CHAPTER VI

Cryococonservation
(a) Viability & Vigour
Introduction

Storage of seeds is arguably the most effective and efficient method for the ex situ preservation of plant genetic resources (Pritchard, 1995). Low storage costs combined with ease of seed distribution and regeneration of whole plants from genetically diverse material, offer distinct advantages for the storage of seeds for conservation compared with other types of plant tissues, such as meristems and pollen (Pritchard, 1995). Of all the strategies, cryopreservation has proved to be one of the novel methods for the conservation of seeds of various forest tree species (King and Roberts, 1980; Bonner, 1990). This has been considered as one of the scopes for long term storage of orthodox, intermediate as well as recalcitrant seeds (King and Roberts, 1979; Roberts et al., 1984; Chin, 1988; Farrant et al., 1988; Bonner, 1990; FAO, 1993). Though conventional seed banks maintain seeds at about 5% (w/w) moisture content (% fw) and at -18°C, cryopreservation using liquid nitrogen appears to have a bright future because of its adaptability, mechanical and cost advantages (FAO, 1993). Under such storage conditions it is predicted that high levels of viability will be retained for many years (Stanwood and Bass, 1981). Storage at ultra-low temperatures has the potential to eliminate or reduce (Stanwood and Bass, 1981) unfavourable enzymatic reactions (Ashwood and Friedman, 1979).

Most of the desiccation-sensitive recalcitrant and intermediate seeds are shed from the mother plant with high moisture contents (Engelmann, 1997) as they do not undergo maturation drying (Berjak et al., 1984; Chaitanya and Naithani, 1998). These seeds inevitably require definite drying to comparatively lower moisture contents before being stored in liquid nitrogen (Pritchard, 1995) as high moisture content in the seeds at lower temperatures lead to intracellular freezing and death (King and Roberts, 1980). Since the neem seeds can be dried safely to intermediate moisture content, the possibility of their survival at LN2 temperatures may be explored.
Desiccation of seeds, embryonic axes, embryos and other tissues, using silica gel (Chaudhury and Chandel, 1995), air drier (Chaudhury and Chandel, 1991) and sterile air current under a laminar flow (Engelmann, 1997) may be used effectively. Desiccation of plant tissues including embryos or seeds has been performed before exposure to liquid nitrogen temperatures in *Prunus amygdalus* (Chaudhury and Chandel, 1995), *Camellia sinensis* (Chaudhury et al., 1991), *Araucaria excelsa* (Pritchard and Prendergast, 1986) and *Piper nigrum* (Chaudhury and Chandel, 1994). Thus determination of optimal drying conditions (drying method and drying limit) are vital in the cryopreservation of desiccation-sensitive seeds in maximizing the seed viability and vigour (Staines et al., 1998).

In an attempt to cryostore the neem seeds, Berjak and Dumet (1996) reported that non-desiccated neem seeds, with a moisture content above 20% lose viability after 1 h at -196°C. However, the same seeds were stored successfully in LN2 for 24 hours after dehydration to between 18.5% and 4% moisture content (Chaudhury and Chandel, 1991) and for 4 months when dried to between 0.09 and 0.06 g g⁻¹ (dry mass basis) (Berjak and Dumet, 1996). Survival rates for small seed samples however, were unpredictable: some desiccated neem seeds germinated 40% after 1 day, whereas germination was 75% after one month and 70% after 4 months, considerable fungal proliferation being found in seeds that germinated 40% (Berjak and Dumet, 1996).

While microorganisms may reduce germination of intermediate and recalcitrant seeds after storage under various conditions (Mittal and Hansen, 1998), the cause of loss of neem seed viability at cryotemperatures is unclear. In other seeds, such as tea and jackfruit, viability loss has been associated with membrane perturbations (Chandel et al., 1995). Loss of neem seed viability during storage is preceded by a decrease in several physiological and
biochemical changes (Varghese and Naithani, 2000) that are indicators of seed vigour (Woodstock, 1973). No attempts have been made to evaluate seed quality in neem by measuring vigour index following cryopreservation. Assessment of seed quality is vital for genetic resource conservation because poor quality seeds can produce abnormal seedlings (McDonald Jr., 1994). Hence, the purpose of this study was to explore the long-term (one year, i.e., rotation period) storage of neem seeds, after desiccating the seeds to critical moisture at cryotemperature. Seed vigour is also evaluated to test the quality of seed as it is one of the most desired and important aspects of conservation in seed banks.

Materials and Methods

Seed Treatment and Drying

The freshly harvested mature neem fruits were depulped mechanically and dried under shade to their initial moisture content (as described in materials and methods, chapter 1). These seeds with the hard endocarp were divided into two lots. One of the lots was left with the intact endocarp (non-excised seeds) whereas, the seeds of another lot was slightly hammered to break the hard endocarp, yielding the naked seeds (excised seeds). To optimize the drying technique the excised and non-excised seeds were dehydrated by two different methods of drying; forced-air drying and silica gel drying. In forced-air drying method, the non-excised and/or excised seeds were dried under a current of forced-air (temperature 27-30°C, 20-30% RH) in front of a seed drier (USHA LEXUS model FH423, India). The seeds were shuffled frequently for uniform drying. Rate of desiccation was monitored by harvesting the seed sample at various intervals followed by % moisture content determination. Percentage germination was also examined for seeds desiccated to various moisture contents. In case of silica gel drying, the non-excised and/or excised seeds
118 of neem were covered by self-indicating, coarse silica gel (Qualigens, India) and were placed in a desiccator at room temperature. The silica gel was replenished after every 2-3 hrs with fresh silica gel to achieve faster and uniform drying of seeds. Desiccation rate by silica gel method was determined by recording changes in seed moisture content as described for forced-air drying method together with its effect on % germination. Detailed methodology for determining % moisture content and % germination has been already described in the materials and methods of chapter 1.

