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Development, fatty acid composition, and storage of drupes and seeds from the endangered pondberry (*Lindera melissifolia*)

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ABSTRACT

Pondberry (*Lindera melissifolia* [Walt.] Blume: Lauraceae) is an endangered, dioecious, clonal shrub that grows in bottomland hardwood forests in the southeastern United States. Prior work has emphasized vegetative reproduction associated with the clonal nature of this species. Little has been published about the early morphological and biochemical characteristics of the fruit as they mature. Fruits, drupes originating from the axillary buds, were collected every 30 days after anthesis and examined for seed structure development and fatty acid composition of the fruit and seed. Sixty days after anthesis, fruits had not formed an organized embryo/cotyledon, weighed 0.1 ± 0.001 g, and measured 7.1 ± 0.04 mm × 4.3 ± 0.03 mm. Ninety days after anthesis, a complete seed had formed within the drupe. Of the total drupe weight (average 0.23 ± 0.01 g), the seed comprised 33% of the mass gained from 60 days after anthesis. Overall composition of the seed and pulp lipids changed significantly over the course of development. Myristic, palmitic, steric, oleic, linoleic, and linolenic fatty acids were revealed by the lipid analyses. Lauric acid was not found in any of the early seed lipid samples but it increased in quantity as seed matured to become the dominant fatty acid in this tissue. Conversely, pulp contained only small amounts of lauric acid; its fatty acid profile was dominated by oleic acid. Fully hydrated seeds stored well for 16 months at both 4 °C and −2 °C. Although drying had a deleterious effect on germination when dried seeds were conventionally stored at 4 °C, seeds that had been dried for 24 h to a moisture content of 8.6% were successfully stored in liquid nitrogen.

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1. Introduction

Pondberry (*Lindera melissifolia* [Walt.] Blume: Lauraceae) is a federally listed endangered shrub that may always have been rare in occurrence (Steyermark, 1949; Radford et al., 1968; Kral, 1983). It grows in bottomland hardwood forests in six states in the southeastern United States and has recently been rediscovered in a seventh. Though the species is found

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over a relatively broad range, it is distributed sparsely within this territory, occurring in largely isolated localities. The 1–2 m tall plants are usually found in clonal colonies, a result of rhizomatous sprouting. Colonies are often found in areas that commonly flood in the spring but which are frequently dry in the fall (Devall and Schiff, 2004).

Reproductive information on the species has concentrated on its clonal regenerative capabilities, but it is misleading to consider pondberry only from this aspect. While vegetative reproduction serves to increase colony size, sexual reproduction and seed dispersal are necessary for establishment of new colonies. Smith et al. (2004) suggested hydrochory and zoochory as potential seed dispersal mechanism in wetland forests, noting that the showy, persistent fruit of pondberry may favor dispersal by birds or other animals. However, their research has yet to document an animal dispersal agent capable of disseminating seed relatively long-distances to suitable but uncolonized locales.

The sexual reproductive potential of pondberry is considerable. In Mississippi, flowering typically begins in February, before leaves emerge. The dioecious plants produce small yellow flowers on axillary inflorescences. Though late frosts can damage or destroy flower and seed crops, colonies of female plants often produce an abundant crop of bright red, spicy-scented drupes. Indeed, in some years individual female stems can contain in excess of 250 drupes (Connor; personal observation), representing a large energy investment for the plants. However, little else is known of the species' population ecology and, thus far, its basic fruiting biology. Fundamental information about pondberry fruit development, fruit biochemistry, and seed maturation and storability is unknown.

