

***In Vitro* Growth of Embryo Axes after Long-term Storage in Liquid Nitrogen**



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Summary

In vitro viability testing is a valuable tool for evaluating seeds and embryos with known or suspected low viability, and it has been used successfully to recover tissues for propagating endangered plants. It is a necessity, however, for evaluating and recovering isolated embryo axes that have been stored long-term. Cryostorage of embryo axes of large-seeded temperate tree species has been demonstrated as a potentially useful technique for the long-term storage of seeds with recalcitrant or suborthodox seeds (Pence, 1990; 1992). Axes of *Juglans nigra* (suborthodox), *Aesculus glabra*, *A. hippocastanum*, and *Quercus palustris* (all recalcitrant) have recently been removed from storage to evaluate survival after 8.5–9.5 years. Axes of *J. nigra*, *A. glabra* and *A. hippocastanum*, which had been dried and stored in liquid nitrogen, showed 80 to 100% survival when grown *in vitro*, although growth was affected by the medium used. Axes, dried and then stored in 'desiccated' cryovials at 4°C or at -20°C, showed no survival. No survival of *Q. palustris* embryos was observed under any of the storage or growth conditions tested. These results suggest that liquid nitrogen may provide conditions allowing long-term survival of embryo axes from recalcitrant and suborthodox seeds, although optimization for the growth and recovery of tissues *in vitro* is required for each species.

Introduction

Seeds of many species of large-seeded temperate trees are either recalcitrant (sensitive to desiccation) or suborthodox (desiccation tolerant but short-lived), and as such, are difficult to store. Isolated embryo axes of such seeds, however, can survive drying and subsequent exposure to liquid nitrogen (LN) (Pence, 1990; 1992; Wesley-Smith *et al.*, 2001), a procedure allowing more rapid and uniform drying and cooling than is possible with the large, intact embryo. The recovery of plants from these axes requires growth *in vitro*, under aseptic conditions, with the medium supplying the nutrients normally provided by the cotyledons.

Although isolated axes can survive LN exposure, there has been little work demonstrating the effectiveness of LN for long-term storage. This study examines the recovery of axes of *Aesculus glabra* and *Juglans nigra* after 8.5 and 9.5 years of storage, respectively, at 4°C, -20°C and in LN, as well as axes of *A. hippocastanum* stored in LN for more than 8 years. A few embryos of *Quercus palustris* stored for 7 years in LN were also examined. Although only small numbers of embryos were available for testing, the results demonstrate the potential value of LN storage for isolated embryo axes, and illustrate some of the factors that require consideration when testing for viability *in vitro*.

Materials and Methods

Embryo axes were isolated from seeds of *J. nigra*, *A. glabra*, *A. hippocastanum*, and *Q. palustris* collected after shedding in 1991, 1992, and 1993. The axes were placed on barely moist paper towels in a glass Petri dish sitting on ice, in order to prevent drying and the initiation of germination. Axes were isolated during a 2–3 h period in the evening and then stored overnight at 4°C. They were then surface sterilized in a 1:20 dilution of commercial sodium hypochlorite for 5 min, followed by 2 rinses of sterile water and blotted dry on sterile filter paper. The axes were dried in Petri dishes on sterile filter paper under the air flow of the laminar flow hood and then transferred to 2 ml polypropylene cryovials with screw closures. For storage at 4°C and at -20°C, axes in tightly capped cryovials were placed inside small desiccators, 20 ml scintillation vials containing 3–3.5 g silica gel and closed with polypropylene screw caps. For LN storage, axes in tightly capped cryovials were immersed in LN in a storage vessel (Minnesota Valley Engineering, XC47/11). Eight to 10 embryos were stored per vial.

