PHYSICAL ASPECTS OF FREEZING IN BLACK OAK ACORNS[1]

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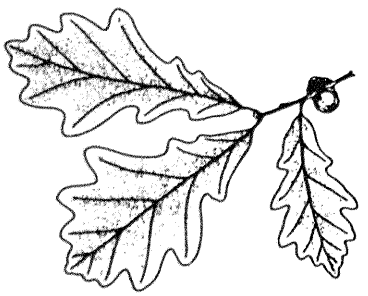

Abstract.--Differential thermal analysis (DTA) and viability tests (on a limited sample size) were utilized to characterize the freezing of tissue water in black oak (Quercus velutina Lam.) acorns after stratification at 2°C. It was determined that fully hydrated acorns with pericarp intact or removed survived ice formation in their tissues to -7°C when cooled at 0.3°C/hr and when artificially nucleated with ice at slightly below 0°C. Freezing appeared to occur in an equilibrium or near equilibrium fashion. Desiccated acorns (approximately 20 percent fresh weight water content) with pericarp intact were observed to supercool and freeze in a lethal nonequilibrium fashion between -11 and -15°C. Desiccated acorns without pericarp, however, did not supercool. Extended storage tests indicated that fully hydrated acorns can survive exposure to -7°C for one month.

INTRODUCTION

Black oak (Quercus velutina Lam.) is an inconsistent seed producer with two to three years between crops (Downs and McQuilkin, 1944; Fowells, 1965; and Olson, 1974). This periodic production in itself is not a problem until it is combined with the problems related to seed storage. Storage recommendations for oaks indicate that acorns of the white oak group should not be stored and acorns of the red oak group should not be stored for more than six months (Olson, 1974). Generally, acorns are stored at temperatures slightly above 0°C and in nearly a fully hydrated state. The limitations in storage time noted above result from the loss of viability during extended storage under these conditions.

Acorns fit into the recalcitrant seed type described by Stanwood and Bass (1978). This seed type is characterized by fleshy seeds that are sensitive to desiccation. Seeds which are insensitive to desiccation are referred to as orthodox. Because of their recalcitrant nature, acorns must be stored at fresh weight moisture contents of 20 percent or greater (Jones, 1962; Olson,1974). As suggested by Becwar et al. (1983), if cellular water can be removed to extracellular or extraorgan ice during freezing without causing desiccation injury, altering the storage temperature to below the melting point of the cell sap may be a tool for prolonging storage of recalcitrant seeds.

Although acorns have been reported to be capable of surviving a few degrees below 0°C (Heit, 1967), no data is available on the physical behavior of water in acorns at subfreezing temperatures. A few recent studies have, however, investigated some of the biophysical details of ice formation in orthodox seeds. For example, Junttila and Stushnoff (1977) found that the minimum temperature survived by lettuce (Lactuca sativa L.) seeds varied with the moisture content. Seeds with lower moisture contents survived lower temperatures. They also utilized differential thermal analysis (DTA) to follow the course of ice formation in the seed tissues. Their results established that seeds with fresh weight water contents greater than approximately 20 percent contained a fraction of water which supercooled to near -20°C. Loss of viability was associated with the crystallization of this supercooled fraction of water. Seeds with water contents between 5 and 16 percent displayed no observable freezing as measured by DTA and survived exposure to temperatures below -30°C without dam-

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age. The cooling rate in these experiments was 20°C/hr. Keefe and Moore (1981) have subsequently shown that slower cooling rates (less than 2°C/hr) allowed water in hydrated lettuce seeds to freeze outside the endosperm causing desiccation of the embryo and prevention of its freezing (i.e. supercooling was eliminated). Hydrated seeds survived this slow freezing to below -25°C with high viability. Ishikawa and Sakai (1982) have utilized extended storage at temperatures between -5 and -10°C as well as slow cooling rates to condition hydrated seeds of lettuce, Celastrus orbiculatus, Oenothera sp. and Vitus coignetiae for cooling to -196°C. Becwar et al. (1983) have also studied dehydration effects on freezing characteristics and low temperature survival in a number of desiccation-tolerant and desiccation-sensitive seeds.

