DEVELOPMENT OF STORAGE TECHNIQUES FOR THAMPAKAM *(Hopea parviflora* Bedd.*)* SEEDS

By

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THESIS

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DEPARTMENT OF SILVICULTURE AND AGROFORESTRY

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DECLARATION

I hereby declare that the thesis entitled **"Development of storage techniques for thampakam (*Hopea parviflora* Bedd.) seeds"** is a bonafide research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any University or Society.

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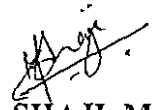

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Shaji, M.

Dedicated to

my mother

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Introduction

INTRODUCTION

Overcoming physiological deterioration of a stored seed sample is one of the most important tasks in the preservation process. Large-scale mechanical refrigeration systems, which hold seed at temperatures down to -20°C have greatly increased the storage life of a seed sample (IBPGR, 1976). However, deterioration and loss of viability can still occur with increased time of storage, resulting in a loss of precious genetic material. Based on storage behaviour, seeds are classified into orthodox and recalcitrant groups (Roberts, 1973). Berjak *et al.* (1989) described them as poikilohydrous and homoiohydrous respectively. More recently a third category called intermediate was identified between the orthodox and recalcitrant groups (Ellis *et al.*, 1990).

Recalcitrant seeds do not undergo maturation drying and are shed at relatively high moisture contents of 30 to 80 per cent on a wet weight basis. They are killed if moisture content is reduced below some relatively high critical value (King and Roberts, 1980, Pammenter *et al.*, 1994) and hence cannot be stored for long by conventional storage methods. They do not conform to the viability equation, which describes relations between longevity and air-dry seed storage environments (Roberts, 1973).

Hopea parviflora Bedd. is one of the most gregarious of evergreen trees of South India, coming under the family Dipterocarpaceae. The wood is hard, heavy and extremely durable. This species had great value as constructional timber suitable for bridges, ship and boat building, platform boards, agricultural

implements, decorative wood works, etc. Wood of this species is superior to teak wood in almost all physical characters (Troup, 1921). Seeds of this species are recalcitrant, which lose their viability within 7 to 10 days under natural conditions, when the seed moisture content reduces below a "high critical value" (Troup, 1921). However, Sunilkumar and Sudhakara (1998) while studying the effect of temperature, media and fungicide on the storage behaviour of *Hopea parviflora* seeds found that seeds could maintain their viability up to a period of 14 days.

The degree of sensitivity of recalcitrant seeds to desiccation varies with physiological maturity of the seed (Pammenter *et al.*, 1991). Immature embryos might be more adaptable to manipulation than mature embryos or mature embryonic axes. Immature embryos might be highly embryogenic than mature embryos (Pence, 1991). There is evidence in the literature on orthodox seeds that the immature seeds of certain species may exhibit dormancy or require after-ripening e.g. *Brassica japonica* Sieb. (Tokumasu, 1970) and *Agrostis gigantea* Roth. (Williams, 1973). A number of workers have suggested that the germination of recalcitrant seeds during storage could be reduced if they were harvested before attaining full maturity, e.g. in cocoa (Pyke *et al.*, 1934), in coffee (Veen van de, 1934) and in citrus seed (Patt, 1953).

Hong and Ellis (1996) suggested that removal of seed covering structures might help to promote speedy germination. This may be because hard seed coats serve as protective mechanisms, which can exclude oxygen and/or water from the embryonic tissue thus extending seed life. Cohen (1958) and Mumford and Grout (1979) suggested that the germination of lemon seed (*Citrus limon* L.) was improved when the testa was removed and they produced evidence to prove

that the testa of lemon seeds has a marked deleterious effect on the seed's response to desiccation. Recalcitrant nature may be also due to their seed coat and storage tissue characteristics (King and Roberts, 1980).

Fu *et al.* (1990) suggested that embryonic axes have higher desiccation tolerance than intact recalcitrant seeds. Because of its organized small structure and its ability to produce a whole plant from the meristematic tissues, embryonic axes are preferred. There are many reports on the advantage of rapid drying over slow drying which would help surpass desiccation injuries if any, inflicted due to the loss of structural water from the cells. Several scientists have proposed rapid drying as one of the methods to reduce desiccation injury. It has been found that the more rapidly dehydration can be achieved, the lower is the water content to which seeds or axes can be dried, without damage accumulation that culminates in viability loss. This is particularly the case when excised axes are dried (Normah *et al.*, 1986; Berjak *et al.*, 1989). Bonner (1996) suggested that the rate of desiccation could influence the expression of recalcitrant behaviour in seeds.

According to Roberts *et al.* (1984), the most promising method of germplasm conservation for recalcitrant seeds is storage in liquid nitrogen. For seeds to survive at very low temperature, they must be dried prior to freezing. If seeds have sufficiently low moisture content, they will not suffer injury even at -196°C (Stanwood and Bass, 1978). Cryopreservation of somatic and zygotic embryos has been reported to be successful for many species, which show orthodox, intermediate and recalcitrant seed storage behaviour (Engelmann *et. al.*, 1995). However, very few true recalcitrant seeds can be dried and stored in liquid

nitrogen and that the desiccation of embryonic axes should be investigated in detail prior to the works on cryopreservation.

Various storage methods *viz.*, storing in moist conditions, sealed containers, perforated polythene bags, burying in soil *etc.*, have been tried at different temperatures to find out an ideal storage method for long term storage of recalcitrant seeds, but in vain. Similarly species trials and plantation programmes involving *Hopea parviflora* were often at doldrums owing to its recalcitrant seed storage behaviour. Suitable storage techniques have to be devised to achieve long-term storage of these seeds without any loss of their viability and longevity to make forestry programmes involving *Hopea parviflora*, a success.

The present study was carried out during 1999-2001 in the College of Forestry, Vellanikkara with the following objectives

1. To find out the best desiccation protocol as a pre-treatment for the purpose of storage of *Hopea parviflora* seeds using cryopreservation techniques.

2. To determine the ideal maturity level of the seeds of *Hopea parviflora* for the purpose of storage using cryopreservation.

3. To investigate the possibility of micro encapsulation of zygotic embryo (synthetic seed) as a storage method of *Hopea parviflora* seeds.

Review of Literature

REVIEW OF LITERATURE

The world plant genetic resources are being drastically reduced through loss of old cultivars and replacement of primitive land races by new genetically uniform cultivars (King and Roberts, 1980). Seed storage is the easiest and least expensive method to arrest this trend (Harrington, 1970). The importance of seed storage has been recognized ever since man began to domesticate plants. The duration of successful storage depends upon both the objectives and the species concerned. Seed longevity varies greatly among species. Not all species' seeds respond to the environment before and during storage in the same way. Roberts (1973) defined two categories of seed storage behaviour - orthodox and recalcitrant. More recently, a third category 'intermediate' between the orthodox and recalcitrant has been identified (Ellis *et al.*, 1990). Seeds of species with orthodox seed behaviour can be maintained satisfactorily *ex situ* over the long term in appropriate environments. Short term storage is usually the best that can be achieved with seeds, which shows recalcitrant storage behaviour. Medium term storage is feasible for seeds of species with intermediate storage behaviour provided the storage environment is well defined and well controlled. Recalcitrant seeds are usually short-lived and very sensitive to desiccation and low temperatures making it very difficult to store them on a long term basis (Chin *et al.*, 1981). Unfortunately, seeds of most of the economically important tree species in the tropics are recalcitrant in nature and cannot be stored for long periods. Their requirement for a high moisture content reduces their storability since they can only be stored in wet medium to avoid dehydration injury and at warm temperature because chilling injury is common to these type of seeds (Chin and Roberts, 1980;

Come and Corbineau, 1992). The current view is that recalcitrant seeds are metabolically active and undergo germination-associated changes in storage. Some of these changes such as extensive vacuolation and increase in cell size imply a requirement for water additional to that present in the seed on shedding (Pammenter *et al.*, 1994). The quick loss of viability may also be due to desiccation sensitivity, chilling injury, microbial contamination and germination during storage (King and Roberts, 1980). Mature orthodox seeds survive desiccation to low moisture contents, at least to 2-6 % depending on the species. Above this value, there is a negative logarithmic relation between seed moisture content and longevity (Ellis and Roberts, 1980).

2.1 Desiccation Sensitivity

Recalcitrant seeds are those that undergo little, or no, maturation drying and remain desiccation sensitive both during development and after they are shed. In reality, however, the situation is far more complex than this perception implies, because of the wide range of variability among recalcitrant seeds of different species (Berjak and Pammenter, 1997). Farrant *et al.* (1993c) proposed a continuum of recalcitrant seeds type mainly based on the sensitivity towards water loss and low temperature. Variation in desiccation sensitivity between species has been reported in many genera such as Dipterocarpus (Tompsett, 1984), *Acer* (Olsen and Gabriel, 1974) and in *Araucaria* (Tompsett, 1984). According to King and Roberts (1980), variation in the moisture content at fully imbibed state is the main reason for apparent variation in susceptibility to desiccation in different species.

Recalcitrant seeds are shed in a hydrated condition, but the water content can be anywhere in a wide range. Shedding water content is partially species characteristic, depending on the degree of dehydration that occurs late during seed development; this goes hand-in-hand with the degree of desiccation tolerance developed by individual species (Finch-Savage, 1996). Desiccation tolerance in recalcitrant seeds increases during seed development on the mother plant; however, unlike orthodox seeds, maturation drying to low moisture contents does not occur (Hong and Ellis, 1990). Fresh recalcitrant seeds have high levels of moisture contents at maturity/ shedding between, for example, 36 per cent for rubber (Chin *et al.*, 1981) and 90 per cent for Choyote (*Sechium edule*) (Ellis, 1991). When acorns of *Quercus nigra* L. were dried at three different rates and two temperature, there were losses of both germination and moisture content as desiccation increased and critical moisture content of 10-15 per cent was identified by Bonner (1996).

There are marked differences in the rate at which water will be lost from seeds of various species under the same dehydrating conditions (Farrant *et al.*, 1989). There are other factors too, that influence the post-harvest responses of recalcitrant seed, e.g. developmental status (Berjak *et al.*, 1993; Finch-Savage and Blake, 1994) and chilling sensitivity (Berjak and Pammenter, 1997).

Recalcitrant seeds are sufficiently hydrated at shedding and germination commences without any additional water (Berjak *et al.*, 1989; Farrant *et al.*, 1988). Desiccation sensitivity of recalcitrant seeds is intimately associated with their persistent state of metabolic activity.

Generally the axes of the recalcitrant seeds are at considerably higher water content than are the cotyledons (Berjak *et al.*, 1989; Maithani *et al.*, 1989; Fu *et al.*, 1993). Finch-Savage (1992a) has demonstrated that for *Quercus robur*, there is a higher proportion of matrix bound water in the cotyledons, which may underlie the greater desiccation sensitivity of the cotyledons relative to the axes.

Methods of drying seeds using silica gel have been described by Hanson (1985). Dry (newly regenerated) silica gel is in equilibrium with about 5 per cent moisture content at 20°C. The first change of the colour of the indicator (diminishing intensity of blue) occurs when the silica gel moistens to equilibrium with about 12-13 per cent relative humidity. By the time it has become pale blue it is in equilibrium with about 49 per cent relative humidity (and thus very moist): 100 g of regenerated silica gel (in equilibrium with about 5 per cent relative humidity) can absorb about 7 g of moisture from seeds until the first colour change is apparent (in equilibrium with 13.5 per cent relative humidity). At this point the silica gel should be regenerated. It should be done by drying in an oven maintained at about 130°C for 3-4 hours. It should then be stored in a sealed container overnight to cool to ambient temperature before being used to dry the seeds (Hong and Ellis, 1996).

Drying excised embryonic axes by silica gel or an aseptic air current allowed excised embryonic axes to survive desiccation to a lower value than that achieved by the vacuum method (Fu *et al.*, 1993). For example, although the vacuum drying method provided more rapid drying, no excised embryonic axes of *Artocarpus heterophyllus* survived desiccation to 44 per cent moisture content,

while the excised embryonic axes dried with an aseptic air flow and silica gel tolerated desiccation to 26 per cent and 16 per cent moisture content respectively (Fu *et al.*, 1993).

2.2 Seed development and levels of desiccation tolerance

For both recalcitrant and orthodox seeds the relative level of desiccation tolerance changes throughout the development so that embryos become more tolerant as they mature and less tolerant as they germinate (Adams *et al.*, 1983; Berjak *et al.*, 1989). However, only orthodox seeds achieve considerable tolerance of desiccation. The acquisition of tolerance is presumably developmentally controlled (Bewley and Oliver, 1992). Galau *et al.* (1991) divided post differentiation embryogenesis into five stages based on the appearance of molecular markers: (1) maturation (2) post vascular separation (PVS) (3) pre-desiccation (4) desiccation and (5) quiescence. They suggested that desiccation tolerance is acquired during the PVS stage.

Studies on the effect of premature harvest on seed vigour and viability suggest that maximum desiccation tolerance is achieved only upon the successful completion of the first three stages of embryogenesis and the rapid completion of the fourth stage. Complete maturation is required for *Acer platanoides* to survive complete desiccation (Hong and Ellis, 1992). Recalcitrance appears to be a product of either an abbreviated PVS stage (Progression toward germination process following abscission) (Berjak *et al.*, 1989; Farrant *et al.*, 1985) or an early termination of development (Finch-Savage, 1992b).

Hong and Ellis (1996) suggested that removal of seed covering structures, filing or chipping seeds with a scalpel, or nicking with a needle might help to promote germination during prolonged tests. Cohen (1958) and Mumford and Grout (1979) suggested that the germination of lemon seed (*Citrus limon* L.) was improved when the testa was removed and they produced the evidence to prove that the testa of lemon seeds has a marked deleterious effect on the seeds response to desiccation.

At the early stages of development, embryos are extremely sensitive to dehydration stress (Rogerson and Matthews, 1977). There is however little information regarding how much water is actually required, but sufficient quantities to allow cell division are necessary. According to Myers *et al.* (1992) water potentials greater than -1.6 M Pa are required.

During the maturation phase, the embryo accumulates dry matter and become germinable. Coincident with these changes, tolerance to low water potentials increases. In orthodox seeds, there is a transition, at a particular stage in the developmental pathway, from a relatively desiccation intolerant to a tolerant state. This transition can be prematurely induced of prolonged by the environmental of chemical manipulations (Blackman *et al.*, 1991). Once induced the immature embryo can become fairly tolerant of desiccation within a few days (Galau *et al.*, 1991).

Recalcitrant seeds may become increasingly tolerant of drying as maturation proceeds (Berjak *et al.*, 1992; Finch-Savage, 1992b), they remain hydrated and metabolically active throughout development (Berjak *et al.*, 1992;

Farrant *et al.*, 1992). Recalcitrant seeds appear to initiate germination related metabolism shortly after shedding (Farrant *et al.*, 1988; Berjak *et al.*, 1989) and in *Avicennia marina* 10 to 15 days before shedding (Farrant *et al.*, 1993b). As germination events progress, the seeds become increasingly sensitive to drying (Farrant *et al.*, 1986).

2.3 Desiccation, metabolic stresses and membrane damage

Metabolism will continue in recalcitrant seeds even after shedding when water is loosing slowly, but at some stage when the seeds are still at relatively high water contents, metabolism will become unbalanced or out-of-phase as a result of internal water stresses (Senaratna and Mc Kersie, 1986). As water is removed from the cell, the concentration of the solutes is increased, and eventually the fluidity of the aqueous medium declines. These changes affect the metabolic status of the cell. The changes in the metabolic activity are believed to occur at specific moisture levels (Leopold and Vertucci, 1989). Below a moisture level of about -1.5 M Pa, tissues no longer grow and expand (Mc Intyre, 1987) and protein and nucleic acid synthesis patterns change (Dell' Aquila and Spada, 1992). This slight desiccation may induce production of protectants (Close and Chandler, 1990). Greater levels of desiccation can result in metabolic imbalances. At about 0.45 g H_2O/g dm or about -3 M Pa (Dell' Aquila, 1992), protein synthesis ceases and repair processes become inoperative (Dhindsa and Cleland, 1975; Dell' Aquila, 1992). Respiratory activity continues until tissues are dried below about 0.25 g g^{-1} or -11 M Pa (Leopold and Vertucci, 1989; Vertucci, 1989). At moisture levels between -3 and -11 M Pa (about 0.45 to 0.25 g H_2O/g dm), catabolic activities

continue unabated and processes utilizing the high-energy intermediates are impaired (Leopold and Vertucci, 1989).

Dehydration injuries occur primarily on the basis of alterations in membrane structural integrity, function and physico-chemical properties. Sun *et al.* (1994) found that the glossy state of membranes is not sufficient for desiccation tolerance, whereas the ability of membranes to retain the liquid crystalline phase is correlated with desiccation tolerance. Poulsen and Eriksen (1992), in a study using excised embryonic axes of recalcitrant *Quercus robur* acorns proved that critical water potential for the initiation of damage was -5 MPa. Salmen-Espindola *et al.* (1994) studying *Araucaria angustifolia* found that dehydration induced deterioration of cell membranes as evidenced by a high increase in leakage of solutes. Dehydration also resulted in the damage of the nuclei, which was not repaired upon rehydration. During desiccation, respiratory activity was decreased and however, O_2 uptake could not be related as an indication of germination ability. Desiccation also resulted in a rapid decrease in the ability for protein synthesis.