Desiccation and LN2 Storage Trials

In another set of experiment, the excised seeds (without endocarp) were desiccated to various moisture content by silica gel drying method and their LN2 sensitivity was determined. The dehydrated excised seeds of different moisture contents were plunged into liquid nitrogen after being enclosed in polypropylene cryovials (Laxbro, India). Seeds of various moisture contents were stored under LN2 temperatures and retrieved after 24 hours to determine the survival by recording their % germination.

Storage of Seeds at Cryotemperatures

Long-term cryopreservation experiments were performed using excised seeds only. Excised seeds of two moisture regimes were used for storage at LN2 temperatures. The first category comprised of the freshly harvested seeds with 48.2% moisture content (fresh weight basis) referred to as non-desiccated seeds (control). The second category of excised seeds comprised of seeds desiccated over silica gel to optimum lower moisture content (7.53%) with maximum survival in the initial trials of cryoexposure.

Excised neem seeds were enclosed in polypropylene cryovials (Laxbro, India) and plunged into liquid nitrogen in liquid nitrogen containers (cryocan; BA - 35, IBP, India). The
seeds were not treated with any cryoprotectants. These seeds were retrieved at desired intervals from liquid nitrogen and immediately thawed by rapidly immersing the seeds in water on a water bath maintained at 37-38°C (Chaudhury and Chandel, 1991). The thawed seeds were used for the following physiological and biochemical analyses:

**Percentage Germination**

The non-desiccated and desiccated seeds were retrieved from liquid-nitrogen storage and their % germination was tested to assess the survival of seeds after cryoexposure. The germination test was performed by the method described by Chaitanya and Naithani (1994) as already discussed in detail in materials and methods of chapter 1.

**Germination Index**

The seeds kept for testing % germination were also used for the evaluation of seed vigour parameter, germination index as described in materials and methods in chapter 1.

**Mean Germination Time**

The mean germination time, yet another parameter that indicates the seed vigour, was performed by the method of Ellis and Roberts (1981) that has been given in the materials and methods of chapter 1.

**Leachate Conductivity**

The seed steep water of both the non-desiccated and desiccated seeds kept for germination were collected to determine the leachate conductivity. The specific conductance of the leachates was estimated by the method described in the materials and methods in Chapter 2.
Results

Cryopreservation pretreatment: Desiccation trials

Table 1 documents the various changes in moisture content (% f. wt. basis) and percent germination both in the non-excised (with endocarp) and excised (without endocarp) seeds with respect to flash drying using silica gel and forced air drying using a seed drier. Rapid rates of desiccation were achieved in excised seeds, but not in non-excised seeds due to the hard endocarp.

Table 1 The effect of desiccation of non-excised and excised neem seeds by silica gel and forced air, on percent seed moisture content and percentage germination

<table>
<thead>
<tr>
<th>Hours after desiccation</th>
<th>Non-Excised</th>
<th>Excised</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Silica Dried</td>
<td>Forced Air Dried</td>
</tr>
<tr>
<td></td>
<td>% MC</td>
<td>%G</td>
</tr>
<tr>
<td>0 h</td>
<td>46.7 ± 1.3</td>
<td>100</td>
</tr>
<tr>
<td>1 h</td>
<td>45.6 ± 1.4</td>
<td>100</td>
</tr>
<tr>
<td>4 h</td>
<td>45.7 ± 1.5</td>
<td>100</td>
</tr>
<tr>
<td>8 h</td>
<td>48.1 ± 2.2</td>
<td>100</td>
</tr>
<tr>
<td>16 h</td>
<td>43.1 ± 2.8</td>
<td>100</td>
</tr>
<tr>
<td>24 h</td>
<td>41.5 ± 0.7</td>
<td>100</td>
</tr>
<tr>
<td>32 h</td>
<td>39.8 ± 1.9</td>
<td>100</td>
</tr>
<tr>
<td>48 h</td>
<td>37.9 ± 0.3</td>
<td>100</td>
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</table>

The non-excised seeds with initial moisture content of 46.7% showed slow rates of drying and was dried to 46.1% in silica dried and 46.5% in forced air dried seeds after 8 h of desiccation. These seeds registered 37.9% moisture in silica dried and 39.3% moisture content in forced air dried seeds after 48 h of desiccation when the experiment was terminated. These seeds kept for germination after desiccation for various period showed 100% germination right from the beginning to the end of the study, both in the silica dried and forced air dried seeds (Table 1). On the contrary, the excised seeds showed remarkable
reduction in moisture content and concurrent changes in the per cent germination in both the test conditions. The seeds dehydrated very rapidly by silica gel and forced air to 5.6% and 9.2% moisture content respectively after 48 h of desiccation resulting in net losses in moisture content of 88% and 80%, respectively. The seeds with initial moisture content 46.7% (f. wt. basis) recorded a comparatively faster decline in moisture content as compared to the non-excised seeds. The moisture content of the seeds recorded after 16 h of desiccation was 10.3% in silica dried and 18.9% in forced air dried seeds and both the seeds exhibited 100% germination. Thereafter, a decline in germination was discernible in the seeds dried to 5.6% in silica gel dried and 9.2% in forced air dried seeds. These seeds registered 15% and 50% loss in per cent germination respectively. Hence, the excised seeds dried by silica gel method was finally selected for various experiments of cryopreservation.