When removed from storage, cryovials were warmed at ambient temperature, approximately 21°C, for 20 min before transfer to recovery media. Axes were recovered on semi-solid medium in 25 mm × 150 mm culture tubes with 15 ml of medium. Recovery medium consisted of either Murashige and Skoog (1965) (MS), Woody Plant (Lloyd and McCown, 1980) (WP), or Driver and Kuniyuki (1984) Walnut (DKW) basal salts, 3% sucrose, 0.8% agar or 0.22% Phytigel, with either no growth regulators or 2 mg dm⁻³ benzylaminopurine (BAP) and 2 mg dm⁻³ indoleacetylphenylalanine (IAPhe). For the long-term storage recoveries, 100 mg dm⁻³ Benlate and 0.2 cm³ dm⁻³ Plant Preservative Mixture (PPM®, Plant Cell Technology, Inc.) were added to some of the media. Cultures were maintained at 26°C with a 16:8 h light:dark cycle, under CoolWhite fluorescent lights, 20 μmol m⁻² s⁻¹. Moisture determinations were made gravimetrically and were calculated on a % wet weight basis. Viability was evaluated by visual examination of the embryos, two weeks after culture. Embryos were judged to be alive when swelling, elongation or expansion of hypocotyl, root, shoot or leaves occurred.

Results and Discussion

Embryo axes from *J. nigra* seeds harvested in 1991 were dried for 3.75 h, reducing the moisture level from 37% to about 5%, but maintaining 80% of the viability of the axes (Table 25.1). After 9.5 years in storage, moisture levels were maintained at all three storage temperatures, but although 75% of the embryos stored in LN survived, there was no survival of embryos stored at either 4°C or at -20°C. *Juglans nigra* has been classified as being tolerant of desiccation, but has been designated *suborthodox*, to indicate that it is not long-lived, even in the dry state (Bonner, 1986). Good survival of dried embryo axes of *J. nigra*, *J. regia* and *J. cinerea*, through exposure to LN, has been reported (Pence, 1990; Beardmore and Vong, 1998) indicating that the levels to which the embryos can be dried are sufficient to allow survival through freezing. Many desiccation-tolerant seeds are routinely dried and stored long-term at -20°C, and the reason for the loss of viability of *J. nigra* embryo axes at this temperature in long-term storage is unclear. Factors that contribute to the loss of viability in traditional short-term storage may also contribute to a slower decline at -20°C, but this requires further study.

Juglans nigra embryos recovered after 9.5 years were cultured on media with and without antimicrobial agents. A low level of contamination was observed, but this occurred in the presence of the antimicrobial agents. However, there did not appear to be any inhibition of growth by either the contamination or the antimicrobial agents (data not shown). On the other hand, embryos were also recovered on media with and without growth regulators. Whereas the presence of growth regulators did not appear to be necessary for recovery, it did result in abnormal curling and swelling of both the root and the shoot (data not shown). Medium with no growth regulators produced the most normal-appearing growth (Figure 25.1).

Embryo axes from *A. glabra* seeds harvested in 1992 were dried for 2.5 h, reducing the moisture level from 68% to 29% (Table 25.1). Although there was some contamination of the axes upon recovery *in vitro*, viability was maintained at 70%. After 8.5 years of storage in LN, the moisture level and the viability of these axes had not changed although, again, a low level of contamination was observed. As with *J. nigra*, the presence of the two antimicrobial agents tested did not appear to affect either the rate of contamination or the growth of the axes. The presence of growth regulators, however, did cause swellings and abnormal growth, as in the case of the *J. nigra* axes (data not shown).

Table 25.1 Survival of axes of *Juglans nigra* and *Aesculus glabra* after storage at three temperatures (nd signifies not determined)

Treatment	Time in storage	(%) Moisture	Number sown <i>in vitro</i>	(%) Contaminated	(%) Responsive
<i>J. nigra</i>					
Non-dried	0 d	37	10	0	80
Dried	0 d	5.0	8	0	75
4°C	9.5 years	5.8	8	0	0
-20°C	9.5 years	3.7	8	12	0
LN	9.5 years	5.5	8	25	75
<i>A. glabra</i>					
Non-dried	0 d	68	10	40	80
Dried	0 d	29	10	40	70
4°C	1 d	nd	10	40	80
4°C	2 months	nd	8	0	100
4°C	8.5 years	7.6	8	0	0
-20°C	1 d	nd	10	40	40
-20°C	2 months	nd	8	88	0
-20°C	8.5 years	11.8	8	12	0
LN	1 d	nd	10	90	20
LN	2 mo	nd	7	71	14
LN	8.5 y	31.8	8	25	75