2.4 Effect of drying rate on viability of seeds

In experimental manipulation of recalcitrant seeds of excised axes, the rate of dehydration must be equated with the time taken for the material to pass through a series of declining water contents. It has been found that the more rapidly dehydration can be achieved, the lower is the water content to which seeds of axes can be dried, without damage accumulation that culminates in viability loss. This is particularly the case when excised axes are dried (Normah *et al.*,

1986; Berjak *et al.*, 1989). Far from being actually desiccation tolerant axes from recalcitrant seeds will survive only for very short periods (hours to a day or two) at the lowest water contents attainable without immediate deleterious effect (Walters *et al.*, 1997a). Similar effects of drying rate on whole seeds are usually less marked, and generally are harder to attain, because their size often prevents the achievement of suitably rapid dehydration. However, the ability to achieve lower water contents while retaining viability has been recorded for *Avicennia marina* (Farrant *et al.*, 1985).

Pammenter *et al.* (1997) studied the effect of drying rate on whole seeds of *Ekebergia capensis*. Burying it in silica gel after removal of the endocarp performed rapid drying. It was found that slowly dried seeds lose viability at water contents of 1.25 g water per g dry material, while those that were dehydrated rapidly showed unimpaired vigour and full germinability at a water content of 0.7 gg^{-1}. Seeds dried at intermediate rate retained viability to the intermediate water content level of 1.0 gg^{-1}. Electron microscopic observations suggested that different deleterious mechanisms brought about intracellular damage, depending on the drying rate. When rapidly dried, there will be a water content at which material that is desiccation sensitive will sustain injury. This is usually near the range where only structure associated (non-freezable) water remains (Pammenter *et al.*, 1991, 1993). Damage occurring at relatively low water contents is defined as desiccation damage, *sensu stricto* (Walters *et al.*, 1997b) and coincides with the perturbation of the non-freezable water. Desiccation tolerant material on the other hand, can withstand the removal of a considerable proportion of this water (Pammenter *et al.*, 1991).

In contrast to desiccation damage, *sensu stricto*, slowly dried, desiccation sensitive material is held to sustain damage as a result of aqueous based, degradative reactions that are the result of unbalanced metabolism (Pammenter *et al.*, 1991, 1997). The severity of metabolic damage will increase in inverse proportion to the drying rate with viability loss occurring at increasingly high water contents (Pammenter *et al.*, 1997).

2.5 Chilling sensitivity

Most of the recalcitrant seeds belonging to timber, plantation crops and fruit species grow in, and are adapted to a warm and tropical forest habitat (King and Roberts, 1980). Thus it is not surprising that they do not tolerate freezing temperatures, although a failure of seeds of some species to survive at 15°C is hard to understand (Chin, 1988). Some tropical recalcitrant seeds are damaged by chilling injury at temperatures of 10-15°C and below. The longevity of recalcitrant seeds is generally short, particularly for species adapted to tropical environments, typically from a few weeks to a few months (King and Roberts, 1979, 1980). However, the longevity of seeds of species adapted to temperate environments can be maintained for much longer periods.

A study on the hydrated seeds of *Azadirachta indica* indicated that, in response to chilling, a decline in viability was accompanied by ultra structural degeneration; mitochondria and plastids in axis cells lost internal organization and vacuoles generally collapsed. Areas of advanced degradation also occurred,

comprising cells in which the plasmalemma was discontinuous and vesiculated (Berjak *et al.*, 1995).

Two key enzymes of glycolysis, phospho-fructokinase and pyruvatekinase have also been identified as being cold labile (Guy, 1990). The vacuolar collapse reported for cold-exposed *Azadirachta indica* cells might have been a consequence of dismantling of cytoskeleton in response to chilling (Berjak *et al.*, 1995). This would affect glycolysis in view of the structural association between key glycolytic enzymes and actin-microfilaments (Masters, 1984). Maintenance of intracellular spatial organization includes the existence of multi-enzyme particles (Hrazdina and Jensen, 1992), such as those of glycolysis (Masters, 1984). If some key enzymes of both glycolytic pathway and the TCA cycle become impaired, then out-of-phase metabolism must be likely (Lyons, 1973). As a consequence free radical activity might escalate to proportions where considerable damage could accumulate, if enzymatic and anti oxidant scavenging systems operate inefficiently (Senaratna and Mc Kersie, 1986). As a result membrane lipids would be adversely affected.

Chandel *et al.* (1995) found an extremely abrupt fall in viability with declining temperature on cocoa seeds. The primary cause of chilling injury may be due to the physical response of the membrane lipids to low temperature (Lyons, 1973). Species of tropical and subtropical origin, suffer chilling injury when exposed to temperatures above freezing point of tissue but below 15°C (Bedi and Basra, 1993). Chilling injury may be exhibited as a loss of viability of reduced growth during germination at favourable temperatures (Wolk and Herner, 1982).

Incidence of chilling damage is the most marked feature of the effect of temperature on the storage of dipterocarp seeds (Tompsett, 1992). Tang (1971) found that *Shorea curtissi* would be fatally damaged by exposure to only 16 hours of 4°C indicating extreme susceptibility to chilling temperature. Sasaki (1980) and Yap (1981) proposed classification of dipterocarps based on the chilling sensitivity.

2.6 Recalcitrant seed storage

Recalcitrant seeds cannot be dried without damage and so they cannot conform to the viability equation, which describes relations between longevity and air-dry seed storage environments (Roberts, 1973). When fresh recalcitrant seeds begin to dry, viability is first slightly reduced as moisture is lost, but then begins to be reduced considerably at a certain moisture content termed the "critical moisture content" (King and Roberts, 1979, 1980) or "lowest safe moisture content" (Tompsett, 1984). If drying continues further, viability is eventually reduced to zero. Moist storage for recalcitrant seeds should be at moisture content levels between the "lowest safe moisture content" and the "fully imbibed" level at the coolest temperature, which is not damaging to seed viability.

Critical moisture content levels vary greatly among species and even among cultivars and seed lots (King and Roberts, 1979; Chin, 1988). They may also vary with the method of drying (Farrant *et al.*, 1985). Protective mechanisms such as hard seed coats can exclude oxygen and/or water from the embryonic tissue thus extending seed life.

There is no satisfactory method for maintaining the viability of recalcitrant seeds on a long-term basis, owing to the fact that they cannot be dried. They cannot be stored at sub-zero temperatures because they would get killed by freezing injury resulting from ice formation. The longevity of recalcitrant seeds is generally short, particularly for species adapted to tropical environments, typically from a few weeks to a few months (King and Roberts, 1979, 1980).

Srimathi *et al.* (2001) studied the suitability of storage media (sand, sawdust, coir pith, and charcoal) for preserving the viability of recalcitrant jamun (*Syzygium cuminii*) seeds at ambient (31-32°C) and 10°C temperature. They found that under ambient condition the seeds stored in different media showed *in situ* germination, while the seeds without media were not viable after one month of storage. The seeds stored at 10°C in different media in polythene bags were viable upto 3 months to a tune of 76 to 84 per cent. Among the media, the seeds stored in sand medium moistened to 2 per cent level with water and mixed with seed in 4:1 ratio retained the viability at higher order than other media did. Seed storage in single and double polythene bags was also found to be better in maintaining the viability of jamun seed at 10°C.

According to Bonner (1990), recalcitrant seeds can be subdivided into those of tropical origin, and those adapted to temperate climates (temperate latitudes of high altitudes in the tropics); the latter can be stored at cooler temperatures and for longer. Germination during storage is a chief problem identified with storage of recalcitrant seeds as they shed usually at fully imbibed state and is capable of immediate germination in moist ecosystems to which they

belong (King and Roberts, 1980; Tompsett, 1992). Any storage method for recalcitrant seed should give emphasis on preventing desiccation injury, chilling injury, germination during storage, microbial contamination and maintaining adequate oxygen supply (King and Roberts, 1980).

Depending on the species, hydrated, intact recalcitrant seeds can be stored only for periods from days to months (Chin and Roberts, 1980). Storage life can be prolonged by various manipulations but the effective extension of longevity is still not useful for long term conservation of the germplasm. One of the major difficulties even in the short term, is that the high relative humidity conditions necessary to prolong storage life of the seeds, are also conducive to the proliferation of the micro-organisms, especially as chilling is precluded in many instances (Berjak, 1995). As the vigour of the wet stored recalcitrant seeds declines as a result of inherent changes (Pammenter *et al.*, 1994), it has been suggested that natural defense mechanisms fail, facilitating fungal invasion of the debilitated seed tissues. Short of *in situ* conservation and minimal growth storage of seedling of *in vitro* cultures the only option for conservation of germplasm, and thus the biological diversity of the species with recalcitrant seeds, is cryo-storage (Berjak *et al.*, 1995).

The optimum moisture level for storage represents a compromise between slowing aging reactions and preventing lethal ice formation by drying, and retaining the structural integrity of cellular constituents by supplying sufficient structural water. For desiccation sensitive tissues, there may not be a moisture content and temperature combination at which aging reactions are sufficiently

slowed, and lethal freezing injury and desiccation damage are prevented (Vertucci, 1993).

The longevity of recalcitrant seeds is maximal when stored fully or almost fully imbibed when oxygen is freely available. However, germination is either prevented or reduced to a very slow rate. In other words, treatments are akin to slow growth treatments in tissue cultures. It is therefore easier to recalcitrant species with dormant seeds than those with non-dormant seeds under such conditions, because seeds of the latter tend to germinate during storage. Low temperatures can reduce the rates of both seed deterioration and germination provided that they remain above the value, which results in chilling damage, or the lower value at which ice crystallization occur (Hong and Ellis, 1996).

With normal harvesting and storage procedures it is practically impossible to harvest seed free of fungi. Over 50 species of fungi have been isolated from agricultural seed. The optimum temperature for fungal invasion is about 30°C, but certain species grow well at 145°C and slow growth of others has been observed at 5°C (Christensen, 1957). It is rather doubtful that deterioration due to fungal invasion would occur at moisture contents of 10 per cent or below. As per Harrington (1963), at seed moisture contents in excess of 10 to 13 per cent fungal invasion can rapidly diminish seed viability. Since recalcitrant seeds should be stored in a moist condition, microbial contamination could be an important constraint to reckon with in the conservation of recalcitrant seeds. Microbial growth can be reduced to some extent by lowering the temperature of the storage

environment (King and Roberts, 1980). But in the case of recalcitrant seeds, being chilling sensitive, this may not be applicable.

2.7 Synthetic seeds

The use of somatic embryos to produce synthetic seeds was first proposed by Murashige (1978). Synthetic seed is a novel analog to botanic seed capable of development to an entire plant body which comprises of meristematic tissue, encapsulated free from botanical accessory structures in a hydrated gel capsule (Redenbaugh, 1986). The gel acts as a synthetic seed coat and protects embryo from injuries.

It is now common to encapsulate somatic embryos in alginate salt (Gray, 1987). Literature revealed that during the production of artificial seeds, usage of $CaCl_2$ concentration beyond 75 mM adversely affected shoot emergence. Best results in terms of bead quality and per cent shoot emergence were obtained when the beads were formed using a combination of 5 per cent Sodium alginate solution with 75 mM $CaCl_2$ $2H_2O$, while studies conducted on encapsulation of zygotic embryo of cocoa (*Theobroma cacao*) showed that a combination of 4 per cent Sodium alginate and 75 mM $CaCl_2$ $2H_2O$ are appropriate (Sudhakara *et al.*, 2000).

It has been frequently observed that simply encapsulated embryo could not emerge from the gel bead even if the embryo had excellent quality. Possible causes for this inhibition of conversion are unsuitable elasticity and/or strength of the gel bead (Onishi *et al.*, 1994). A novel self breaking or self splitting

gel bead has been developed to overcome the above mentioned difficulty (Sakamoto *et al.*, 1992).

The most important requirement for a synthetic seed to be used for clonal mass propagation of plants is high and uniform conversion under practical sowing condition such as nursery bed in a greenhouse or in the field (Onishi *et al.*, 1994). In an experiment conducted in College of Forestry, Vellanikkara, encapsulated zygotic embryo of cocoa were stored at 4°C on dry cotton and wet cotton and was found that synthetic seeds stored on wet cotton retained viability for longer period (Sudhakara *et al.*, 2000). Seventy one per cent of synthetic seeds stored on wet cotton regenerated to complete plantlets after 25 days while synthetic seeds stored on dry cotton showed a gradual decline in regeneration after 15 days of storage and only 50 per cent germination was obtained after 25 days of storage.

Gupta and Durzan (1987) encapsulated somatic embryos of loblolly pine in alginate. But conversion of embryo into seedling was not achieved after storing at low temperature (4°C) for four months. However, successful development of plantlets was reported by Redenbaugh *et al.* (1986) with encapsulated alfalfa and celery somatic embryos using sodium alginate. This technique was successfully applied for the encapsulation of axillary and apical shoot buds of *Valeriana wallichi* and *Picorrhiza kurroa*.

Encapsulation of protocorm like bodies (plbs) obtained by culturing shoot apices of *Dendrobium wardianum* increased plant regeneration as well as storage period. Increase in storage period of somatic embryos by encapsulation is also reported in *Asparagus cooperi* where the frequency of conversion of artificial

seeds to plants was affected by the concentration of $CaCl_2$ and commercial sources of sodium alginate and nutrient medium (Ghosh and Sen, 1994).

Ganapathi *et al.* (1992) encapsulated shoot tips isolated from multiple shoot cultures of banana in 3 per cent sodium alginate. The encapsulated shoot tips recorded 100 per cent regeneration in White's medium, and also were successfully established in soil. Successful regeneration of artificial seeds in MS medium + 3 ppm 2,4-D + 0.5 ppm kinetin was also reported in *Dendrocalamus strictus* (Mukunthakumar and Mathur, 1992). The germination frequency was 96 per cent and 45 per cent in *in vitro* and in soil respectively. Synthetic seeds of *Hopea parviflora* were successfully stored up to one month at 10°C without significant reduction in germination percentage. Sodium alginate encapsulated synthetic seeds of *Hopea parviflora* retained viability up to four weeks when stored at temperature of 10°C and 4°C. However, synthetic seeds stored at room temperature (27±2°C) retained the viability only up to one week (Sunilkumar *et al.*, 2000).

Twenty days old green protocorms cultured from seeds of *Spathoglottis plicata* were encapsulated in sodium alginate (Senaratna *et al.*, 1990) and found that they could be stored up to six months at 4°C with little loss of viability. Encapsulated protocorms regenerated to complete plantlets in Vacin and Went modified medium, while non-encapsulated protocorms stored at same temperature showed no viability after 30 days of storage.

Artificial seeds can also be directly sown to artificial media like vermiculite, agar, soil, filter paper, greenhouse mix etc. under *in vitro* conditions.

But sowing of beads directly into soil under *in vivo* conditions generally resulted in failure of germination. To solve this problem Mathur *et al.* (1989) working in *Valeriana wallichi* suggested removal of sucrose from liquid media and incorporation of antimicrobial agents like Chloramphenicol or Bavistin in the encapsulation medium. Bapat and Rao (1990) have also reported the beneficial effect of adding fungicides in the encapsulation medium.

2.8 Desiccation tolerance

The use of somatic embryos from cell culture systems in the clonal propagation of plants would be greatly facilitated if the somatic embryos could be dried and stored in a dormant state in a similar way to true seeds (Senaratna *et al.*, 1990). Application of abscisic acid (ABA), heat shock, high sucrose concentration (Mc Kersie *et al.*, 1990), water or nutrient stresses (Mc Kersie *et al.*, 1989) applied to the embryoids at the cotyledonary stage of development resulted in acquiring desiccation tolerance. The embryoids could be subsequently air dried slowly (over 7 days) or rapidly (over 1 day) to moisture contents of less than 15 per cent and retained viability up to 8 months (Mc Kersie *et al.*, 1989).

The rate of drying also affects survival of somatic embryos. Senaratna *et al.* (1989) reported that slow drying (1.2 g H_2O/g/day over six days) gave higher and more consistent embryoid survival, compared to fast drying (6 g H_2O/g/day over one day). Desiccation tolerance was also induced by exposing somatic embryos to sub lethal levels of low temperature. However, some of the stress pre treatments had other deleterious effects on embryoid maturation and seedling vigour after inhibition. Treating the embryos with ABA for ten days before

encapsulation is also reported to be enhancing germination even when the embryo is dehydrated to five per cent moisture content (Liu *et al.*, 1992).

Anandarajah and Mc Kersie (1990) investigated the effect of sucrose concentration in the maturation medium in combination with a heat shock treatment at 36°C to improve the vigour of seedlings grown from dry somatic embryos of *Medicago sativa*. It was found that with 3 per cent sucrose in the maturation medium the somatic embryos germinated precociously and were unable to survive desiccation. At higher sucrose concentration germination was delayed and after 13 days on 6 per cent sucrose medium, the somatic embryos tolerated drying to 12 per cent moisture content without exposure to exogenous ABA. Heat shock, which presumably stimulated endogenous ABA synthesis, improved the desiccation tolerance of somatic embryos if applied prior to 27 days after sieving.