The objectives of the work reported below were to ascertain if water in acorn tissues of black oak remains supercooled at subfreezing temperatures or if it freezes in an equilibrium fashion and to determine if acorns could survive ice formation in their tissues.

MATERIALS AND METHODS

Acorns were collected during October, 1983 from selected black oak (Quercus velutina Lam.) trees near the University of Missouri-Columbia campus. The acorns were floated in tap water for 24 hours at 20°C. Those which had a density greater than water were placed in plastic bags and stratified at 2°C for three months before low temperature testing was initiated.

Freezing of tissue water was monitored by DTA. The DTA method involves cooling a sample and reference at the same rate, but in separate chambers (Pope and Judd, 1977). The sample is attached to a temperature sensor and placed in one chamber. The reference is an inert sample attached to a similar temperature sensor. Freezing events occurring in the sample cause a warming of the sample with respect to the reference. This temperature differential is recorded in a suitable fashion.

A DTA system utilizing thermistor sensors similar to that described by George (1982) was used; however, the chamber was modified to accommodate the large size of the acorns. In the experiments reported here, acorns were wrapped in a thin strip of damp cheesecloth and then placed in an aluminum foil cup which held the sample in contact with the thermistor. Part of the cheesecloth extended from the DTA chamber and provided an external ice nucleation source for the acorns tissues. The reference sample was an empty foil cup. Samples were equilibrated at -3 to -5°C for approximately three hours before cooling was initiated. The DTA cooling rate was 0.3°C/hr. Preliminary experiments by George and Mitchell (1982, unpublished data) found that faster cooling rates led to extensive supercooling in acorns regardless of the presence of an external ice nucleating source. This rate is also in keeping with the slow rates suggested for freezing studies in other seeds (Keefe and Moore, 1981; Ishikawa and Sakai, 1982).

Differential thermal analysis was performed on fully hydrated and desiccated acorns with and without pericarp. Tissues were hydrated by soaking stratified acorns in tap water for 24 hrs at 25°C. These acorns were considered to be fully hydrated. For desiccation studies, acorns were dried to the desired water content at 25°C on a lab bench.

Viability testing was carried out on hydrated and desiccated acorns with the pericarp intact. Acorns (10 to 17 per test temperature) were wrapped in wet cheesecloth and surrounded with aluminum foil. These were placed in a low temperature freezer and cooled in a stepwise fashion to -4, -7, -10, -14 and -18°C. The samples were held at each temperature for approximately 24 hours. Copper-constantan thermocouples were attached to selected samples and temperatures were recorded on a multipoint recorder. Temperature was maintained within 1°C of the set point. After exposure at each temperature was complete, the acorns were placed in a thermos bottle chilled to the same temperature and then transferred to a refrigerator at 2°C. After thawing was complete (typically 24 hours) the acorns were germinated between damp blotter paper in a growth chamber at 25°C. Emergence and elongation of the radicle was used as an indicator of low temperature survival.

In addition to viability testing, fully hydrated acorns with pericarp intact were exposed to extended storage at -7°C. Five sets of ten acorns each were prepared as above. These samples were placed in thermos bottles and exposed to -7°C for one week, two weeks, one month, three months and six months. After thawing, survival was evaluated as described above.

RESULTS AND DISCUSSION

Differential thermal analysis curves of fully hydrated black oak acorns (43 percent fresh weight water content) indicated that freezing of tissue water began above -4°C regardless of pericarp intactness (Fig. 1 and 2). A typical DTA trace for a fully hydrated acorn with pericarp (Fig. 1) contained two broad freezing peaks. The first peak began just below 0°C and was due to ice formation in the damp cheesecloth. The second peak resulted from crystallization of tissue water. This peak began slightly below -2°C and ended at approximately -6°C. This slow high temperature freezing is representative of an equilibrium or a near equilibrium freezing process where intracellular water migrates to ice in extracellular spaces and cells are slowly dehydrated (Burke et al., 1976). In seeds, extraorgan freezing may also be possible under slow freezing conditions (Keefe and Moore, 1981; Ishikawa and Sakai, 1982); however, no microscopic observations were made of frozen acorns to determine if the freezing was extracellular or extraorgan. Differential thermal analysis does not discriminate between extraorgan and extracellular freezing.