% Moisture Content & % Germination after Desiccation and LN2 Exposure

Fig. 6.1 exhibits the changes in the moisture content (% f. wt. basis) and percentage germination with respect to desiccation and exposure to cryotemperatures for 24 h to determine the optimum moisture for storage of the seeds under LN2. The excised seeds with an initial high moisture content (48.2 %) didn't survive LN2 exposure and all the seeds were recorded dead when retrieved from LN2 and put to germination. Similarly, the seeds dried to different other moisture regimes (41.5% after 2 h of desiccation, 31% after 5 h and 24.9% moisture content after 7 h of desiccation) also succumbed to storage at ultra-low temperatures. The first sign of survival in the experiment was shown by seeds dried to 16.5% moisture content after 10 h of desiccation. These seeds exhibited 16% germination as compared to the maximum survival (90%) by seeds dried to 7.1% moisture content after 16 h of desiccation. On further desiccation to 5.2% and 3.7% moisture content after 20 h and 24 h of desiccation
Fig. 6.1 Germination of excised neem seeds after desiccation at various intervals to different moisture regimes and retrieval after immersion in liquid nitrogen for 12 hours. Moisture content values are mean of four replicates on fresh wt. basis.

respectively, a slight decline in per cent germination (80% and 60% germination respectively) was discernible. Hence, seeds with moisture content close to 7.1% obtained after 16 h of desiccation was selected for further experiments of storage under LN2 temperatures and other cryopreservation experiments and parameters as well.

Percentage Germination

The survival of neem seeds with high moisture content (non-desiccated) and low moisture content (desiccated by silica gel) seeds was tested and is exhibited in Fig. 6.2. The non-desiccated seeds (48.2% moisture content) did not survive exposure to LN2 temperatures
Fig. 6.2 Percentage germination of excised, non-desiccated and desiccated neem seeds after cryostorage for different time intervals. Each value of germination is a mean of 45 observations. The value for each data point has been plotted on the bar.

Even for 5 minutes. The fresh excised seeds exhibited 100% germination and registered absolute loss of germination when stored in liquid nitrogen for 5 min to 12 h (Fig. 6.2). On the contrary, the desiccated seeds not only showed high survival but also for prolonged periods (12 months) and maintained 80% germination after 3 months of storage under LN2 temperatures. Hereafter, a slight decline in percent germination was recorded. The seeds stored for 6 months showed 70% germination and 60% germination was registered for the seeds stored for 12 months.

Mean Germination Time

Delayed germination measured as mean germination time showed extremely contrasting patterns in non-desiccated and desiccated seeds (Fig. 6.3). The fresh non-desiccated seeds
registered a mean germination time of 24 h and in regard to no germination in the seeds thereafter, no mean germination time could be calculated. On the other hand, the desiccated seeds recorded slight increase in mean germination time with increase in duration of LN2 exposure. These seeds registered a MGT of 42 h at zero hour which increased to 49 h on 3 months of storage and to 55.3 h after 12 months of storage.

![Bar chart showing mean germination time registered for excised, non-desiccated and desiccated neem seeds after cryostorage for different periods. The value for each data point has been plotted on the bar.]

**Fig. 6.3** Mean germination time registered for excised, non-desiccated and desiccated neem seeds after cryostorage for different periods. The value for each data point has been plotted on the bar.

**Germination Index**

The vigour of the seed during storage was calculated by the data obtained in the germination test and is presented in Fig. 6.4. Though, both the desiccated and non-desiccated seeds showed a similar trend, there was difference in the magnitude of expression as the non-desiccated seeds with high moisture content did not show any germination after
storage in LN2. Initially, the GI recorded for non-desiccated seeds at zero hour was 17.31 which after LN2 storage for 5 minutes reduced to zero due to no survival of the seeds. The GI of desiccated seeds, initially recorded, was 3.69 which declined to 3.44 after 2 days of storage and 1.34 after 8 months of storage in liquid nitrogen. The seeds retrieved after 12 months of LN2 storage registered a GI of 1.21 which was even less than half of what was recorded in the beginning of the experiment.

**Fig. 6.4** Showing decline in germination index (GI) values in excised, non-desiccated and desiccated neem seeds on germination after retrieval from cryostorage at various intervals. The value for each data point has been plotted on the bar.

**Leachate Conductivity**

The membrane deterioration in the seeds studied as conductivity of the leachates in the germinating medium was recorded and in presented in Fig. 6.5. Initial leachate conductivity registered for non-desiccated seeds was 0.21 mMhos/ml leachate which showed
Fig. 6.6 Effect of cryopreservation on electrolyte leakage measured as specific conductance of the leachates from non-desiccated up to 12 h and desiccated neem seeds up to 12 months after cryostorage. Each value is a mean of 3 replicates. The value for each data point has been plotted on the bar. The maximum value of SD for non-desiccated and desiccated seeds was 1.9 and 0.2 respectively.

an enormous increase just after even 5 minutes of storage at ultra-low temperatures. The specific conductance of the leachates increased around 34 folds as no seeds exhibited germination thereafter. On the contrary, the magnitude of increase in specific conductance of the leachates in desiccated seeds was comparatively very low. The leachate conductivity of the desiccated seeds recorded at zero hour was 0.47 mMohs/ml which showed a slight increase to 0.6 mMohs/ml after 1 month of storage. The maximum increase recorded after 12 months of storage was just 2.5 fold of the initial levels.
Discussion

Drying rate can affect desiccation-tolerance which in turn affects the seed viability, such that desiccation-sensitivity is inversely related to dehydration (Farrant et al., 1989). Rapid rates of desiccation can be achieved in excised seeds, but not in non-excised seeds due to the hard and relatively impermeable endocarp (Table 1). Drying of non-excised neem seeds for 48h, both by silica gel and forced air reduced moisture contents by approximately 9% and 7%, respectively. Slow drying rates in non-excised neem seeds have been reported elsewhere (DFSC, 1997 and Pukkitayacamee, 1997). In contrast, excised neem seeds with an initial moisture content of 46.7% were dehydrated very rapidly by silica gel and forced air to 5.6% and 9.2% moisture contents, respectively. In several other tropical and temperate species with recalcitrant seeds, rapid drying permitted seed survival to lower water contents than does slower drying (Berjak et al., 1984; Grabe, 1989; Pritchard, 1991). The faster rate of desiccation of excised seeds, as compared to non-excised seeds, may be suitable therefore, for cryo-storage of neem seeds.

