

LONG-TERM STORAGE OF HAZELNUT EMBRYONIC AXES IN LIQUID NITROGEN

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Abstract

Whole hazelnut (*Corylus L.*) seeds do not survive freezing in liquid nitrogen (LN₂) because of large size and high oil content. However, excised embryonic axes can survive with appropriate treatment. Although freshly harvested axes are easily cryopreserved, secondary dormancy inhibits growth soon after harvest. A short stratification treatment of stored nuts followed by dehydration and LN₂ storage was successful for long-term storage of hazelnut germplasm. Whole seeds dried to very low moisture contents were frozen and the embryonic axes survived LN₂ exposure although cotyledons were badly damaged. Dried whole hazelnuts can be stored in LN₂ if the axes are excised and cultured after thawing. This technique avoids the problems of cotyledon damage and subsequent contamination and death of whole seeds during germination. A two-week stratification of stored seed before excision of axes greatly improved shoot growth and decreased callusing. Drying isolated embryonic axes to 8-10% moisture, either by laminar flow or over silica gel prior to freezing, produced high survival and shoot growth from stratified-stored *Corylus* seed. *C. americana* Marshall, *C. colurna L.*, *C. heterophylla* Fisch. Ex Trautv., and *C. sieboldiana* Blume axes were prepared, cryopreserved, shipped to the National Seed Storage Laboratory in Fort Collins, Colorado, and stored in liquid nitrogen. This is the first base (long-term) conservation of hazelnuts in world germplasm collections. Seed of additional species will be added as they become available.

1. Introduction

Hazelnuts cannot be stored for more than one year without substantial loss in viability (Mehlenbacher, 1991). The development of new cultivars resistant to insects or diseases or with improved nut characteristics may include the use of breeding material from wild species (Mehlenbacher, 1991). Wild hazelnut germplasm must be preserved as growing plants in orchards or as refrigerated or frozen pollen due to the short viability of the seeds. In vitro cold storage of hazelnut is an alternative method for medium-term storage of clonal germplasm, good for 8 months to 2.7 years (Reed and Chang, 1997). In addition to their large size, hazelnut seeds have a hard seed coat, internal dormancy, and irregular germination with increasing length of storage (Williams *et al.*, 1973). Thus, storage of *Corylus* germplasm in liquid nitrogen (LN₂) would provide a long-term base collection for the species. Hazelnut seeds are considered desiccation-tolerant but they are LN₂ sensitive due to their large size and high oil content (Stanwood, 1985). The first *Corylus* cryopreservation attempts were made by Pence (1990) who reported that embryonic axes of mature stored *Aesculus*, *Castanea*, *Corylus*, and *Quercus* seeds remained viable following exposure to LN₂, but most callused and did not produce viable plants. Gonzales-Benito and Perez (1994) later found that embryonic axes of freshly harvested hazelnuts (*Corylus avellana L.* cvs. Butler and Morell) could be successfully stored in LN₂ with good regrowth potential. Hazelnut seeds have a high coefficient of variation for moisture content, but can be desiccated to 5% moisture without loss of germination (Normah *et al.*, 1994). Whole desiccated seeds do not survive LN₂ exposure

due to damage to cotyledonary tissue, however the embryonic axes may be removed and grown in culture (Normah *et al.*, 1994; Normah and Reed, 2001). This technique avoids the problems of cotyledon damage and subsequent contamination of whole seeds during germination, but may be complicated by decreased shoot production in axes from stored seed. A 2-wk stratification of stored seeds before excision of axes greatly improves shoot growth and decreases callusing of cultured axes (Reed *et al.*, 1994). Drying isolated embryonic axes to 8-10% moisture, either by laminar flow or over silica gel, produces high survival and shoot growth from stratified-stored *Corylus* seed. If fresh axes are not readily available, short stratification treatment of stored nuts followed by dehydration and LN₂ storage is a relatively simple, alternative technique for long-term storage of hazelnut germplasm. We applied this technique to the storage of hazelnut germplasm at the National Clonal Germplasm Repository in Corvallis, Oregon.

2. Materials and Methods

Isolated embryonic axes from the following species were stored in LN₂: *Corylus americana* Marsh., *C. colurna* L., *C. heterophylla* Fisch. ex Trautv., and *C. sieboldiana* var. *mandshurica* (Maxim.) C. Schneider.

