Chapter 10

Cryopreservation of Excised Embryos and Embryonic Axes

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10.1 Introduction

Seeds are categorized into two main groups according to their response to desiccation and their storage physiology: orthodox (desiccation-tolerant) and recalcitrant (desiccation-sensitive) seeds (Roberts 1973). A third category of seeds are those that are relatively desiccation tolerant but do not withstand desiccation down to water contents as low as those tolerated by orthodox seeds. These seeds are freezing sensitive and are referred to as intermediate seeds (Ellis et al. 1990, 1991).

Seed is the most preferred plant propagule for *ex situ* germplasm conservation due to low storage cost, ease of seed handling and regeneration of whole plants from genetically diverse materials (Chin 1994; Pritchard 1995). While orthodox seeds are acquiescent to storage under conventional gene bank conditions for centuries, (i.e. 3–7% seed water content at –20°C) (FAO/IPGRI 1994), cryopreservation is the only available option for long-term storage of non-orthodox seeds. However, in circumstances where storage of the whole seed of a non-orthodox species is constrained by desiccation and freezing sensitivity on one hand, and by its relatively large seeds on the other, excised embryos and embryonic axes are an alternative option. In other cases where viability of lipid-rich orthodox seeds under conventional storage conditions is drastically reduced due to the thin seed

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*

coat coupled with lipid peroxidation, as in the case of peanuts (*Arachis hypogaea*), germplasm curators can resort to cryopreservation of the excised embryonic axes (Gagliardi et al. 2002). Several studies on recalcitrant and intermediate species empirically determined that excised embryos and embryonic axes (in most cases) are more tolerant to desiccation and subsequent cryoexposure than whole seeds (e.g. Bajaj 1984; Radhamani and Chandel 1992; Normah et al. 1994; Makeen et al. 2005).

The removal of a large amount of water from the tissues by desiccation coupled with appropriate cooling–rewarming rates, will probably preclude the formation of lethal intracellular ice crystals (e.g. Mazur 1984; Steponkus 1985). Desiccation of excised embryos and embryonic axes is one of the simplest techniques for cryopreservation, whereby explants are dehydrated by airflow in a laminar flow cabinet, over silica gel or by flow of sterile compressed air prior to cryoexposure. Though not commonly used, embryos and embryonic axes can be desiccated via equilibration to a series of controlled relative humidities created by saturated salt solutions at a given temperature. In view of its simplicity and practicability, desiccation is the most commonly used procedure for successful cryopreservation of zygotic embryos and embryonic axes excised from seeds of many non-orthodox plant taxa. These plant taxa are exemplified by rubber (Normah et al. 1986), chayote (Abdelnour-Esquivel and Engelmann 2002), hazelnuts (Normah et al. 1994), coffee (Normah and Vengadasalam 1992; Dussert et al. 2001), olive (Gonzaléz-Rio et al. 1994), almond (Chaudhury and Chandel 1995), tea (Kim et al. 2002) and *Citrus* (Radhamani and Chandel 1992; Cho et al. 2002, 2003; Makeen et al. 2005).

Whole plant recovery from frozen embryos and axes is generally attained at an optimal range of water contents between 7 and 20% on a fresh weight basis (equivalent to 0.08–0.25 g $H_2O.g^{-1}$dw). However, embryos and embryonic axes excised from seeds of several recalcitrant species such as tea, chayote and almond can survive cryogenic exposure after dehydration down to far lower water contents. In contrast, excised embryos of recalcitrant *Zizania palustris* survived cryoexposure at extremely high water content (30–47%; 0.36–0.56 g $H_2O.g^{-1}$dw) obtained under ultrarapid desiccation conditions (Touchell and Walters 2000).

Though moisture content (MC) of the excised embryos and embryonic axes is the most critical factor for a successful cryopreservation protocol, manipulations of desiccation conditions, particularly the rate of desiccation (Pammenter and Berjak 1999), physiological status, preculture and cryoprotectant treatments, cooling/rewarming rates, recovery medium and viability assessment require optimization for recovery of vigorous plantlets from the cryogen.

10.2 Desiccation Rate

Desiccation rates influence the extent of water loss that recalcitrant seed tissues can tolerate. Excised embryonic axes from non-orthodox seeds survive cryogenic storage at recovery rates commensurate with the acquired desiccation tolerance obtained under rapid (Normah et al. 1986; Berjak et al. 1993; Pammenter and Berjak 1999; Makeen et al. 2005), or ultra rapid drying (Berjak et al. 1990; Vertucci et al. 1991; Berjak et al. 1999; Walters et al. 2001, 2002). Acquisition of desiccation tolerance under fast and ultra-rapid desiccation conditions is likely a result of minimizing the time of intermediate water content in the metabolically active tissues during which deleterious degradative processes take place (Pammenter et al. 1991, 1998; Pritchard and Manger 1998).

Desiccation curves of whole seeds are characterized by their monotonic pattern following a simple exponential function (e.g. Tompsett and Pritchard 1998; Pammenter et al. 1998; Dussert et al. 1999; Makeen et al. 2006). In contrast, for small explants such as excised embryos and embryonic axes, initial desiccation is expected to be faster than exponential, giving rise to biphasic desiccation curves. This is, in fact, seen in excised embryonic axes of *Theobroma cacao* (Liang and Sun 2000), seeds of *Ekebergia capensis* (Pammenter et al. 1998) and excised embryonic axes of *Citrus suhuiensis* cv. Limau langkat (Makeen et al. 2005). This biphasic desiccation curve should be determined for each seed type before cryopreservation.