Results of viability tests on fully hydrated acorns showed that 86.7 percent (13 of 15) survived -4°C, 93 percent (14 of 15) survived freezing to -7°C, but none (0 of 15) survived -10°C. Despite

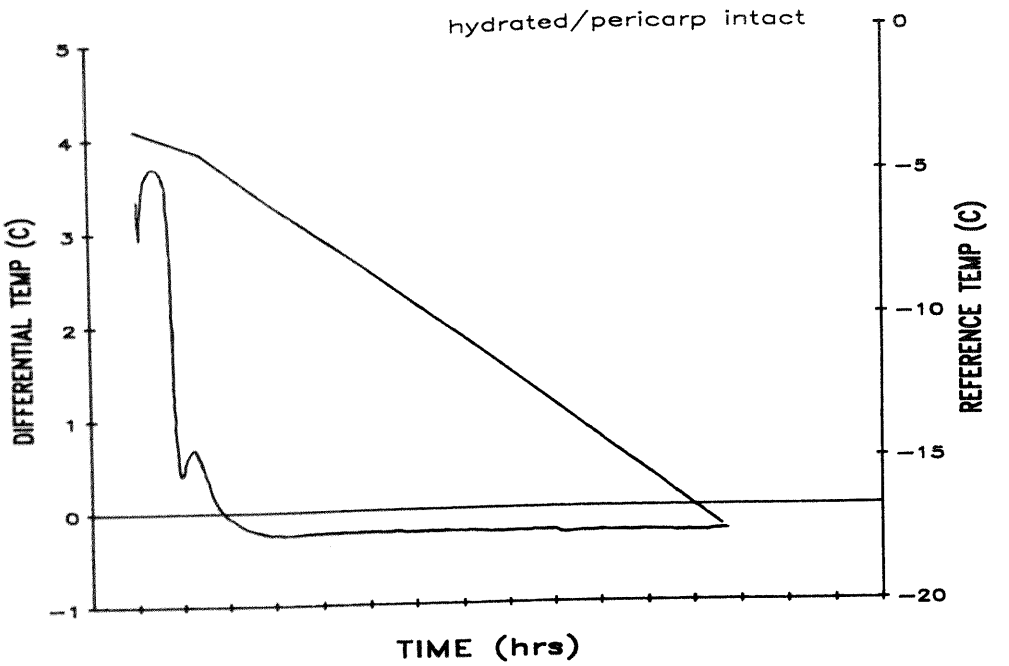

FIGURE 1.--Freezing curve determined by DTA for a fully hydrated black oak acorn with pericarp intact. The high temperature peak is due to ice formation in the damp cheesecloth surrounding the acorn and provided an external ice nucleation source. The low temperature peak is from ice formation in the acorn. Ambient temperature of DTA reference is also shown. Each time increment on the abscissa is equal to five hours. See text for discussion.