Plant species conservation often depends on the storability of its seeds (Touchell and Dixon, 1993). Stored seeds or plant tissues can provide a safety net for species found in low numbers in the wild and can be used as a source for reintroduction if a population fails (Touchell and Dixon, 1993; Wilkinson et al., 2003). The majority of seeds from temperate forest species are 'orthodox' (Roberts, 1973), meaning they can be dried to moisture contents of less than 12%. They become metabolically quiescent when dried and are conventionally stored at temperatures ranging from 4° to −18 °C or cryostored in liquid nitrogen at −196 °C. However, there are seeds that are desiccation-sensitive. Sub-orthodox (or intermediate) and recalcitrant seeds show sensitivity to drying and/or cold temperatures (Roberts, 1973; Ellis et al., 1990). If carefully handled, sub-orthodox seeds can retain viability and still be dried to as low as 10–12% moisture content. Recalcitrant seeds, however, must be stored fully hydrated; thus both they and fungi found on their outer surfaces are metabolically active. Metabolically active seeds can continue to mature and germinate while in storage, and loss of recalcitrant seeds to fungal infection is not uncommon. These processes serve to drastically shorten any useful period of seed storage and complicate germplasm conservation. Additionally, sensitivity to moisture loss can vary among genera in a family or even among species in the same genus (Thompset, 1984; Hong and Ellis, 1990; Hor et al., 2005). It is unknown if the seeds of pondberry are orthodox, sub-orthodox, or recalcitrant.

Cryopreservation protocols have been developed for some seeds that prove difficult to store (Towill and Bajaj, 2002; Engelmann, 2003). Techniques used include two-step freezing (Withers and King, 1980), encapsulation-dehydration (Dereuddre et al., 1991; Shibli and Al-Juboory, 2000), rapid cooling (Mycock et al., 1995), flash drying (Berjak et al., 1989; Wesley-Smith et al., 1992), and vitrification (Touchell et al., 2002). Researchers have been successful in preserving embryonic axes and somatic and zygotic embryos (Shibli and Al-Juboory, 2000; Towill and Bajaj, 2002; Fang et al., 2004). Alternatively, cryopreservation of shoot tips, buds, and apical meristems has also proven successful for many species (Towill et al., 2004). While encouraging, these labor-intensive processes are primarily undertaken for valuable agricultural species, fruit trees, or economically important forest tree species. Additionally, the cryopreservation of shoot tips and buds requires destruction of entire plants or plant parts, generally unacceptable when working with endangered species except under extreme circumstances.

Our purpose in this work was to examine (1) the morphological development of pondberry fruit and seeds; (2) fatty acid composition of the storage lipids of seeds and fruit pulp as development progresses and (3) maturation and storability of the seeds. Such information will provide basic life history and biochemical information about the species and can be used to evaluate the potential for establishing artificial germplasm reserves.

2. Materials and methods

Two studies were initiated to address the objectives. The first investigated drupe development, from pollination through maturity. In addition, we tracked the fatty acid biochemistry of drupes as they developed. The second study determined the storability of pondberry seeds and suggested a successful seed handling protocol.

2.1. *Study site*

Plant colonies studied for seed development were located on the Delta National Forest, Sharkey County, Mississippi, in the Yazoo River Basin of the Lower Mississippi Alluvial Valley, USA. The site supports a typical bottomland hardwood forest cover for the area. Overstory trees are primarily sweetgum (*Liquidambar styraciflua* L.), sugarberry (*Celtis laevigata* Willd.), and red maple (*Acer rubrum* L.). Nuttall oak (*Quercus nuttallii* E.J. Palmer) and boxelder (*Acer negundo* L.) also contribute to the canopy but are of lesser importance (Hawkins et al., 2004). Climate in the area is typical of the humid, subtropical region of the Northern Temperate Zone. The growing season averages 229 days long, with 27 °C as the average temperature in July and 7.5 °C as the average January temperature (Scott and Carter, 1962; Scott et al., 1975). Site topography is relatively flat, and soils are of the Sharkey series – very-fine, montmorillonitic clay, nonacid, thermic Vertic Haplaquepts (Scott et al., 1975). Some seasonal flooding can occur on the site from winter to spring. Ponding is evident after heavy rainfall or when flood waters recede.

