Chapter 19

Cryopreservation of Orthodox (Desiccation Tolerant) Seeds

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19.1 Introduction

Although there are several methods of *ex situ* plant conservation, seed banking is the most efficient for many species, particularly for ease of application and the amount of diversity conserved (Linington and Pritchard 2001). Indeed, seed storage is the main form of *ex situ* plant genetic resources (PGR) conservation globally, representing about 90% of all collections, the vast majority of which are crops, including cultivars (FAO 1996). More than half the world's PGR accessions are held in medium-term or long-term storage conditions. For long-term storage, the international standards are drying at 10–25°C and 10–15% RH to 3–7% moisture content, followed by storage at about –18°C (FAO/IPGRI 1994). Whilst less than expected seed longevity at about –20°C is known for "intermediate" or Type II seeds (see Pritchard 2004), 'orthodox' Type I seeds can also age quicker at seed bank temperatures than predicted by the seed viability equations (for explanation see Pritchard and Dickie 2003). This was revealed by an elegant experiment in which orthodox *Hordeum vulgare* ssp *distichium* cv. Proctor seed, ageing at warm temperatures, was interrupted by transfer to –20°C,

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which indicated longevity parameters associated with storage at –6°C (Roberts and Ellis 1977). This observation, combined with comparisons between actual performance and extrapolation of longevity to sub-zero temperatures, suggests that the benefits of all sub-zero storage temperatures may be less than previously thought (Dickie et al. 1990; Pritchard 1995; Pritchard and Dickie 2003; Walters et al. 2004). Although the modelling of seed longevity at sub-zero temperatures is a challenge, such cooling generally enhances dry seed longevity (Dickie et al. 1990; Pritchard and Seaton 1993; Walters et al. 2004). Consequently, cryopreservation may be of particular importance for the long-term (10-100s years) storage of otherwise inherently short-lived orthodox seeds (Pritchard 1995; Pritchard and Seaton 1993; Pritchard et al. 1999b; Walters et al. 2004; Pritchard 2007).

The extra biological insurance of cryopreserving seeds was recommended about 30 years ago (Stanwood and Bass 1978). Since then, numerous studies have reported dry seed tolerance of liquid nitrogen exposure of hundreds of species (see citations in Pritchard 1995, 2007). Cryopreservation studies on genetic resources in general have increased significantly in recent times (Pritchard 2002), including on dry seeds (Pritchard 2007). Most of the studies in about the last 10 years have been on species of socio-economic importance, particularly woody (about 40), horticultural (about 9) and agriculture (about 13) (for review see Pritchard 2007). Native, endangered or endemic species have also been studied. Moreover, greater collective efforts to cryopreserve unique PGR are evident recently. Such initiatives include: technology transfer of methods for vegetatively propagated crops (Reed et al. 2004), the establishment of a regional cryo centre of excellence in Africa (Darwin Initiative 2005; Wood et al. 2005), and functional regional cryo networks (EU 2006; Pritchard 2006). However, interest in the low temperature extremophily of seeds through experimentation with liquefied gases extends back to the nineteenth century, with a golden era between the 1830s and 1930s (e.g. Thiselton-Dyer [1899]; see "The latent life of seeds").

19.2 The Latent Life of Dry Seeds

As early as 1834, Edwards and Colin (1934) subjected dry seeds of barley, broad beans, rye and wheat to a temperature which "froze mercury" after which "their power of germination was unchanged." Similarly, Wartman (1860) explored the effects of different temperatures on dry seeds of nine species (*Avena sativa*, *Clarkia elegans*, *Eschscholtzia californica*, *Hordeum vulgare*, *Lepidium sativum*, *Linaria bipartite*, *Nemophila insignis*, *Portulaca oleracea*, *Triticum sativum*); in all cases, germination was "unimpaired"

after 20–30 min exposure to –110°C. Also De Candolle and Pictet (1879), De Candolle (1865) and Dewar and McKendrick (1892) noted no loss of seed vitality after exposure to liquefied gases. Brown and Escombe (1897–1898) treated seeds of 12 species in liquid air for 110 h, with no ill effects. Selby (1901) followed a similar approach by exposing seeds of 12 species (*Chenopodium album*, *Cucumis sativus*, *Helianthus annuus*, *Linum usitatissimum*, *Lupinus luteus*, *Mimosa pudica*, *Onobrychis sativa*, *Pinus sylvestris*, *Ricinus communis*, *Secale cereale*, *Triticum sativum* and *Zea mays*) to liquid air; concluding that "it is not apparent that any marked unfavourable effect on germinable seeds may be traced to the immersion." The effects of even lower temperature (1.35 K; –272°C) were assessed by Lipman (1936); with vetch, wheat, barley, tobacco, flax, buckwheat, spinach, milo, maize and *Melilotus* seeds showing no ill effects when germinated and grown in a greenhouse.