2.9 Cryopreservation

As habitat loss threatens the diversity of the worlds' flora, interest in seed banks as one method for germplasm preservation ex situ has developed. Seed banks have traditionally been used to preserve genetic lines of agricultural, horticultural and silvicultural species and temperatures used for storing seeds range from 10°C to 20°C (Ellis and Roberts, 1980). When dried, many seeds are stable for long periods of time under these conditions, but seed deterioration in storage does occur, depending on species, temperature and moisture level (Roberts and Ellis, 1989). A technique, which may overcome this problem, is cryostorage - the storage of seeds in liquid nitrogen (LN_2) (-196°C). At this temperature, biological processes are brought to a halt, and it is presumed that the deterioration which can

occur in seeds at high temperatures will not take place, making very long term storage possible (Stanwood, 1985).

Storage in liquid nitrogen seems to be the most promising method and many of the current difficulties in maintaining the viability of stored recalcitrant seed could be overcome by this method (Roberts *et al.*, 1984). Dry orthodox seeds at 2 - 18 % moisture content often survive exposure to liquid nitrogen. A quick method for "identifying" orthodox seeds, which has been used to some extent is the determination of survival following short duration of exposures to liquid nitrogen (from 1 hour to 6 days). However, this method is not always reliable since both moisture content and the rates of cooling and rewarming need to be optimized for survival in each species separately (Hong and Ellis, 1996). Moreover, such quick tests cannot distinguish between orthodox and intermediate seeds.

Preservation of seed germplasm is achieved by holding seeds at low temperature (5 to 20°C) coupled with low seed moisture content (Roberts and Ellis, 1989). Increasing seed moisture content results in reduced germination capacity of most seeds at storage temperature above 0°C, while freezing injury and related damage to the germination process may occur at temperatures below 0°C (Roberts and Ellis, 1989).

All seeds whether orthodox or recalcitrant are also damaged at sub-zero temperatures when their moisture content is high because of abundant and rapid extra- and intracellular ice formation (Levitt, 1980), with the critical moisture content varying with temperature (Roberts, 1973).

Experiments subjecting seeds to LN_2 exposure have shown that many species can survive cryostorage if they are exposed in the dry state. Seeds surviving exposure have ranged in moisture content from 2.2 to 17.5 % (Stanwood and Roos, 1979; Stanwood, 1980; Styles *et al.*, 1982). Moisture content is the single most important factor affecting the ability of germplasm to be stored in the LN_2 (Stanwood and Bass, 1981; Stanwood, 1985). In general, the seeds that are shed with high moisture content show a considerable decline in viability below a certain level of moisture and increased seed moisture content leads to a decline in survival after LN_2 storage (Stanwood, 1985; Fu *et al.*, 1990; Chandel *et al.*, 1995).

In vitro techniques have a clear role in conserving specific genotypes such as recalcitrant seeds. These involve the use of conventional micropropagtion, restricted growth techniques and cryopreservation. Although these techniques have been used primarily with herbaceous species, increasing attention is being given to woody species (Blakesley *et al.*, 1996).

Cryostorage of recalcitrant germplasm involves the maintenance of zygotic embryonic axes, explants of various kinds, or somatic embryoids in liquid nitrogen (-196°C) or liquid nitrogen vapour (-150°C). At such temperatures, low energy levels should preclude molecular movement and thus reactions, although events such as free radical generation and macromolecule damage by ionizing radiation cannot be eliminated (Grout *et al.*, 1983). While it may seem enigmatic to suggest that freezing chilling sensitive material will succeed, there are distinct differences between chilling and freezing that allows this. It is essential in the first instance that the material is rapidly dehydrated to a range of water contents that

obviates both dehydration damage and the lethal injury that results from ice crystal formation. This can be achieved by a combination of flash drying (or other, relatively rapid means of lowering the water content) and very rapid freezing (Berjak *et al.*, 1995). Additionally, the specimen to be frozen must be as small as possible and when necessary, cryoprotectants of other appropriate pre-treatments are required. Nearly 100 % survivals of zygotic axes from a variety of recalcitrant seed species, as well as somatic embryoids have been reported by Berjak *et al.* (1995).

Cryopreservation of somatic and zygotic embryos has been reported to be successful for many species, which show orthodox, intermediate and recalcitrant seed behaviour (Engelmann *et al.*, 1995). For successful cryopreservation, excised embryos from recalcitrant seeds must survive desiccation below the threshold freezable moisture contents (Hor *et al.*, 1990) below which value there is no freezable water for ice formation by cooling to ultra-low temperatures.

As per Vertucci *et al.* (1991), a survival rate of 90 % following freezing to -70°C and storage at that temperature for 6 months was achieved for embryonic axes excised from desiccation sensitive seeds of *Landolphia kirkii*. These had been very rapidly dehydrated to the optimal moisture level prior to freezing. A preferred technique of cryopreservation requires optimizing the moisture level of embryonic axes so that there is minimal damage by desiccation or freezing (Grout *et al.*, 1983; Normah *et al.*, 1986). This method has been demonstrated to work well for seeds, which have minimally recalcitrant

characteristics and can be dried to moisture contents as low as 10 per cent. At this moisture content, water freezing and melting transitions are not detected in seed tissues (Vertucci, 1989).

Though storage of seeds like *Theobroma cacao*, *Hevea braziliensis*, *Mangifera indica*, *Juglans* sp., *Camellia sinensis*, *Cinnamomum zeylanicum*, etc. cannot be preserved under ordinary conditions for long periods due to degeneration of the embryos (Roberts and King, 1980). In such case where seeds are short lived, the germplasm could possibly be conserved through the cryopreservation of excised embryos or their segments. In wide scale hybridization programmes, especially those dealing with intergeneric crosses, which are incompatible due to degeneration or abortion of the embryos, possibly they can be dissected out at immature stages and cryopreserved. There are a number of reports on the cryobiology of zygotic embryos of rice (Bajaj, 1981), barley, mustard (Withers, 1974). Although entire plants could be regenerated from the retrieved embryos in the first four cases, only callus was obtained with coconut. In all these experiments, quick-freezing followed by thawing at 35 to 40°C was employed; however, their ability varied considerably.

Cryopreservation techniques provide the potential for "indefinite" preservation by reducing metabolism to such a low level (Ashwood-Smith and Farrant, 1980) that all biochemical processes are significantly reduced and biological deterioration virtually stopped. The longevity of seeds or the maintenance of seed viability is a balance between extrinsic and intrinsic deleterious factors and repair or protective mechanisms. Deleterious factors may

include depletion of essential metabolites, denaturation of macromolecules, accumulation of toxic metabolites, attack by microorganisms and insects, and effects of ionizing radiation (Osborn, 1977, 1980).

Storage in liquid nitrogen (LN_2) appears to be both practical and desirable for long term preservation of numerous kinds of seed. The utilization of LN_2 as a storage medium is predicted on the capability of seeds to survive LN_2 exposure without significant damage to viability. Seeds fall into three general categories with regard to exposure to LN_2 temperatures (Kartha, 1985). They are (1) Desiccation-tolerant, LN_2-tolerant seeds (comprises most common agricultural and horticultural species, with which considerable success had been there in cooling such seeds to, LN_2 temperatures and rewarming them to ambient temperature (20°C) without loss of viability as in *Allium cepa* (Harrison and Carpenter, 1977), (2) Desiccation-tolerant, LN_2-sensitive seed (consists of seeds of many fruit and nut crops such as *Prunus* sp., *Juglans* sp., *Corylus* sp., and *Coffea* sp. Most seeds can be dried to moisture contents less than 10 per cent, but cannot withstand temperatures lower than -40°C. Seeds fall into this category are also noted for their short storage life of usually less than 5 years) and (3) Desiccation-sensitive, LN_2-sensitive seed (These are most difficult to preserve and listed by Chin and Roberts (1980). Several attempts have been made to develop long term preservation techniques for desiccation sensitive seeds with minimal success (Merymann and Williams, 1980).

2.9.1 Cryoprotectants

Di-Methyl Sulphoxide (DMSO) has been extensively used and proved to be an excellent cryoprotectant, for animal and plant cultures. An efficient cryoprotectant should (1) have low molecular weight, (2) be easily miscible with the solvent, (3) be non-toxic even at low concentration, (4) be easily washed from the cells and (5) permeate rapidly into the system. The long term freeze storage of germplasm requires special caution with regard to the choice and concentration of the cryoprotectant. It should not bring about any genetic aberrations in the form of chromosome breakage or mutations.

Many of the cryoprotectively effective substances, especially those that penetrate the cell only slowly, or not at all, display osmotic character, i.e., they withdraw water from and plasmolyse the cells. The accompanying shrinkage of both the cell contents and plasma membrane, particularly the danger of irreversible shrinkage, has often called for a cautious rate of addition of cryoprotective compounds, to avoid an osmotic shocking effect and a damaging excess of pressure across the cell membrane (Kartha, 1982). Because of the toxic effects the cryoprotective additives may have on growth (Dougall and Wetherell, 1974), it often appears desirable to dilute out or remove the cryoprotective compounds after the cells are frozen and thawed. In achieving this, the rate of post-thaw dilution (deplasmolysis) has also been assigned a critical role in cell survival (Towill and Mazur, 1976).

Cryoprotective compounds are being used in both structural and viability studies. Cells that have been treated with cryoprotectants are frozen for

the purpose of preserving ultra-structural detail, are not viable after the cells are thawed (Farrant *et al.*, 1977). Cells cryoprotected and frozen in order to preserve viability are for the most part not typical in their ultra-structural appearance (Withers and Davey, 1978). The interactions of cryoprotective compounds with themselves and with cells are not well understood (Moiseyer *et al.*, 1982; Franks, 1977). Cells are often cooled before they are cryoprotected as part of the pre-freezing protocol. Plant tissues that are susceptible to low temperatures may show changes in cell structure following only brief exposures to lowered temperatures (Patterson *et al.*, 1979). Chilled but unfrozen cells undergo reversible as well as irreversible structural changes (Niki *et al.*, 1979). The presence of cryoprotectants results in apparent cellular disorganization and this does not always lead to cell death. Cryoprotectants are, indeed, often necessary when frozen to -196°C, thawed and recovered, even though there are exceptions (Nei, 1978; Sakai and Otsuka, 1967). The effects of cryoprotectants on the structure of plant cells when treated for preservation of viability have not been extensively studied (Zavala and Finkle, 1980, 1981). Two basic approaches have been used in ultra-structural investigations; (1) to fix cells in the presence of cryoprotectants, or (2) to freeze cells according to normal cryogenic routine and to freeze substitute then with the cryoprotectant *in situ*. Such studies can provide clues as to how cryoprotectants function to promote viable freezing.

Cryoprotective additives are used in the freezing procedure; most often as single compounds and lists of such compounds have been compiled for plant and animal tissues (Sakai and Yoshida, 1968; Karrow, 1969). Of these compounds DMSO (Di-methyl Sulphoxide) has dominated the plant literature, with glycerol or

sucrose appearing prominently in other experiments. DMSO fulfils all the criteria of being an ideal cryoprotectant also.

After freezing in LN_2 in the presence of protective compounds, higher plants have in several cases given rise to a whole plant after thawing in contrast with freezing without any cryoprotectant, a treatment that would usually kill the tissue. The compounds considered as "cryoprotectants" and usually used just prior to freezing have often been found useful in the 5 to 20 per cent range (Withers, 1980). Yet as little as 0.3 per cent DMSO has been reported to give maximal protection to conidia of *Neurospora crassa* (Banhart and Terry, 1979). For the somatic embryos and embryogenic suspensions of carrot, DMSO in the range of 5 to 10 per cent has been used (Dougall and Wetherall, 1974; Bajaj, 1976). Withers (1982) claims 15 per cent DMSO to be optimal for Hordeum embryos. Dimethyl sulfoxamine has been reported as a successful cryoprotectant for Chlamydomonas (Gresshoff, 1977).

2.9.2 Slow freezing

Method of freezing plant meristems slowly is based on the physicochemical events occurring during freezing. Mazur (1969) has identified the factors to which a cell is subjected during freezing and thawing. With progressive temperature reduction, the cell and its external medium initially supercool, followed by ice formation in the external milieu. In the case of plant cells, the cell wall and the plasma membrane act as barriers and prevent the ice from seeding the cell interior at temperatures above ca. -10°C, and thus the cell remains unfrozen

but supercooled. As the temperature is further lowered, an increasing fraction of extra-cellular solution is converted into ice, resulting in the concentration of extra-cellular solutes. Since the cell remains supercooled and its aqueous vapour pressure exceeds that of frozen exterior, the cell equilibrates by loss of water to external ice (dehydration). Slowly cooled cells reach equilibrium with the external ice efflux of water and will remain shrunken, provided the cell is sufficiently permeable to water. In such cases, intracellular ice formation, considered to be one of the most important factors responsible for causing freezing injury, will not occur. This phenomenon is utilized in devising slow freezing cryopreservation techniques with meristems (Kartha, 1985).

According to Farrant (1980) an intermediate rate of cooling generally protects the cells from freezing injury. The cells are generally cooled at optimum rates to -30 or -40°C by which time all the freezable water from the cells has escaped to become external ice and a subsequent drop in temperature to that of liquid nitrogen has very little adverse effect. Therefore, it should be theoretically possible to reduce the water content of the cells by osmotic agents prior to freezing and subsequently increasing the post freezing survival. The intermediate cooling rates used for cryopreservation of plant cells are in the range of 0.5 to 2°C/min, which is often referred to as slow cooling. Slow freezing has been extended with success to a number of different types of plant material such as meristems, somatic embryos and clonal plantlets (Withers, 1979).

2.9.3 Rapid freezing

The cooling rates imposed during rapid freezing of plant meristems are of the order of several hundred degrees per minute and often there exists no control on the rate at which the cells are frozen. During rapid lowering of temperature, in contrast to freezing by regulated slow cooling, the cells do not have time to equilibrate with the external ice or the vapour pressure deficit by efflux of cellular water. The cells attain equilibrium by intracellular freezing which is lethal to biological specimens. Therefore, rapid freezing techniques may not be advisable for cryopreservation of meristems.

The rapid freezing method is based on the mechanism of Luyet (1937) which suggests that viability may be maintained by preventing the growth of intracellular ice crystals formed during rapid cooling by rapidly passing the tissue through the temperature zone in which lethal ice crystal growth occurs (Seibert and Wetherbee, 1977). In most of the examples of rapid freezing, the viability of the cryopreserved meristems is not very high and it has been demonstrated that better survival rates could be obtained by slow freezing as was the case with potato (Towill, 1981), strawberry and cassava (Kartha *et al.*, 1980). Moreover, rapid freezing has resulted in complete loss of viability of pea meristems and a very low rate of survival of strawberry meristems (Kartha *et al.*, 1979, 1980). The advantage of using rapid freezing technique mainly lies in the simplicity of the operation.

Materials and methods

MATERIALS AND METHODS

The present investigation on improving storage techniques of the seeds of *Hopea parviflora* by selecting different maturity levels, desiccation techniques, embryo encapsulation (synthetic seeds) and cryopreservation methods was carried out at the College of Forestry, Vellanikkara.

3.1 Materials

3.1.1 Seed source

Seeds were collected from the *Hopea parviflora* stand at the Kerala Forest Research Institute, Subcentre located at Karimpuzha, Nilambur Taluk of Malappuram District. This stand was established in 1920's. Eventhough establishment of the plantation was a failure; it became successful as a result of profuse natural regeneration when adequate protection was given. Other tree species found in the area are *Swietenia macrophylla, Xylia xylocarpa, Vateria indica, Terminalia tomentosa* etc. The area is located at 11°17'N and 76°4'E and has a warm humid tropical climate with mean annual temperature of 18°C to 30°C. Mean annual precipitation of this area is 2400 mm.

3.2 Methods

3.2.1 Seed collection

The site was visited at weekly intervals and observations recorded on the date of onset of flowering, date of fruit set and date of fruit shedding. Inflorescences were observed daily and upon anthesis, they were tagged.

Seeds were collected at six and seven weeks after anthesis from the site during June-July 2000 and 2001 and brought to the College of Forestry on the same day for conducting the experiment.

3.2.2 Preparation of the propagule

Extreme hygiene was maintained while collecting seeds to avoid culture contamination. Seeds were first washed clean in running tap water. After dewinging, seeds were blot dried, transferred to 0.1 per cent $HgCl_2$ solution for sterilization and moved to laminar airflow cabinet. Seeds were removed from the chemical after 15 minutes and rinsed with sterilized distilled water to remove traces of the sterilant if any, sticking to the surface. Sterile surgical blade was used for removing seed coat from the seed (seeds without seed coat) and also for extracting embryonic axes. All materials used were sterilized prior to work and the entire work was done under aseptic conditions.

3.2.3 Maintenance of relative humidities

Different relative humidities were maintained in desiccators of uniform size by pouring 100 ml each of the solutions as shown in Table 1. Distilled water (100 ml) was used to maintain 100 per cent relative humidity. The propagules were kept in sterilized petri plates inside the desiccators and covered with the lid after smearing the edges with petroleum jelly to keep the desiccators airtight.

