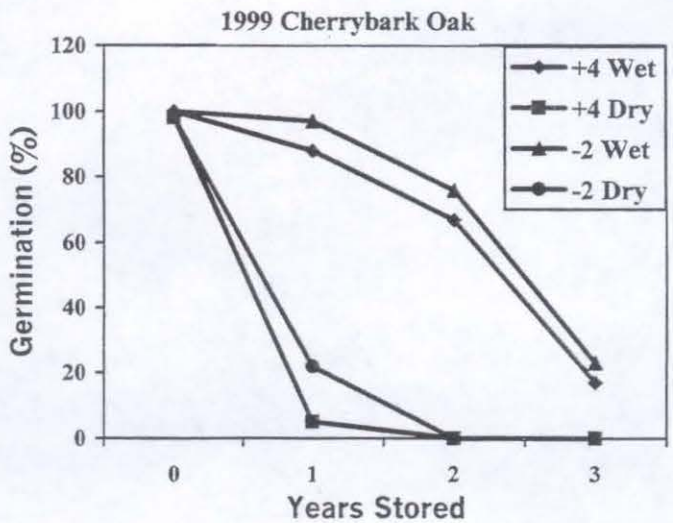

Figure 2. Germination (%) of water oak acorns and cherrybark oak acorns stored at two temperatures and two moisture contents for three years.

Experiment 2

Water oak acorn mc was 30.5% fr wt for the fresh acorns and decreased to only 25.6% after 2d of drying prior to storage. However, this slight reduction in mc reduced initial acorn viability by 9% (Fig. 2). After 1 yr, temperature of storage had a greater effect on seed viability than did initial mc. Both fully hydrated and dried acorns stored at -2°C maintained a higher viability than those stored at 4°C. This was not the case after 2 yrs of storage, when mc was the more important factor. Acorns, which had been dried prior to refrigeration, had lower viability than those stored fully hydrated. This was still the case after 3 yrs in storage, although deterioration had progressed in seeds stored fully hydrated. Mc did not change significantly from the original amount throughout the course of the experiment.

Cherrybark oak acorn mc was 29.6% for the fresh acorns and 19.9% for those dried 2d. However, drying reduced initial viability by only 2% (Fig. 2). Unlike water oak acorns, moisture content, and not temperature, was the most important factor in all test years. Only acorns stored in the fully hydrated condition retained high viability after 1 yr in storage. Those dried for 48 h prior to storage were severely affected after 1 yr of storage and were dead after 2 yrs. Changes in mc during storage were not significant.

Experiment 3

Cherrybark acorn germination was highly dependent on mc and severely declined when seed mc dropped below 17% (Table 3). Changes in molecular structure due to drying and rehydration were measured by changes in the frequency (and bandwidth) of the infrared absorbance of lipid and protein functional groups. Membrane lipid structure was measured by the frequency and bandwidth of the symmetric CH_2 stretch at 2850/cm (Sowa *et al.* 1991). An increase in vibrational frequency corresponds to increased fluidity (phase change from gel to liquid crystalline). In the liquid crystalline phase, membranes are fluid and in their normal state; when in the gel phase, membranes may leak cell solutes and cause irreparable damage to seeds. In this experiment, fresh tissues exhibited reversible shifts between gel and liquid crystalline phases upon drying and rehydration in the cotyledon tissue (Fig. 3). After drying for 8d, membrane lipids changed to gel phase and did not recover their fluidity upon rehydration.

Table 3. Cherrybark germination (%) and moisture content (fr wt) for FT-IR experiments.

Days	1999		2000	
dried	Germ.	MC	Germ.	MC
0	100	29.7	99	33.5
2	98	20.8	98	23.8
4	81	16.7	65	17.5
6	16	10.8	10	13.5
8	---	---	0	13.4

Protein secondary structure was measured using the amide I and II vibrations near 1650 and 1550/cm (Sowa *et al.* 1991). Changes in amide frequency correspond to changes in secondary structure. Alpha-helix structures absorb at higher frequencies, while beta-sheets absorb near 1630cm; denatured protein typically exhibits extended beta-sheet conformation, with infrared absorbances common at frequencies less than 1630/cm. Irreversible changes in the protein secondary structure, illustrated by shifts in the amide absorbance near 1650/cm, occurred in the cherrybark acorn cotyledon tissue (Fig. 4). Secondary structure was completely lost upon dehydration (day 8) and remained so upon rehydration of these samples (day 9).