The effect of storage time at –196°C was assessed for a range of species by Lipman and Lewis (1934). Neither sugarcane, spinach, cucumber, sugar beet, buckwheat, barley, purple vetch, oat, onion, mustard and *Melilotus* seeds stored for 30 days, nor pea, corn, squash, alfalfa and sunflower seeds stored for 60 days, showed any detrimental effects with respect to laboratory/greenhouse germination or vigour. Similarly, sweet clover seeds tolerated 176 days storage in liquid air, yielding 74% germination, compared to 64% for the unfrozen control (Busse 1930). As germination of these stored seeds was not checked until 6 months after thawing, at which point the controls had only about 50% germination, Busse (1930) felt that "storing in liquid air….may be beneficial in retarding normal aging of …seeds," a sentiment that has subsequently been shown to be true. Wartman (1860) also compared seed responses (short-term exposure) at –110°C with –57°C, observing no systematic differences.

Finally, two-step freezing ("gradual transition" from vapour to immersion) was pioneered by Selby (1901), in a comparison with rapid cooling ("sudden transition" by immersion) to liquid air (–190°C). Selby (1901) also considered storage time (6–48 hours), but a full factorial design was not used, making specific insights difficult other than most of the treatments yielded similar germination to the controls.

In summary, the basis of modern seed cryopreservation was established between 170 and 70 years ago with basic experiments on the short-term (hours) tolerance of mainly crop seeds to various cryogenic temperatures, including the first consideration of two-step freezing. Only at the end of this period did attention turn to the importance of seed moisture content on survival of cryogenic temperatures.

19.3 Moisture Content

In 1934 Busse and Burnham noted that "the percentage of abnormal plants decreases with increasing moisture content up to 10 or 15 per cent" when treated with liquid air. The authors had dried oilseeds of flax for 10 h at 51°C and then manipulated the uptake of additional moisture above the dried level in an atmosphere of high humidity. The impact on seedling quality was observed as a decrease in seedlings with a double growing point and double or fasciated hypocotyls compared with the driest cryopreserved seeds. The abnormalities produced by low temperature were not inherited, however, but due to "temporary changes in the developmental processes." Similarly, cotton seeds treated with liquid air subsequently germinated but had "cotyledonary leaves filled with cracks" and the driest flax seeds also had slightly reduced germination (–12%) after cryopreservation. Busse and Burnham (1934) opined that such injury was "due to the simple mechanical stresses and strains set up in the seeds owing to different coefficients of expansion and different rates of cooling of the different parts." Interestingly, both rapid warming (transfer from liquid air immediately onto a piece of metal at room temperature) and slower warming (allowing liquid air to evaporate) had similar effects on the number of abnormalities in flax seed (Busse and Burnham 1934). Rapid temperature changes cause similar stresses and strains in dry soybean, with cracking of dry beans (Sakai and Noshiro 1975). Use of such stresses to positive effect is also known; a few minutes exposure to liquid air or liquid nitrogen is known to reduce hardseededness (impermeability to water), for example in seeds of sweet clover and alfalfa (Busse 1930) and trifolium (Pritchard et al. 1988). Cooling at about 10°C min^{-1} tends to overcome these stresses (Vertucci 1989a).