2.2. Seed development

2.2.1. Field measurements

The purpose of this component of the study was to assess the physical development of pondberry seeds from 1 month after pollination until maturity. Measurements were taken at 30-day intervals. We noted the time of flowering and, 30 days after anthesis, selected 10 female plants and permanently tagged them with plastic flagging and a metal tag (a 'plant' was considered to be a single stem). Because of the distribution pattern of the female colonies and the necessity to find plants with at least 50 fruits, it was impractical to use a grid line to systematically sample plants. Therefore, for this experiment and all subsequent studies, we selected plants from many different female colonies throughout the two study areas. Each plant was measured for height, basal diameter, number of shoots and shoot length, total number of branches, and the number of drupes. To track fruit abortion and individual fruit development, each fruit cluster on the plant was tagged with telephone wire. This is small diameter copper wire with insulation in a variety of distinct colors and patterns that do not fade upon exposure to the elements. After tagging, we measured length and diameter of each drupe on the plant at 30-day intervals, using digital calipers (model CD 6″CS, Mitutoyo America Corp.).

We tagged an additional 30 female pondberry plants for fruit monitoring and for the biochemical analyses. Each plant selected for this study had at least 50 drupes to ensure enough maturing fruit for the entire length of the study. Five drupes were collected monthly from each plant from April through October for the various analyses. Drupes were placed in polyethylene bags and immediately put in a cooler. All drupes were weighed and measured within 3 h after removal from the plant. A subsample of 5 drupes was examined under a dissecting scope to determine if seed structures had formed. When visibly defined seeds were found, 90 days after anthesis, drupes were dissected in the laboratory; pulp was removed, and seed diameters were measured.

2.2.2. Lipid extraction and analysis

We performed lipid analyses on a subsample of 10 drupes collected for the development study. For the first two sample periods, 30 and 60 days after anthesis, we could not separate seed from pulp since seeds were not yet formed. Therefore, the fatty acid analysis was for the entire fruit. By 90 days after anthesis, seed could be separated from pulpy exterior, and seed and pulp tissues were analyzed separately.

Immediately after weighing and measuring the drupes (and, when possible, seeds), tissues were freeze-dried and then ground in a Wiley mill. The ground samples were then funneled into cryovials and placed in liquid nitrogen storage until ready for analysis. Lipid analyses were according to the following procedure. The tissue sample was stirred for 30 min in a 2:1 solution of chloroform and methanol. The sample was then filtered, the filtrate measured volumetrically, washed, and purified in the manner of Folch et al. (1957) using one wash of 0.9% sodium chloride and 2 washes of a 1:1 solution of methanol and 0.9% sodium chloride. Butylated hydroxytoluene (5 mg/L) was added as an antioxidant to all solvents used in the extractions (Pearce and Abdel Samad, 1980). Lipids were esterified using 1.5% concentrated sulfuric acid in methanol (Christie, 1990). Samples were placed in a 50 °C waterbath overnight, cooled, and vortexed for 15 s with 3 ml water and 2 ml hexane (Murrieta et al., 2003). The hexane phase was removed, dried over anhydrous sodium sulfate, and analyzed on a Hewlett–Packard® 5890 gas chromatograph (GC) using an HP-5 column. The initial oven temperature, for 5.2 min, was 110 °C. Oven temperature was then increased at 30 °C per minute to 140 °C and held for 22 min; temperature was then elevated at 30 °C per minute to 170 °C and held for 18 min. Total run time was 47.2 min. Injector and detector temperatures were 200 °C. Response factors were calculated from injections of low erucic rapeseed oil and AOCS oil reference mix no. 3 (Sigma Chemical Co., St. Louis, MO).

2.2.3. Seed storage

2.2.3.1. General information. Previous results from a thermogradient plate study using temperature combinations ranging from 10 to 40 °C indicated that the best temperature/light regime for germinating pondberry seeds was 16 h of light at 30 °C with 8 h of dark at 35 °C (Connor, unpublished results). We used this information to set the temperatures and lights on the Stults® germination cabinets in order to optimize results in all germination tests. It appeared that some stratification was necessary to enhance germination, so before testing, freshly collected seeds were cold stratified for 6 months followed by 7 days in wet sand at room temperature. Seeds were also washed for 1 min in a 10% sodium hypochlorite solution and then rinsed 3 times with distilled water prior to germination testing. We determined fresh weight moisture content from five replications of 4 seeds (minus pulp) each. Seeds were placed in metal weighing tins and oven-dried at 103 °C for 17 ± 1 h (Bonner, 1981; ISTA, 1993).