10.3 Physiological Status

For a successful cryopreservation protocol, the excised embryos and embryonic axes should be in a physiological and developmental state suitable for acquisition of desiccation and freezing tolerance, with the ability to regenerate vigorous plantlets after cryogenic storage. Because recalcitrant seeds do not undergo maturation drying and they are shed wet with variable water contents (Berjak et al. 1990, 1993), there are subtle metabolic differences between seeds that are immature compared with those that are fully mature (Farrant et al. 1993). Developmental stage and metabolic activity may contribute variability among axes of the same seed lot and from one seed lot to another (Berjak et al. 1996). At the intracellular level variability in the degree of vacuolation and nature of vacuole constituents and extent of membrane development may significantly affect response of individual axes to ultra-rapid drying and subsequent cryoexposure (Berjak

et al. 1993, 1996). Therefore it is important to select uniform embryos and embryonic axes at a developmental state that can ensure maximal tolerance to desiccation and cooling. The importance of choosing the proper stage was shown for embryonic axes excised from mature seeds of tea (*Camellia sinensis* L.) and *Zizania texana* (Kim et al. 2002; Walters et al. 2002) that yielded better plantlet recovery compared to early mature and late mature seeds. The choice of maturity stage of the fruit affects recovery after cryopreservation. Higher plantlet recovery was obtained when fruits of *Citrus suhuiensis* cv. Limau langkat (intermediate) and *Prunus amygdalus* (desiccation-sensitive seed with inherently poor germination) were collected at horticultural maturity rather than from less mature fruits (Makeen et al. 2005; Chaudhury and Chandel 1995).

10.4 Cryopreservation

10.4.1 Preculture and Cryoprotectant Treatment

For several recalcitrant species desiccation of embryos and excised embryonic axes under ultra-rapid drying conditions allows their survival to very low water contents (0.28–0.44 g $H_2O.g^{-1}$ dw), close to the point of only non-freezable water remaining in tissues (Berjak et al. 1993; Pammenter et al. 1993). Axes of other species are injured at water contents that are appreciably higher. Freeze drying upon cryoexposure may take place at water contents far above the previously mentioned hydration level (Wesley-Smith et al. 1992, Berjak et al. 1996), a factor that indicates the need for a preculture treatment after embryo and axis excision and before cryopreservation (Berjak et al. 1996).

Preculture involves the culture of the excised embryos and embryonic axes on medium containing sucrose or sugar alcohols followed by exposure to cryoprotectants such as dimethylsulfoxide (DMSO), glycerol and ethylene glycol for few hours to several days. Samples are partially dried to appropriate water contents prior to immersion into the cryogen (Engelmann 1997, 2000; Walters et al. 2002). Osmoprotection is a technique where the embryos and embryonic axes are subjected to a short duration (normally from minutes to few hours) of incubation on medium consisting of various sugars or other osmotically active substances. Preculture and osmoprotection treatments have positive influences on the recovery of the frozen embryos and embryonic axes (Engelmann 1997; Walters et al. 2002). Medium content and exposure duration are two key factors for successful cryopreservation.

Embryos and embryonic axes of several plant species survived cryogenic storage after vitrification treatments using cryoprotectants. *Zizania texana* (Walters et al. 2002) embryos showed a substantial increase in recovery (from 5% to 75%) following preculture in high concentrations of sugars and sugar alcohols (sucrose, glucose, raffinose, sorbitol, mannitol, xylitol and ribitol) and treatment in a cryoprotectant (PVS2), followed by partial drying to the appropriate water content.

10.4.2 Cooling and Warming Rate

Successful cryopreservation protocols entail optimization of cooling rates in conjunction with seed-tissue hydration level, to eliminate or at least minimize, nucleation of lethal intracellular ice crystals. Water loss enhances cytoplasmic viscosity (Buitink et al. 1998; Leprince et al. 1999) and rapid cooling of sufficiently dehydrated embryos and embryonic axes minimizes ice nucleation (Berjak et al. 1996; Wesley-Smith et al. 2004). Vertucci (1989) demonstrated that at higher hydration levels where freezable water is present (> 0.25 g H_2O g^{-1}dw), faster cooling rates are required to maintain plantlet vigor.

Ultra-rapid cooling prevents the growth of intracellular ice crystals (Sakai 1986) and enables flash-dried embryonic axes to successfully survive cryoexposure at moderate and high water contents (Berjak et al. 1996). Substantial recovery of plantlets was obtained from axes of the recalcitrant seed of *Quercus robur* (60%) that were flash-dried and ultra-rapidly cooled (Berjak et al. 1999). Successful cryopreservation of embryonic axes excised from recalcitrant seed can be attained if water content and cooling rates are effectively balanced. In a recent study on embryonic axes excised from a putative intermediate seed of *Citrus suhuiensis* cv. Limau langkat (Makeen et al. 2005), high recovery (83%) was attained under rapid cooling (at about –200°C min^{-1}) at axes with a water content of 0.10 g H_2O g^{-1}dw.

Rewarming is one of the most important post-cryopreservation processes that affect survival. During warming, small ice crystals can coalesce into larger ones inflicting damage to the plasma membrane (Mazur 1984; Bajaj 1985). Rapid cooling followed by slow warming is almost invariably detrimental to standard plant tissues, but slow warming is considered less damaging to slowly-cooled embryonic axes (e.g. Wesley-Smith et al. 2004). In the majority of cases, embryos and embryonic axes are warmed rapidly by immersing the cryotubes in a water-bath at 38±2°C. Most protocols use the fast warming method, immersing cryovials or aluminum envelopes in a water bath at 37–40°C for 1–5 min.

10.4.3 Recovery

It is widely accepted that suboptimal recovery conditions of the cryopreserved embryos and embryonic axes adversely affect their recovery. Factors that arise in the recovery medium adversely influence recovery rates of frozen axes that are already suffering desiccation and freezing stresses. A variety of culture media are used for recovery of frozen embryos and axes. MS medium (Murashige and Skoog 1962) with varying growth regulators is the most commonly used. Modification of growth regulator balance in the recovery medium was beneficial for coffee zygotic embryos (Normah and Vengadasalam 1992). Damage incurred by photo oxidative stress and free radicals are suggested to adversely influence recovery (Touchell and Walters 2000). Touchell and Walters (2000) demonstrated that recovery of *Zizania palustris* axes improved from 35% to 56% when cultures were maintained in the dark as compared to light. Recovery media that suppress production of free radicals, or provide free radical scavenging elements will sustain high recovery rates (Touchell and Walters 2000). Culture medium with suitable antioxidants and frequent subcultures are also important (Chandel et al. 1996).