Table 1. Solutions to maintain different relative humidities

Relative humidities	Salts	Concentration
100%	--	100 ml distilled water
85.3%	KCl	100 ml saturated solutions
75.6%	NaCl	100 ml ----do----
46.3%	$Ca(NO_3)_2.4H_2O$	100 ml ----do----
30%	KOH	42.3 g/100 ml distilled water
20%	KOH	47.0 g/100 ml distilled water

3.3 Effect of desiccation on seed viability

The seeds were brought to the laboratory on the same day of collection itself. The seeds were then dewinged and homogeneous samples of dewinged seeds, seeds without seed coat and embryonic axes were subjected to desiccation in the desiccators set at different relative humidities. The seeds collected in the year 2000 were pretreated in desiccators set at relative humidities of 100%, 85.3%, 75.6%, 46.6%, 30% and 20% each for time duration 3, 6, 12 and 24 hr. The experiment was conducted in four replications each with ten seeds. In the year 2001, the intact seeds and seeds without seed coat were subjected to desiccation at 100%, 85.3%, 75.6%, 46.6% and 30% relative humidities for 3, 6, 9 and 12 hr duration. The embryonic axes were desiccated at 100%, 85.3%, 75.6% and 46.6% relative humidities only for 3 and 6 hours duration. In this case, the experiment was conducted in three replications each with five seeds/ seeds without seed coat/ embryonic axes. In the whole experiment, desiccation for 9 hr. duration was excluded in the case of embryonic axes. In the case of seeds and seeds without seed coat, 9 hr. duration was excluded in 20% relative humidity. The treatments were excluded because of the scarcity of seeds as very few trees flowered in the whole stand in both years of study. The whole work was done under sterile conditions inside a laminar flow cabinet. Germination characteristics of the propagules were studied before and after the treatment.

3.4 Germination methods

Seeds/ seeds without seed coat were germinated on a double layer of filter paper (Borosil No.1) inside petri dishes having a diameter of 9 cm.

The petri dishes were covered and kept for germination at room temperature (29 ± 1°C). The filter paper was moistened with distilled water on daily basis. The embryonic axes were inoculated into ½ MS media in test tubes and incubated in a culture room provided with cool white fluorescent lamps to give a light intensity of 2000 lux for 16 hours light period. The temperature was maintained at 27 ± 2°C. Germination was observed daily.

The propagules were considered to have germinated when the radicle reached about one centimeter length and the green hypocotyls became visible. All germinated seeds were collected and removed at every assessment to prevent double counting. At the end, cumulative germination percentage was calculated for each treatment. Vigour parameters were also calculated using Germination Value (GV), Mean Daily Germination (MDG) and Peak Value (PV) (Czabator, 1962).

3.5 Culture media

Regeneration potential of the embryonic axis was studied using ½ MS medium. The technical composition of MS medium is given in Table 2. Standard procedures (Gamborg and Shyluck, 1981) were followed for the preparation of the media. Stock solutions of major and minor nutrients were prepared by dissolving the required quantity of the chemical in distilled water and were stored in amber coloured bottles in refrigerator. While the nutrient stock solutions were prepared afresh every four weeks, that of vitamins, amino acids and growth regulators were prepared every week.

Table 2. Chemical composition of Murashige and Skoog medium

Compound	Quantity (mg/l)
INORGANIC	
Ammonium nitrate	1650.0
Boric acid	6.2
Calcium chloride - 2 hydrate	440.0
Cobalt chloride - 6 hydrate	0.025
Copper sulphate - 5 hydrate	0.025
Ferrous sulphate - 7 hydrate	27.8
Manganese sulphate - 1 hydrate	22.3
Magnesium sulphate - 7 hydrate	370.0
Na_2EDTA - 2 hydrate	37.3
Potassium dihydrogen phosphate	170.0
Potassium iodide	0.83
Potassium nitrate	1900.0
Sodium molybdate - 2 hydrate	0.25
Zinc sulphate - 7 hydrate	8.6
ORGANIC	
Inositol	100.0
Nicotinic acid	0.5
Thiamine HCl	0.1
Pyridoxine HCl	0.5
Glycine	2.0
OTHERS	
Sucrose (in per cent w/v)	3.0
Agar (in per cent w/v)	0.7

½ MS denotes half the amounts of the inorganic constituents per litre.

Specific quantities of the stock solution of the chemicals were pipetted out in to a 1000 ml beaker previously rinsed with distilled water. Sucrose and inositol were added afresh and dissolved subsequently. Required quantities of the growth regulators and other supplements were also added for each media and the solution was made up to the required volume. The pH of the solution was adjusted to the range 5.6 to 5.8 (using 1N NaOH or 1N HCl). Agar was then added to the media except in cryoprotection medium.

The solutions for MS and Recovery media were then boiled for melting the agar. 20 ml each of the melted media was poured hot to the oven-dried culture tubes (15 x 2.5 cm), which were previously washed, rinsed in distilled water and dried. The tubes with the media solidified were then tightly plugged with cotton plugs and autoclaved. After sterilization, the culture tubes were stored in culture room maintained at a temperature of 27± 2°C for further use.

The cryoprotection medium is neither boiled nor added with agar like the other two media. This is poured to propagules after sterilizing it under laminar flow in three increments over 45 minutes. The composition of the cryoprotection media is given in Table 3.

The viability of intact seeds, seeds without seed coat, embryonic axes and synthetic seeds, after cryopreservation was tested using recovery medium, the composition of which is given in Table 4.

Table 3. Chemical composition of Cryoprotection medium (I + II)

Medium I	Medium II
1. Basal M	1. Basal MS
2. Sucrose 3 %	2. Sucrose 1M
3. pH 5.6	3. pH 5.6
	4. DMSO 20 %

Table 4. Chemical composition of Recovery medium

Salts	Concentration
1. Basal MS	
2. Casein hydrolysate	1 g/l
3. Coconut water (De -proteinized)	10 %
4. ABA	10 mM
5. NAA	3 ppm
6. Charcoal (Activated)	0.05 %
7. Agar	0.8 %
8. Sucrose	3 %
9. pH	5.6

Sodium alginate was used for embryo encapsulation and basic tissue culture medium was used for studying their viability. Wet cotton was used for storing the synthetic seeds.

3.6 *Seed moisture content*

Low constant temperature oven method (ISTA, 1985) was used to determine the moisture content of the seeds/ seeds without seed coat and embryonic axes before desiccation. In the year 2000, the moisture content of the seeds was tested using only one replication of five seeds, as the seeds were available in very low quantities. The study was repeated in the year 2001 using five seeds in three replications. After determining the initial weight, seeds/ seeds without seed coat and embryonic axes were treated in desiccators as described before. Moisture content of the seeds/ seeds without seed coat and embryonic axes after desiccation was determined on wet weight basis and for this the following formula was used (Hong and Ellis, 1996). Use of this formula does not need the oven-dried weight of the materials. Thus the seeds/seeds without seed coat and embryonic axes used for determining the moisture content could also be used for testing the leachate conductivity of the material.

$$\text{Weight of seed (g) at Desired Moisture Content Per cent.(DMC\%)} = \frac{(100 - \text{Initial MC\%})}{(100 - \text{DMC\%})} \times \text{Initial seed weight (g)}$$

3.7 Leachate conductivity measurement

In the year 2000, the leachate conductivity of the seeds was tested using only one replication of five seeds, as the seeds were available in limited quantities. The study was repeated in the year 2001 using five seeds in three replications. Five seeds/ seeds without seed coat/ embryonic axes were immersed in 50 ml of distilled water overnight and leachate conductivity was measured using a conductivity meter (Elico, CM 180).

3.8 Microencapsulation studies

The seeds were sterilized as described in Section 3.2.2. Seeds were split open using a sharp razor blade and the embryonic axis carefully excised. The embryos were then kept in desiccators set at relative humidities of 100%, 85.3%, 75.6% and 46.6% for time duration of 3 and 6 hr. After desiccation, the embryonic axes were subjected to encapsulation.

3.8.1 Encapsulation media

The best combination of sodium alginate and calcium chloride standardized for cocoa (Nagaraj, 1994) was used for the production of synthetic seeds of *Hopea parviflora*. Sodium alginate slurry was prepared by adding 40 g of sodium alginate (Sd fine, 40105) to one litre of distilled water and heating slowly with constant stirring. Calcium chloride (75 mM) solution was prepared by adding 11.026 g of $CaCl_2$ $2H_2O$. to one litre of distilled water. The above two solutions were sterilized in an autoclave at 15 psi pressure and 121°C temperature for 20

minutes. Fresh solutions of sodium alginate and calcium chloride were used for encapsulation whenever required.

3.9.1 Encapsulation of the propagule

The desiccated embryos were dropped into sodium alginate solution and stirred well. Each of these embryo was then carefully sucked using a glass tube having an inner diameter of 4 mm. The sucked gel coated embryos were then dropped into calcium chloride solution for complexation. The encapsulated embryos (synthetic seeds) were allowed to stay in the calcium chloride solution for 25 – 30 minutes with intermittent stirring and then transferred to a petri plate with sterilized distilled water to remove excess calcium ions. All the process was done under aseptic conditions.

3.9.1 Storage of synthetic seeds

Sixty synthetic seeds each were placed on wet sterilized cotton kept at the bottom of twenty seven Erlenmeyer flasks (100 ml capacity). Mouth of the Erlenmeyer flask was plugged with sterilized cotton. The flasks were then stored at 4°C, 20°C and at room temperature (27±2°C).

3.9.1 Testing of viability

The effect of storage of synthetic seeds was studied at weekly intervals by taking fifteen synthetic seeds from each flask and observing for their germination percentage, number of days taken for initiation of germination

and number of days taken for completion of germination. Transfer of synthetic seeds to and from the Erlenmeyer flask was done inside the laminar airflow cabinet and all the operations were done under aseptic conditions.

The effect of desiccation of embryonic axes before encapsulation on germination of synthetic seeds was studied by comparing the germination of excised embryonic axes with or without encapsulation. The influence of encapsulating the excised embryonic axes in sodium alginate was studied by comparing the germination of excised embryonic axes with or without encapsulation. Similarly, the effect of storage temperature was also studied by comparing the germination of synthetic seeds stored at different temperatures at weekly intervals.

3.9.1 Inoculation and culturing of synthetic seeds

To inoculate the synthetic seeds in the culture medium, the cotton plug of the culture tube was removed and the neck was first flamed over a gas burner kept in the chamber. After transferring the synthetic seed to the medium, the neck of the culture tube was again flamed and the cotton plug was replaced. All the inoculation procedures were carried out under aseptic conditions. The cultures were incubated in a culture room provided with cool white fluorescent lamps to give a light intensity of 2000 lux for 16 hours light period. The temperature was maintained at 27±2°C.

3.10 Cryopreservation techniques

3.10.1 Pretreatment for cryoprotection

Seeds/ seeds without seed coat/ embryonic axes subjected to the desiccation treatments as described in section 3.3, were given a cryoprotection pretreatment. Similarly, synthetic seeds prepared from the desiccated embryonic axes were also given this pretreatment.

The propagules were treated in a liquid cryoprotection medium for 45 minutes at room temperature. For this, the propagules were added to a medium consisting of MS salts and 3 % sucrose at a pH of 5.6. Over a period of 45 minutes an equal volume of MS medium with 1 M sucrose and 20 % DMSO was added in three increments. This will give a final concentration of 0.5 M sucrose and 10 % DMSO. After the pretreatment the propagules were transferred to cryovials of 1.2 ml capacity filled with the liquid cryoprotection medium.

3.10.2 Direct plunging of propagules in liquid nitrogen

The cryovials containing seeds/ seeds without seed coat/ embryonic axes/ synthetic seeds along with cryoprotection medium were kept inside canisters and quickly plunged into liquid nitrogen contained in 30 litre cryocans filled with liquid nitrogen. At the end of 24 hour duration, these materials were taken out of liquid nitrogen and were rapidly thawed in a water bath at 37°C for 30 minutes. Afterwards, their regeneration potential was studied. Due to limited availability owing to poor seed year, five seeds/ seeds without seed coat/ embryonic axes/ synthetic seeds were used with three replications for the study.

3.10.3 Slow freezing of propagules

After the pretreatment, the propagules were transferred aseptically to 1.2 ml cryovials filled with cryoprotection medium and slow freezing was accomplished in two stage freezing protocol using the Programmable Biofreezer model PTC 1000C of Apex Instruments, Calcutta. The freezing was accomplished using the freezing programme PTC 1000C version-1.22 released on 14-04-1998 supplied by M/s Apex Instruments, Calcutta. Initially the propagules were cooled to 4°C from room temperature, at a rate of 5°C per minute. Now there was a pause in which the propagules were kept at 4°C for 30 minutes and then they were cooled down to -40°C at a rate of 0.4°C per minute. After keeping the propagules for 30 minutes at –40°C in the Biofreezer, the cryovials containing the propagules were immediately transferred to canisters and plunged into liquid nitrogen contained in 30 litre cryocans. The freezing protocol and the progress of freezing are shown in Fig. 1.

At the end of 24 hours, the propagules were removed from liquid nitrogen and immediately thawed in warm water bath at 37°C for 30 minutes. The thawed seeds/seeds without seed coat were inoculated on to the recovery medium contained in a petri dish and embryonic axes/ synthetic seeds were inoculated into test tubes containing the recovery medium. Control propagules were placed directly on the recovery medium without freezing. The entire propagules were incubated in a culture room at 26°C with a 16:8 hour light: dark cycle.

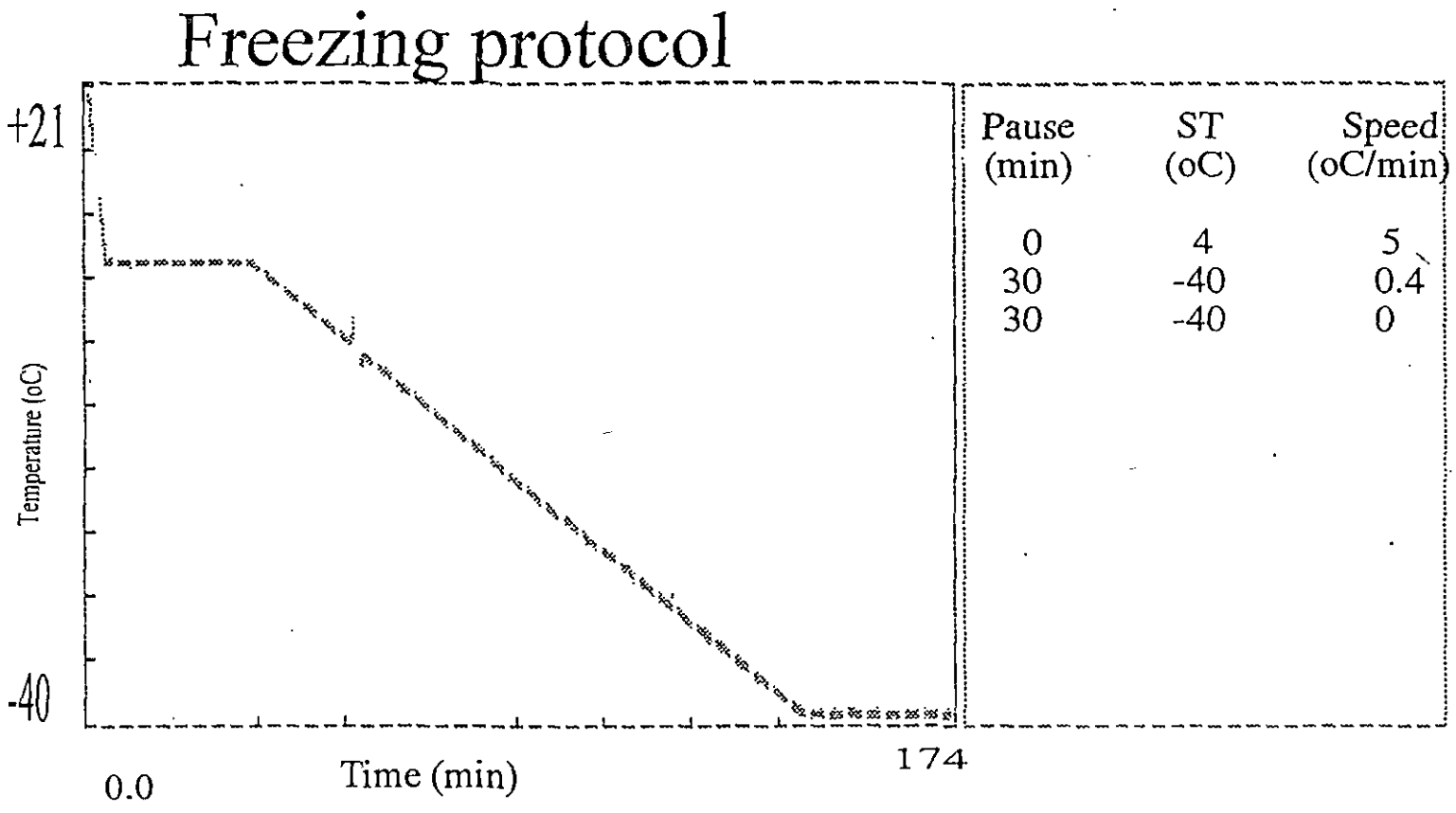


Fig. 1. Freezing protocol and the progress of freezing

3.11 Statistical analysis

All the observations recorded were statistically analyzed following the methods suggested by Panse and Sukhatme (1978). The data were subjected to repeated analysis using the statistical package SPSS version-10 for Windows. If the maturity level of the intact seeds/seeds without seed coat/embryonic axes were found to be non-significant on preliminary analysis, the data for the two maturity levels were combined to do the analysis taking only two factors as the variables. Such analysis was done for finding out the effect of desiccation of the intact seeds/seeds without seed coat/embryonic axes and in the analysis of the data of storage studies. The treatments were set up in Completely Randomized Block Design. The analysis was done using Completely Randomized Block Design with unequal number of replications. For this, the data were combined together by excluding the effect of year and including it as replications. Logarithmic and square root transformed data were used wherever necessary.