2.2.4. Experiments

1. We mixed all the freshly collect pondberry seeds into one batch. We removed the pulp and dried the seeds on the laboratory bench at room temperature so that surface moisture was removed (no longer than 1 h). We divided these fully hydrated seeds into 10 lots of 100 seeds each; half of the lots were stored at 4 °C in a walk-in cooler and half at −2 °C in a Precision 818 low temperature incubator. At intervals of 6, 9, 12, 16, and 24 months, we removed one lot from each storage unit and tested for viability. Sample seed lots were germinated for 4 weeks as two replications of 50 seeds.
2. We removed pulp from freshly collected pondberry seeds and dried the seeds on the laboratory bench at room temperature. Randomly selected samples of 170 seeds were collected from the bench after 0, 6, 18, and 24 h. One hundred of the seeds from each sample were conventionally stored in plastic bags at 4 °C in a walk-in cooler. Fifty seeds were cryostored in a liquid nitrogen sample container. The remaining 20 seeds were used to determine moisture content of the sample at time of storage. Upon removal from either conventional or cryostorage, seeds were warmed to room temperature, stratified in wet sand for 7 days, and then placed in the germination cabinet. We removed the 100-seed samples from conventional storage after 2

years and placed them in a germination cabinet for 4 weeks as four replications of 25 seeds. The 50-seed cryostored samples were removed from storage after 7 months and placed in a germination cabinet for 4 weeks as two replications of 25 seeds.

2.2.5. *Statistics*

Averages and standard errors were calculated for all germination tests performed using SigmaStat. Differences among seed size measurements and between fresh samples and stored seeds were determined by simple *t*-tests, level of significance $P = 0.05$. The abortion/abscission data represent actual counts of seeds present.

3. Results and discussion

Fruit abortion was especially prevalent in the first 3 months of the study (May to July) (Fig. 1). The number of developing drupes dropped 20% in May and was down 40% of the original count by June; over half the crop was gone by July. However, fruit loss leveled off for the next 3 months (July through September). There was a final drop in drupe number between September and October that may not have been strictly from abscission but rather to drupes that were removed from the plants by predators (Smith et al., 2004) or that may have matured and dropped before the count was completed.

Although tagged plants were widely separated in the study area, variation in fruit and seed size was low within samples collected at each development phase (Fig. 2). Seeds did not visibly form in pondberry drupes until between 60 and 90 days after anthesis. Before this, the interiors of the drupes were undifferentiated tissue with no visible delineation between seed and pulp and no discernable structures. At 90 days after anthesis, when seeds were first visible, they had already gained 40% of their final maturity mass; and at 90 days after anthesis, drupes had attained over 85% of their mature size and 55% of their mature weight. Drupes reached full size by July and the seeds by August. We attempted to germinate a few seeds collected in August from green drupes and were

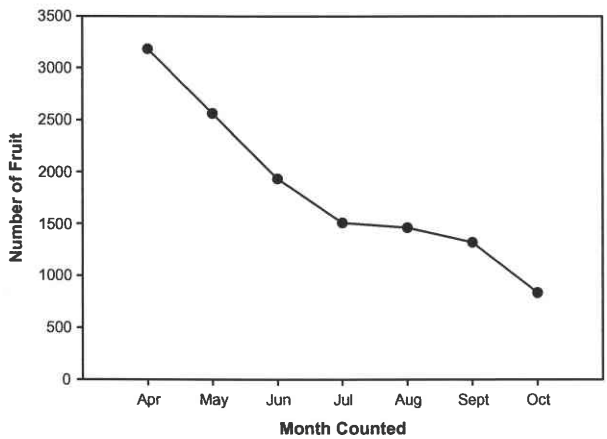

Fig. 1 – Fruit abscission on monitored pondberry plants from 30 days after anthesis until maturity.