10.4.4 Viability Assessment

The only definitive assessment of viability is regrowth of the embryos and embryonic axes into normal seedlings. However, it is very important to know as soon as possible if the material is living after cryopreservation, because in many cases regrowth is very slow. Vital stains such as fluorescein diacetate (FDA) and triphenyl tetrazolium chloride (TTC) are frequently used to determine viability. TTC is often used for embryos and axes. TTC is reduced into red colored formazan by respiration in mitochondria of the living cells. This test is qualitative for large tissues and organs.

10.5 Summary

Air desiccation, either in a laminar airflow cabinet or over silica gel, is normally used for cryopreservation of embryos and embryonic axes. This is the simplest method and should be tried first before going on to other techniques. The optimum moisture for cryopreservation ranges from 8 to 20% (period of desiccation, usually 1–2 h) depending on the species. Vitrification is used for embryos and axes that are sensitive to desiccation (i.e. *Citrus macroptera*). Most protocols use cryovials as storage containners.

The protocol for rubber however, requires the use of aluminum foil envelopes. This is one of the other alternatives that can be considered when low survival is obtained after cryoexposure. Rubber embryos did not survive cryoexposure when stored in cryovials (Normah et al. 1986).

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10.6 Protocols

10.6.1 Almond (Prunus amygdalus) *Air Desiccation Cryopreservation*

By R Chaudhury based on Chaudhury and Chandel 1995

Items required a day before initiating cryopreservation

1. Sterile glass Petri dishes, 100 ml flasks, filter paper discs
2. Scalpel blade holder, scalpel blades, forceps, needles
3. Sterile cryovials and cryomarkers
4. MS (Murashige and Skoog 1962) culture medium in glass test tubes
5. Dewar flasks containing liquid nitrogen (LN)

The procedure

1. Crack open the hard endocarp to extract the seeds. Surface sterilize in sodium hypochlorite (2–2.7%) for 10–15 min and rinse four times with sterile distilled water.
2. Remove brown seed coat and gently separate out the cotyledons, snapping one of the attached connections with embryonic axes.
3. Make an incision at the other connection to separate it out.
4. Determine the fresh weight of 10 axes replicated three times each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
5. Spread rest of the axes on sterile filter paper discs in the laminar flow cabinet for desiccation to between 6.8 and 7.5% moisture (fresh weight basis).
6. Place 10–15 axes in sterile 2 ml cryovial and plunge into LN.
7. Rewarm cryovials in a water bath at +38°C for 5 min.
8. Culture on MS medium with 1 $mg \cdot ml^{-1}$ each of 6-benzylamino purine and napthelene acetic acid, 2 $g \cdot l^{-1}$ charcoal and 0.7% agar. Maintain at 25±2°C with a 16 h photoperiod under light intensity of 35 $\mu E\ m^{-2}s^{-1}$.

Notes

1. If the seeds are hard and it is not easy to separate out the cotyledons; they may be first soaked in water for 20 min.
2. The excision of attachment points of axis to cotyledons should be done carefully to avoid damage to shoot or root apex.

The highest survival (normal seedling percentage) of axes was 66.6% at a water content of 7% (= 0.075 g $H_2O\ g^{-1}$dw) after 3 h desiccation.

10.6.2 Cryopreservation of Arachis (A. hypogaea *and Wild Relatives*)

By RF Gagliardi and E Mansur based on Gagliardi et al. 2002

Air desiccation

Checklist for desiccation
Items needed to dissect embryonic axes

1. Tools
2. Sodium hypochlorite (5%) for surface sterilization of seeds
3. Sterile distilled water
4. Sterile Petri dishes

Prepare in advance
MS (Murashige and Skoog 1962) medium with 8.8 μM BAP (6-benzylaminopurine) and 0.7% agar

The procedure
This is a 1-day procedure.

1. Surface sterilize seeds by immersion in sodium hypochlorite (5%) with agitation for 20 min. This may need to be adjusted if there is a high level of contamination.
2. Wash the seeds four times with sterile distilled water.
3. Carefully separate cotyledons with a scalpel and dissect embryonic axes from the seeds.
4. Place axes in Petri dishes for exposure to the laminar flow cabinet for 1–2 h to reach 60–65% moisture reduction.
5. For MC determination: Determine the fresh weight of 10 axes replicated three times each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
6. Place the embryonic axes in cryovials and submerge in LN.
7. Warm cryovials in 38–40°C water for 2 min.
8. Culture the embryonic axes individually in culture tubes (25 × 150mm) containing 10 ml of MS medium with 8.8 μM BAP and 0.7% agar under light.

Vitrification

Checklist for vitrification

Items needed to dissect embryonic axes

1. Tools
2. Sodium hypochlorite (2–2.7%) for surface sterilization of seeds
3. Sterile distilled water
4. Sterile Petri dishes

Prepare in advance

1. Osmotic loading solution (MS medium with 2 M glycerol and 0.4 M sucrose) (Sakai et al. 1991)
2. PVS2: [30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulphoxide] (Sakai et al. 1991)
3. Rinsing solution: Liquid MS medium with 1.2 M sucrose
4. Liquid MS medium with no growth regulators (MS0)
5. MS medium with 8.8 μM BAP and 0.7% agar

The procedure

This is a 1-day procedure.

1. Surface sterilize seeds and dissect axes as described above.
2. Place axes in cryovials with 1 ml osmotic loading solution and hold for 15 min at 25°C.
3. Remove the solution and add 1 ml PVS2, hold for 1–2 h to reach 60–65% moisture reduction. Test the time for new accessions.
4. Submerge cryovials in LN.
5. Warm in 38–40°C water for 2 min.
6. Replace the PVS2 immediately with rinsing solution for 10 min.
7. Gradually reduce the sucrose concentration by removing 0.5 ml of this solution and adding 0.5 ml of liquid MS0. Repeat this procedure successively six times.
8. Culture the embryonic axes individually in culture tubes (25×150mm) containing 10 ml of MS medium with 8.8 μM BAP and 0.7% agar.

Possible problems

Controls should be regrown after the steps involving air desiccation and incubation with PVS2. Excessive dehydration may result in undesired callogenesis during the recovery step.