Discussion

No one single temperature was best for storage of recalcitrant seeds. In previous experiments, chinkapin (*Q. muehlenbergii* Engelm.), northern red (*Q. rubra* L.), and Shumard (*Q. shumardii* Buckl.) oak acorns favored the lower storage temperature of -2°C (Connor and Bonner 1999). While Durand oak acorns and red buckeye seeds exhibited significantly higher viability when stored at -2°C, live oak acorns were harmed by the low

temperature. Also, sprouting during storage was a problem in red buckeye (17% after 180d), live oak (18% after 120d), Durand oak (16% after 120d), and white oak (96% after 90d) seeds stored at 4°C. Sprouting remained below 2% in seeds stored at -2°C for the same lengths of time.

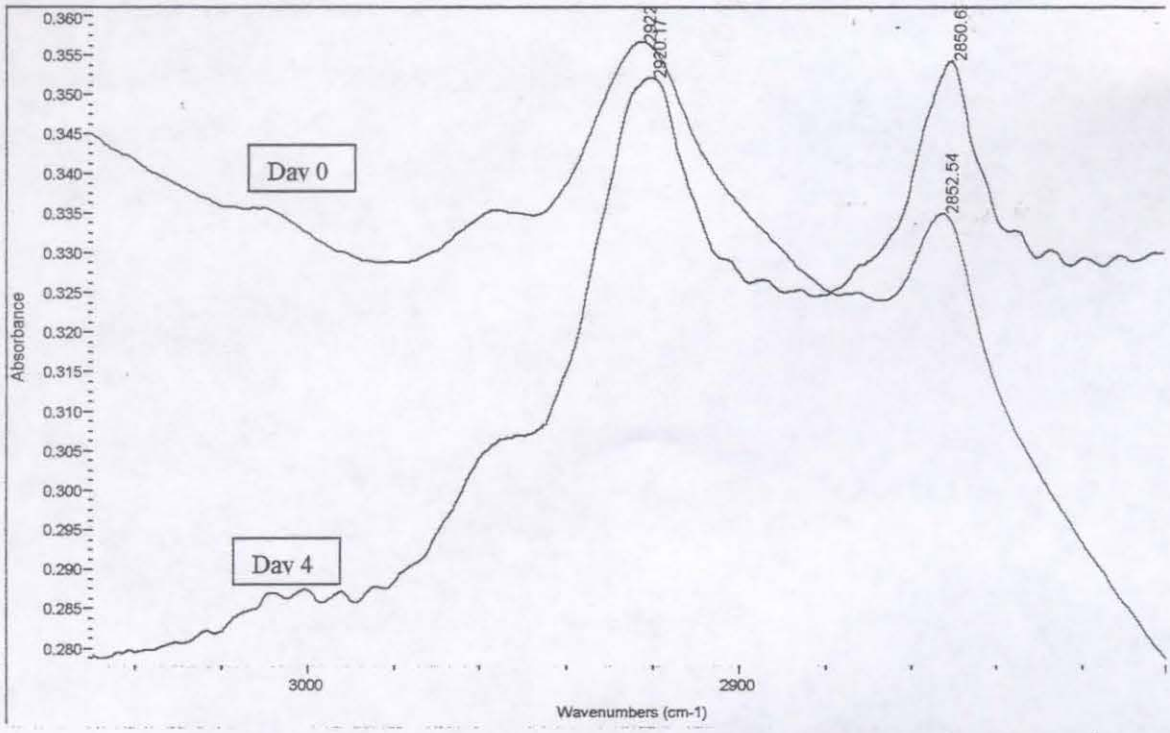

Figure 3. Membrane lipid $-CH_2-$ vibrations in cherrybark embryonic axes, symmetric (2850 cm^{-1}) and asymmetric (2920 cm^{-1}).