Results

RESULTS

The results of the study on the moisture content, leachate conductivity and germination parameters of *Hopea parviflora* seeds, seeds without seed coat and excised embryonic axes as affected by their maturity levels, different levels and duration of desiccation are presented in this chapter. Germination parameters of synthetic seeds prepared from the desiccated embryos as affected by different treatments and the results of the cryopreservation studies using seeds, seeds without seed coat, excised embryonic axes and synthetic seeds are also presented here.

4.1 Flowering and fruiting

Flowering was extremely rare during 1999 in the whole stand and hence, the work could not be conducted during that year. The onset of flowering during the year 2000 was in the first week of May. Anthesis of the flowers occurred in the last week of May and fruitset in the first week of June. After anthesis, the fruits took six weeks to attain maturity and started shedding during the third week of July. Desiccation studies on seeds, seeds without seed coat and excised embryonic axes were carried out with the seeds collected during this year. In the year 2001, flowering was noticed in the second week of March and anthesis occurred in the first week of April. The seeds attained maturity by the last week of May and started shedding in the first week of June. The seeds collected during this season were used to prepare synthetic seed for conducting storage studies. Few trees in the stand flowered during mid June and seeds were collected from these trees during August. Synthetic seeds were prepared from these seeds for

conducting the cryopreservation studies. Both during 2000 and 2001, less than five trees flowered in the whole stand. It was also observed that with the onset of intense downpour associated with the monsoon, mature seeds exhibited vivipary by and large.

4.2 Moisture content, leachate conductivity and germination parameters of intact seeds of *Hopea parviflora* as affected by maturity levels, and levels of desiccation for different duration

4.2.1 Moisture content

Significant differences were found in the moisture content of intact seeds due to the effect of levels of desiccation ($P<0.01$) for various duration ($P<0.01$). Maturity levels of intact seeds and interaction effects were not significant. The data pertaining to this are given in Table 5. The intact seeds kept at 100% relative humidity had moisture content of 54.1 per cent. Decline in moisture content of seeds due to desiccation at 85.3 and 75.6 per cent relative humidities were not significantly different. When desiccated at 46.6 per cent relative humidity, the moisture content decreased significantly to 48.1 per cent. The moisture content again decreased significantly to 42 per cent when the seeds were subjected to desiccation at 30 per cent relative humidity. Reduction in moisture content was observed due to desiccation at 20 per cent relative humidity also. But this reduction was not significant.

A significant reduction in moisture content was observed only after 6 hours of desiccation. From the initial moisture content of 49.8 per cent, it decreased to 46.3 per cent. Moisture content decreased gradually with increasing

Table 5. Moisture content (%) of intact seeds of *Hopea parviflora* as affected by levels of desiccation for different duration

Time (hours)	Relative humidity (%)						Mean
	100	85.3	75.6	46.6	30	20	
0	53.0 ±4.202	53.0 ±4.202	53.0 ±4.202	53.0 ±4.202	53.0 ±4.202	33.8 ±1.165	49.8^{ab} ±1.811
3	52.9 ±2.564	52.3 ±2.064	52.8 ±2.340	52.4 ±2.662	49.3 ±3.286	32.9 ±3.850	48.8^{ab} ±1.254
6	53.2 ±2.081	53.3 ±1.715	51.6 ±2.066	48.5 ±2.457	44.5 ±3.561	27.0 ±2.510	46.3^{b} ±1.359
9	57.1 ±2.081	55.6 ±0.948	54.6 ±0.769	51.3 ±0.798	45.6 ±1.536	*	52.8^{a} ±0.936
12	55.3 ±1.843	53.8 ±1.489	52.8 ±1.525	43.9 ±2.370	35.8 ±2.755	21.3 ±3.815	43.8^{b} ±1.667
24	53.4 ±0.125	49.8 ±2.520	46.5 ±2.950	39.4 ±4.610	24.0 ±5.335	18.6 ±3.800	38.6^{c} ±4.079
Mean	54.1^{a} ±1.136	53.0^{ab} ±1.020	51.9^{ab} ±1.077	48.1^{b} ±1.326	42.0^{c} ±1.801	26.7^{c} ±2.280	46.0 ±0.703

	Relative humidity	Time	Interaction
P level	0.01	0.05	NS

*treatment not studied

**Values with the same superscripts do not differ significantly

duration of desiccation, but the difference was not significant. However, at the end of 24 hours, a significant reduction in moisture content, which was equal to 22.5 per cent compared to the control, was observed.

4.2.2 Leachate conductivity

Leachate conductivity of the intact seeds was significantly affected by the duration of desiccation only ($P<0.01$). The effects of maturity levels, levels of desiccation and interaction were not significant. The data pertaining to this are given in Table 6. The initial leachate conductivity of the intact seeds was 0.061 mhos cm^{-1}. Increasing the duration of desiccation up to 6 hours did not affect the leachate conductivity significantly. After 9 hours of desiccation, there was a significant decrease in the leachate conductivity. However, further increase in the duration of desiccation significantly increased the leachate conductivity. Desiccating the seeds for 24 hours increased the leachate conductivity to 0.139 mhos cm^{-1}. This increase was equivalent to 128 per cent compared to the control.

4.2.5 *Germination parameters*

4.2.5.1 Cumulative germination percentage

Significant differences were found in the cumulative germination percentage due to the effect of maturity levels ($P<0.01$) and levels of desiccation ($P<0.05$) for various duration ($P<0.01$). Interaction effects were not significant. The data pertaining to this are given in Table 7. Germination percentage was significantly higher in the case of seeds collected at seven weeks after anthesis (94.8 per cent) compared to that of seeds collected at six weeks after anthesis (93.0

Table 6. Leachate conductivity (mhos cm^{-1}) of intact seeds of *Hopea parviflora* as affected by levels of desiccation for different time duration.

Time (hours)	Relative humidity (%)						Mean
	100	85.3	75.6	46.6	30	20	
0	0.061 ±0.010	0.061 ±0.010	0.061 ±0.010	0.061 ±0.010	0.061 ±0.010	0.062 ±0.004	0.061[b] ±0.004
3	0.050 ±0.015	0.052 ±0.014	0.048 ±0.013	0.055 ±0.013	0.044 ±0.013	0.101 ±0.018	0.058[b] ±0.006
6	0.054 ±0.018	0.050 ±0.016	0.049 ±0.015	0.050 ±0.015	0.038 ±0.010	0.075 ±0.009	0.053[b] ±0.006
9	0.022 ±0.002	0.027 ±0.001	0.028 ±0.002	0.029 ±0.004	0.026 ±0.003	*	0.026[c] ±0.001
12	0.063 ±0.024	0.064 ±0.023	0.062 ±0.024	0.064 ±0.022	0.038 ±0.008	0.055 ±0.008	0.058[b] ±0.009
24	0.192 ±0.009	0.189 ±0.001	0.187 ±0.006	0.182 ±0.006	0.041 ±0.009	0.047 ±0.006	0.139[a] ±0.021
Mean	0.074 ±0.009	0.073 ±0.008	0.072 ±0.008	0.074 ±0.008	0.041 ±0.004	0.068 ±0.007	0.066 ±0.003

	Relative humidity	Time	Interaction
P level	NS	0.01	NS

*treatment not studied

**Values with the same superscripts do not differ significantly

Table 7. Cumulative germination percentage of intact seeds of *Hopea parviflora* at six and seven weeks after anthesis as affected by levels of desiccation for different duration

	Time (hours)	Relative humidity (%)						Mean
		100	85.3	75.6	46.6	30	20	
6 weeks after anthesis	0	96.5 (4.6±0.013)	96.5 (4.6±0.013)	96.5 (4.6±0.013)	96.5 (4.6±0.013)	96.5 (4.6±0.013)	93.8 (4.6±0.000)	96.0^{ab} (4.6±0.005)
	3	96.5 (4.6±0.013)	95.5 (4.6±0.018)	93.9 (4.5±0.028)	95.0 (4.6±0.020)	96.5 (4.6±0.013)	93.8 (4.6±0.000)	95.2^{ab} (4.6±0.008)
	6	96.5 (4.6±0.013)	90.8 (4.5±0.049)	91.3 (4.5±0.033)	93.9 (4.5±0.028)	91.7 (4.5±0.035)	93.8 (4.6±0.000)	93.0^{b} (4.5±0.013)
	9	100.0 (4.6±0.000)	100.0 (4.6±0.000)	100.0 (4.6±0.000)	100.0 (4.6±0.000)	93.2 (4.4±0.170)	*	98.6^{ab} (4.6±0.034)
	12	96.0 (4.6±0.016)	94.5 (4.6±0.021)	92.6 (4.5±0.038)	95.0 (4.6±0.020)	89.8 (4.5±0.053)	92.8 (4.5±0.010)	93.5^{b} (4.5±0.013)
	24	88.8 (4.5±0.034)	75.3 (4.3±0.039)	86.1 (4.4±0.063)	87.7 (4.5±0.029)	88.8 (4.5±0.034)	80.7 (4.4±0.051)	84.6^{d} (4.4±0.020)
	Mean	95.7^{a} (4.6±0.008)	92.1^{bc} (4.5±0.018)	93.4^{abcd} (4.5±0.015)	94.7^{abc} (4.6±0.010)	92.7^{bcd} (4.5±0.019)	91.0^{d} (4.5±0.017)	93.0 (4.5±0.006)
7 weeks after anthesis	0	96.5 (4.6±0.013)	96.5 (4.6±0.013)	96.5 (4.6±0.013)	96.5 (4.6±0.013)	96.5 (4.6±0.013)	93.8 (4.6±0.000)	96.0^{ab} (4.6±0.005)
	3	96.5 (4.6±0.013)	96.5 (4.6±0.013)	96.5 (4.6±0.013)	96.5 (4.6±0.013)	96.5 (4.6±0.013)	93.8 (4.6±0.000)	96.0^{ab} (4.6±0.005)
	6	93.9 (4.5±0.028)	93.9 (4.5±0.028)	96.5 (4.6±0.013)	96.0 (4.6±0.016)	96.5 (4.6±0.013)	93.8 (4.6±0.000)	95.1^{ab} (4.6±0.008)
	9	100.0 (4.6±0.000)	100.0 (4.6±0.000)	100.0 (4.6±0.000)	94.6 (4.5±0.074)	100.0 (4.6±0.000)	*	98.9^{a} (4.6±0.015)
	12	96.5 (4.6±0.013)	96.5 (4.6±0.013)	93.5 (4.5±0.029)	95.6 (4.6±0.018)	95.6 (4.6±0.018)	91.0 (4.5±0.010)	94.8^{ab} (4.6±0.008)
	24	91.0 (4.5±0.010)	90.0 (4.5±0.000)	87.7 (4.5±0.029)	88.8 (4.5±0.034)	85.3 (4.5±0.034)	85.3 (4.5±0.034)	88.0^{c} (4.5±0.011)
	Mean	95.7^{a} (4.6±0.008)	95.6^{ab} (4.6±0.008)	95.1^{abc} (4.6±0.010)	94.7^{abc} (4.6±0.010)	95.0^{abc} (4.6±0.010)	91.5^{cd} (4.5±0.011)	94.8 (4.6±0.004)

	Age	Relative humidity	Time	Interaction
P level	0.01	0.05	0.01	NS

*treatment not studied **Values with the same superscripts do not differ significantly
***log transformed values are given in parenthesis

per cent). At six weeks after anthesis, desiccating the seeds at 85.3, 30 and 20 per cent relative humidities significantly reduced the germination percentage compared to the control. The other desiccation levels were at par. At seven weeks after anthesis, lowest desiccation level of 20 per cent only significantly reduced the germination percentage. On average, desiccating the intact seeds of *Hopea parviflora* at 20 per cent relative humidity resulted in 5 per cent reduction in germination percentage.

Both at six and seven weeks after anthesis, desiccation for 24 hours resulted in largest and highly significant reduction in germination percentage. Compared to the control, this was equal to 10 per cent reduction in germination percentage. Only at six weeks after anthesis, desiccation for 6 and 12 hours resulted in significant reduction in germination percentage. All the other treatments were at par.

4.2.5.2 Mean Daily Germination (MDG)

MDG of intact seeds of *Hopea parviflora* was significantly affected by the maturity levels ($P<0.01$) and duration of desiccation ($P<0.01$). Levels of desiccation and interaction effects were not significant. The data pertaining to this are given in Table 8. The MDG of the seeds collected at seven weeks after anthesis was 20.8, which was significantly higher than the MDG of 14.2 of seeds at six weeks after anthesis.

The MDG of seeds at six and seven weeks after anthesis showed the largest and highly significant reduction when desiccated for 24 hours. However, the reduction was more in the case of seeds collected at six weeks after anthesis.

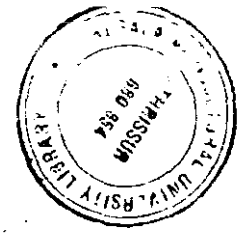
Table 8. Mean Daily Germination of intact seeds of *Hopea parviflora* at six and seven weeks after anthesis as affected by levels of desiccation for different duration

6 weeks after anthesis								7 weeks after anthesis							
Time (hours)	Relative humidity (%)						Mean	Time (hours)	Relative humidity (%)						Mean
	100.0	85.3	75.6	46.6	30.0	20.0			100.0	85.3	75.6	46.6	30.0	20.0	
0	21.7 ±6.513	21.7 ± 6.513	21.7 ± 6.513	21.7 ± 6.513	21.7 ±6.513	8.8 ±1.838	19.5^{abc} ±2.538	0	23.1 ±2.217	23.1 ±2.217	23.1 ±2.217	23.1 ±2.217	23.1 ±2.217	27.1 ±2.075	23.8^{a} ±.877
3	10.2 ± 2.956	15.4 ± 1.921	14.2 ± 3.444	13.6 ± 3.115	10.9 ±3.066	6.3 ±1.250	11.7^{de} ±1.200	3	21.0 ±2.760	21.7 ±2.205	22.9 ±2.074	22.9 ±1.010	21.6 ±2.507	21.7 ±2.034	21.9^{a} ±.854
6	16.7 ± 1.409	15.3 ± 2.145	11.3 ± 1.613	12.9 ± 3.433	11.1 ±2.666	5.0 ±0.981	12.0^{de} ±1.044	6	16.7 ±0.731	17.8 ±1.124	25.2 ±2.249	20.4 ±1.747	23.9 ±2.838	23.8 ±1.250	21.3^{a} ±.898
9	26.1 ±3.879	20.0 ± 0.00	19.8 ± 3.091	20.0 ± 0.00	17.2 ±4.338	*	20.6^{ab} ±1.362	9	24.4 ±4.433	30.5 ±2.767	19.8 ±3.091	15.9 ±2.087	27.8 ±2.767	*	23.7^{a} ±1.845
12	21.2 ± 3.055	17.4 ± 4.999	13.5 ± 2.821	12.8 ±3.221	11.1 ±3.00	6.7 ±2.253	13.8^{cd} ±1.509	12	24.8 ±4.756	17.3 ±0.772	19.6 ±1.470	19.5 ±1.535	17.5 ±1.139	16.2 ±.728	19.2^{abc} ±1.014
24	11.7 ± 2.882	5.1 ± 1.318	7.3 ± 1.040	7.5 ±3.170	6.5 ±2.170	6.8 ±1.759	7.5^{e} ±0.900	24	15.7 ±1.509	14.0 ±0.606	13.5 ±0.501	15.8 ±1.022	16.7 ±2.012	15.6 ±0.982	15.2^{d} ±.498
Mean	17.9 ±1.778	15.8 ±1.826	14.6 ±1.720	15 ±1.817	13.1 ±1.799	6.7 ±0.722	14.2 ±0.744	Mean	20.9 ±1.313	20.7 ±1.000	20.7 ±0.990	19.6 ±0.800	21.8 ±1.060	20.9 ±1.179	20.8 ±0.433

	Age	Relative humidity	Time	Interaction
P level	0.01	NS	0.01	NS

*treatment not studied

**Values with the same superscripts do not differ significantly



In this case, the MDG decreased by 62 per cent compared to the initial MDG of 19.5. In the case of seeds collected at seven weeks after anthesis, this reduction was equal to 36 per cent. Desiccation for 3, 6 and 12 hours also significantly reduced the MDG of intact seeds at six weeks after anthesis compared to the control.

4.2.5.3 Peak Value (PV)

Significant differences were observed in the PV of intact seeds due to levels ($P<0.01$) and duration ($P<0.01$) of desiccation and their interaction effects ($P<0.05$). Maturity levels had no significant effect. The data pertaining to this are given in Table 9. Highly significant reduction in PV was observed when the seeds were desiccated at 85.3, 75.6 and 20 per cent relative humidities for 24 hours. When desiccated at 85.3 per cent relative humidity. This reduction was equal to 53 per cent compared to the control. The reduction in PV due to other desiccation levels was not significant.