10.6.3 Air-Desiccation Cryopreservation of Embryos of Chayote (Sechium edule)

By A Abdelnour-Esquivel based on Abdelnour-Esquivel and Engelmann 2002

Items needed to excise and cryopreserve the embryos

1. Tools (scalpels, blades and forceps), detergent
2. Liquid nitrogen
3. 1.5 ml sterile cryotubes
4. A water bath preset at 40°C
5. An oven preset at 103°C

Prepare in advance

1. Freshly harvested fruits
2. MS medium (Murashige and Skoog 1962) with 0.5 $mg \cdot l^{-1}$ benzyladenine (BA), 30 $g \cdot l^{-1}$ sucrose and 2 $g \cdot l^{-1}$ Phytagel
3. Sterile distilled water, Petri dishes and filter papers
4. Calcium hypochlorite solution (~4% active chlorine)

The procedure

Day 1: Seed extraction and disinfection

1. Thoroughly wash the fruits with running tap water and detergent.
2. Extract the seeds and immerse in calcium hypochlorite for 10 min.
3. Rinse three times with sterile distilled water.
4. Excise and place the embryos on semi-solid MS in the dark at 24°C for 24 h before cryopreservation.

Day 2: Cryopreservation of the excised zygotic embryos

1. Desiccate embryos in laminar airflow cabinet for 4 h (19% moisture content MC). The highest regrowth of embryos was 30% with a water content of 19% (= 0.23g H_2O g^{-1}dw) after 4 h desiccation.
2. For MC determination: Determine the fresh weight of 10 axes replicated 3 times each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
3. Place the desiccated embryos inside cryotubes (10 embryos in each cryotube) and directly immerse into LN.
4. Rewarm by rapid immersion in a water bath at 40°C for 60–90 sec. Culture warmed embryos at 24±1°C in the dark for 2 days, then under a 16 h light/8 h dark photoperiod (34 $\mu E\ m^{-2}\ s^{-1}$). Assess the growth after 3 weeks.

10.6.4 Vitrification of Embryonic Axes of Citrus macroptera

By R Chaudhury based on Malik and Chaudhury 2006

Items required a day before initiating cryopreservation

1. Sterile glass Petri dishes, 100 ml flasks, filter paper discs
2. Scalpel blade holder, scalpel blades, forceps, needles
3. Sterile cryovials and cryomarkers, Dewar with LN

Prepare in advance

1. Preculture medium: MS medium (Murashige and Skoog 1962) with 0.3 M sucrose, 2 M glycerol and 0.7% agar
2. Osmotic loading solution: 0.4 M sucrose, 2 M glycerol in MS
3. PVS2: 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4 M sucrose in MS medium
4. Rinsing solution: 1.2 M sucrose in MS medium
5. Culture medium: MS with 1 $g \cdot l^{-1}$ activated charcoal, 0.17 $g \cdot l^{-1}$ NaH_2PO_4 and 1 $mg \cdot l^{-1}$ each of 6-benzylamino purine (BAP) and naphtlene acetic acid (NAA) after Chin et al. (1988)

The procedure

1. Collect seeds from ripe fruits within 4–5 days of harvest.
2. Remove the seed coat from the seeds just before experimentation.
3. Surface sterilize the seeds with sodium hypochlorite (2–2.7%) for 10 min.
4. Rinse four times with sterile distilled water.
5. Gently separate out the cotyledons, snapping one of the attached connections with the zygotic embryonic axes. Scoop it out by making another incision at the other joining point.
6. For moisture content determination: Determine the fresh weight of 10 axes replicated three times each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
7. Preculture axes on preculture medium for 16 to 24h.
8. Transfer 15–25 axes to 1.2 ml sterile cryovials and treat with 0.5 ml loading solution for 20 min at 25°C.
9. Replace loading solution with 0.5 ml PVS2 for 30 min at 25°C.
10. Plunge cryovials into LN (LN).
11. Rewarm in a 38±1°C water bath for 1 min with vigorous shaking.
12. Replace the PVS2 with 0.5 ml rinsing solution at 25°C for 20 min.
13. Drain the solution and blot the axes dry on sterile filter papers.
14. Culture the axes in culture medium (above) and maintain at 25±2°C with a 16 h photoperiod under light intensity of 35 $\mu E\ m^{-2}s^{-1}$.

10.6.5 Cryopreservation of Embryonic Axes of Citrus suhuiensis *cv. Limau langkat through Desiccation*

By AM Makeen and MN Normah based on Makeen et al. 2005

Desiccation under laminar flow

Items needed

1. Sterile plastic Petri dishes, glass Petri dishes and beakers/ bottles
2. Sterile propylene cryovials, cryocanes and cryovials rack
3. Sterile filter papers
4. Tools (scalpels, sterile blades and forceps)
5. 95% (v/v) ethanol for flaming the tools
6. Stereomicroscope

Prepare in advance

1. Freshly harvested fruits
2. Sterile distilled water
3. 200 ml 80% (v/v) ethanol (for seed sterilization)
4. 200 ml of 20% (v/v) bleach (~5% sodium hypochlorite) and a few drops of Tween 20
5. Aluminum foil boats for water content determination
6. Growth medium: MS medium with 0.1 $g \cdot l^{-1}$ 6 benzylaminopurine (BAP) and 0.7% agar in Petri dishes
7. Liquid nitrogen
8. Water bath preset at 40°C
9. An oven preset at 103°C
10. A desiccator to cool down the dried embryonic axes (for moisture content determination)

The procedure

This is a 1-day procedure.

1. Remove the seeds and wash in running tap water for 1 h.
2. Place the whole seeds inside a bottle or a beaker and pour the 200 ml 80% ethanol, leave for 2 min, shake gently. Pour off the ethanol and submerge the seeds in 20% bleach solution for 20 min with shaking every 5 min.

