RECALCITRANT BEHAVIOR OF TEMPERATE FOREST TREE SEEDS: STORAGE, BIOCHEMISTRY, AND PHYSIOLOGY

Kristina F. Connor and Sharon Sowa[1]

Abstract—The recalcitrant behavior of seeds of live oak (*Quercus virginiana* Mill.), and Durand oak (*Quercus durandii* Buckl.) was examined after hydrated storage at two temperatures, +4° C and -2° C for up to 1 year. Samples were collected and analyses performed at monthly intervals. At each sampling time, seeds were tested for viability and moisture content. Red buckeye (*Aesculus pavia* L.) seeds were similarly stored but analyzed at intervals of 3 months, while those of cherrybark oak (*Quercus pagoda* Raf.) and water oak (*Quercus nigra* L.) were tested yearly. Durand oak, live oak, and red buckeye seeds stored at -2° C maintained higher viability for a longer period of time than did those stored at +4° C. However, live oak acorns were damaged by the colder storage temperature. Sprouting during storage occurred at the higher storage temperature, but not at -2° C. After 2 years, water oak and cherrybark oak acorns which had been dried prior to refrigeration had lower viability than those stored fully hydrated. The damage was especially apparent in cherrybark acorns, with viability reduced after 1 year to 22 percent in those dried and then stored at -2° C and to 5 percent in those stored at +4° C. It is suggested that all precautions against desiccation be taken when collecting cherrybark and water oak acorns 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 year. Fourier transform infrared spectrometry (FT-IR) studies have shown that cherrybark acorns subjected to severe desiccation exhibit irreversible changes in membrane lipid and protein secondary structure. This change was the most sensitive indicator of viability loss as yet encountered in these experiments. Future studies will examine the role of protein denaturation in seed deterioration.

INTRODUCTION

Early studies on low temperature storage of hardwood tree seeds resulted in the division of seeds into two storage behavior classes (Roberts 1973): 'Orthodox' seeds undergo a period of desiccation before being shed from the tree and can easily be stored at low temperatures for long periods of time at moisture contents of less than 12 percent. Temperate 'recalcitrant' seeds, however, do not undergo this final maturation drying and are thus very sensitive to moisture loss, making storage for any useful period extremely difficult. Immediate causes of seed viability loss are attack by pathogens and premature germination. Recent work has modified both Roberts' initial definition of recalcitrance and our perspective of the nature of recalcitrance. Pammenter and others (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. Thus, while much progress has been made in understanding the nature of recalcitrance, the storage of some recalcitrant tree seeds over a long period remains an insurmountable problem. North American genera containing species with recalcitrant seeds are *Castanea* (Pritchard and Manger 1990), and some *Acer*, *Aesculus*, and *Quercus* (Bonner 1990).

Acorns of the red oaks and of *Quercus robur* have reportedly been stored at -1° C or -2° C for periods up to 5 years in Europe (Suszka and Tylkowski 1980, 1982). Experiments here have been less successful, suggesting a varying degree of dormancy between European and U.S. species. We are reporting the results from three studies: (1) a 1 year storage study of Durand oak (*Quercus durandii* Buckley), live oak (*Quercus virginiana* Mill.), and red buckeye (*Aesculus pavia* L.) at 2 temperatures; (2) second year results of a water oak (*Quercus nigra* L.) and cherrybark oak (*Quercus pagoda* Raf.) acorn storage experiment at 2 temperatures and 2 moisture contents; and (3) a Fourier transform infrared (FT-IR) spectroscopy study of desiccating cherrybark oak acorns.

METHODS

Durand oak and red buckeye seeds were collected locally in Oktibbeha County, MS. The water oak and cherrybark oak acorns were purchased from a local supplier, while the live oak acorns were collected in Washington County, MS. All seeds were cleaned by floatation, soaked overnight, and then stored at 4° C until the start of the experiment. Original moisture contents for each drying regime were determined by drying 2-4 samples of seeds at 105° C for 16-17 hours. In preparation for germination tests, acorns were cut in half horizontally. The seed coat was removed from the half

[1]Plant Physiologist, USDA Forest Service, Southern Research Station, Starkville, MS; Biochemist, Indiana University of Pennsylvania, Indiana, PA, respectively.