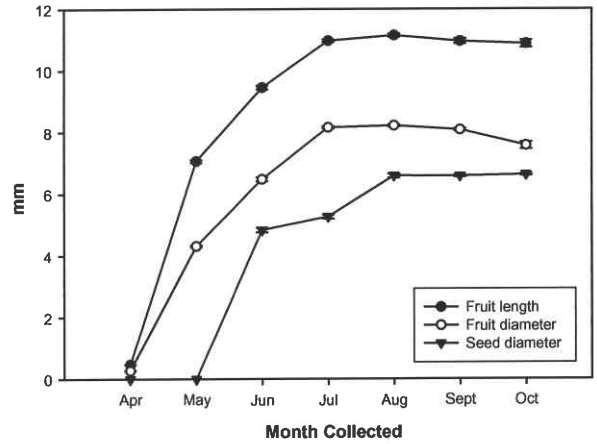

Fig. 2 – Development of pondberry drupes and seeds from 30 days after anthesis until maturity. Error bars represent one standard error beyond the mean.

unsuccessful; thus we know that attainment of mature size is not an indication of seed maturity. In the case of pondberry, the bright red seed color is still the best indicator of maturity although not necessarily of viability.

3.1. *Fatty acids*

Lipids, which are made up of fatty acids, are an important component of cell function, involved in membrane structure, photosynthesis, cellular signalling, cell differentiation, transformation, and proliferation (Dey and Harborne, 1997; Buchanan et al., 2000). Seed lipids we analyzed primarily serve as an important energy source for seedling establishment and growth.

The quantities of individual fatty acids in the early stages of pondberry fruit development were, as expected, very low (Fig. 3a) but certain fatty acids increased rapidly as drupes developed and matured. Most notably in the pulp, oleic acid was present in very large quantities, averaging 312.7 ± 2.6 mg/g dry weight and representing 79.8% of the pulp's total fatty acid makeup. Palmitic acid and linoleic acid were also present, with a maximum of 34.2 ± 0.5 mg/g (8.7%) and 22.7 ± 0.1 (5.8%) mg/g, respectively.

In seeds, lauric acid was present in very large quantities, reaching a peak in August at 234.4 ± 17.2 mg/g dry weight of tissue and representing 71.0% of the pulp's total fatty acid makeup (Fig. 3b). Oleic acid and linoleic acid were also present, with a maximum of 47.1 ± 0.9 mg/g (14.2%) and 18.0 ± 0.2 mg/g dry weight (5.4%), respectively. The quantity of lauric acid peaked in August and, unlike oleic acid in the pulp, declined as the seeds matured. This may, like color change, be an indication of seed maturation. Bonner (1973) reported that crude fat content of *Fraxinus pennsylvanica* Marsh samaras increased from immaturity to physiological maturity. When analyses are performed on individual fatty acids, however, results can be conflicting. Rahamatalla et al. (2001) found that both saturated and unsaturated fatty acids fluctuated in developing safflower (*Carthamus tinctorius* L.) cultivars. Palmitic acid content increased toward maturity in 3 of the 4