3. Remove the disinfectant and rinse the seeds with sterile distilled water three times until the foam made by Tween 20 disappears.
4. Keep the sterilized seeds in Petri dishes sealed with Parafilm.
5. Place one or two layers of filter paper on a glass Petri dish and place a few seeds on the filter paper. Under a stereomicroscope with the aid of a pair of forceps and a scalpel excise the biggest embryonic axes (seeds are highly polyembryonic) leaving a small block of the cotyledon attached to the axes. Place the excised embryonic axes (in a row of 10 axes) inside another Petri dish with a filter paper moistened with few drops of sterile distilled water. Close the Petri dish with its cover. This is done until all axes are excised for desiccation.
6. Transfer the axes to glass Petri dishes with dry filter papers and desiccate for 2 h in laminar airflow cabinet (using high airflow).
7. For moisture content (MC) determination: Determine the fresh weight of 10 axes (replicated three times) in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh (Fig. 10.1).
8. Place 10–30 axes inside cryovials (5 axes in each vial), mount the cryovials in the cryocanes and plunge into the LN.
9. Rapidly warm the cryovials in a 40°C water bath for 2 min, then culture the axes on growth medium.
10. Seal all Petri dishes with Parafilm and keep the cultures in the culture room (Culture conditions: 25±1°C under 16 hours light/ 8 h dark photoperiod with light intensity of 25 μmol $m^{-2}s^{-1}$).
11. Score viability when axes develop into normal seedlings (shoot and root).

Desiccation over silica gel

Items needed to dissect embryonic axes
Use the same list as for air desiccation in laminar flow hood.

Prepare in advance
1. Silica gel: wrap batches of 15 g silica gel in aluminum-foil bags; place them in an autoclavable container and autoclave for 20 min. After that place silica gel packages inside the oven set at 103°C for 24 h or until use.
2. Follow the same procedures for embryonic axis excision as for the desiccation under laminar airflow.

The procedure
1. Place 15 g of sterile, dry silica gel in a glass Petri dish (7 cm diameter) and cover with a sterile filter paper.
2. Place the axes on the filter paper covering the silica gel.
3. Seal each Petri dish with Parafilm and leave for the prescribed desiccation period.
4. For moisture content (MC) determination: Determine the fresh weight of 10 axes replicated three times each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh (Fig. 10.2).
5. Follow the previously described procedure for cryopreservation, rewarming and culture.

Optimal hydration for cryopreservation
The most normal seedlings regrown from cryopreserved axes was 83.3% obtained at MC of 7.8 (= 0.095 g H_2O g^{-1}dw) after 2 h of desiccation in the laminar airflow cabinet and 62% at 11.5% MC (0.139 g H_2O g^{-1}dw) after one hour of desiccation over silica gel.

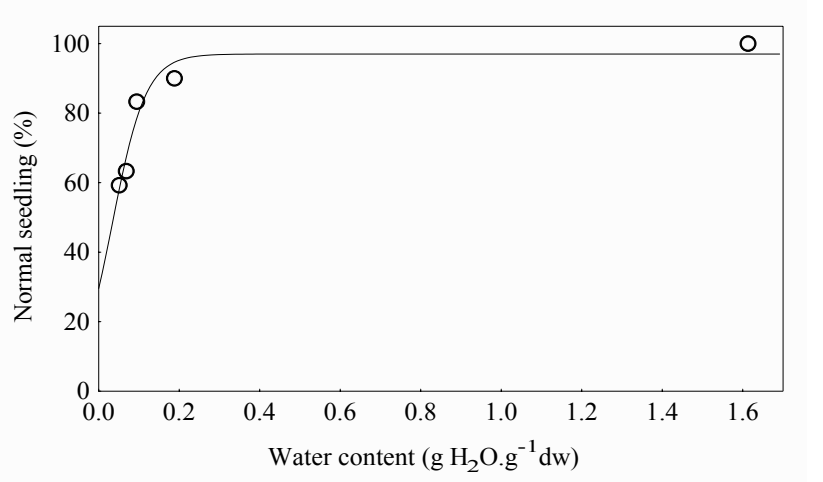

Fig. 10.1 Observed normal seedling percentage (o) of the excised embryonic axes of *C. suhuiensis* cv Limau langkat desiccated in laminar airflow cabinet to various water contents. The fitted pattern of the desiccation sensitivity model was computed by Quasi-Newton method. (From Makeen et al. 2005)

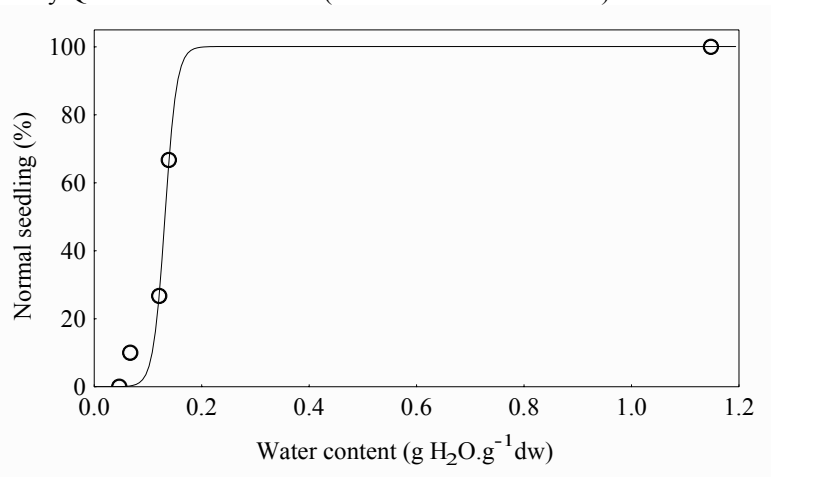

Fig. 10.2 Observed normal seedling percentage (o) of the excised embryonic axes of *C. suhuiensis* cv Limau langkat desiccated over silica gel to various water contents. The fitted pattern of the desiccation sensitivity model was computed by Quasi-Newton method. (From Makeen et al. 2005)

10.6.6 Cryopreservation of Coffee (Coffea liberica) *Embryos through Air Desiccation*

By MN Normah based on Normah and Vengadasalam 1992

Items needed to excise and to cryopreserve the embryos

1. A laminar airflow cabinet
2. Tools (forceps, blades, scalpels)
3. Polypropylene cryovials
4. LN tank
5. Sterile glass Petri dishes and beakers/bottles
6. An oven preset at 103°C, desiccator for moisture determination

Prepare in advance

1. Sterile distilled water
2. A 40°C water bath
3. 5% solution of commercial bleach (final concentration 0.27% sodium hypochlorite)
4. Recovery medium: MS medium (Murashige and Skoog 1962) with 0.1 mg·l^{-1} kinetin, 0.1 mg·l^{-1} 2,4-D, 30 g·l^{-1} sucrose, 2 g·l^{-1} activated charcoal and 8 g·l^{-1} Difco Bacto Agar
5. Subculture medium: MS with 0.1 mg·l^{-1} 6 benzyl adenine (BA), 0.5 mg·l^{-1} indole 3 butyric acid (IBA), 7 g·l^{-1} agar

The procedure

This is a 1-day procedure.