Citation for proceedings: Outcalt, Kenneth W., ed. 2002. Proceedings of the eleventh biennial southern silvicultural research conference. Gen. Tech. Rep. SRS–48. Asheville, NC: U.S. Department of Agriculture, Forest Service, Southern Research Station. 622 p.

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 at the start of each germination test of the number of seeds in a sample which had sprouted during storage. Experiments were conducted as follows:

Experiment 1

This experiment was conducted on 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 germinations and moisture content were determined for the fresh acorns and every 30 days thereafter for one year, as acorn supplies permitted. A subsample of acorns was dissected, and the embryos and cotyledons cryostored for future chemical and FT-IR spectroscopic analyses. Acorns were germinated as two replications of 50 seeds each per sampling period and were rehydrated overnight in tapwater prior to germination testing. Red buckeye seeds were stored as above; however, they were tested only at fresh, 90-, 180-, and 360-day intervals and were stored in batches of 59 seeds per bag. Germination tests consisted of 2 replications of 15 seeds each per sampling period.

Experiment 2

High and low moisture levels for water and cherrybark oak acorns were imposed by either soaking in tapwater for 16 hours or by drying on a lab bench for 48 hrs. 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 moisture contents 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 tapwater 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. Cotyldeon samples of fresh seeds and those that had been dried for 2, 4, 6, and 8 days 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 moisture content dropped below 15 percent, and 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.

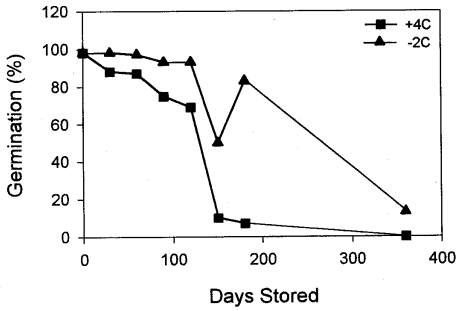

Figure 1—Viability of Durand oak (*Quercus durandii* Buckley) acorns stored for 1 year at 4° C and at -2° C.

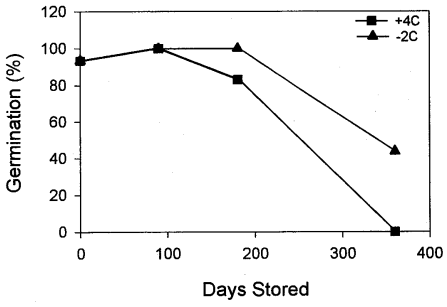

Figure 2—Viability of red buckeye (*Aesculus pavia* L.) seeds stored for 1 year at 4° C and at -2° C.

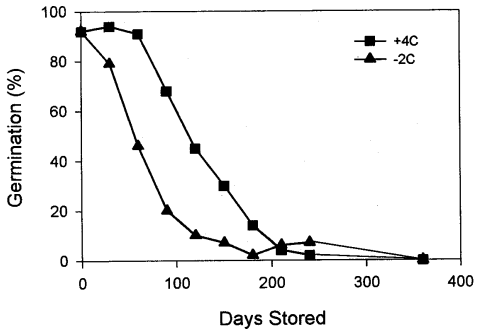

Figure 3—Viability of live oak (*Quercus virginiana* Mill.) acorns stored for 1 year at 4° C and at -2° C.

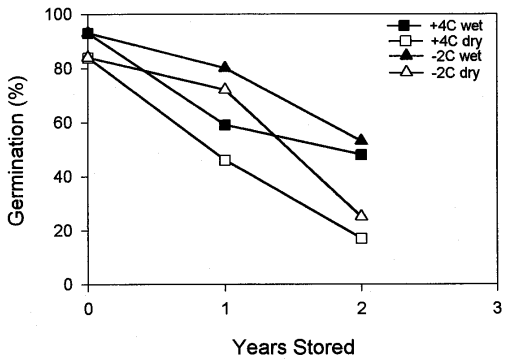

Figure 4—Water oak (*Quercus nigra* L.) acorns stored at two moisture contents and two temperatures.