Germination of excised seeds with moisture contents between 48.2% and 24.9% was reduced after immersion in liquid nitrogen (Fig. 6.1). When seeds were dried to between 16.5% and 3.7% moisture, germination ranged from 16% to 90%. Highest survival, 90% germination, was recorded for seeds dried to 7.1% moisture content. For subsequent evaluations of liquid nitrogen tolerance, excised seeds were dehydrated to 7.5% moisture content.

The freshly harvested viable neem seeds with 48.2% moisture content (non-desiccated seeds) lost absolute viability within 5 minutes of cryostorage (Fig. 6.2). Storage of hydrated seeds at cryotemperatures results in cellular damage due to the formation of ice crystals in
the intercellular spaces (Ahuja, 1986; Jorgensen, 1990; Wesley-Smith et al., 1992). In contrast, desiccated (7.5% moisture content) excised seeds showed significantly high percentage of survival during cryostorage. For the first time we report survival of neem seeds for 12 months of cryostorage with 60% viability. So far, dried neem seeds have been shown to survive cryotemperatures not more than 4 months with 65% viability (Berjak and Dumet, 1996). In our study, the dried excised neem seeds tolerated exposure of cryotemperature at least for 1 month period without significant loss of viability (93% to 90% viability). But cryostorage for subsequent period lead to gradual loss in germination.

Decline in survival percentage of seeds have been demonstrated in neem seeds (Berjak and Dumet, 1996) at cryotemperatures and olive embryos (Gonzalez et al., 1994). Berjak and Dumet (1996) recorded more than 50% loss in survival rate within one month of cryostorage in neem seeds. Similar loss in survival percentage was reported following cryostorage in several other recalcitrant or intermediate seeds like Aesculus hippocastanum (Pence, 1992), Aesculus gabra (Pence, 1992), Araucaria excelsa (Pritchard and Prendergast, 1986), Atrocarpus heterophyllus (Chandel et al., 1995) and Baccaurea polyneura (Normah and Marzalina, 1996).

Germination index (GI) and mean germination time (MGT) are physiological tests for quantifying seed vigour (Kraak and Vos, 1987; Tarquis and Bradford, 1992). Reduced MGT (Fig. 6.3) and maintenance of higher values of GI (Fig. 6.4) in excised desiccated neem seeds cryostored for 12 months further substantiate the higher germinability and survival of these seeds. Higher values of GI along with reduced MGT has been described to be associated with high vigour neem seeds (see chapter 1). Reduction in GI values was often discussed and linked with loss of vigour in deteriorating seeds (Farrant et al., 1986). Survival of neem seeds at cryotemperature for one rotation period (fruiting once in an year) with significantly high viability and vigour make it a most suitable proposition for effective conservation. Therefore, experiments are being undertaken to further extend the survival period of neem.
seeds at cryotemperature, after pretreatment of desiccated excised seeds, to achieve high viability and vigour.

Monitoring of the seed leachates showed considerable difference between the desiccated and non-desiccated seeds during cryostorage. Significantly high amounts of electrolytes in leachates (7.22 to 8.61 mMhos/ml) were characteristics of non-viable excised neem seeds retrieved 5 minutes and 2 days after cryostorage of hydrated seeds, whereas remarkably low levels of leachate conductivity in survived excised desiccated seeds (Fig. 6.5) suggests its close association with seed viability. Adverse storage conditions leading to loss of viability is invariably linked to cellular membrane damage and increased leakage loss (Senaratna and McKersie, 1983). Roberts (1979) proposed that loss of membrane integrity, resulting in the enhanced efflux of cellular constituents in the seed leachates, was one of the earliest events associated with the loss of seed viability. A gradual accumulation of leachates in survived excised seeds with advance in cryostorage period and loss of germination percentage strongly supports the loss of membrane integrity in neem seeds after 12 months of cryostorage. Majority of the recalcitrant seeds such as tea, jackfruit and intermediate seeds like Coffea arabica and Fagus sylvatica do not survive LN2 exposure and exhibited progressive damage with increased electrolyte leakage.

It is concluded that neem seeds showing intermediate storage may be cryopreserved after rapid dehydration essentially to below intermediate moisture content (7.5%) for 12 months with an acceptable level (60% viability) of success. These seeds also exhibited sufficiently high vigour although loss of viability and vigour was also evident along with increased membrane perturbations. Vigour tests on bean, pea and lettuce seeds revealed no adverse effects from exposure to LN2 (Stanwood and Roos, 1979). The next chapter further discusses the changes in vigour and possible causes at biochemical level.
(b) Oxygen Metabolism
Introduction

A multitude of studies have been done already on oxidative stress in stored seeds (Stewart and Bewley, 1980; Senaratna and McKersie, 1986; Leprince et al., 1990; Hendry, 1993; Chaitanya, 1997). But the involvement of free radicals in the cryopreservation of plant germplasm is, to date, virtually unknown (Benson, 1990). Although free radical damage has been recognized as a major factor in seed deterioration (Niehaus, 1978; Senaratna and McKersie, 1983; Finch-Savage et al., 1994), its importance in the cryopreservation of plant germplasm has yet to be substantiated (Benson, 1990). However, free radical toxicity is seen increasingly as an important source of injury in mammalian tissues stored at low and ultra low temperatures (Fuller et al., 1988). In addition, work on low temperature biology of plants has produced several reports implicating oxidative stress in freezing and cold injury (Benson and Withers, 1987; Benson and Noronha-Dutra, 1988).