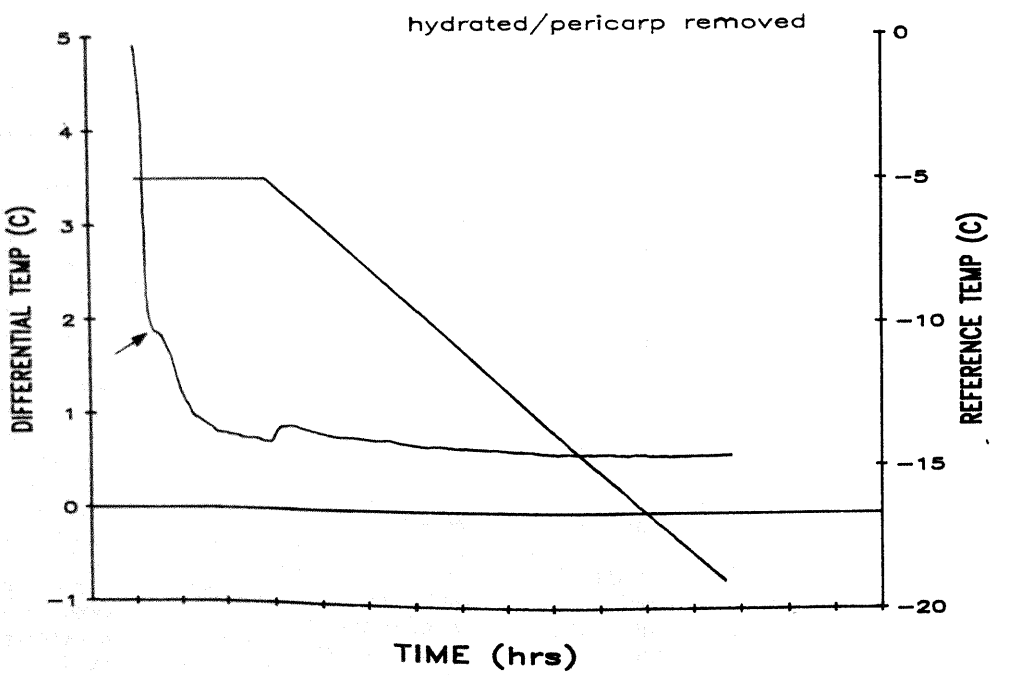

FIGURE 2.--Differential thermal analysis curve for a fully hydrated acorn without pericarp. See text for discussion. Initiation of ice formation in the acorn is indicated by the arrow. The deflection on the trace near -5°C was due to a heat capacity difference between the sample and reference chambers as cooling at 0.3°C/hr was initiated and is not associated with ice formation in the acorn.

this relatively high lethal freezing temperature, it is apparent that acorns can survive ice in their tissues. No detailed viability tests were conducted on fully hydrated acorns without pericarp, but previous work by George and Mitchell (1982, unpublished) found survival to similar temperatures in hydrated black oak acorns without pericarp. The similarity of the DTA traces for acorns with and without pericarp (Figs. 1 and 2) at minimum suggests that the pericarp is not a significant barrier to ice formation in fully hydrated seeds.

Differential thermal analysis curves for intact acorns desiccated to approximately 20 percent fresh weight water content displayed sharp freezing peaks at considerably lower temperatures than for fully hydrated acorns (Fig. 3). Peak start temperatures in four acorns tested were -11.6, -12.5, -14.3 and -14.6°C, respectively. This type freezing response is typical of the supercooling observed in seeds studied by other workers (Juntilla and Stushnoff, 1977; Keefe and Moore, 1981; Becwar et al., 1982; Becwar et al., 1983). In particular, the freezing response was analogous to that observed by Becwar et al. (1982) in seeds of conifer dwarf mistletoe (Arceuthobium spp.) where stable supercooling of the entire seed occurred to a temperature of -35°C. At this temperature a lethal crystallization of tissue water was observed. In general, the freezing curves for desiccated acorns are similar to those observed for other plant parts such as the floral primordia of certain tree fruits and the vegetative buds of various conifers. These plant parts avoid freezing in some or all of their tissues by supercooling. See George et al. (1982) and George and Burke (1984) for a review of supercooling in plants.