1. Rinse coffee fruits in water and remove the exocarp and mesocarp.
2. Isolate the embryos under laminar airflow.
3. Desiccate embryos in open Petri dishes in the laminar airflow for 30–50 min.
4. Determine the moisture content (MC) of the excised embryos after desiccation treatment (optimal is 20% = 0.25 g H_2O g^{-1}dw). Determine the fresh weight of 10 axes replicated three times each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
5. Place embryos in cryovials and immerse directly into LN.
6. To warm: rapidly immerse vials in a 40°C water bath for 2–3 min.
7. Sterilize embryos with bleach for 6 min followed by 5 rinses with sterile distilled water and directly culture on the recovery medium.
8. Maintain cultures at 27±1°C with 8 h photoperiod (25 μmol $m^{-2}s^{-1}$).
9. Score viability when embryos develop into normal seedlings (shoot and root). The highest regrowth was 83–86% at 25–20% MC.

10.6.7 Cryopreservation of Embryonic Axes of Hazelnut (Corylus avellana) *by Air and Silica Gel Desiccation*

By MN Normah and BM Reed based on Reed et al. 1994 and Normah et al. 1994

Air desiccation

Items needed to dissect and cryopreserve the embryonic axes

1. Sterile propylene cryovials, cryocanes and cryovials rack
2. Tools (scalpels, sterile blades and forceps)
3. Sterile filter papers
4. Welled culture plates or Petri dishes of recovery medium
5. LN in a storage dewar
6. Commercial bleach (5.3% sodium hypochlorite)

Prepare in advance

1. Freshly harvested hazelnuts (nuts can be stored for several months in 70 l burlap bags at 20°C and relative humidity of 20–40%)
2. Aluminum foil boats for weighing the embryonic axes
3. An oven preset at 103°C, a desiccator
4. Basal NCGR-COR medium (Yu and Reed 1993)
5. Perlite, moistened with tap water, autoclaved for 30 min and cooled

The procedure

Week 1: Stratification of whole seeds

1. Surface sterilize fresh or stored whole nuts in 20% bleach for 10 minutes and rinse in tap water.
2. Plant intact seeds in the perlite, close tray with plastic bag and chill at 4°C for 2 weeks.

Week 3: Cryoexposure of hazelnut excised embryonic axes

1. Crack nuts to remove the pericarps and excise the embryonic axes.
2. Surface sterilize the embryonic axes in 10% bleach (final concentration 0.53% sodium hypochlorite) for 10 min and rinse in sterile water.
3. Blot dry on sterile filter paper. Dry in an open Petri dish in a laminar flow hood for 1.5–2 h (8–10% moisture = 60% regrowth).
4. Moisture content (MC): Determine the fresh weight of 10 axes replicated three times each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
5. Place 10 axes in each cryovial, immerse directly into LN for 1 h.
6. Warm cryovials in a water bath at 45°C for 1 min.

7. Culture axes on NCGR-COR medium (Yu and Reed 1993) at 25°C with a 16 hr photoperiod (25 μmol m^{-2} s^{-1}).
8. Score viability when axes develop into normal seedlings.
9. This technique was used to store axes from four species of *Corylus*.

Silica gel dehydration

Embryonic axes can also be excised from directly cryopreserved seeds of *Corylus*. The protocol is as follows:

Items needed for cryopreservation of seeds and excision of embryonic axes

1. A seed germinator with alternating temperatures of 10°C for 14 h and 25°C for 10 h under 12 h photoperiod
2. An oven preset at 103°C, a desiccator
3. Cryostorage rack
4. Tools (scalpels, sterile blades and forceps), sterile filter papers
5. Recovery medium: Basal NCGR-COR medium (Yu and Reed 1993)
6. LN storage unit

Prepare in advance

1. Freshly harvested or stored hazelnuts
2. Silica gel in batches of 125 g
3. Gibberellic acid (GA_3) solution (10 mg/100 ml) for soaking the embryonic axes
4. Basal NCGR-COR medium (Yu and Reed 1993)
5. TTC solution: 2,3,5-triphenyltetrazolium chloride (1.0%)

The procedure

Day 1: Desiccation of hazelnut seeds

1. Desiccate whole nuts (with pericarp) over the silica gel for 32 to 48 h.
2. Use 10 nuts with four replicates for moisture content (MC) determination and 25 nuts in triplicates for desiccated-frozen nuts and germination tests (in a seed germinator).
3. For MC determination: Determine the fresh weight of nuts (cut into small pieces) in an aluminum foil boat, then dry in an oven set at 103°C for 16 h, cool in a desiccator and reweigh.

Day 2: Cryoexposure of hazelnut seeds and survival of excised embryonic axes

1. Place nuts into a cryo storage rack and immerse directly into the LN tank.
2. Warm the nuts at room temperature.
3. Crack nuts to remove the pericarps.
4. Perform the tetrazolium test on a sample of the axes to determine their viability.
5. Excise the embryonic axes of the remaining nuts.
6. Surface sterilize the embryonic axes in 10% bleach (final concentration 0.53% sodium hypochlorite) for 10 min and rinse in sterile water.
7. Soak embryonic axes in the GA_3 solution for 5 min and culture on NCGR-COR medium.

Optimal hydration for cryopreservation

The highest regrowth of axes was 97% attained by cryopreserving whole hazelnuts dried to a water content of 15% (= 0.176 g H_2O g^{-1}dw) after desiccation over silica gel for 32 h. Optimal water content for excised axes was 3% (= 0.03 g H_2O g^{-1}dw).