Oxidative stress and related metabolism has been shown to be highly active and detrimental in several plant and animal tissues during low and ultra freezing temperature (Wise and Naylor, 1987; Prasad, 1996). Lipid degrading enzymes in non-blanched tissues are active even at temperatures of -18°C (Benson, 1990). Apparently, free fatty acid production from frozen vegetables is mediated by lipid acyl hydrolases (Duden, 1985). These reactions can produce hydroxyperoxy acids which then enter oxidative pathways probably mediated by free radicals. Similarly, lipid peroxidation has been found to be one of the major mechanisms in frozen meat. Examination of lipid breakdown in the microsomal fraction of fish muscle has shown that oxidative reactions can proceed at -12°C as measured by malondialdehyde (MDA) and hydroperoxide production (Benson, 1990). This was thought to be due to a concentration of reactants during freezing dehydration. Thawing in particular
did not affect the rates of lipid peroxidation (Mouradian et al., 1985). Measurements of oxidized products of lipid like MDA and hydroperoxide showed that oxidative injury was the major factor in destabilization. Analysis of heart muscle by chemiluminescence has demonstrated free radical activity during the freezing and thawing of tissues held at -40°C, and between -70°C and -80°C (Lange et al., 1980). Activation of xanthine oxidase reactions occurs, leading to the formation of superoxide radicals (Halliwell and Gutteridge, 1984; Bowler et al., 1992). Further disruption causes cellular decompartmentalization, the liberation of iron stores and catalysis of Fenton reaction. All these degenerative pathways can cause changes in the synthesis of metabolites and loss of important substrates from the leaky membranes (Simon and Raja Harun, 1972). Studies on cryopreserved Daccus carota cells suggest that lipid peroxidation and OH production can occur after freezing injury (Benson and Withers, 1987). After freezing and thawing this type of injury can persist for extended periods.

In the first instance, freezing injury is mainly manifested as membrane damage (Patterson et al., 1976; Paull, 1981). Studies have shown that free radical mediated lipid peroxidation occurs during early post-thaw recovery in Daccus carota (Benson and Withers, 1987). Studies on cryopreserved shoot tips of Brassica napus have shown that singlet oxygen may be involved in post thaw injury (Benson and Noronha-Dutra, 1988). Similarly, parallel studies in mammalian systems have shown that ultra-low temperature stress to be mediated by oxidative damage (Benson, 1990). In contrast to the field of cryopreservation of in vitro cultures, which has only recently involved the studies of the role of oxidative stress, seed storage studies already offer important pointers for the improvement of storage procedures (Benson, 1990; Hendry, 1993; Chaitanya and Naithani, 1994). In the category of basic research, a free radical approach to assessing deterioration may help elucidate the destabilising effects
of oxidative damage on the genome. This also includes the phenomenon of seed ageing and recovery/repair mechanisms. The latter are particularly associated with repair of damaged membranes, the primary site of free radical attack (Pauls and Thompson, 1981; Gutteridge and Halliwell, 1990). Indeed the establishment of a direct link between free radical damage and genome instability would make a most beneficial contribution to the field of germplasm conservation (Benson, 1990).

Studies on the post-storage recovery of seeds have shown that the operation of repair mechanisms can influence seed viability. Similar observations have been made in the recovery of cryopreserved germplasm (Withers, 1980; Withers, 1985). Repair and recovery are greatly affected by dehydration treatments. Thus, it is not only storage damage which must be considered but protection mechanism also. As discussed, the importance of biochemical stability in seeds, it is revealing that many studies performed over a number of years have linked free radical injury, directly or indirectly, to deterioration during seed storage (Senaratna and McKersie, 1986; Leprince et al., 1990; Hendry et al., 1992; Chaitanya and Naithani, 1994). Indirect and direct evidence presented in this regard has shown that freezing can damage a number of enzymes (Zhang and Kirkham, 1994) and affect the protein content (Prasad, 1996; Sgherri et al., 1996) of tissues. Apart from obvious effects of temperature on enzyme kinetics, certain enzymes show distinct cold-labile properties (Graham and Patterson, 1982). The protection against free radical damage is the major means of preventing oxidative stress (Pacific and Davis, 1991; Zhang and Kirkham, 1994). Thus study of free radical biology and its processing enzymes during long-term cryostorage of neem seeds may provide an answer to their role in cryoinjury.
Materials and Methods

Superoxide Radical Liberation

The liberation of superoxide radical in the embryonic axes and cotyledons of cryostored non-desiccated and desiccated neem seeds was determined by the method of Sangeetha et al., 1990. Details of the extraction and estimation of superoxide radicals are given in materials and methods of chapter 3.

Lipid Peroxidation

The accumulation of TBARS (lipid peroxidation products) during LN2 storage was estimated by the method of Heath and Packer (1968). Refer materials and methods of chapter 3 for detailed methodology of extraction and estimation of lipid peroxidation products.

Extraction of Enzyme and Estimation of Activities of Antioxidant Enzymes

Superoxide Dismutase, Catalase and Peroxidase

Enzyme extraction from the embryonic axes and cotyledons of cryostored neem seeds and the estimation of activities of antioxidant enzymes SOD, CAT and POD were performed by their respective procedures presented in chapter 4 (Materials and Methods).