Moisture contents at ≤8% tend not to affect seed survival following cryopreservation. At less than 13% moisture content (fresh weight basis) no damage to germination was observed in 42 species of commonly cultivated plants (Stanwood and Bass 1978). Stushnoff and Juntilla (1978) noted that seeds with a moisture content of 5–13% were not injured by ultracooling but seeds at 13–16% moisture content were damaged. Moreover, as moisture content increases, the rate of warming (and cooling) can extend the moisture content range for survival (Sakai and Noshiro 1975). In Italian rye grass seed, rewarming rapidly (25°C s^{-1}; 1500°C min^{-1}) rather than slowly (0.5°C s^{-1}; 30°C min^{-1}) following immersion in liquid nitrogen increased the critical moisture content for survival from 24 to 30% moisture content (dry basis; 19–23% fresh weight basis). Similarly, this "critical moisture content" increased from about 16–18% to 19–21% dry basis (i.e. from about 15– 17%

fresh weight [FW] basis) in unhulled rice and winter wheat seeds following comparable treatment. Moreover, it was shown for wheat seeds at 16–18% moisture, that 30 min during warming at an intermediate sub-zero temperature (about –30°C), decreased germination by 38–50%, probably due to ice formation and growth (Sakai and Noshiro 1975).

Subsequent studies aimed to identify this upper moisture content limit for seed cryopreservation using rapid cooling and warming; including those of Stanwood (1985), who coined the term high moisture freezing limit (HMFL). HMFL in oilseeds varies, being about 9% moisture content for sesame (about 50% oil) and about 14% in soybean (about 18% oil) (Pritchard 1995). Overall, HMFL (MC, % FW) is inversely related to oil content (% dry weight [DW]); thus, HMFL = 23.1 to 0.21 oil (Pritchard 1995). Validation of this relationship was confirmed with oily seeds (37–52% DW) of four citrus species, combined with data for 12 other species, including seven *Coffea* sp. (Hor et al. 2005). Using differential scanning calorimetry, it was shown that the seed unfrozen water content, WCu, (% FW) was also related to seed (oil) lipid content (LC); % DW), thus: WCu = 23.4 – 0.28 LC (Hor et al. 2005).

The HMFL and WCu approximate to precooling relative humidities of 74–77% RH in *Citrus* (Hor et al. 2005) and about 85% in other species (see Pritchard 1995 and references therein). Thus drying below the HMFL or the WCu prior to cooling is the best way to ensure successful cryopreservation. Moisture content optima for oilseed cryopreservation are equivalent to about 65–75% RH (Pritchard 1995). It should be noted however that pre-cooling desiccation below this RH may have little negative impact on seed survival, whereas higher moisture levels significantly increase the risk of ice formation.

19.4 Repeated Cooling and Storage Temperature

As noted above, liquid nitrogen exposure can reduce the physical impermeability of hard seeds (irrespective of oil content), the effectiveness increasing with number of cooling–warming cycles (Pritchard et al. 1988). Conceptually, there may be a point at which the number of temperature cycles becomes too stressful, thereby reducing germinability. Gonzalez-Benito et al. (1998) assessed this risk in seeds of *Centaurea hyssopifolia* and *Limonium dichotomum*, finding that they were unaffected by weekly removal from liquid nitrogen compared to constant immersion for 21 weeks.

The optimal temperature in the sub-zero range for the long-term storage of dry seeds remains to be elucidated. This is particularly true of seeds that

can be dried only to intermediate moisture contents (about 8–10%). For these Type II seeds—so called because they can tolerate drying into zone II of the sorption isotherm—it has been hypothesised that storage at –18°C risks devitrification of the aqueous glass (Pritchard 2004). Consequently, it has been suggested that all dried seeds are stored at least 70°C (or K) below the glass transition temperature (Tg); in other words, at ≤ Tg–70. For orthodox seeds, Tg–70 is around 10°C, at which temperature longevity can be around 200 years (Daws et al. 2007); however, further cooling, particularly cryopreservation, has substantial benefits for seed longevity. For example, the reduction in germinability of relatively low initial quality *Dactylorhiza fuchsii* seeds during sub-zero storage was about three times slower in liquid nitrogen compared to –20°C over a 6-year period, although viability loss was not completely stopped (Pritchard and Seaton 1993). Moreover, cryogenic storage clearly prolonged shelf life of lettuce seeds, with half-lives projected as ca. 500 for seeds stored in the vapor and ca. 3400 years in liquid phases of liquid nitrogen (Walters et al. 2004), far in excess of the performance predicted at conventional storage temperature (Pritchard 2007).