4.2.5.4 Germination Value (GV)

GV of intact seeds of *Hopea parviflora* was found to be significantly affected by duration of desiccation ($P>0.01$) only. Effects of maturity levels, levels of desiccation and interaction were not significant. The data pertaining to this are given in Table 10. GV of seeds reduced significantly after 3 hours of desiccation. Compared to the control, this decline was equal to 27 per cent. Even though the GV increased significantly when desiccated for 9 hours, further desiccation resulted in significant reduction and after 24 hours of desiccation, lowest GV of

Table 9. Peak Value of germination of intact seeds of *Hopea parviflora* as affected by levels of desiccation for different duration

Time (hours)	Relative humidity (%)						Mean
	100.0	85.3	75.6	46.6	30.0	20.0	
0	26.3^{bc} ±2.602	26.3^{bc} ±2.602	26.3^{bc} ±2.602	26.3^{bc} ±2.602	26.3^{bc} ±2.602	23.7^{bcd} ±1.898	25.9^{a} ±1.0.34
3	22.8^{bcd} ±2.126	24.1^{bcd} ±2.165	23.9^{bcd} ±1.873	24.4^{bcd} ±1.922	21.2^{bcde} ±1.288	21.1^{bcde} ±1.294	22.9^{ab} ±0.765
6	22.3^{bcde} ±1.829	20.3^{bcde} ±1.929	22.2^{bcde} ±1.805	23.4^{bcd} ±1.475	21.5^{bcde} ±1.819	21.7^{bcde} ±1.633	21.9^{b} ±0.718
9	37.2^{a} ±1.811	30^{ab} ±2.849	23.1^{bcd} ±2.699	18.2^{cde} ±1.181	24.2^{bcd} ±2.783	*	26.5^{a} ±1.557
12	29.4^{ab} ±3.167	20.6^{bcde} ±2.212	21.2^{bcde} ±2.007	20.5^{bcde} ±1.311	17.3^{cde} ±1.294	18.3^{cde} ±1.153	21.2^{b} ±0.945
24	21^{bcde} ±2.358	12.4^{e} ±1.127	14.3^{de} ±0.644	16.7^{cde} ±0.759	17.7^{cde} ±1.055	14.3^{de} ±0.895	16.1^{c} ±0.639
Mean	26.5^{b} ±1.146	22.3^{ab} ±1.064	21.8^{ab} ±0.936	21.6^{ab} ±0.837	21.4^{b} ±0.847	19.8^{b} ±0.792	22.4 ±0.418

	Relative humidity	Time	Interaction
P level	0.01	0.01	0.05

*treatment not studied **Values with the same superscripts do not differ significantly

Table 10. Germination Value of intact seeds of *Hopea parviflora* as affected by levels of desiccation for different duration

Time (hours)	Relative humidity (%)						Mean
	100.0	85.3	75.6	46.6	30.0	20.0	
0	834.2 (25.9±2.524)	834.2 (25.9±2.524)	834.2 (25.9±2.524)	834.2 (24.4±2.524)	834.2 (25.8±2.524)	823.6 (23.4±1.812)	832.5[a] (25.2±1.001)
3	543.3 (20.3±1.68)	601.2 (21.5±1.343)	638.5 (22.3±1.606)	663.0 (22.7±1.121)	550.1 (20.4±1.353)	665.4 (20.5±1.226)	610.3[bc] (21.3±0.581)
6	536.3 (20.1±1.095)	450.0 (18.2±1.276)	576.9 (21.0±1.805)	584.0 (21.2±1.279)	568.0 (20.8±1.804)	697.8 (21.1±1.458)	568.8[c] (20.4±0.606)
9	1403.2 (30.4±1.741)	1179.9 (27.3±2.206)	805.0 (21.3±2.204)	630.8 (18.1±1.243)	920.6 (23.3±3.052)	*	987.9[ab] (24.1±1.212)
12	870.3 (26.5±2.663)	516.0 (19.7±2.019)	499.0 (19.3±1.439)	503.4 (19.4±1.141)	393.5 (16.8±1.176)	499.5 (17.1±0.905)	547.0[c] (19.8±0.791)
24	582.9 (18.9±2.05)	289.2 (11.7±1.111)	361.6 (13.7±0.378)	460.5 (16.2±0.565)	492.3 (16.9±0.935)	565.6 (18.5±4.678)	458.7[d] (16.0±0.921)
Mean	795.0 (23.7±0.962)	645.1 (20.7±0.925)	619.2 (20.6±0.859)	612.7 (20.3±0.738)	626.5 (20.7±0.840)	650.4 (20.1±1.089)	658.1 (21.3±0.367)

	Relative humidity	Time	Interaction
P level	NS	0.01	NS

*treatment not studied **Values with the same superscripts do not differ significantly

*** square root transformed values are given in parenthesis

459.0 was observed. In this case the reduction in GV was equal to 45 per cent, compared to the control.

4.3 Moisture content, leachate conductivity and germination parameters of seeds without seed coat of *Hopea parviflora* as affected by maturity levels, and levels of desiccation for different duration

4.3.1 Moisture content

Significant differences were observed in the moisture content of seeds without seed coat due to the effect of maturity levels ($P<0.01$) and levels of desiccation ($P>0.01$) for different duration ($P<0.01$). The interaction effects were not significant. The data pertaining to this are given in Table 11. The moisture content of seeds without seed coat was significantly higher (47.3 per cent) at seven weeks after anthesis, compared to that at six weeks after anthesis (42.3 per cent).

At both maturity levels, when the seeds without seed coat were desiccated at 20 per cent relative humidity a highly significant reduction in moisture content was observed. On average, this reduction was equal to 52 per cent. But at seven weeks maturity, desiccation at 30 per cent relative humidity also resulted in significant reduction in moisture content. All other treatments were at par.

Largest and highly significant reduction in moisture content due to duration of desiccation was observed when the seeds without seed coat

Table 11. Moisture content (%) of seeds without seed coat of *Hopea parviflora* at six and seven weeks after anthesis as affected by levels of desiccation for different duration

6 weeks after anthesis								7 weeks after anthesis							
Time (hours)	Relative humidity (%)						Mean	Time (hours)	Relative humidity (%)						Mean
	100.0	85.3	75.6	46.6	30.0	20.0			100.0	85.3	75.6	46.6	30.0	20.0	
0	49.5 ±4.243	49.5 ±4.243	49.5 ±4.243	49.5 ±4.243	49.5 ±4.243	36.8 ±0.000	47.4^{abc} ±1.714	0	56.2 ±5.943	56.2 ±5.943	56.2 ±5.943	56.2 ±5.943	56.2 ±5.943	38.4 ±0.000	55.4^{a} ±2.401
3	45.4 ±1.665	47.6 ±2.529	47.5 ±3.085	47.9 ±2.822	44.9 ±3.823	27.8 ±0.000	43.5^{bcd} ±1.435	3	55.1 ±4.279	52.2 ±3.156	52.2 ±3.653	52.0 ±4.748	50.4 ±4.698	31.4 ±0.000	48.9^{abc} ±1.891
6	47.3 ±3.700	46.6 ±1.891	47.5 ±2.684	44.2 ±1.940	40.8 ±3.933	23.6 ±0.000	41.7^{cde} ±1.620	6	56.1 ±4.067	52.7 ±3.623	50.6 ±3.814	50.4 ±3.485	47.1 ±4.412	27.0 ±0.000	47.3^{abc} ±1.982
9	48.9 ±2.014	48.9 ±1.810	49.5 ±1.066	41.9 ±2.621	40.6 ±2.915	*	46.0^{abcd} ±1.328	9	60.7 ±0.685	56.7 ±3.781	54.3 ±0.668	50.4 ±2.293	47.8 ±2.386	*	54.0^{ab} ±1.489
12	49.6 ±1.445	48.6 ±1.882	46.8 ±1.817	36.8 ±1.150	32.6 ±2.403	20.1 ±0.000	39.1^{cde} ±1.967	12	56.8 ±3.644	53.5 ±3.750	52.2 ±2.962	46.9 ±2.937	38.6 ±3.496	24.8 ±0.000	45.4^{abcd} ±2.224
24	49.9 ±0.000	46.6 ±0.000	41.5 ±0.000	38.8 ±0.000	23.2 ±0.000	18.5 ±5.205	36.4^{e} ±0.000	24	51.9 ±0.000	53.3 ±0.000	45.9 ±0.000	42.3 ±0.000	20.8 ±0.000	20.8 ±0.000	39.2^{de} ±6.023
Mean	48.5^{bcd} ±1.166	48.0^{bcd} ±1.053	47.1^{bcd} ±1.179	43.2^{cd} ±1.490	38.6^{d} ±2.080	25.4^{e} ±3.276	42.3 ±0.802	Mean	56.1^{a} ±1.691	54.1^{ab} ±1.636	51.9^{ab} ±1.614	49.7^{abc} ±1.803	43.5^{bcd} ±2.543	28.5^{e} ±3.008	47.3 ±0.992

	Age	Relative humidity	Time	Interaction
P level	0.01	0.01	0.01	NS

*treatment not studied **Values with the same superscripts do not differ significantly

(irrespective of maturity levels) were desiccated for 24 hours. On average, this reduction was equal to 32 per cent, compared to the control.

4.3.2 Leachate conductivity

Significant differences were observed in the leachate conductivity of seeds without seed coat due to the main effects of maturity levels ($P<0.01$) and levels of desiccation ($P<0.01$) for various duration ($P<0.01$). Interaction effects were not significant. The data pertaining to this are given in Table 12. Seeds without seed coat at seven weeks after anthesis had a leachate conductivity of 0.117 mhos cm^{-1}, which was significantly higher than that of seeds without seed coat at six weeks after anthesis. Desiccation of seeds without seed coat at seven weeks maturity at 46.6 per cent relative humidity resulted in largest leachate conductivity of 0.135 mhos cm^{-1} and this was significantly higher compared to that resulted due to desiccation at 20 per cent relative humidity. The reduction in leachate conductivity due to other desiccation levels was not significantly different.

Leachate conductivity increased significantly due to 6 hours of desiccation of seeds without seed coat at six weeks after anthesis. Largest and highly significant increase was observed after 24 hours of desiccation. In this case, there was a three-fold increase, compared to the initial leachate conductivity of 0.045 mhos cm^{-1}. At seven weeks after anthesis, leachate conductivity of seeds without seed coat increased significantly after 12 and 24 hours of desiccation. At

Table 12. Leachate conductivity (mhos cm^{-1}) of seeds without seed coat of *Hopea parviflora* at six and seven weeks after anthesis as affected by levels of desiccation for different duration

6 weeks after anthesis								7 weeks after anthesis							
Time (hours)	Relative humidity (%)						Mean	Time (hours)	Relative humidity (%)						Mean
	100.0	85.3	75.6	46.6	30.0	20.0			100.0	85.3	75.6	46.6	30.0	20.0	
0	0.041 ±0.0098	0.041 ±0.0098	0.041 ±0.0098	0.041 ±0.0098	0.041 ±0.0098	0.069 ±0.0000	0.045^{d} ±0.0040	0	0.087 ±0.0085	0.087 ±0.0085	0.087 ±0.0085	0.087 ±0.0085	0.087 ±0.0085	0.077 ±0.0000	0.085^{bc} ±0.0033
3	0.073 ±0.0301	0.093 ±0.0227	0.088 ±0.0181	0.078 ±0.0204	0.063 ±0.0059	0.077 ±0.0000	0.078^{cd} ±0.0083	3	0.082 ±0.0188	0.126 ±0.0093	0.118 ±0.0069	0.135 ±0.0096	0.129 ±0.0098	0.112 ±0.0000	0.117^{ab} ±0.0060
6	0.087 ±0.0338	0.086 ±0.0296	0.118 ±0.0171	0.093 ±0.0228	0.060 ±0.0039	0.061 ±0.0000	0.084^{bc} ±0.0100	6	0.109 ±0.0187	0.126 ±0.0105	0.135 ±0.0152	0.131 ±0.0099	0.103 ±0.0069	0.070 ±0.0000	0.112^{ab} ±0.0061
9	0.034 ±0.0095	0.047 ±0.0020	0.078 ±0.0035	0.058 ±0.0015	0.057 ±0.0024	*	0.055^{cd} ±0.0043	9	0.123 ±0.0020	0.122 ±0.0104	0.112 ±0.0021	0.124 ±0.0113	0.130 ±0.0134	*	0.122^{ab} ±0.0038
12	0.062 ±0.0431	0.070 ±0.0374	0.107 ±0.0251	0.089 ±0.0259	0.064 ±0.0047	0.046 ±0.0000	0.073^{cd} ±0.0121	12	0.128 ±0.0222	0.124 ±0.0219	0.134 ±0.0143	0.151 ±0.0100	0.121 ±0.0170	0.069 ±0.0000	0.121^{a} ±0.0077
24	0.196 ±0.0000	0.187 ±0.0000	0.180 ±0.0000	0.175 ±0.0000	0.042 ±0.0000	0.039 ±0.0000	0.137^{a} ±0.0305	24	0.209 ±0.0000	0.199 ±0.0000	0.193 ±0.0000	0.181 ±0.0000	0.055 ±0.0000	0.045 ±0.0000	0.147^{a} ±0.0309
Mean	0.082^{cd} ±0.0139	0.087^{cd} ±0.0122	0.102^{abcd} ±0.0101	0.089^{bcd} ±0.0100	0.054^{d} ±0.0031	0.058^{d} ±0.0071	0.079 ±0.0045	Mean	0.123^{abc} ±0.0092	0.131^{ab} ±0.0074	0.130^{ab} ±0.0069	0.135^{a} ±0.0067	0.104^{abc} ±0.0064	0.075^{cd} ±0.0108	0.117 ±0.0033

	Age	Relative humidity	Time	Interaction
P level	0.01	0.01	0.01	NS

*treatment not studied

**Values with the same superscripts do not differ significantly

both maturity levels, the leachate conductivity was highest after desiccating the seeds without seed coat for 24 hours.

4.3.5 Germination parameters

4.3.5.1 Cumulative germination percentage

Seeds without seed coat showed significant differences in cumulative germination percentage due to the effect of levels of desiccation ($P<0.05$) for different duration ($P<0.01$) and their interaction effects ($P<0.01$). Maturity levels had no significant effect. The data pertaining to this are given in Table 13. There was a significant reduction in the germination percentage of seeds without seed coat when they were desiccated for 9 hours at 46.6 per cent relative humidity. However, further increase in the desiccation levels and duration did not affect the germination percentage significantly.

4.3.5.2 Mean Daily Germination (MDG)

Significant differences were found in the MDG of seeds without seed coat due to the effect of maturity levels ($P<0.01$) and duration of desiccation ($P<0.01$). The effects of levels of desiccation and interaction were not significant. The data pertaining to this are given in Table 14. MDG of seeds without seed coat at seven weeks after anthesis was 30.3, which was significantly higher compared to that of seeds without seed coat at six weeks after anthesis. Increasing the duration of desiccation significantly reduced the MDG of seeds without seed coat at both maturity levels. At both maturity levels, the MDG of seeds without seed coat reduced significantly due to 3 hours of desiccation. Further increase in duration of

Table 13. Cumulative germination percentage of seeds without seed coat of *Hopea parviflora* as affected by levels of desiccation for different duration

Time (hours)	Relative humidity (%)						Mean
	100.0	85.3	75.6	46.6	30.0	20.0	
0	96.5^{ab} (4.6 ±0.009)	96.5^{ab} (4.6 ±0.009)	96.5^{ab} (4.6 ±0.009)	96.5^{ab} (4.6 ±0.009)	96.5^{ab} (4.6 ±0.009)	93.9^{abc} (4.6 ±0.000)	96.0^{a} (4.6 ±0.004)
3	96.5^{ab} (4.6 ±0.009)	93.4^{abc} (4.6 ±0.019)	93.4^{abc} (4.6 ±0.019)	96.5^{ab} (4.6 ±0.009)	85.7^{bc} (4.5 ±0.068)	93.9^{abc} (4.6 ±0.000)	93.2^{a} (4.5 ±0.014)
6	96.2^{ab} (4.6 ±0.010)	81.6^{bc} (4.4 ±0.114)	93.4^{abc} (4.6 ±0.019)	91.5^{abc} (4.5 ±0.036)	94.4^{ab} (4.6 ±0.016)	93.9^{abc} (4.6 ±0.000)	91.8^{ab} (4.5 ±0.022)
9	100.0^{a} (4.6 ±0.000)	100.0^{a} (4.6 ±0.000)	93.0^{abc} (4.5 ±0.047)	81.4^{c} (4.4 ±0.102)	100.0^{b} (4.6 ±0.000)	*	95.0^{a} (4.6 ±0.026)
12	93.8^{ab} (4.6 ±0.017)	96.1^{ab} (4.6 ±0.010)	95.6^{ab} (4.6 ±0.012)	89.7^{abc} (4.5 ±0.022)	93.6^{abc} (4.6 ±0.017)	91.5^{abc} (4.5 ±0.007)	93.4^{a} (4.5 ±0.007)
24	91.1^{abc} (4.5 ±0.019)	91.1^{abc} (4.5 ±0.019)	86.3^{bc} (4.5 ±0.022)	88.9^{bc} (4.5 ±0.024)	87.6^{bc} (4.6 ±0.027)	88.4^{bc} (4.5 ±0.022)	88.9^{b} (4.5 ±0.009)
Mean	95.7^{a} (4.6 ±0.006)	93.2^{ab} (4.5 ±0.024)	93.0^{ab} (4.5 ±0.008)	90.8^{b} (4.5 ±0.013)	93.0^{ab} (4.5 ±0.016)	92.3^{b} (4.5 ±0.006)	93.1 (4.5 ±0.006)

	Relative humidity	Time	Interaction
P level	0.05	0.01	0.01

*treatment not studied **Values with the same superscripts do not differ significantly