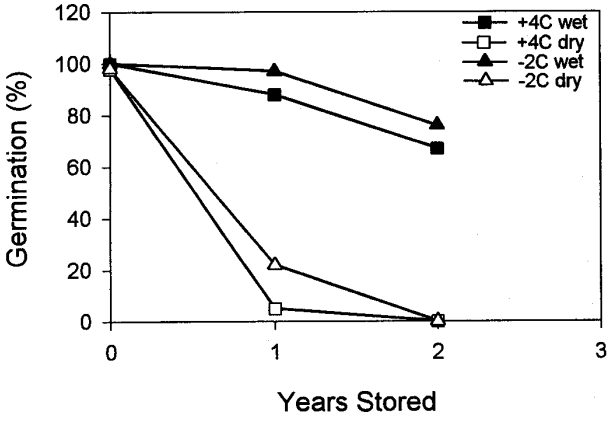

Figure 5—Cherrybark oak (*Quercus pagoda* Raf.) acorns stored at two moisture contents and two temperatures.

RESULTS

Experiment 1

Durand oak acorns stored at -2° C had significantly higher viability than those stored at 4° C in as little as 30 days (figure 1). After 210 days, acorns stored at -2° C averaged 83 percent viability, while only 6 percent of those stored at 4° C survived. Red buckeye seeds also remained viable longer if stored at -2° C (figure 2). The differences in viability did not occur, however, until after 90 days in storage. Acorns of live oak were the only ones tested to date that survive longer if stored at 4° C (figure 3). Storage at -2° C resulted in significant damage to the acorns. Fresh moisture contents were 38.1, 60.6, and 56.6 percent for Durand oak, red buckeye, and live oak, respectively, and did not change greatly during storage.

Experiment 2

Water oak acorn moisture content was 30.5 percent (on a fresh weight basis) for the fresh acorns and 25.6 percent for those dried 2 days prior to storage. Drying reduced initial acorn viability by 9 percent (figure 4). After 1 year, temperature of storage had a greater effect on seed viability than did initial moisture content. 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 years of storage, when moisture content was the more important factor. Acorns which had been dried prior to refrigeration had lower viability than those stored fully hydrated. Moisture content did not change significantly throughout the course of the experiment.

Cherrybark oak acorn moisture content was 29.6 percent for the fresh acorns and 19.9 percent for those dried 2 days. However, drying reduced initial viability by only 2 percent (figure 5). Unlike water oak acorns, moisture content, and not temperature, was the important factor after both 1 and 2 years of storage. Only acorns stored in the fully hydrated condition retained high viability; dried acorns experienced significant losses in viability. after only 1 year in storage and were dead after 2 years. Changes in moisture content during storage were not significant.

Experiment 3

Cherrybark acorn germination was highly dependent on moisture content, and severely declined when seed moisture dropped below 17 percent (table 1). 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 and others 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 (figure 6). After drying for 8 days, membrane lipids changed to gel phase and did not recover their fluidity upon rehydration.

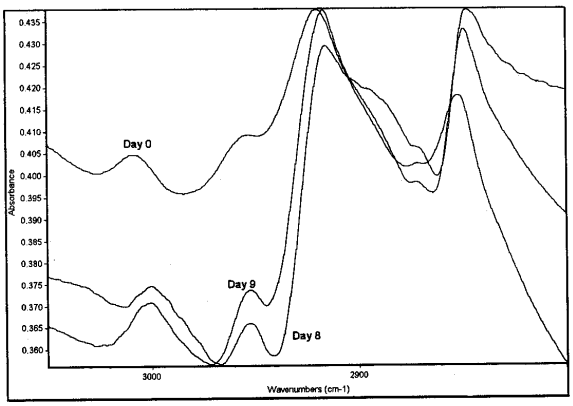

Figure 6—Membrane lipid vibrations in fresh (day 0) cherrybark acorn cotyledon tissue dried for 8 days, and then rehydrated (day 9). Peak frequencies are at 2850.9, 2847.2, and 2848.8 cm^{-1}.