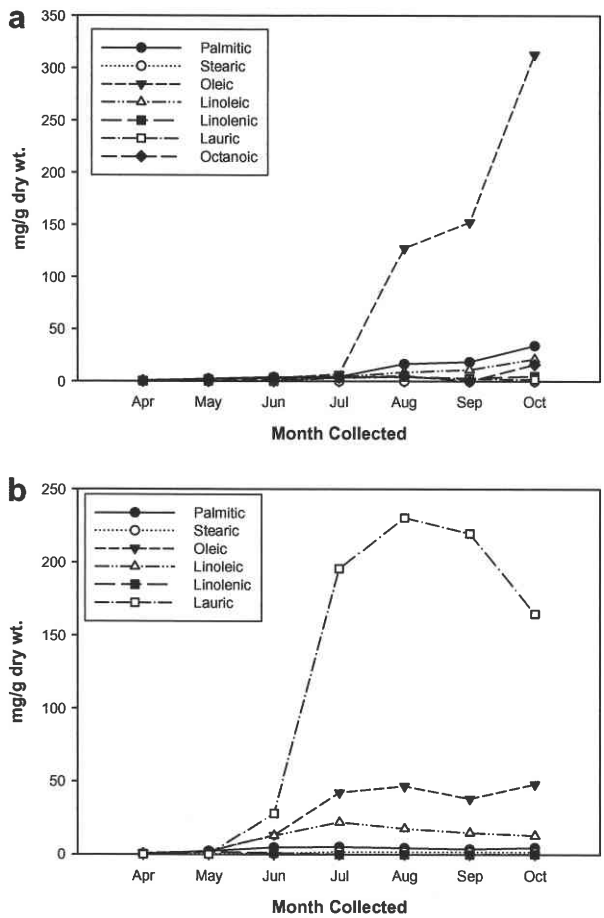

Fig. 3 – Fatty acids from 30 days after anthesis until maturity in the (a) pulp and (b) seeds of pondberry drupes. The drupes collected in April and May did not contain differentiated tissue, and fatty acids were extracted from the entire drupe.

cultivar lines studied but decreased in the fourth. Similarly, linoleic acid decreased with seed growth and development in 3 of the 4 lines but increased significantly in the fourth. Obendorf et al. (1993) reported that shortly after pollination, 80% of the fatty acids in developing buckwheat (*Fagopyrum esculentum* Moench) seeds are saturated, with palmitic acid 3–5 times higher in concentration than any other fatty acid. As the seed matures, 70–80% of the fatty acids are unsaturated, with linoleic, oleic, and palmitic representing 85% of the total. In the achene storage lipids, palmitic acid is the first to accumulate, followed by stearic, oleic, linoleic, and linolenic acids. But, as noted by Taira et al. (1986), fatty acid concentration in buckwheat can be altered simply by lower temperatures during achene maturation. This observation suggests that fatty acid composition may be too sensitive to environmental conditions to be a reliable signal of physiological maturity.

The ratio of unsaturated to saturated fatty acids in the pondberry drupe pulp is typical of that found in seeds of many other temperate-zone species (Table 1). In most cases, unsaturated fatty acids, especially oleic and linoleic acids, dominate the storage lipid composition, and the ratio of unsaturated/saturated fatty acids exceeds 3.0.

Table 1 – Percentages and ratio of unsaturated to saturated fatty acids in pondberry (*Lindera melissifolia*) pulp and seed tissue and in bulk storage lipids from other tropical and temperate zone seeds

Species	Unsat.	Sat.	Ratio
Quercus nigra[a]	83.7	16.3	5.1
Quercus alba[a]	82.9	17.1	4.8
Aesculus pavia[b]	77.8	22.2	3.5
Acer saccharinum[b]	77.0	23.0	3.4
Guarea guidonia[c]	72.0	27.1	2.7
Carapa guianensis[d]	34.7	63.1	1.8
Olive oil[e]	80.0	20.0	4.0
Palm kernel oil[f]	19.0	81.0	0.2
Coconut oil[e]	8–14	86–92	0.1–0.2
Lindera benzoin pulp[g]	76.6	23.4	3.3
Lindera benzoin seeds[g]	4.5	95.5	0.1
Lindera melissifolia pulp	86.8	13.2	6.6
Lindera melissifolia seeds	6.6	93.4	0.1

a Connor et al. (1996).
b Connor and Bonner (2001).
c Connor and Bonner (1998).
d Connor et al. (1998).
e From http://www.welch-holme-clarkl.com, accessed 1.5.06.
f From http://www.wellnaturally.ca, accessed 1.5.06.
g Conway et al., 1994.