Statistical Analyses

The various statistical analyses including linear correlation, non-linear (polynomial) correlations, calculation of coefficient of determination etc. were carried out using computer softwares such as Genstat developed by Indostat Software Services, Hyderabad, India; Microsoft Excel version 97, Microsoft Incorporation, USA and SPSS version 6.0, SPSS Incorporation, USA.
Results

Superoxide Radicals - Embryonic axes

The analyses of superoxide radicals in the embryonic axes of non-desiccated and desiccated neem seeds stored under liquid nitrogen temperatures were performed and the results obtained have been exhibited in Fig. 6.6. The liberation of $O_2^-$ was many fold higher in the non-desiccated seeds as compared to the desiccated seeds. Initially, the levels of $O_2^-$ liberated was 33.6 $\mu$MO$_2^-$/min/g f wt in the non-desiccated and 55.5 $\mu$MO$_2^-$/min/g f wt in the desiccated seeds. Thereafter on storage under ultra low temperatures the generation of superoxide radical was escalated in both type of seeds though with differing magnitude.
In non-desiccated seeds, a steep increase in superoxide generation was recorded from 33.6 μMO₂/min/g fwt at 0 h to 315.8 μMO₂/min/g fwt at 5 minutes and a highest of 410 μMO₂/min/g fwt after 12 h of cryostorage. About 12 fold increase in the O₂⁻ liberation was recorded in the non-desiccated seeds. On the contrary, only a 3 fold increase was registered in the desiccated seeds though stored for 12 months at same conditions of storage. The superoxide liberation increased from 55.5 μMO₂/min/g fwt at 0 h to 145.5 μMO₂/min/g fwt after 1 month and to 178.2 μMO₂/min/g fwt after 12 months of storage.

![Graph showing superoxide liberation in non-desiccated and desiccated seeds during storage at cryotemperatures for different time.](image)

**Fig. 6.7** Showing the liberation of superoxide radicals in cotyledon of non-desiccated and desiccated neem seeds during storage at cryotemperatures for different time. Each value is a mean of 6 replicates. The value for each data point has been plotted on the bar. The maximum value of SO for non-desiccated and desiccated seeds was 27 and 18 respectively.

**Superoxide Radicals - Cotyledon**

The liberation of superoxide radicals in the cotyledon of non-desiccated and desiccated neem seeds stored under cryotemperatures has been shown in Fig. 6.7. In the cotyledon too,
the generation of $O_2^-$ was estimated to be many fold higher in the non-desiccated seeds as compared to the desiccated seeds. On storage of neem seeds under cryo temperatures, the superoxide radical generation showed a significant increase with different magnitude in both type of seeds. In non-desiccated seeds, comparatively very steep increase in superoxide generation was recorded in the cotyledons from $18.2 \mu \text{MO}_2/\text{min/g f wt}$ at 0 h to $224 \mu \text{MO}_2/\text{min/g f wt}$ at 5 minutes and a 16 fold increase to $298.8 \mu \text{MO}_2/\text{min/g f wt}$ after 12 h of cryostorage. On the other hand, just about a 3 fold increase was registered in the desiccated seeds even after storage for 12 months of cryostorage. The superoxide liberation increased from $41\mu \text{MO}_2/\text{min/g f wt}$ at 0 h to $92.8 \mu \text{MO}_2/\text{min/g f wt}$ after 1 month and finally to $135.6 \mu \text{MO}_2/\text{min/g f wt}$ after 12 months of storage.

**Lipid Peroxidation - Embryonic axes**

The changes in lipid peroxidation measured as the accumulation of TBARS in the embryonic axes of non-desiccated and desiccated neem seeds is presented in Fig. 6.8. The levels of TBARS increased significantly in both the seed types but the magnitude of increase was high in the non-desiccated seeds as compared to the desiccated seeds at cryo temperatures. In the embryonic axes of non-desiccated seeds, there was a 4 fold upsurge in lipid peroxidation in the embryonic axes within just 5 minutes of storage under ultra low temperatures. These values recorded showed an increase from $1.48 A_{450}/\text{g fwt}$ in the fresh seeds to $6.55 A_{450}/\text{g fwt}$ after 30 minutes and $6.6 A_{450}/\text{g fwt}$ after 12 hours of storage. Accumulation of TBARS in the desiccated seeds also showed a gradual increase but comparatively lower magnitude. It registered an increase from $3.58 A_{450}/\text{g fwt}$ in the freshly
Desiccated seeds to 4.2 A$_{560}$/g fwt in the seeds 6 days after storage and a high of 4.92 A$_{540}$/g
fwt after 12 months of storage at cryo temperatures.

Fig. 6.8 Estimation of lipid peroxidation (measured as accumulation of TBARS) in embryonic axes of excised, non-desiccated and desiccated neem seeds after cryostorage for various time periods. Each value is a mean of 3 replicates. The value for each data point has been plotted on the bar. The maximum value of SD for non-desiccated and desiccated seeds was 0.16 and 0.21 respectively.

Lipid Peroxidation - Cotyledon

Fig. 6.9 demonstrates the changes in the accumulation of TBARS in the cotyledon of non-desiccated and desiccated neem seeds during storage at ultra low temperatures. Like the embryonic axes, the cotyledon of non-desiccated seeds also recorded a 4 fold upsurge in lipid peroxidation. These values recorded showed an increase from 0.24 A$_{540}$/g fwt in the fresh non-desiccated seeds to 0.94 A$_{540}$/g fwt after 2 h of storage and 1.02 A$_{540}$/g fwt after 12 hours of storage. The levels of lipid peroxidation in the desiccated seeds also showed a
gradual increase from 0.43 A₅₄₀/g fwt in the desiccated seeds at 0 h to 0.57 A₅₄₀/g fwt in the seeds 6 days after storage and a high of 0.72 A₅₄₀/g fwt after 12 months of storage at cryo temperatures. In the desiccated seeds the cotyledons registered just a 1.5 fold increase in lipid peroxidation activity even after 12 months of storage.

Superoxide Dismutase - Embryonic axes

The changes in the levels of antioxidant enzyme superoxide dismutase in the non-desiccated and desiccated neem seeds at cryo temperatures are shown in Fig. 6.10. The embryonic axes of non-desiccated seeds on cryopreservation recorded a rapid and regular decline in the levels of SOD, whereas, these levels were maintained in the axes of seeds stored after desiccation to optimum moisture content 7.1%. The levels of SOD in the non-desiccated
seeds declined from 6132 units / g fwt. to 1525 units / g fwt. after 2 h and 965 units / g fwt. after 12 h of cryostorage. On the contrary, the levels of the enzymes was maintained from 6975 units / g fwt. in the axes of desiccated seeds at 0 h to 8606 units / g fwt. after 6 days 7985 units / g fwt. after 12 months of storage.