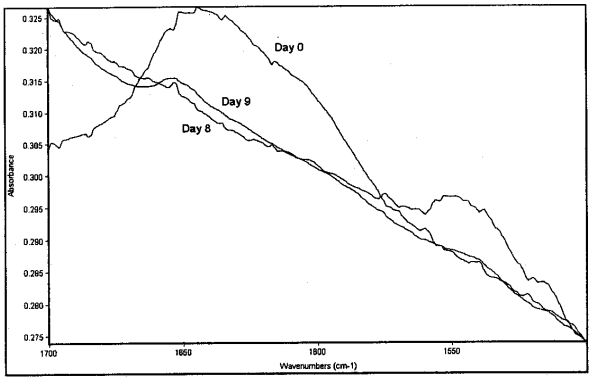

Figure 7—Amide protein vibrations in fresh (day 0) cherrybark acorn cotyledon tissue and in tissue dried for 8 days and then rehydrated (day 9).

Protein secondary structure was measured using the amide I and II vibrations near 1650 and 1550/cm (Sowa and others 1991). Changes in amide frequency correspond to changes in secondary structure. Alpha-helix structures absorb at higher frequencies, while beta-sheets absorb near 1630/cm; 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 (figure 7). 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 (*Quercus muehlenbergii* Engelm.), northern red (*Quercus rubra* L.), and Shumard (*Quercus 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 percent after 180 days), live oak (18 percent after 120 days), and Durand oak seeds (16 percent after 120 days) stored at +4° C. Sprouting remained below 2 percent in seeds stored at -2° C for the same lengths of time.

Both water oak and cherrybark oak acorns retained high viability after 2 years when stored fully hydrated. To date, sprouting and changes in moisture content are not factors in the successful storage of either species. However, unlike a previous report (Connor and Bonner 1999), we did not find significant differences in viability caused by temperature of storage (figs. 4,5). Also, while drying of water oak and cherrybark acorns for 2 days before storage did not affect original viability, the damage was significant in water oak acorns stored for 1 year at +4° C and in cherrybark oak acorns after 1 year 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 year. 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 year) 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 embryos; since embryos in recalcitrant seeds maintain a fairly high water content (Connor and others 1996, 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 and others 1997) and will be addressed in future investigations.

LITERATURE CITED

Berjak, P.; Pammenter, N.W. 1997. Progress in the understanding and manipulation of desiccation-sensitive (recalcitrant) seeds. 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 September 10-15. Reading, U.K.: Kluwer Academic Publishers: 689-703.

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

Connor, K.F.; Bonner, F.T. The effects of desiccation on seeds of *Acer saccharinum* and *Aesculus pavia*: recalcitrance in temperate tree seeds. Trees - Structure and Function.

Connor, K.F.; Bonner, F.T.; 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. 26: 1813-1821.

Golovina, E.A.; Wolkers, W.F.; 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 September 10-15. Reading, U.K.: Kluwer Academic Publishers: 787-796.

Pammenter, N.W.; Berjak, P.; Farrant, J.M.; Smith, M.T.; Ross, G. 1994. Why do stored hydrated recalcitrant seeds die? Seed Science Research. 4: 187-191.

Pritchard, H.W.; 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. 41: 1549-1557.

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

Sowa, S.; Connor, K.F.; Towill, L.E. 1991. Temperature changes in lipid and protein structure measured by Fourier Transform infrared spectrophotometry in intact pollen grains. Plant Science. 78: 1-9.

Suszka, B.; Tylkowski, T. 1980. Storage of acorns of the English oak (*Quercus robur* L.) over 1-5 winters. Arboretum Kornickie. 25: 199-229.

Suszka, B.; Tylkowski, T. 1982. Storage of acorns of the northern red oak (*Quercus borealis* Micz. = *Q. rubra* L.) over 1-5 winters. Arboretum Kornickie. 26: 253-306.