The most dominant saturated fatty acid in the seeds of temperate-zone *Quercus nigra* L., *Q. alba* L., *Acer saccharinum* L. and *Aesculus pavia* L. is palmitic acid (Connor et al., 1996; Connor and Bonner, 2001). The two tropical species in the table, *Carapa guianensis* Aubl. (Connor et al., 1998) and *Guarea guidonia* (L.) Sleumer (Connor and Bonner, 1998), have a higher saturated fatty acid content than the four temperate species, but the unsaturated/saturated ratio is still greater than 1.0, and the most common saturated fatty acid is still palmitic acid.

Unlike the majority of species in Table 1 and unlike the pulp enclosing them, pondberry seeds have a very low unsaturated/saturated fatty acid ratio. This is because 93.4% of the seed storage fatty acids are saturated; lauric acid accounts for 88.3% of these saturated fatty acids. This is similar to the lipid composition found in the drupes of another North American *Lindera* species, spicebush (*Lindera benzoin* [L.] Blume). Like pondberry, spicebush seeds are high in saturated fatty acids (Conway et al., 1994), resulting in a low unsaturated/saturated ratio. Spicebush seed tissue contains 47.5% lauric acid while the fruits contain little lauric acid (0.9%) but, again like those of pondberry, are high in oleic acid (63.9%).

Anecdotal information on oils with high quantities of medium-chain saturated fatty acids suggests that their presence confers benefits ranging from protection of the skin against aging to weight loss in humans. However, published studies support claims that lauric acid in particular does have anti-fungal and anti-microbial properties (Kitahara et al., 2006). While research linking the benefits of this fatty acid to pondberry seed longevity, survivability in the seed bank, or resistance to microbial activity is not in evidence, it does represent a possible area for future research.

3.2. *Seed storage*

More seeds held at −2 °C germinated than those stored at 4 °C at every sampling time (Figs. 4 and 5), but the differences were not significant. After 16 months at either storage temperature, germination had not significantly declined from the fresh values. Germination decreased to 77% at 4 °C after 12 months, but the sample tested after 16 months had 92% germination. We believe that fungal infection may have adversely affected the 12 month sample. Germination of the sample stored for 24 months at 4 °C decreased significantly from the fresh germination percentage.

Germination was still 92% after 12 months and 100% after 16 months for samples stored at −2 °C. It wasn't until the 24 month sample was tested that any decline in percent germination was observed in −2 °C samples but the difference was not significant. Additionally, germination was not significantly higher for seeds stored at −2 °C for 24 months than for those stored at 4 °C.

Insets on Figs. 4 and 5 illustrate that if the length of time in the germinator was extended 3 weeks beyond the standard 4 week test, germination of the fully-hydrated sample stored for 2 years at −2 °C was equivalent to that of the fresh sample. However, even with the extension, we saw a decline in germination of the 4 °C-two year sample; unlike the standard 4 week test, the decrease was not significant.

Mature pondberry seeds contained, on average, 28.6% moisture (g/g fresh weight). Average moisture contents for seeds dried 6, 12, and 24 h before storage were 17.0%, 13.4%, and 8.6%, respectively. Drying had a significant effect on seed viability (Fig. 6). Germination of seed stored fully hydrated averaged 50% after 2 years in storage at 4 °C. Seeds dried for 6, 12, and 24 h averaged 20%, 10.5%, and 8% germination, respectively, after 4 weeks in the germinator. If pondberry seeds were orthodox, we would expect drying to have no deleterious effect on storage; if truly recalcitrant, no seeds would have survived drying to 8.6% moisture content. We suggest, therefore, that pondberry seeds are sub-orthodox.