Keefe and Moore (1981) indicate that supercooling in lettuce seeds is eliminated by slow cooling (less than 2°C/hr). In the experiments reported here, desiccated acorns remain supercooled at a cooling rate of 0.3°C/hr and in the presence of an external ice nucleus (the frozen cheesecloth). As noted above, this response was similar in character to that found in conifer dwarf mistletoe seeds by Becwar et al. (1982). Upon desiccation, the acorn pericarp apparently becomes an effective barrier to ice penetration, at least to the freezing point, and also prevents the rapid sublimation of supercooled tissue water to ice in the surrounding cheesecloth. A metastable equilibrium may be established between external ice and liquid tissue water due to surface chemical effects associated with the porosity of the woody pericarp. From a purely thermodynamic standpoint it can be shown that pore size can influence the melting point of water within the pore in the case of an ice/liquid interface or can lower the vapor pressure over a gas/liquid interface (Everett, 1961). Either situation may occur during freezing of an intact acorn and could be important in permitting the observed supercooling. Cell wall porosity has been suggested to be an important factor which allows supercooling in woody xylem and floral primordia (George, 1983; Ashworth and Abeles, 1984). At this point, it is unclear whether ice penetrates the pericarp at the nucleation temperature or if nucleation is due to internal heterogeneous ice nucleators. This is currently under investigation.

When the pericarp was removed from desiccated acorns the DTA freezing curve did not display supercooling as observed above (Fig. 4). A broad peak similar to that found for fully hydrated acorns was recorded indicating that an equilibrium or near equilibrium freezing process was again taking place. The freezing peak was depressed approximately 2 to 4°C below that measured for fully hydrated acorns. This is probably due to a decreased melting point of the tissue water associated with desiccation, but this was not verified experimentally.

Viability tests indicate that supercooled acorns (20% moisture content) do not survive the low temperature crystallization of tissue water. Survival was 80 percent (8 of 10) at -4°C, 86.7 percent (13 of 15) at -7°C and was 73.3 percent (11 of 15) at -10°C. No acorns (0 of 15) survived -14°C. This result is consistent with the behavior of other supercooled plant parts including seeds where the crystallization of supercooled tissue water is lethal (Burke et al., 1976; Juntilla and Stushnoff, 1977; Keefe and Moore, 1981; Becwar et al., 1982; George et al., 1982; Becwar et al., 1983; George and Burke, 1984). It is believed that when supercooled tissue water freezes it does so intracellularly and disrupts internal cell structure in an irreparable fashion. No viability tests were conducted on desiccated acorns without pericarp. The survival of freezing by desiccated acorns without pericarp, therefore, remains to be established. Additionally, extended storage of supercooled acorns above their freezing points needs to be conducted to determine the long-term stability of the supercooled tissue water.

Results of extended storage of fully hydrated acorns at -7°C found 80 percent survival (8 of 10) after one month, but no freezing survival after three months. It has been suggested elsewhere that acorns be tested for storage at subfreezing temperatures (Heit, 1967). The results presented here indicate that fully hydrated acorns can survive in a frozen state for a considerable period of time. Without modification, however, the freezing protocol utilized in these experiments would be of little use for long-term storage.

CONCLUSION

Fully hydrated black oak acorns have the capacity to survive ice in their tissues at -7°C for periods up to one month. Differential thermal analysis indicated that freezing in fully hydrated acorns was an equilibrium or near equilibrium process. Desiccated acorns supercool and freeze in a lethal nonequilibrium fashion when the pericarp is left intact, but freeze in a near equilibrium fashion when the pericarp is removed. The observation that acorn tissues can survive freezing suggests that long-term cryopreservation of intact acorns or perhaps excised embryos at extreme low temperatures may be possible; however, this will require other freezing protocols than simple slow freezing in the presence of an external ice nucleator.