19.5 Recovery

For all dried seeds there is the risk of damage during rehydration in the early stages of the germination test. This is best avoided by humidification of seeds above water. Damage is most often observed when dried seeds are placed in water, rather than on moistened (not soaking wet) filter paper or on water agar. Germination should progress under optimum conditions empirically derived for the species under investigation.

19.6 The Future

The kinetics of seed viability loss at sub-zero temperatures have been predicted from the warmer temperature responses of a range of species (Dickie et al. 1990; Pritchard 1995) and determined for one species, lettuce (Walters et al. 2004). Clearly, much remains to be elucidated about seed storage performance at low temperatures. Of particular interest is the increasing number of observations of sub-optimal performance of seeds under conventional seed bank conditions (drying and storage temperature). Examples are prevalent in tropical species (Ellis et al. 1990, 1991; Pritchard and Dickie 2003; Pritchard 2004), which form the vast majority of global plant diversity. In papaya, drying *per se* (Wood et al. 2000) and short-term

"freezing" in the dry state (Pritchard et al. 1999b) compromises germinability.

Associations have been made with the conformational state of seed lipids, particularly crystallisation at –18°C, in *Cattleya aurantiaca* (Pritchard and Seaton 1993) and some *Cuphea* species (Crane et al. 2003; Crane et al. 2006) and reduced storage performance. Such sensitivity in dry oilseeds may reflect a predilection for oil-body coalescence and massive cellular disruption during rehydration (Leprince et al. 1998; Volk et al. 2006). Slower seed imbibition over water, imbibition at high temperature, or preheating of dry seeds before imbibition (Crane et al. 2003) appear to reduce such problems. However, in some orchid species exhibiting seed cold-shock, germinability appeared independent of imbibition temperature (Pritchard et al. 1999a). Other observations that the range of sub-zero temperatures inducing cold shock in dry seeds varies in some orchids species, i.e. is not limited to –18°C (Pritchard et al. 1999b), emphasises the need for future studies in this area.

Cryogenic storage prolongs dry seed longevity compared to conventional freezer storage. Such cryopreservation of 'dry' seeds requires careful attention at three procedural steps: (1) seed dehydration to water contents below the high moisture freezing limit (~ 70–80% relative humidity [RH]); (2) relatively slow cooling and rewarming of the dry samples (~10°C –20°C min^{-1}); and (3) gentle imbibition in the germination test for viability assessment. As critical steps in the process, step (1) is important for all seeds, whilst steps (2) and (3) can be particularly important for oilseeds.

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19.7 Protocols

19.7.1 General Seed Cryopreservation

Based on Sakai and Noshiro 1975

High survival of air-dried crop (and other) seeds after immersion in liquid nitrogen may be easily obtained. Physical damage to seeds post-cryopreservation (see Pritchard et al. 1988) can be overcome usually by using slower cooling rates (Vertucci 1989).

Checklist

1. Protective clothing (gloves, apron and face shield)
2. Wide neck LN dewar (1–2 l capacity) for cooling
3. LN storage vessel and inventory system (i.e. containers and racks)
4. Long handle forceps for placing containers in and extracting from liquid nitrogen
5. Seed storage vessels/packets or polypropylene screw-cap vials. Alternatively, packets can be shaped from aluminium foil.
6. Water bath at 35–40°C

The procedure

1. Dehydrate to the correct range of moisture content: 8–15% dry weight [DW] (8–13% fresh weight [FW]) using a desiccant, such as silica gel, low relative humidity saturated salt solutions (see Protocol 19.7.3), an air conditioned room, or drying room operating at ca. 15% RH. Drying under ambient conditions where the RH is < 70% will also enable such moisture contents to be achieved.
2. Enclose seeds in aluminium foil or plastic vessel with a screw cap.
3. From room temperature, immerse the foil/vessel enclosing seeds in liquid nitrogen. The plastic vessel will enable slower cooling (and warming) as heat transfer is slower compared with foil.
4. After storage, transfer the foil/vessel to air at 0°C or room temperature (slow warming). More rapid warming can be achieved by placing the container in a 40°C water bath; this is generally not harmful to the seeds and may be important if the seeds are close to the high moisture freezing limit (see Protocol 19.7.2).

Possible problems

Ultra-dry seeds may crack when rapidly cooled/ warmed. To reduce this risk, use rates of about 10°C min^{-1}, achieved by using plastic cryotubes and vapour phase cooling, combined with warming in air at room temperature (rather than using a hot water bath).