10.6.8 Air-Desiccation Cryopreservation of Embryonic Axes of Neem (Azadirachta indica)

By R Chaudhury based on Chandel et al. 1996

Items required a day before initiating cryopreservation

1. Sterile glass Petri dishes and 100 ml flasks
2. Sterile filter paper discs
3. Scalpel blade holder, scalpel blades, forceps, needles
4. Sterile cryovials and cryomarkers
5. MS medium (Murashige and Skoog 1962) with 0.1 mg ml^{-1} each of 6-benzylamino purine (BAP) and naphthlene acetic acid (NAA), 0.7% agar in test tubes
6. Dewar flasks containing LN
7. Seeds: The seeds should be harvested only from ripe yellow fruits. Complete the extraction process within 4–5 days of harvest to obtain optimal results in cryopreservation.

The procedure

1. Collect seeds from ripe yellow neem fruits. Break open the endocarp to extract the seeds just before experimentation. Surface sterilize the seeds using sodium hypochlorite (2–2.7%) for 10 min.
2. Rinse four times with sterile distilled water.
3. Remove brown seed coat and gently separate out the cotyledons snapping one of the attached connections with the embryonic axis.
4. Make an incision at the other attached connection of the axis and scoop it out.
5. For moisture content determination: Determine the fresh weight of 10 axes replicated three times each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
6. Spread rest of the axes on sterile filter paper discs in the air current of a laminar flow cabinet for desiccation to moisture levels between 11 and 16% (on fresh weight basis).
7. Moisture content (MC): Determine the fresh weight of 10 axes replicated three times each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
8. If the initial moisture content of neem axes is about 45%, it may require 3.5–4 h of desiccation to achieve target moisture level.
9. Place about 10–15 desiccated axes in sterile 1.2 ml cryovial and plunge rapidly in LN.
10. Warm the cryovials in a water bath at 37–38°C for 5 min, and culture on MS medium under growth room conditions.

10.6.9 Desiccation Cryopreservation of Isolated Embryos of Olive (Olea europaea)

By MA Revilla based on Gonzaléz-Rio et al. 1994

Items needed

1. Sterile tools (scalpels, blades, forceps)
2. A laminar airflow cabinet
3. An oven set at 70°C for moisture content determination
4. Sterile 2 ml polypropylene cryovials
5. LN tank
6. A 45°C water bath

Prepare in advance

1. Fruits with intact mesocarps (to reduce chances for contamination)
2. A solution of 70% ethanol
3. A solution of 1.2% active sodium hypochlorite
4. Sterile distilled water, filter papers
5. MS (Murashige and Skoog 1962) medium with 30 $g \cdot l^{-1}$ sucrose and 7.5 $g \cdot l^{-1}$ agar

The procedure

Day 1: Remove the fleshy mesocarp of the fruit and break the endocarp using a vice. Soak seeds in water overnight.

Day 2

1. Make a longitudinal excision in the endosperm and remove embryo.
2. Immerse embryos in 70% ethanol for 2 min, followed by 1.2% sodium hypochlorite for 10 min, then rinse twice with sterile water.
3. Place embryos on filter paper and desiccate for 13 and 18 h in the laminar flow cabinet. Determine the moisture content.
4. The highest normal seedling percentages (70%) were obtained when embryos were desiccated for 18 h to a moisture content of 3% (0.03g $H_2O.g^{-1}$dw) and for 13 h with recovery of 53% and 10% MC (0.11g $H_2O.g^{-1}$dw).
5. For moisture content determination: take fresh weight of 10 axes replicated three times each in an aluminum foil boat, then dry in the oven for 24 h, cool in a desiccator and reweigh.
6. Place five embryos in each cryovial and plunge into LN.
7. Rewarm by immersing the cryovials in a 45°C water bath for 2 min.
8. Culture embryos on MS medium at 26°C with 16 h light (30–45 μE $m^{-2}s^{-1}$)/8 h dark. Embryos turn green after 15 days of culture.

10.6.10 Desiccation Cryopreservation of Embryonic Axes of Rubber (Hevea brasiliensis)

By MN Normah based on Normah et al. 1986

Items needed

1. An oven set at 103–105°C, desiccator
2. Sterile tools for excision
3. Sterile aluminum foil envelopes
4. LN
5. A water bath set at 37±2°C

Prepare in advance

1. Fresh seeds: Seeds from fruits collected from trees give better regrowth compared to those dispersed on the ground.
2. 20% solution of commercial bleach (final concentration 1% sodium hypochlorite) with a few drops of Tween 20 per 500 ml.
3. Sterile distilled water
4. MS medium (Murashige and Skoog 1962) with 0.6–0.7 μM kinetin, 1.0 μM naphthelene acetic acid (NAA), 1.4 μM gibberellic acid (GA_3) and 4 $g \cdot l^{-1}$ activated charcoal. Softer agar (6–7 $g \cdot l^{-1}$) for the recovery medium produces better growth of the seedlings.

The procedure

This is a 1-day procedure.

1. Immerse the seeds in bleach solution for 30 min. Rinse with sterile distilled water three to four times.
2. Crack the seeds and isolate the embryonic axes by completely removing the endosperm and the cotyledons (in laminar airflow cabinet).
3. Desiccate the excised embryonic axes for 3–4 h at 26–28°C. (Optimal MC 16.2% = 0.19 g H_2O g^{-1} dw)
4. After each desiccation period, determine moisture content of the axes; use 40 axes (10 in 4 replicates).
5. For moisture content determination: Determine the fresh weight axes in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
6. Place desiccated axes inside sterile aluminum foil envelopes and directly immerse into the LN tank for 16 h.
7. Warm the frozen axes by immersing the foil envelopes in the water bath (at 37± 2°C) for 1–2 min.
8. Culture the axes under a 12 h photoperiod with light intensity of 25 μmol $m^{-2}s^{-1}$.