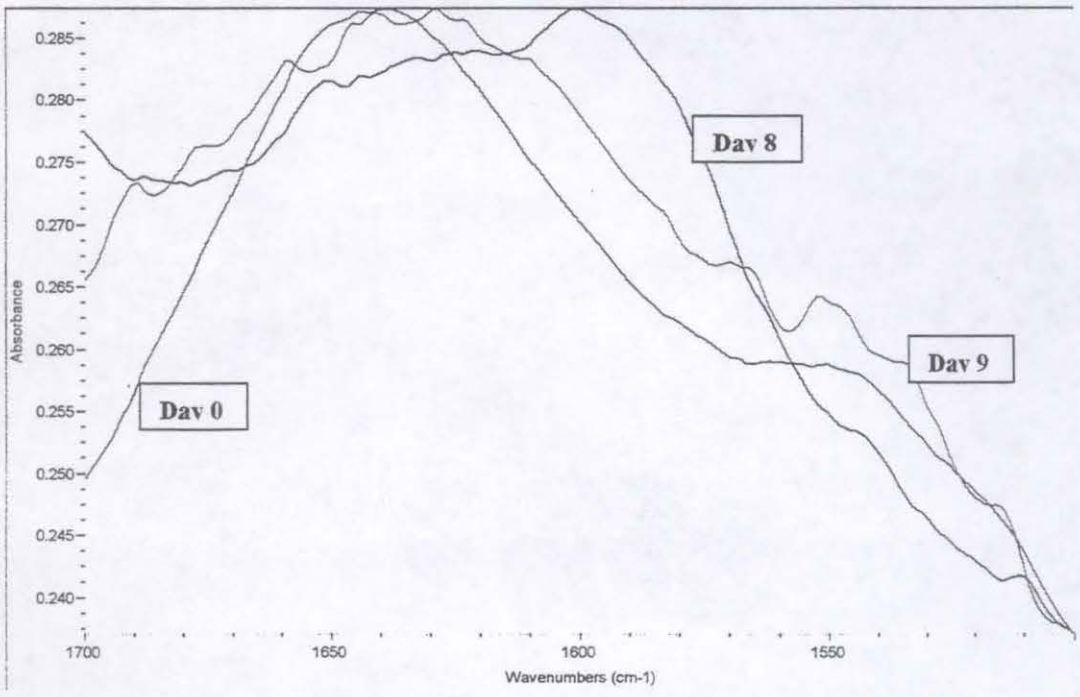

Figure 4. Protein (amide) vibrations in cherrybark oak embryonic axes. Peak frequencies at 1638.5, 1635, and 1629.9 cm^{-1}.

Both water oak and cherrybark oak acorns retained high viability after 2 yrs when stored fully hydrated. To date, sprouting and loss of moisture during storage are not factors in the successful storage of either species. While drying of water oak and cherrybark acorns for 2d before storage did not affect original viability, the damage was significant in water oak acorns stored for 1 yr at 4°C and in cherrybark oak acorns after 1 yr at either storage temperature. It is, therefore, strongly suggested that all precautions against moisture loss be taken when collecting acorns of these species that are not for immediate use. Unless the acorns are collected when fresh and maintained in a fully hydrated state, severe losses can arise when stored for only 1 yr. Orchard managers and seed processors must place emphasis on careful handling of acorns during the collection process. Also, the sooner acorns can be collected after dropping from the tree, and placed under refrigeration, the higher the probability of successful long-term (1 yr) storage.

Membrane lipids changed phase from liquid crystalline to gel upon drying and did not recover upon rehydration as viability was lost. Ions can pass indiscriminately through cell membranes in the gel phase, and this loss of selective permeability ultimately results in seed mortality. In this experiment, the change occurred first in the cotyledon tissue and then in the embryonic axes; since axes in recalcitrant seeds maintain a fairly high water content (Connor *et al.* 1996, Connor and Bonner 2001), this was not unexpected. It was interesting to note that after severe desiccation, rehydration did not restore membranes to their original fluid state.

Changes in protein secondary structure occurred in cotyledons as moisture was lost. Secondary structure was completely lost upon dehydration and remained so upon rehydration of nonviable samples. This evidence of protein denaturation occurring in the cytosol and/or cellular membranes was the most sensitive indicator of viability loss as yet encountered in these experiments. It is also contrary to behavior observed in orthodox seeds using infrared techniques (Golovina *et al.* 1997) and will be addressed in future investigations.

Literature Cited

Berjak, P. and Pammenter, N.W., 1997. Progress in the understanding and manipulation of desiccation-sensitive (recalcitrant) seeds. In: Basic and Applied Aspects of Seed Biology. Proceedings of the Fifth International Workshop on Seeds, Reading, 1995. Eds. R.H. Ellis, M. Black, A.J. Murdoch, and T.D. Hong. Kluwer Academic Publishers, London, pp 689-703.

Bonner, F.T., 1973. Storing red oak acorns. *Tree Planters' Notes*, vol. 24, no. 3, pp. 12-13.

Bonner, F.T., 1990. Storage of seeds: potential and limitations for germplasm conservation. *Forest Ecology and Management*, vol. 35, pp. 35-43.

Bonner, F.T. and Vozzo, J.A., 1987. Seed biology and technology of *Quercus*. USDA Forest Service General Technical Report SO-66, 21 p.

Connor, K.F. and Bonner, F.T., 1998. Physiology and biochemistry of recalcitrant *Guarea guidonia* (L.) Sleumer seeds. *Seed Technology*, vol. 20, no. 1, pp. 32-42.