19.7.2 Cryopreservation of Australian Wild Citrus Seed

By Kim Hamilton based on Hamilton et al. 2005

Cryopreservation of seeds after controlled drying to low moisture contents has been demonstrated as an alternative option for long-term *ex situ* storage of some cultivated *Citrus* species, for example *Citrus aurantifolia*, *C. aurantium*, *C. limon* and *C. sinensis* (Lambardi et al. 2004; Hor et al. 2005). A simple desiccation-based cryopreservation protocol for seeds has also been demonstrated in *C. australasica* (Finger lime), a southeastern Australian wild *Citrus* species grown as a bushfood. *C. australasica* seeds desiccated down to 5% moisture content had a germination level of 82% and normal morphology after short-term storage in liquid nitrogen (Hamilton et al. 2005).

Procedure

1. Extract seeds from mature fruits.
2. Dry seeds by incubation at 15% relative humidity (15°C) for 7 days (~5% moisture content). Alternatively, seeds can be dried over activated silica gel for 5–10 days (~3% moisture content).
3. Place dried seeds in cryotubes on canes and directly immerse in liquid nitrogen for storage.
4. Rapidly rewarm seeds by plunging cryotubes into water at 40°C for 1–2 min.
5. Surface sterilize seeds by rinsing in 2% sodium hypochlorite for 30 s, followed by two rinses in water. Seeds need to be cleaned to reduce fungal infection during germination testing, most likely caused by presence of seed coat mucilage. This may be a feature of seeds from other fruits.
6. Place seeds on moist filter papers (or 1% agar) in Petri dishes and incubate at 30°C with an 8 h day length. Seeds must be kept moist throughout germination testing.
7. Record germination after at least a 2 month incubation period.

19.7.3 Manipulation of Seed Moisture Content

Based on Sun 2002

An understanding of seed water (moisture) content (i.e. how much water) and an understanding of water relations terms is essential for any studies on seed longevity (Roberts and Ellis 1989), including cryopreservation experiments. Water (moisture) content on a wet (fresh) weight basis is widely used in the literature and can be easily converted to dry weight basis. Because water content is a function of seed chemical composition, particularly oil (lipid) content, it is important to consider that seeds of different species may vary in dependency of water content on relative humidity (sorption isotherm). Fortunately, the relative humidity of an environment used to pretreat seeds can be converted to water potential (i.e. how available the water is), as long as the temperature conditions are known, allowing wider comparisons to be made between seed responses and the wider literature on plant water relations.

Prepare in advance
Saturated salt solutions

Checklist
1. Saturated salt solutions in jars that can be sealed hermetically and with raft to keep seeds above the solution.
2. Forceps and scalpel (in case seed parts require dissection)
3. Electronic balance (accuracy depending on sample mass)
4. Oven (for sample drying)
5. Equilibrium relative humidity meter

Procedure
1. Place seed at 25°C over saturated salt solutions at prescribed humidity level. Higher relative humidities may hydrate seeds above the high moisture freezing limit or unfrozen water content (see Protocol 19.7.4).

   a. KOH (8%)
   b. K acetate (23%)
   c. K_2CO_3 (43%)
   d. NH_4NO_3 (63%)
   e. NaCl (75%)
   f. NH_4Cl (78%)

2. Regularly weigh seed sample over many weeks until equilibrated.
3. Determine sample dry weight by drying 1 day in oven at 103°C and calculate water content (WC).
   WC (% weight basis [w.b.]) = (fresh weight – dry weight)/fresh weight × 100.
4. To convert moisture content to dry weight basis:

$$\text{WC (\% w.b.)} \times 100 / [100 - \text{WC (\% w.b.)}].$$

5. Determine equilibrium relative humidity of seeds in closed sample container of a humidity meter over about 30 min (record temperature of sample).
6. Co-plot water content with RH of environment (i.e. construct a water sorption isotherm).
7. Convert RH of environment to water potential:

$$\Psi_W = (RT/V_W) \ln (\% \text{ RH}/100)$$

   Where R is the universal gas constant (8.314 J K^{-1} mol^{-1}), T the absolute temperature and V_W the partial molar volume of water (18 cm^3 mol^{-1}).