10.6.11 Flash Drying Tea Zygotic Embryos (Camellia sinensis)

By HH Kim based on Kim et al. 2002, 2005

Checklist for flash drying of tea zygotic embryos

Items needed to prepare plant material

1. Sterile filter papers in sterile Petri dishes
2. Tea seeds
3. Sterile (autoclaved) distilled water
4. Scalpel, forceps for inoculation
5. 80% ethyl alcohol for surface sterilization
6. 1% sodium hypochlorite solution with 3 drops of detergent

Items needed to dehydrate and cryopreserve

1. Sterile standard sieves (850 μm) to dry embryos
2. Petri dishes with sterile filter paper to remove moisture following surface sterilization
3. Cryovials (2 ml) and markers
4. Recovery medium: ½ strength MS medium (Murashige and Skoog 1962) with 0.5 $mg \cdot l^{-1}$ benzylaminopurine (BAP), 0.01 $mg \cdot l^{-1}$ indole butyric acid (IBA), 1.5 $g \cdot l^{-1}$ activated charcoal and 2.5 $g \cdot l^{-1}$ Phytagel
5. Canes, canisters and long-term storage Dewar
6. A 40°C water bath
7. Vertical laminar airflow to dry embryos

The procedure

Step 1. Plant material

1. Harvest tea seeds at mid-maturity (mid-October in Korea).
2. Store the seeds with pericarp at 5–8°C under humid conditions.
3. Use seeds within 1 month after harvest. Germination of dried cotyledonary embryonic axes (CEAs) decreases rapidly after seed storage.
4. Remove the pericarp and seed coats with a scalpel.
5. Dissect axes with fragments of cotyledons attached to the embryonic axis (CEAs) forming explants of pyramidal shape 5–6 mm long.
6. Sterilize in 1% sodium hypochlorite with 2–3 drops of detergent for 15 min with shaking, and then rinse three times with sterile water.
7. Place CEAs on sterile filter paper in a Petri dish to absorb moisture.

Step 2. Dehydration and cryopreservation of CEAs

1. Place sterilized standard sieve (pore size: 850 μm) in the air current of the vertical laminar airflow.
2. Place the CEAs on sieves and dry for ~4 h to embryo moisture content (MC) of 16–17% (fresh weight basis).
4. Place CEAs in 2 ml cryovials (10 CEAs per vial) and submerge in vapor phase LN.
5. Rapidly warm samples in a 37°C water-bath for 2 min.
6. Culture at 22–25°C with 16 h light (35 μmol m^{-2} s^{-1})/8 h dark.
7. Expansion of embryonic axes is noticeable after 1 week.
8. CEAs expand and produce a small green shoot of at least 3 mm by 10 days after inoculation. CEAs develop normal shoots and roots within 1 month.

Notes

The following are critical for successful cryopreservation:

1. Seed harvesting stage: Early- to mid-maturity seeds showed higher survival than late-maturity seeds.
2. Dissection of CEAs: Dissection area and dissection angle (depth) should be precisely controlled. The location (in depth) is different among individual seeds.
3. Drying to moisture of 16–17%: The MC of CEAs is the most critical factor. The hydration window for tea zygotic embryos is narrow. CEAs with higher or lower moisture content are damaged due to freezing or desiccation injury.

10.6.12 Desiccation of Zygotic Embryo Axes of Chestnut (Castenea)

By AM Vieitez based on Corredoira et al. 2004

Items needed

1. Tools
2. Sterile filter paper
3. Decoated chestnut seeds for excision of embryonic axes
4. 70% ethanol
5. Chlorine solution at 5% (Millipore® chlorine tablets) plus a few drops of Tween 80
6. Sterile distilled water
7. Sterile 2-l beakers for seed sterilization
8. Sterile Petri dishes
9. Cryovials and markers
10. Recovery medium

Prepare in advance

1. Sterile 2-l beakers for surface sterilization of chestnut seeds
2. Sterile distilled water in flasks or bottles
3. Sterile Petri dishes (one for each 25 axes)
4. Recovery medium: Murashige and Skoog (1962) medium (with half-strength nitrates), 0.5 mg l^{-1} 6-benzyl adenine (BA), 1 ml l^{-1} Plant Preservative Mixture (PPM) and 0.09 M sucrose. Dispense in Petri dishes (25 ml of 5 g·l^{-1} agar and 20 × 150 mm tubes (16 ml of 6 g·l^{-1} agar).

The procedure

1. Collect seeds and store in paper bags at 4°C for up to 1 month.
2. Remove seed coats and surface sterilize in 70% ethanol (2 min) and 5% chlorine solution (30 min). Shake from time to time.
3. Rinse three times with sterile distilled water.
4. Aseptically dissect embryonic axes from the cotyledons.
5. Desiccate axes on open Petri dishes in a laminar flow hood for 4–5 h (20–24% moisture content on a fresh weight basis).
6. For moisture content determination: Determine the fresh weight of 10 axes replicated 3 times each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
7. Place axes into 2 ml cryovials (five axes to a vial) plunge into LN.
8. Warm vials in a 40°C water bath for 2 min.

9. For rehydration place embryonic axes in Petri dishes of recovery medium, and incubate in darkness at 25°C for 24 h.
10. Transfer axes to culture tubes (one axis per tube) with fresh recovery medium, but with agar concentration increased to 6 $g \cdot l^{-1}$.
11. After 2 weeks place under a 16 h photoperiod (30 $\mu mol\ m^{-2}s^{-1}$).
12. Assess plant recovery (whole plants developing directly from embryonic axes) 8 weeks after warming (Table 10.1).

Comments

Replicate samples should be enough to determine the fresh and dry weights before and after Step 5. Controls (desiccated and non-cryopreserved axes) should be included. Appropriate procedures should be developed for the *in vitro* culture of embryonic axes in recovery medium prior to the cryopreservation experiments.

In this protocol, most non-cryostored embryonic axes develop as whole plantlets (100% when post-desiccation moisture content was 29–35%). Following cryopreservation plant recovery increases from zero for non-desiccated axes to 63% for those with moisture contents ≅ 20% (5 h desiccation). In addition, 36% of the axes produce only roots. Desiccation to moisture contents less than 18% results only root development.

Table 10.1 Shoot recovery (% ± standard error) of cryopreserved shoot apices of six chestnut genotypes from juvenile- and mature-tree origin. Assessment made 8 weeks after cryopreservation and plating on recovery medium

Genotype	Origin	Shoot recovery (%)
812	Juvenile	53.2 ± 1.6
818	Juvenile	53.3 ± 3.3
12	Juvenile	37.5 ± 9.8
LA1	Mature	35.4 ± 4.4
LA3	Mature	54.4 ± 8.1
Pr5	Mature	42.5 ± 3.8