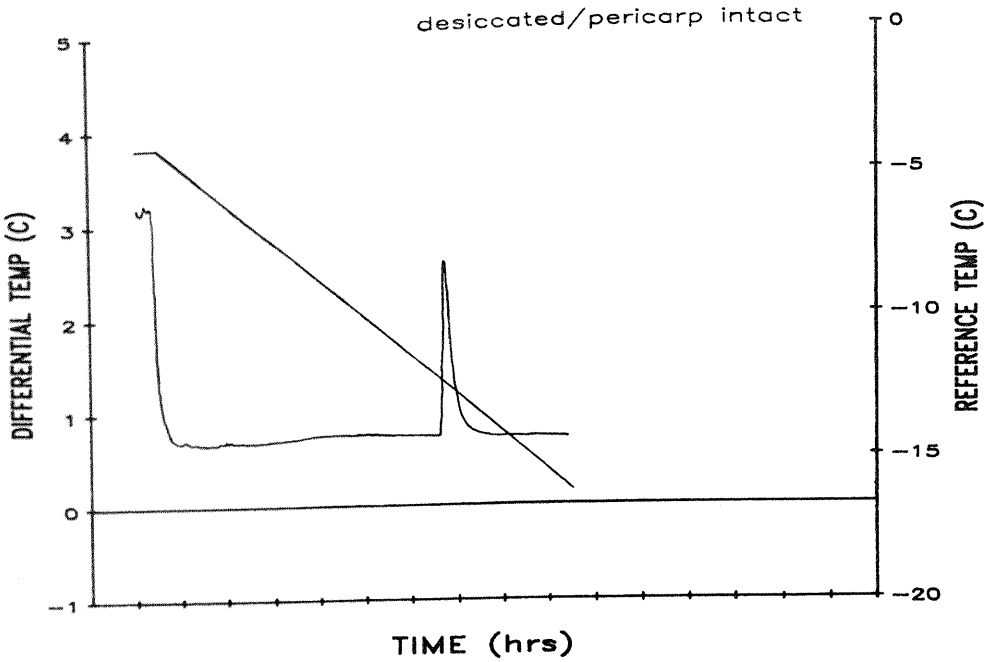

FIGURE 3.--Differential thermal analysis curve for a desiccated acorn with pericarp. See text for discussion.

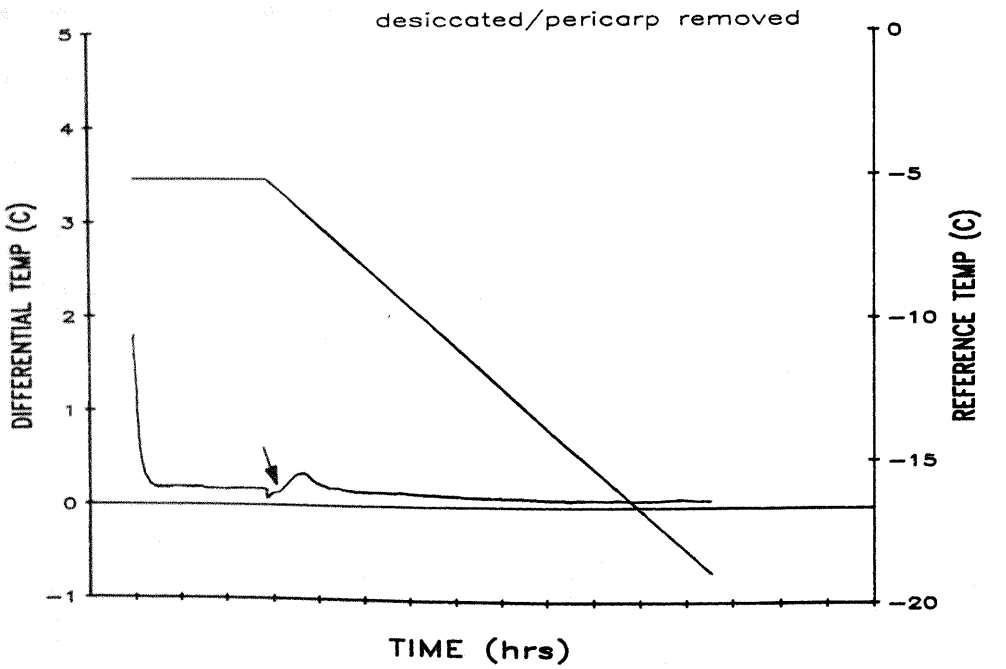

FIGURE 4.--Differential thermal analysis curve for a desiccated acorn without pericarp. See text for discussion. Initiation of ice formation in the acorn is indicated by the arrow. The deflection on the trace near -5°C was due to a scale change and heat capacity difference between the sample and reference chamber as cooling at 0.3°C/hr was initiated and is not associated with ice formation in the acorn.

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