Possible problems
Seeds in lower RH treatments may take a considerable time to equilibrate.

19.7.4 Determination of the Unfrozen Water Content

Based on Hor et al. 2005

Pre-drying orthodox seeds below the high moisture freezing limit (HMFL) or unfrozen water content (Vertucci 1989; Hor et al. 2005) is a pre-requisite for successful cryopreservation**.** Theses moisture limits to cryopreservation can be determined using differential scanning calorimetry.

Checklist

1. Forceps and scalpel (in case seed parts require dissection)
2. Saturated salt solutions in jars that can be sealed hermetically and with raft to keep seeds above the solution
3. High accuracy balance (for sample weight determinations)
4. Oven (for sample drying)
5. Differential scanning calorimeter (DSC), with proprietary software

The procedure

1. Equilibrate seed for 4 week at 25°C over saturated salt solutions providing a RH spectrum between 8% and about 93%.

   a. KOH (8%)
   b. K acetate (23%)
   c. K_2CO_3 (43%)
   d. NH_4NO_3 (63%)
   e. NaCl (75%)
   f. NH_4Cl (78%)
   g. $(NH_4)_2SO_4$ (80 %)
   h. KCl (85%)
   i. $BaCl_2$ (90%)
   j. KNO_3 (93%)

2. To achieve even higher moisture levels, partially rehydrate in a 100% RH atmosphere, i.e. over water in a closed container.
3. For thermal analysis, seal seed samples (about 15 mg) in aluminium pans.
4. In the DSC: Cool pans to about –120°C at maximum cooling rate of machine (often about 200°C min^{-1}).
5. Heat pans at about 10°C min^{-1} from –120°C to 20°C; record thermograms.

6. Puncture pans and determine sample dry weight by drying about 1 day in oven at 103°C.
7. Analyse heating thermograms for the determination of the peak and onset temperatures and the enthalpy of endothermic transitions.
8. Determine transition enthalpy (joules per gram DW) from the area above the baseline.
9. Estimate the unfrozen water content (WC_u) by plotting enthalpies against seed sample water content (g H_20 g^{-1} DW).
10. In oily seeds, two linear relationships will exist: for equilibrium moisture contents below and above about 80% RH.
11. Solve the two linear relationships to estimate the WCu. For non-oily seeds, WCu is the *x*-intercept for the linear regression.

Possible problems

Long-term (many weeks) equilibration of seed samples at high humidity may result in fungal contamination of the sample. Use slightly lower equilibration temperatures or shorter times.

19.7.5 Seed Rehydration and Heating Based on Lipid Melting Characteristics

Based on Crane et al. 2003

Oilseeds in particular appear to be sensitive to the rehydration phase of the germination test, possibly as a result of physical state of the lipids on ingress of water. Induction of such sensitivity appears in some species to be a function of seed thermal, especially sub-zero, history. Although in its infancy as a practice, thermal properties of seed lipids may be used as a marker for sensitivity to storage at –18°C [e.g. *Cattleya aurantiaca* (Pritchard and Seaton 1993)] and possibly exposure to other sub-zero temperatures. For example, following –18°C treatment *Cuphea* seeds with lipid peak melting at ≥27°C require heating to >45°C prior to the germination test to ensure high germination (Crane et al. 2003).

Checklist

1. Container of water for humidification of seeds
2. Oven (for sample drying)
3. Differential scanning calorimeter (DSC), with proprietary software.

The procedure

1. Dry seed to about 5% moisture content (see Protocol 19.7.3 or 19.7.4) or over silica gel.
2. Place seeds at sub-zero temperatures for at least 18 h.
3. To limit imbibitional damage, pre-hydrate samples over water for at least 18 h or treat dry seed sample at 45°C for at least 1 h before germination test (without pre-hydration).
4. Germinate under normal growth conditions for the seeds of species under investigation.
5. To determine thermal behaviour of seeds, seal about 4 mg of sample into aluminium pans.
6. Cool pans from 20°C to–100°C at 10°C min^{-1}, hold for 1 min and rewarm to 50°C at the same rate.
7. Record crystallisation events on cooling and peak temperature at the apex of the lipid transition during warming, using the highest transition peak temperature when several peaks are observed.