Connor, K.F. and Bonner, F.T., 1999. Effects of temperature and moisture content on the storability of hardwood seeds. In: Proceedings of the Tenth Biennial Southern Silvicultural Research Conference, Shreveport, LA, 1999. Ed. J.D. Haywood.USDA Forest Service Gen. Tech. Rept. SRS-30, pp. 123-126.

Connor, K.F. and Bonner, F.T., 2001. The effects of desiccation on seeds of *Acer saccharinum* and *Aesculus pavia*: recalcitrance in temperate tree seeds. *Trees*, vol. 15, pp. 131-136.

Connor, K.F., Bonner, F.T., and Vozzo, J.A., 1996. Effects of desiccation on temperate recalcitrant seeds: differential scanning calorimetry, gas chromatography, electron microscopy, and moisture studies on *Quercus nigra* and *Quercus alba*. *Canadian Journal of Forest Research*, vol. 26, pp. 1813-1821.

Connor, K.F., Kossmann-Ferraz, I., Bonner, F.T. and Vozzo, J.A., 1998. Effects of desiccation on the recalcitrant seeds of *Carapa guianensis* Aubl. and *Carapa procera* DC. *Seed Technology*, vol. 20, no. 1, pp. 71-82.

Farrant, J.M., Berjak, P. and Pammenter, N.W., 1985. The effect of drying rate on viability retention of recalcitrant propagules of *Avicennia marina*. *South African Journal of Botany*, vol. 51, pp. 432-438.

Farrant, J.M., Pammenter, N.W. and Berjak, P., 1988. Recalcitrance - a current assessment. *Seed Science and Technology*, vol. 16, pp. 155-166.

Flood, R.G. and Sinclair, A., 1981. Fatty acid analysis of aged permeable and impermeable seeds of *Trifolium subterraneum* (subterranean clover). *Seed Science and Technology*, vol. 9, pp. 475-477.

Golovina, E.A., Wolkers, W.F. and Hoekstra, F.A., 1997. Behavior of membranes and proteins during natural seed aging. In: Ellis, R.H., Black, M., Murdoch, A.J., Hong, T.D. (Eds.). Basic and applied aspects of seed biology: Proceedings of the Fifth International Workshop on Seeds: 1995 Sept. 10-15, Reading, U.K., Kluwer Academic Publishers, pp. 787-796.

Jaynes, R.A., 1969. Long-term storage of chestnut seed and scion wood. *Annual Report of Northern Nut Growers Association*, vol. 60, pp. 38-42.

Pammenter, N.W., Vertucci, C.W. and Berjak, P., 1991. Homeohydrous (recalcitrant) seeds: dehydration, the state of water and viability characteristics in *Landolphia kirkii*. *Plant Physiology*, vol. 96, pp. 1093-98.

Pammenter, N.W., Berjak, P., Ross, G. and Smith, M.T., 1994. Why do hydrated, recalcitrant seeds die? *Seed Science Research*, vol. 4, pp. 187-191.

Priestly, D.A. and Leopold, A.C., 1983. Lipid changes during natural ageing of soybean seeds. *Physiologia Plantarum*, vol. 59, pp. 467-470.

Pritchard, H.W., 1991. Water potential and embryonic axis viability in recalcitrant seeds of *Quercus rubra*. *Annals of Botany*, vol. 67, pp. 43-49.

Pritchard, H.W. and Manger, K.R., 1990. Quantal response of fruit and seed germination rate in *Quercus robur* L. and *Castanea sativa* Mill. to constant temperatures and photon dose. *Journal of Experimental Botany*, vol. 41, pp. 1549-57.

Roberts, E.H., 1973. Predicting the storage life of seeds. *Seed Science and Technology*, vol. 1, pp. 499-514.

Seewaldt, V., Priestly, D.A., Leopold, A.C., Feigenson, G.W. and Goodsaid-Zaluondo, F., 1981. Membrane organization of soybean seeds during hydration. *Planta*, vol. 152, pp. 19-23.

Sowa, S., Connor, K.F. and Towill, L.E., 1991. Temperature changes in lipid and protein structure measured by Fourier Transform infrared spectrophotometry. *Plant Science*, vol. 78, pp. 1-9.

Wesley-Smith, J., Vertucci, C.W., Berjak, P., Pammenter, N.W. and Crane, J., 1992. Cryopreservation of desiccation-sensitive axes of *Camellia sinensis* in relation to dehydration, freezing rate and the thermal properties of tissue water. *Journal of Plant Physiology*, vol. 140, pp. 596-604.