*** log transformed values are given in parenthesis

Table 14. Mean Daily Germination of seeds without seed coat of *Hopea parviflora* at six and seven weeks after anthesis as affected by levels of desiccation for different duration

6 weeks after anthesis								7 weeks after anthesis							
Time (hours)	Relative humidity (%)						Mean	Time (hours)	Relative humidity (%)						Mean
	100.0	85.3	75.6	46.6	30.0	20.0			100.0	85.3	75.6	46.6	30.0	20.0	
0	40.5 ±3.374	40.5 ±3.374	40.5 ±3.374	40.5 ±3.374	40.5 ±3.374	41.7 ±4.821	40.7[a] ±1.343	0	39.3 ±5.051	39.3 ±5.051	39.3 ±5.051	39.3 ±5.051	39.3 ±5.051	50 ±0.000	41.1[b] ±1.973
3	22.9 ±2.074	26.2 ±2.823	21.7 ±2.543	23.9 ±2.838	26.0 ±4.190	32.1 ±6.574	25.5[bc] ±1.353	3	32.1 ±3.366	33.5 ±4.804	29.5 ±3.994	28.6 ±1.677	23.8 ±3.831	27.1 ±2.075	29.1[b] ±1.495
6	23.7 ±1.813	25.9 ±2.079	23.6 ±0.922	30.7 ±3.942	22.1 ±1.010	41.7 ±4.821	27.9[bc] ±1.328	6	29.7 ±1.677	27.1 ±5.617	31.7 ±3.617	29.8 ±4.104	28.6 ±1.677	29.2 ±2.396	29.3[b] ±1.415
9	21.7 ±1.667	33.3 ±8.333	19.4 ±3.389	14.8 ±1.009	21.7 ±1.667	*	22.2[c] ±2.275	9	30.5 ±2.767	33.3 ±0.000	33.3 ±0.000	16.7 ±4.410	29.8 ±10.578	*	28.7[b] ±2.590
12	28.9 ±4.157	22.9 ±1.093	28.8 ±4.059	24.8 ±3.317	26.7 ±2.166	29.8 ±2.547	27.0[bc] ±1.281	12	34.7 ±2.707	28.6 ±1.677	28.6 ±1.677	25.0 ±1.703	26.3 ±1.935	23.1 ±0.625	27.7[bc] ±0.947
24	19.3 ±1.974	19.0 ±0.577	25.4 ±2.571	20.9 ±1.625	20.2 ±2.323	26.5 ±1.536	21.9[c] ±0.917	24	27.1 ±2.075	29.2 ±2.396	19.6 ±1.068	24.4 ±0.625	23.1 ±1.197	27.7 ±2.432	25.2[bc] ±0.926
Mean	26.1 ±1.690	28.0 ±1.638	26.6 ±1.683	25.9 ±1.837	26.2 ±1.652	34.3 ±2.272	27.8 ±0.728	Mean	32.3 ±1.480	31.8 ±1.884	30.3 ±1.714	27.3 ±1.724	28.5 ±1.780	31.4 ±2.296	30.3 ±0.729

	Age	Relative humidity	Time	Interaction
P level	0.01	NS	0.01	NS

*treatment not studied **Values with the same superscripts do not differ significantly

desiccation did not reduce the MDG very significantly in both cases. The reduction in MDG was equal to 46 per cent in the case of six weeks mature seeds without seed coat when they were desiccated for 24 hours. In the same way, there was 39 per cent decline in MDG of seeds without seed coat at seven weeks after anthesis after 24 hours of desiccation.

4.3.5.3 Peak Value (PV)

Levels of desiccation ($P < 0.01$) for different duration ($P < 0.01$) significantly affected PV of germination of seeds without seed coat. Effects of maturity levels and interaction were not significant. The data pertaining to this are given in Table 15. PV of germination was significantly reduced due to desiccation at 46.6 and 30 per cent relative humidities. The effects of other desiccation levels were not significantly different. PV was highest (45.5) in the case of seeds without seed coat not subjected to any desiccation treatment. This reduced significantly after 3 hours of desiccation, which was equal to 35 per cent, compared to the control. Desiccating for 24 hours resulted in 43 per cent reduction in PV.

4.3.5.4 Germination Value (GV)

Significant differences were found in the GV of seeds without seed coat due to the effect of levels of desiccation ($P<0.05$) for different duration ($P<0.01$). The effect of maturity levels and interaction effects were not significant. The data pertaining to this are given in Table 16. Significant reduction in GV was found when the seeds without seed coat were desiccated at 46.6 and 30 per cent relative humidities, compared to other levels of desiccation. The effects of other levels of desiccation were at par. Desiccating for 6 hours significantly reduced the

Table 15. Peak Value of germination of seeds without seed coat of *Hopea parviflora* as affected by levels of desiccation for different duration

Time (hours)	Relative humidity (%)						Mean
	100.0	85.3	75.6	46.6	30.0	20.0	
0	44.9 ±3.287	44.9 ±3.287	44.9 ±3.287	44.9 ±3.287	44.9 ±3.287	48.5 ±2.628	45.5^{a} ±1.310
3	28.9 ±2.254	32.8 ±3.450	27.9 ±2.525	26.5 ±1.535	24.2 ±2.242	37.1 ±4.592	29.6^{bc} ±1.149
6	29.0 ±1.079	31.4 ±4.713	31.1 ±2.227	34.1 ±3.818	29.7 ±1.787	42.3 ±6.092	32.9^{b} ±1.389
9	30.6 ±2.774	35.3 ±3.561	27.8 ±4.007	16.0 ±2.048	28.1 ±4.562	*	27.5^{bc} ±1.874
12	33.4 ±2.634	27.7 ±1.251	33.0 ±2.891	25.0 ±1.737	31.0 ±1.662	29.8 ±2.581	30.0^{bc} ±0.932
24	24.5 ±1.880	25.0 ±1.962	25.0 ±1.545	28.4 ±2.628	23.1 ±0.847	29.3 ±1.532	25.9^{c} ±0.770
Mean	31.9^{ab} ±1.274	32.9^{ab} ±1.576	31.6^{ab} ±1.382	29.2^{b} ±1.517	30.2^{b} ±1.331	37.4^{a} ±2.014	31.9 ±0.610

	Relative humidity	Time	Interaction
P level	0.01	0.01	NS

*treatment not studied **Values with the same superscripts do not differ significantly

Table 16. Germination Value of seeds without seed coat of *Hopea parviflora* as affected by levels of desiccation for different duration

Time (hours)	Relative humidity (%)						Mean
	100.0	85.3	75.6	46.6	30.0	20.0	
0	2252.1 (42.2 ±2.922)	2252.1 (42.2 ±2.922)	2252.1 (42.2 ±2.922)	2252.1 (42.2 ±2.922)	2252.1 (42.2 ±2.922)	3177.0 (47.1 ±2.542)	2406.3[a] (43.0 ±1.176)
3	1120.3 (28.2 ±2.252)	1335.2 (31.2 ±3.105)	1034.8 (26.9 ±2.484)	1026.0 (26.7 ±1.637)	836.0 (23.6 ±2.077)	1764.1 (32.7 ±3.255)	1186.0[ab] (28.2 ±1.024)
6	1148.7 (28.6 ±1.576)	1160.9 (28.8 ±3.652)	1205.3 (29.4 ±2.108)	1397.3 (32.1 ±3.197)	1085.0 (27.6 ±1.398)	2285.0 (38.5 ±4.433)	1380.4[b] (30.8 ±1.138)
9	1630.0 (28.0 ±2.044)	2171.8 (34.3 ±3.594)	1551.9 (27.0 ±3.686)	795.8 (15.9 ±2.053)	1527.6 (26.7 ±4.787)	*	1535.4[ab] (26.4 ±1.791)
12	1602.5 (34.7 ±3.086)	1023.6 (26.7 ±1.220)	1294.3 (30.7 ±2.414)	890.1 (24.5 ±1.690)	1104.9 (27.9 ±1.354)	1428.3 (28.5 ±2.256)	1223.9[ab] (28.9 ±0.919)
24	1093.1 (23.8 ±1.908)	1139.8 (24.5 ±2.081)	1082.6 (23.6 ±1.445)	1162.7 (24.8 ±1.791)	1000.1 (22.4 ±1.087)	1354.7 (27.5 ±1.428)	1138.9[c] (24.4 ±0.681)
Mean	1474.4[ab] (30.9 ±1.249)	1513.9[ab] (31.3 ±1.372)	1403.5[ab] (30.0 ±1.256)	1254.0[b] (27.7 ±1.350)	1301.0[b] (28.4 ±1.207)	2001.8[a] (34.9 ±1.707)	1478.5 (30.3 ±0.549)

	Relative humidity	Time	Interaction
P level	0.05	0.01	NS

*treatment not studied **Values with the same superscripts do not differ significantly

*** square root transformed values are given in parenthesis

GV compared to the control. In this case the reduction in GV was equal to 43 per cent. Largest and highly significant reduction in GV was observed after 24 hours of desiccation. In this case, the reduction in GV was equal to 53 per cent, compared to the control.

4.4 Moisture content, leachate conductivity and germination parameters of excised embryonic axes of *Hopea parviflora* as affected by maturity levels, and levels of desiccation for different duration

4.4.1 Moisture content

Significant reduction was observed in the moisture content of embryonic axes due to the effect of levels of desiccation ($P<0.01$) for different duration ($P<0.05$). Maturity levels and interaction effects were not significant. The data pertaining to this are given in Table 17. Embryonic axes kept at 100 per cent relative humidity had moisture content of 52.9 per cent. Desiccation of embryos at 85.3 and 75.6 per cent relative humidities did not reduce the moisture content significantly. A significant reduction in moisture content was observed when the embryos were desiccated at 46.6 per cent relative humidity. In this case the moisture content declined by 16 per cent, compared to the control. The reduction in moisture content due to desiccation at 30 and 20 per cent relative humidities were also significant. Largest and highly significant reduction in moisture content was observed when the embryos were rapidly desiccated at 20 per cent relative humidity. In this case, the decline was equal to 44 per cent, compared to the control.

Table 17. Moisture content (%) of excised embryonic axes of *Hopea parviflora* as affected by levels of desiccation for different duration.

Time (hours)	Relative humidity (%)						Mean
	100	85.3	75.6	46.6	30	20	
0	53.2 ±3.381	53.2 ±3.381	53.2 ±3.381	53.2 ±3.381	38.7 ±0.630	38.7 ±0.630	48.4[a] ±1.621
3	51.6 ±2.424	50.8 ±2.236	48.2 ±2.049	45.5 ±2.164	37.6 ±1.110	32.9 ±2.390	44.4[ab] ±1.260
6	54.2 ±1.661	49.5 ±2.731	41.5 ±4.860	37.3 ±4.480	33.9 ±0.135	30.9 ±0.990	41.2[b] ±2.000
12	52.3 ±2.560	49.1 ±3.770	48.9 ±3.575	43.0 ±0.095	30.3 ±2.450	22.8 ±1.230	41.1[b] ±3.371
24	53.0 ±2.900	51.9 ±2.660	51.3 ±2.455	42.7 ±2.825	31.6 ±7.585	22.8 ±1.240	42.2[b] ±3.639
Mean	52.9[a] ±1.250	50.9[ab] ±1.384	48.6[ab] ±1.929	44.4[b] ±2.017	34.4[c] ±1.630	29.6[c] ±2.105	43.5 ±0.942

	Relative humidity	Time	Interaction
P level	0.01	0.05	NS

*treatment not studied

**Values with the same superscripts do not differ significantly

Desiccating the embryos for 3 hours did not reduce the moisture content significantly. However, 6, 9 and 12 hours of desiccation resulted in significant reduction. The reduction in moisture content due to 6 hours of desiccation was equal to 15 per cent, compared to the control. Increasing the duration beyond 6 hours did not reduce the moisture content significantly.

4.4.2 Leachate conductivity

Significant differences were found in the leachate conductivity of embryonic axes due to duration of desiccation ($P<0.01$) and interaction ($P<0.01$) of the main effects. Maturity levels and levels of desiccation had no significant effect. The data pertaining to this are given in Table 18. In general, the leachate conductivity of embryos kept at 100, 85.3, 75.6 and 46.6 per cent relative humidities increased significantly after 12 hours of desiccation. On average, an eight-fold increase was observed in the leachate conductivity in all these cases. The increase in leachate conductivity due to 24 hours of desiccation was not significantly different from that of 12 hours of duration. Leachate conductivity was not significantly affected by increasing the duration of desiccation at 30 and 20 per cent relative humidities.

4.4.5 Germination parameters

4.4.5.1 Cumulative germination percentage

Significant differences were observed in the germination percentage of embryonic axes due to levels of desiccation ($P<0.01$) for different duration ($P<0.01$) and their interaction ($P<0.05$). The effect of maturity levels was not significant. The data pertaining to this are given in Table 19. Keeping the

Table 18. Leachate conductivity (mhos cm^{-1}) of excised embryonic axes of *Hopea parviflora* as affected by levels of desiccation for different duration.

Time (hours)	Relative humidity (%)						Mean
	100	85.3	75.6	46.6	30	20	
0	0.023^{f} ±0.0097	0.023^{f} ±0.0097	0.023^{f} ±0.0097	0.023^{f} ±0.0097	0.067^{bcdef} ±0.0095	0.067^{bcdef} ±0.0095	0.037^{b} ±0.0047
3	0.041^{def} ±0.0225	0.043^{def} ±0.0220	0.042^{def} ±0.0199	0.037^{ef} ±0.0178	0.107^{abcdef} ±0.0045	0.101^{abcdef} ±0.0055	0.062^{b} ±0.0093
6	0.045^{def} ±0.0254	0.049^{def} ±0.0260	0.051^{cdef} ±0.0253	0.042^{def} ±0.0216	0.094^{abcdef} ±0.0030	0.083^{abcdef} ±0.0005	0.061^{b} ±0.0106
12	0.194^{ab} ±0.0045	0.189^{ab} ±0.0090	0.180^{abc} ±0.0065	0.162^{abcde} ±0.0075	0.077^{abcdef} ±0.0050	0.064^{bcdef} ±0.0000	0.144^{a} ±0.0161
24	0.202^{a} ±0.0075	0.194^{ab} ±0.0090	0.189^{ab} ±0.0100	0.172^{abcd} ±0.0005	0.066^{bcdef} ±0.0070	0.046^{def} ±0.0000	0.145^{a} ±0.0193
Mean	0.101 ±0.0146	0.100 ±0.0143	0.097 ±0.0136	0.087 ±0.0121	0.082 ±0.0057	0.072 ±0.0063	0.090 ±0.0058

	Relative humidity	Time	Interaction
P level	NS	0.01	0.01

**Values with the same superscripts do not differ significantly

Table 19. Cumulative germination percentage of excised embryonic axes of *Hopea parviflora* as affected by levels of desiccation for different duration

Time (hours)	Relative humidity (%)						Mean
	100.0	85.3	75.6	46.6	30.0	20.0	
0	87.6^{a} (4.5 ±0.033)	87.6^{a} (4.5 ±0.033)	87.6^{a} (4.5 ±0.033)	87.6^{a} (4.5 ±0.033)	85.5^{a} (4.4 ±0.041)	85.5^{a} (4.4 ±0.041)	86.9^{a} (4.4 ±0.014)
3	74.3^{ab} (4.3 ±0.059)	61.8^{abc} (4.1 ±0.132)	23.4^{cdef} (3.1 ±0.469)	13.8^{ef} (2.6 ±0.546)	39.6^{abcde} (3.6 ±0.153)	16.5^{def} (2.8 ±0.240)	38.2^{b} (3.4 ±0.162)
6	62.0^{abc} (4.1 ±0.106)	27.3^{bcdef} (3.3 ±0.479)	14.0^{ef} (2.6 ±0.550)	10.9^{f} (2.4 ±0.574)	21.1^{cdef} (3.0 ±0.247)	14.0^{ef} (2.6 ±0.248)	24.9^{bc} (3.0 ±0.194)
12	46.9^{abcd} (3.8 ±0.131)	46.9^{abcd} (3.8 ±0.131)	44.6^{abcd} (3.8 ±0.110)	34.6^{abcde} (3.5 ±0.123)	14.0^{ef} (2.6 ±0.248)	10.1^{f} (2.3 ±0.215)	32.8^{bc} (3.3 ±0.112)
24	22.9^{cdef} (3.1 ±0.272)	22.9^{cdef} (3.1 ±0.272)	24.8^{cdef} (3.2 ±0.195)	16.5^{def} (2.8 ±0.240)	10.1^{f} (2.3 ±0.215)	10.1^{f} (2.3 ±0.215)	17.9^{c} (2.8 ±0.107)
Mean	58.7^{a} (4.0 ±0.075)	49.3^{ab} (3.8 ±0.140)	38.9^{abc} (3.4 ±0.194)	32.7^{c} (3.1 ±0.218)	34.0^{bc} (3.2 ±0.148)	27.2^{c} (2.9 ±0.155)	40.1 (3.4 ±0.071)

	Relative humidity	Time	Interaction
P level	0.01	0.01	0.05

* log transformed values are given in parenthesis

**Values with the same superscripts do not differ significantly

embryonic axes at 100 per cent relative humidity decreased the germination percentage only after 24 hours. Compared to the control, this decline was equal to 74 per cent. At 85.3 per cent relative humidity, even 6 hours of desiccation reduced the germination percentage significantly. In this case, the reduction was equal to 69 per cent, compared to the control. The decline in germination percentage due to 24 hours of desiccation at 85.3 per cent relative humidity was equal to that at 100 per cent relative humidity. Significant reduction in germination percentage was observed due to 3 hours of desiccation at 75.6, 46.6 and 20 per cent relative humidities. These desiccation levels resulted in highly significant reduction in germination percentage with increasing duration. Largest and highly significant reduction was observed due to 12 hours of desiccation at 20 per cent relative humidity and 24 hours of desiccation at 30 and 20 per cent relative humidities. These desiccation treatments resulted in the lowest germination percentage of 10.1. This decline was equal to 88 per cent, compared to the control.