![Graph showing changes in SOD levels](image)

**Fig. 6.10** Showing the changes in the levels of superoxide dismutase in the embryonic axes of non-desiccated and desiccated neem seeds during storage at liquid nitrogen temperatures. Each value is a mean of 6 replicates. The value for each data point has been plotted on the bar. The maximum value of SD for non-desiccated and desiccated seeds was 0.21 (thousands) and 0.08 (thousands) respectively.

**Superoxide Dismutase - Cotyledon**

The variation in the levels of SOD in the cotyledon of non-desiccated and desiccated neem seeds at cryo temperatures is exhibited in Fig. 6.11. Like the embryonic axes, the cotyledons of non-desiccated seeds also showed a rapid decline in the levels of SOD during cryostorage, whereas, these levels increased and was maintained in the axes of seeds stored after desiccation to optimum moisture content 7.1%. The levels of SOD in the non-desiccated
seeds declined from 465 units / g f wt. to 170 units / g f wt. after 2 h and 160 units / g f wt. after 12 h of cryostorage. But, the levels of these enzyme increased from 868.11 units / g f wt. at 0 h to 1356 units / g f wt. after 6 days and to almost similar levels of 1352 units / g f wt. even after 12 months of storage.

![Bar graph showing changes in enzyme activity over time.]

**Fig. 6.11** Showing the changes in the levels of enzyme superoxide dismutase in the cotyledon of non-desiccated and desiccated neem seeds during storage at liquid nitrogen temperatures. Each value is a mean of 6 replicates. The value for each data point has been plotted on the bar. The maximum value of SOD for non-desiccated and desiccated seeds was 0.09 (thousands) and 0.05 (thousands) respectively.

**Catalase - Embryonic axes**

The activity of the antioxidant enzyme catalase was studied in the embryonic axes of neem seeds which showed a trend reverse to that of SOD. The levels of the enzymes showed a gradual and consecutive decline (Fig. 6.12) not only in the non-desiccated seeds while also in the desiccated seeds during cryostorage for different periods of time. The magnitude of
The levels of CAT declined from 2425 A$_{240}$/min/g fwt. in the fresh seeds to 855 A$_{240}$/min/g fwt. just after 30 minutes and a 6 fold decrease to 405 A$_{240}$/min/g fwt. after 12 h of cryostorage. Similarly, the desiccated seeds also showed a decline in CAT activity though with a slow rate. The levels of CAT declined from 2785 A$_{240}$/min/g fwt. in desiccated seeds at 0 h to 2686 A$_{240}$/min/g fwt. after 1 month and 1654 A$_{240}$/min/g fwt. after 12 months of storage.

![Graph showing changes in catalase levels over time for non-desiccated and desiccated seeds](image)

**Fig. 6.12** Changes registered in the levels of catalase in embryonic axes of non-desiccated and desiccated neem seeds during storage at ultra low temperatures. Each value is a mean of 6 replicates. The value for each data point has been plotted on the bar. The maximum value of SD for non-desiccated and desiccated seeds was 154.55 and 101.2 respectively.

**Catalase - Cotyledon**

The decline in the levels of catalase in the cotyledon of seeds during storage at liquid nitrogen temperatures is presented in Fig. 6.13. Almost a 14 fold decrease in CAT levels was recorded in the cotyledon of non-desiccated seeds within storage for 12 h at LN2.
temperatures. The levels decreased from 481 A260/min/g fwt. in the fresh seeds to 172 A260/min/g fwt. after 30 minutes and 35 A260/min/g fwt. after 12 h of cryo storage. On the contrary, only a 2 fold decrease in enzyme levels was discernible in the cotyledon of desiccated neem seeds and that too after 12 months of storage (Fig. 6.13).

![Graph showing changes in catalase levels](image)

**Fig. 6.13** Displaying the changes in the levels of catalase in cotyledon of non-desiccated and desiccated neem seeds during storage at liquid nitrogen temperatures. Each value is a mean of 6 replicates. The value for each data point has been plotted on the bar. The maximum value of SD for non-desiccated and desiccated seeds was 34.25 and 40.75 respectively.

**Peroxidase - Embryonic axes**

Non-desiccated and desiccated neem seeds during storage under liquid nitrogen temperatures recorded a decline in guaiacol peroxidase activity in the embryonic axes of the seeds throughout the period of the study (Fig. 6.14). In the non-desiccated seeds, storage at LN2 temperatures lead to a rapid decline in peroxidase levels. Within 12 h of storage a 23 fold decline in peroxidase levels were discernible. The levels decreased from 255 A470/
Fig. 6.14 Showing changes in guaiacol peroxidase activity in embryonic axes of non-desiccated and desiccated neem seeds during cryostorage. Each value is a mean of 6 replicates. The value for each data point has been plotted on the bar. The maximum value of SD for non-desiccated and desiccated seeds was 54.56 and 25.9 respectively.

min/g fwt to 25.66 A₄₇₀/min/g fwt in 2 h and a low of 10.8 A₄₇₀/min/g fwt after storage for 12 h at ultralow temperatures. The axes of the desiccated seeds also registered decline in the POD levels but with a lower magnitude. The levels declined from 380 A₄₇₀/min/g fwt at 0 h to 300 after 6 days and 122 after 12 months of storage.