In addition to the *A. glabra* axes, 10 axes of *A. hippocastanum* were cultured after 9.5 years of storage in LN. Ninety percent of the axes responded in culture, while 20% were contaminated slightly. Several studies have demonstrated the ability of dried *Aesculus* axes to survive exposure to liquid nitrogen (Pence, 1990; 1992; Wesley-Smith *et al.*, 2001). The results with these two species indicates the potential of this procedure for long-term maintenance of *Aesculus* germplasm in LN.



Figure 25.1 Embryo of *Juglans nigra* growing *in vitro* after 9.5 years of storage in LN. (Bar = 2 mm)

As a comparison with LN storage, axes of *A. glabra* were also stored in closed cryovials inside small desiccators at -20°C and 4°C for 8.5 years. The moisture contents of these axes was reduced to 11% and 7%, at -20°C and 4°C , respectively, and, in contrast with LN storage, there was no survival of axes stored at these two temperatures. Although the cryovials were tightly closed, it is likely that the ambient humidity in the desiccator reduced the humidity and moisture content of the embryos inside the cryovials. It has been shown that embryos of *Aesculus glabra*, *A. hippocastanum*, and *A. pavia* lose viability when dried below a level of about 30–40% moisture (Pence, 1992; Tompsett and Pritchard, 1993; 1998; Farrant and Walters, 1998; Connor and Bonner, 2001). Further experiments are needed to distinguish whether mortality at 4°C and -20°C was related to the temperature of storage, as appeared to be the case with *J. nigra* axes, or to the loss of moisture during storage.

Only a very few embryo axes of *Q. palustris* were available and three of each were cultured after more than 7 years at 4°C, -20°C and in LN. There was no survival of any of the axes, although all appeared to be free of contamination. *Quercus* species are sensitive to drying and *Q. palustris* has shown only a low level of survival as callus after LN exposure in the past (Pence, 1992). Others have also noted the sensitivity of *Quercus* axes to drying and freezing (Gonzalez-Benito and Perez-Ruiz, 1992; Gonzalez-Benito *et al.*, 1999; Berjak *et al.*, 1999), and further work is needed to improve LN storage procedures for germplasm of *Quercus*.

Recovery and growth of these isolated embryos is dependent on an *in vitro* culture system which can supply the nutrients and energy normally provided by the cotyledons. Because these systems are aseptic, bacteria and fungi in the embryo can compromise recovery. In these experiments, however, although there was contamination from some of the axes, it did not appear to inhibit the early growth of the embryos. The antimicrobial agents used in these experiments also did not appear to inhibit embryo growth, although they were not effective against the contaminants encountered. They have been shown to be of value in controlling contamination in other systems (Pence and Sandoval, 2002) and may be of use against some microbial agents in the culture of embryo axes. Growth regulators were added to some of the media in order to help stimulate growth, but they did not appear to be necessary, and were, in fact, inhibitory to normal growth. Such abnormal growth has been noted in other experiments (Pence, 1992) and suggests that these embryo axes do not need additional growth regulators even after years of storage in LN.

The technique of embryo or embryo axis cryopreservation was studied by several researchers in the 1980s (e.g., Grout, *et al.*, 1983; Grout, 1986; Normah *et al.*, 1986; Pritchard and Prendergast, 1986) and work has continued with this approach for seeds with large cotyledons or endosperm and suborthodox or recalcitrant tissues. Although further research is needed for optimizing normal growth, the *in vitro* viability testing described here demonstrates the potential of LN for long-term storage of isolated embryo axes of temperate, large-seeded trees.

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