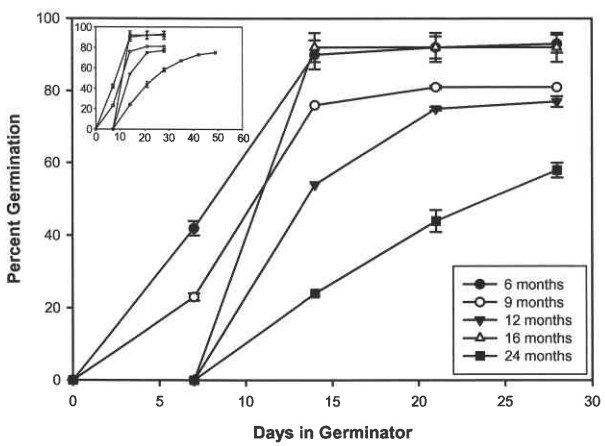

Fig. 4 – Cumulative germination percentages of pondberry seeds stored for 6, 9, 12, 16, and 24 months at 4 °C. After 6 weeks in the germinator, the 24-month sample showed a significant decrease in germination from that of the fresh sample. After 9 weeks in the germinator, the difference in percent germination between fresh seeds and those stored for 24 months was no longer significant. Error bars represent one standard error beyond the mean.

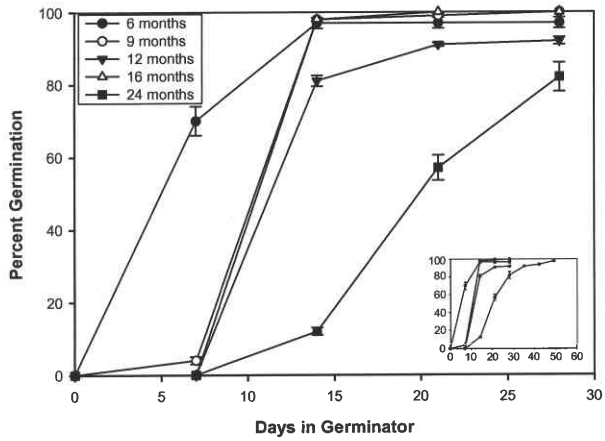

Fig. 5 – Cumulative germination percentages of pondberry seeds stored for 6, 9, 12, 16, and 24 months at −2 °C. At this storage temperature, no significant decreases in germination were observed. Error bars represent one standard error beyond the mean.

This is reinforced by the cryostorage survival of some dried seeds. Not surprisingly, seeds that were fully hydrated or dried for only 6 h did not survive immersion in liquid nitrogen. Of the seeds dried for 12 h, 4% (2 seeds) survived and germinated. However, seeds dried for 24 h to a moisture content of 8.6% before immersion in liquid nitrogen averaged 70% germination, much higher than seeds conventionally stored for 2 years either fully hydrated or at 8.6% moisture content.

We conclude that although the regenerative abilities of pondberry are overshadowed by its clonal nature, fruit and seed production may play an important role in survival of the species. Under ideal environmental conditions, individual female stems can produce several hundred drupes; and each

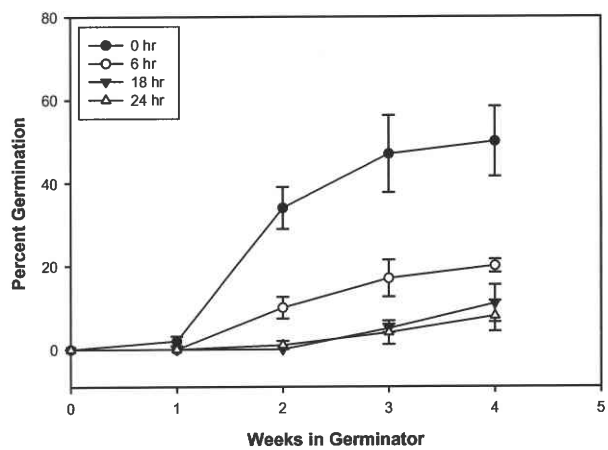

Fig. 6 – Cumulative germination percentages of pondberry seeds dried for 0, 6, 18, or 24 h before storage for 2 years at 4 °C. Error bars represent one standard error beyond the mean.

of these represents a potential future pondberry colony. Additionally, storing fully hydrated pondberry seeds is practicable if seeds are used within a 16 month period. More importantly, with careful drying pondberry seeds can be stored in liquid nitrogen. While sensitivity to moisture loss is a complicating factor in pondberry seed survival, it is easily overcome by cryostorage. This is encouraging for longterm germplasm conservation of the species.

Acknowledgements

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