Peroxidase - Cotyledon

Similar to the axes, the non-desiccated and desiccated neem seeds under LN2 temperatures exhibited a gradual decline in guaiacol peroxidase activity in the cotyledon of the neem
seeds (Fig. 6.15). In the non-desiccated seeds, storage at LN2 temperatures lead to a rapid decline in peroxidase levels in the cotyledon. Within 12 h of storage no peroxidase levels were registered in the cotyledon. The levels decreased from 52.42 $A_{370}/\text{min/g f wt.}$ to 8.08 $A_{370}/\text{min/g f wt.}$ in 2 h and nil activity by 6 h after storage. The cotyledon of the desiccated seeds also registered decline in the POD levels. The levels lowered from 125.75 $A_{370}/\text{min/g f wt.}$ in seeds at 0 h to 92.05 $A_{370}/\text{min/g f wt.}$ after 6 days of storage. These values again decline to 37.77 $A_{370}/\text{min/g f wt.}$ after 12 months of storage.
Discussion

In the previous chapter (chapter - 6a), successful cryostorage of neem seeds was reported. Neem seeds was shown to survive LN2 temperature only after desiccation to critical moisture content (7.5%) for one year with 60% viability (Fig. 6.2). Loss of viability and vigour, although very marginal, was discernible in the cryostored neem seeds. The viability loss in seeds is frequently associated with free radical attack and peroxidative damage (Halliwell and Gutteridge, 1984; Wilson and McDonald, 1986; Cakmak et al., 1993; Finch-Savage et al., 1994). In the present study, the hydrated seeds when subjected to cryostorage could not survive and became non-viable with brief exposure (5 minutes) (Fig. 6.2). These seeds exhibited sudden, sharp and phenomenal accumulation of superoxide and its mediated lipid peroxidation products (TBARS) which increased further with the extension of storage period (12 h). In contrast, the desiccated excised seeds showed longer period of survival with sufficiently high viability and vigour (Fig. 6.3 and Fig. 6.4) perhaps because of significantly reduced levels of superoxide (Fig. 6.6 and 6.7) and TBARS (Fig. 6.8 and 6.9) in these seeds. Studies on cryopreserved carrot cells (Benson and Withers, 1987) and oilseed rape meristems (Benson and Noronha-Dutra, 1988) provided evidence for the production of ROS during early post-thaw recovery. Cryoinjury has frequently been related to free radical pathology in mammalian and plant tissues viz., heart tissues (Benson, 1990), vegetables (Duden, 1985) and Daccus carota (Benson and Withers, 1987). Free radicals and activated oxygen species are themselves highly toxic, however, their damaging effects are further enhanced by secondary oxidative reactions (Benson et al., 1992). Thus free radicals attack lipid membranes and cause the formation of lipid peroxides (Dhindsa, 1982; Wilson and McDonald, 1986) which then break down further in the cells to form a wide range of cytotoxic
compounds (Funes and Karel, 1981; Dupont et al., 1982). These compounds impair cell function, interfere with DNA (Pan and Yau, 1991) and protein synthesis (Roubal, 1970) and may be mutagenic (Frankel, 1987; Esterbauer et al., 1988). Thus it is suggested that the drying of excised neem seeds to critical levels (i.e., 7.5% moisture content) before cryostorage offered potential to these seeds to tolerate cryotemperature. Since these frozen-thawed seeds produce remarkably low levels of superoxide and lipid peroxidized products both in the embryonic (Fig. 6.6 and Fig. 6.8) axes and cotyledons (Fig. 6.7 and Fig. 6.9), they continue to survive with high viability percentage. At the same time, marginal loss of viability and vigour in these seeds corresponds to equally slow rate of accumulation of superoxide and TBARS both in the axes and the cotyledons.

Higher viability and vigour in neem seeds have been associated with reduced production of free radical and lipid peroxidized products (chapter - 3). These viable seeds have been shown to have relatively high levels of free radical processing enzymes which renders them tolerance during dehydration to intermediate moisture contents. Cryostorage of desiccated excised seeds induced remarkably high levels of antioxidant enzymes (SOD, CAT and GPOD) thus providing protection against any oxidative damage due to accumulation of ROS in these seeds. In contrast, massive reduction in antioxidant enzymes in response to cryostorage in non-desiccated seeds favours non-viability in these seeds. Protective role of SOD, CAT and GPOD has been extensively discussed in free radical damage of cellular membranes (Winston, 1990; Smirnoff, 1993; Zhang and Kirkham, 1994) and failure of these antioxidant enzymes leads to impairment of cellular metabolism (Leprince et al., 1992; Chantanya and Naithani, 1994; 1999; Zhang and Kirkham, 1994).
The excised desiccated neem seeds may be described as cryotolerant as these seeds survived cryotemperatures for one year. Accomplishment of cryotolerance in desiccated neem seeds resulted partly due to enhanced activities of antioxidant enzymes both in the embryonic axes (Fig. 6.10, Fig. 6.12 and Fig. 6.14) and the cotyledons (Fig. 6.11, Fig. 6.12 and Fig. 6.13) which offer protection against cryotemperature-induced ROS damage, accumulated in these seeds although, at a slow rate. In the present study, active metabolism exhibited by desiccated excised neem seeds constantly for one year at cryotemperatures overrule the hitherto regarded view that cryopreservation offers the opportunity of significantly reducing the damage of the tissue by suppressing all the metabolic activities (Stanwood and Bass, 1981). Gradual increase in antioxidant enzymes activity as well as free radical production from 12 h to 12 months is clearly an indicator of active metabolism in these seeds.

Therefore, we warrant short-term (1 h to 1 month) studies performed to evaluate the suitability of cryostorage at least for desiccation-sensitive recalcitrant and intermediate seeds as these seeds may exhibit altered viability and vigour due to active metabolism even at cryotemperatures during long term storage. Therefore monitoring of viability and vigour in desiccation-sensitive seeds for longer periods (6 months to 8 months) is recommended for germplasm conservation of seeds at cryotemperatures in seed banks.