Techniques developed earlier for storage of *Corylus avellana* 'Barcelona' seeds were used (Normah *et al.*, 1994; Reed *et al.*, 1994). Seeds were stratified for 2 wk in moist perlite at 4-5°C unless freshly gathered from the ground in November to January. Seeds were cracked without breaking the cotyledons and embryonic axes were removed. Axes were collected in covered petri dishes, surface sterilized in 10% bleach for 10 min, rinsed and drained on dry filter paper. Axes were arranged in rows in a sterile petri dish, 20 per dish in a 5 x 4 grid and dried uncovered for 2 hrs (~ 9% moisture) in the laminar flow hood. After drying, 20 axes were placed in each vial labeled as *Corylus* "species" embryonic axes, local number, and the date. Twenty dried but not frozen control axes were plated as controls when sufficient numbers were available. Storage vials are placed on canes in a dewar or travel container for shipment. One control vial was thawed after 24 hr by immersing for 1 min in 45° C water then 25° C water, then plated on NCGR-COR recovery medium in multiwelled plates (Yu and Reed, 1995).

Seeds of *C. americana* and *C. colurna* were gathered from the ground at the Oregon State University Smith Horticulture Farm in the winter of 1994-95 and cryopreserved without further stratification (Normah and Reed, 2001). A few seeds of *C. sieboldiana* (COR 636) were received from China in December 1997. Half of these seeds were stratified for 8 wk for regular seed germination. The other half (22 seeds) of the seeds were removed after 2 wk of stratification and stored as described above. One cryopreserved vial (4 axes) was removed from storage after one day to test viability. *C. heterophylla* (COR 703.001) seeds were stratified for 2 wk. About 60 axes were dried for 2 hr and stored in LN₂. Ten axes were thawed as controls.

3. Results and Discussion

The moisture contents of the species tested declined to below 10% after 2-hr in the laminar flow hood (Table 1). The moisture content of *C. americana* axes was reduced from 22% to 4% moisture, and regrowth was 96% (of 23 axes) after 1 hr LN₂ exposure. The moisture content of *C. colurna* axes was reduced from 33% to 9% moisture, and regrowth was 80% (of 20 axes) after 1 hr LN₂ exposure. Standard seed germination with a GA₃ presoak was 70% for *C. americana* and 28% for *C. colurna*. Three-yr storage resulted in 100% regrowth of *C. americana* axes and 45% for *C. colurna*. The limited regrowth of *C. colurna* axes is probably due to less than optimum stratification (November collection) prior to removal of the axes, but was still higher than the dried control. *C. sieboldiana* recovery after 1 hr LN₂ exposure was 75% (3 of 4 axes) and seed germination was 40% (6 of 15 seed) in the greenhouse.

Ten vials (100 axes) each of *C. americana* and *C. colurna*, 5 vials (10 axes) of *C.*

heterophylla, and 2 vials (10 axes) of *C. sieboldiana* have been placed in long-term storage at the National Seed Storage Laboratory at Fort Collins, Colorado. Additional vials of each accession are being kept at NCGR.

Axes from stratified seed dried to moisture contents below 10% were successfully cryopreserved in LN₂. Control recovery was high for two of the three species tested and acceptable for the other. The low recovery for *C. colurna* was likely due to insufficient stratification; axes do not grow well in culture if the stratification requirement has not been met. This problem can be avoided by harvesting the seeds directly from the trees in September and immediately cryopreserving them (Gonzalez-Benito and Perez, 1994). When collecting germplasm it is often not possible to immediately process samples. In this case, stratification to break the secondary dormancy becomes necessary (Reed *et al.*, 1994). Controls exposed to LN₂ for 1 hr all had good recovery except for *C. heterophylla*. The poor recovery recorded for *C. heterophylla* may be inaccurate due to the small number of seeds tested. For long-term storage, we found that *C. americana* maintained recovery similar to the controls even after a 3-yr storage. *C. colurna* recovery declined but was similar to the dried controls, and thus possibly attributable to culture conditions or variation between vials.

4. Conclusions

Base collections of important crop germplasm should be preserved for future generations. Clonal crops and those with recalcitrant seeds are especially in need of preservation. The techniques needed to store clonal crops in liquid nitrogen are now available and should be used for appropriate species preservation. The techniques described for the long-term storage of these four species of hazelnut can also be applied to other species. This is the first base (long-term) conservation of hazelnuts in the world. Embryonic axes of additional species will be added to the long-term storage collections as they become available.

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