4.4.5.4 Germination Value

Germination value of embryonic axes was significantly affected by the effect of levels of desiccation ($P<0.01$) for different duration ($P<0.01$) and their interaction ($P<0.05$). The effect of maturity levels was not significant. The data pertaining to this are given in Table 20. Keeping the embryos at 100 per cent relative humidity for 12 hours resulted in significant reduction in GV. Highly significant reduction was observed after 24 hours. This reduction was equal to 64 per cent, compared to the control. GV was significantly reduced due to 6 hours of desiccation at 85.3 per cent relative humidity. Significant reduction was also observed after 24 hours of desiccation. Compared to the control, this decline was

Table 20. Germination Value of excised embryonic axes of *Hopea parviflora* as affected by levels of desiccation for different duration

Time (hours)	Relative humidity (%)						Mean
	100.0	85.3	75.6	46.6	30.0	20.0	
0	248.8^{a} (12.8 ±0.582)	248.8^{a} (12.8 ±0.582)	248.8^{a} (12.8 ±0.582)	248.8^{a} (12.2 ±0.870)	307.4^{a} (12.2 ±0.518)	307.4^{a} (12.3 ±0.518)	265.5^{a} (12.5 ±0.265)
3	226.1^{a} (12.0 ±0.966)	172.2^{ab} (10.1 ±1.012)	95.9^{bcdefg} (6.8 ±1.352)	74.4^{defgh} (5.6 ±1.301)	131.9^{defgh} (6.2 ±0.899)	62.7^{hi} (2.6 ±0.763)	127.2^{b} (7.2 ±0.579)
6	164.9^{abc} (9.8 ±0.835)	123.0^{bcd} (8.1 ±1.319)	82.6^{defgh} (6.1 ±1.417)	89.9^{cdefg} (6.5 ±1.579)	76.2^{ghi} (3.5 ±0.834)	53.6^{i} (2.0 ±0.763)	98.4^{bc} (6.0 ±0.587)
12	172.8^{bcd} (7.9 ±0.932)	165.3^{bcde} (7.6 ±0.793)	155.0^{bcdef} (7.2 ±0.642)	122.3^{defgh} (5.8 ±0.759)	51.5^{i} (1.9 ±0.706)	38.5^{i} (0.9 ±0.584)	117.6^{c} (5.2 ±0.496)
24	89.9^{efghi} (4.2 ±1.134)	87.3^{efghi} (4.1 ±1.118)	84.9^{fghi} (3.9 ±0.700)	77.5^{ghi} (3.5 ±1.244)	40.4^{i} (1.1 ±0.682)	38.5^{i} (0.9 ±0.584)	69.7^{d} (3.0 ±0.418)
Mean	180.5^{a} (9.3 ±0.536)	159.3^{ab} (8.5 ±0.579)	133.4^{ab} (7.3 ±0.635)	119.1^{bc} (6.7 ±0.682)	121.5^{cd} (5.0 ±0.718)	100.2^{d} (3.8 ±0.741)	135.7 (6.8 ±0.286)

	Relative humidity	Time	Interaction
P level	0.01	0.01	0.05

* square root transformed values are given in parenthesis

**Values with the same superscripts do not differ significantly

equal to 65 per cent. Significant reduction in GV was also observed due to 3 hours duration when the embryos were desiccated at 75.6, 46.6, 30 and 20 per cent relative humidities. Desiccating the embryos at these relative humidities for increasing duration resulted in significant reduction in GV. Embryos desiccated at 20 per cent relative humidity for 12 hours showed the largest and highly significant reduction in GV. In this case, the reduction in GV was equal to 88 per cent, compared to the control. Increasing the duration beyond 12 hours resulted in no further significant reduction in GV.

4.5 Germination parameters of synthetic seeds as affected by levels of desiccation for different duration

4.5.1 Cumulative germination percentage

Levels of desiccation ($P<0.01$) of the embryonic axes for different duration ($P<0.01$) had significant effect on the germination percentage of synthetic seeds. Maturity levels of the embryos and interaction effects were not significant. The data pertaining to this are as shown in Fig. 2. Synthetic seeds had a germination of 66.1 percentage when they were developed from embryos kept at 100 per cent relative humidity. Significant reduction of 18 per cent was observed when the embryos were desiccated at 46.6 per cent relative humidity. Synthetic seeds had a germination percentage of 70.2 per cent when the embryos were not subjected to any desiccation treatment. Subjecting the embryos to three hours of desiccation resulted in significant reduction in germination percentage of the synthetic seeds. This reduction was equal to 19 per cent, compared to the control.

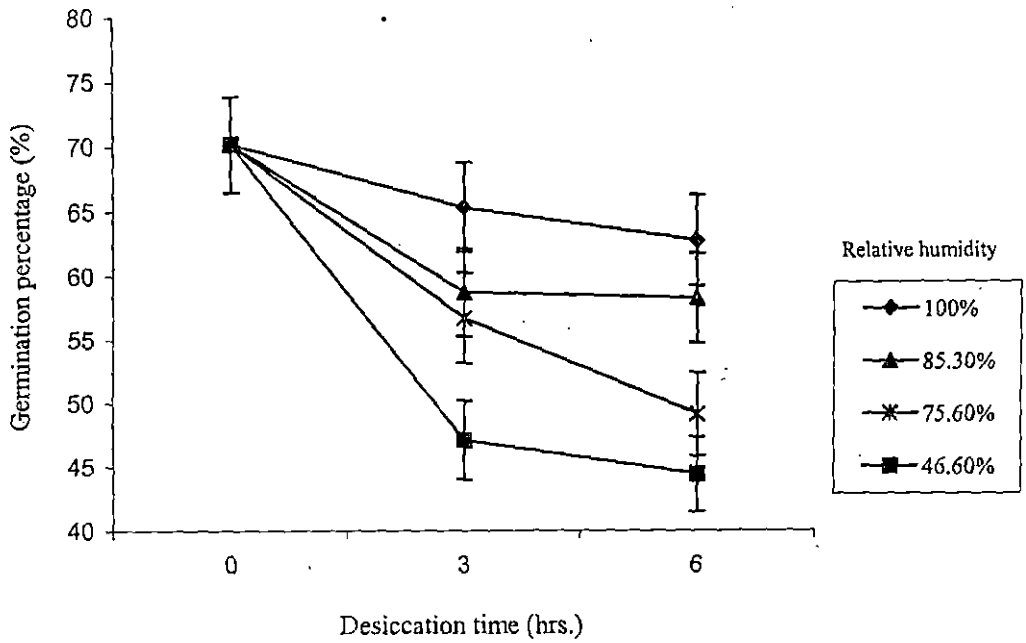


Fig. 2. Cumulative germination percentage of synthetic seeds of *Hopea parviflora* as affected by desiccation of embryos for different duration

Further reduction in germination percentage of synthetic seeds due to six hours of desiccation of the embryos was not significant.

4.5.2 Germination Value (GV)

GV of synthetic seeds was significantly affected by maturity levels ($P<0.01$) and levels of desiccation ($P<0.01$) of the embryonic axes for different duration ($P<0.01$). Interaction effects were not significant. The data pertaining to this are as shown in Fig. 3 and 4. Significantly higher GV of 79.8 was observed in the case of synthetic seeds developed from six weeks mature embryos. At seven weeks after anthesis, the GV was only 55.0

GV of synthetic seeds was largest (93.7) when they were developed from six weeks mature embryos kept at 100 per cent relative humidity. This reduced significantly to 65.4 due to desiccation of the embryos at 46.6 per cent relative humidity. Compared to the control, this reduction was equal to 30 per cent. At seven weeks after anthesis, the reduction in GV due to levels of desiccation was not significant.

GV of synthetic seeds developed from fresh embryos at six weeks after anthesis was 114.4. In this case, significant reduction in GV was observed due to three hours of desiccation. This reduction was equal to 42 per cent, compared to the control. The largest and highly significant reduction in GV of synthetic seeds was observed at seven weeks after anthesis due to six hours of desiccation of the excised embryonic axes. This reduction was equal to 59 per cent, compared to the control.

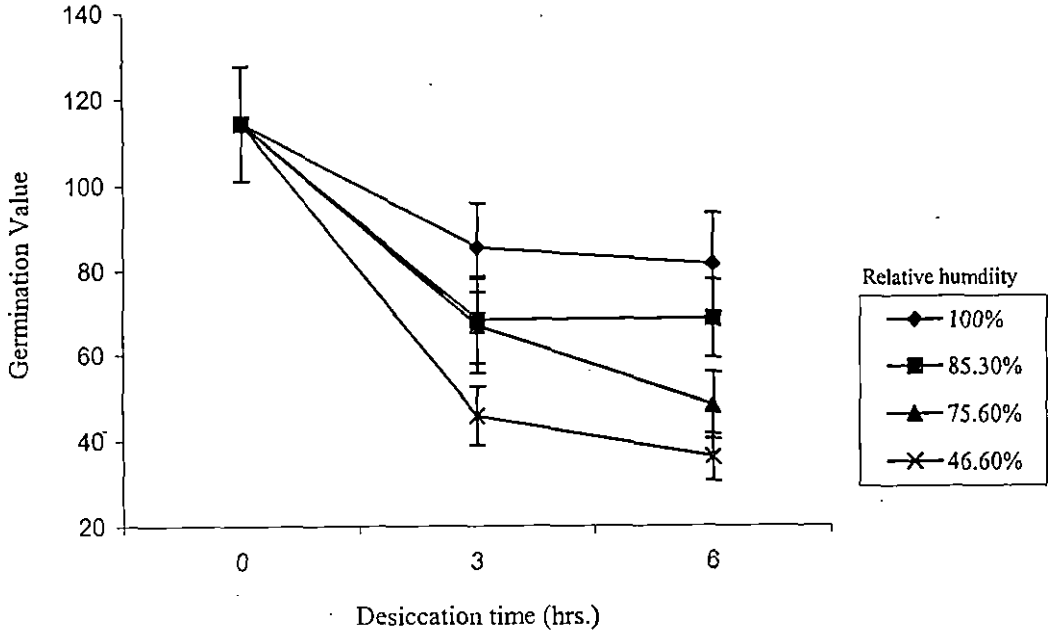


Fig. 3. Germination Value of synthetic seeds of *Hopea parviflora* as affected by levels and duration of desiccation of embryos at six weeks after anthesis

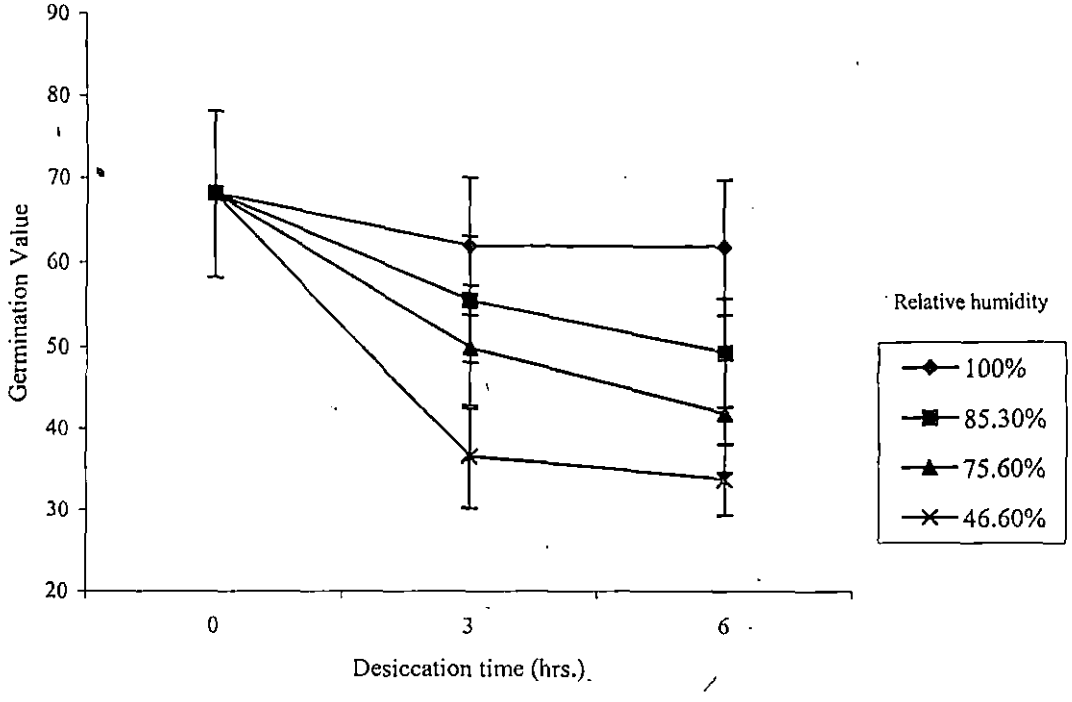


Fig. 4. Germination Value of synthetic seeds of *Hopea parviflora* as affected by levels and duration of desiccation of embryos at seven weeks after anthesis

4.6 Germination parameters of synthetic seeds as affected by storage at different temperatures for different periods

4.6.1 Cumulative germination percentage

Cumulative germination percentage of synthetic seeds was significantly affected by maturity levels ($P<0.01$) of the embryonic axes, storage of the synthetic seeds at different temperatures ($P<0.01$) for different periods ($P<0.01$) and their interaction ($P<0.01$). The data pertaining to this are as shown in Fig. 5 and 6. Significantly higher germination of 60.6 per cent was observed in the case of synthetic seeds developed from six weeks mature embryos. The germination percentage of synthetic seeds at seven weeks after anthesis was only 58.9 per cent. At six weeks after anthesis, fresh synthetic seeds had a germination of 87.4 per cent. In this case, storage at different temperatures resulted in significant reduction in germination percentage after two weeks. Largest reduction was observed due to four weeks of storage. This decrease was equal to 38 per cent and 65 per cent respectively, compared to the control. When stored at 27°C, the synthetic seeds at six weeks after anthesis failed to germinate completely after fours weeks of storage.

At seven weeks after anthesis, fresh synthetic seeds gave a germination of 94.3 per cent. When stored at 4°C, significant reduction was observed after three weeks of storage. But, storage at 20°C and 27°C resulted in significant reduction due to two weeks of storage itself. In all the cases, largest and highly significant reduction was observed due to four weeks of storage. However, storage

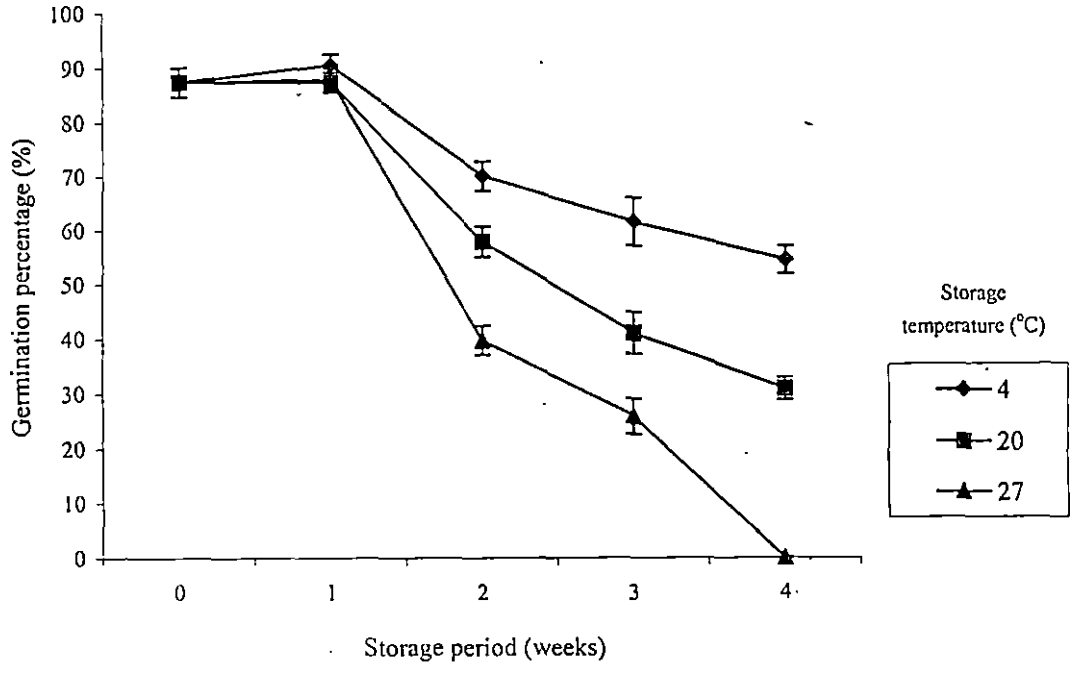


Fig. 5. Cumulative germination percentage of synthetic seeds at six weeks after anthesis of *Hopea parviflora* as affected by their storage at different temperatures for different periods

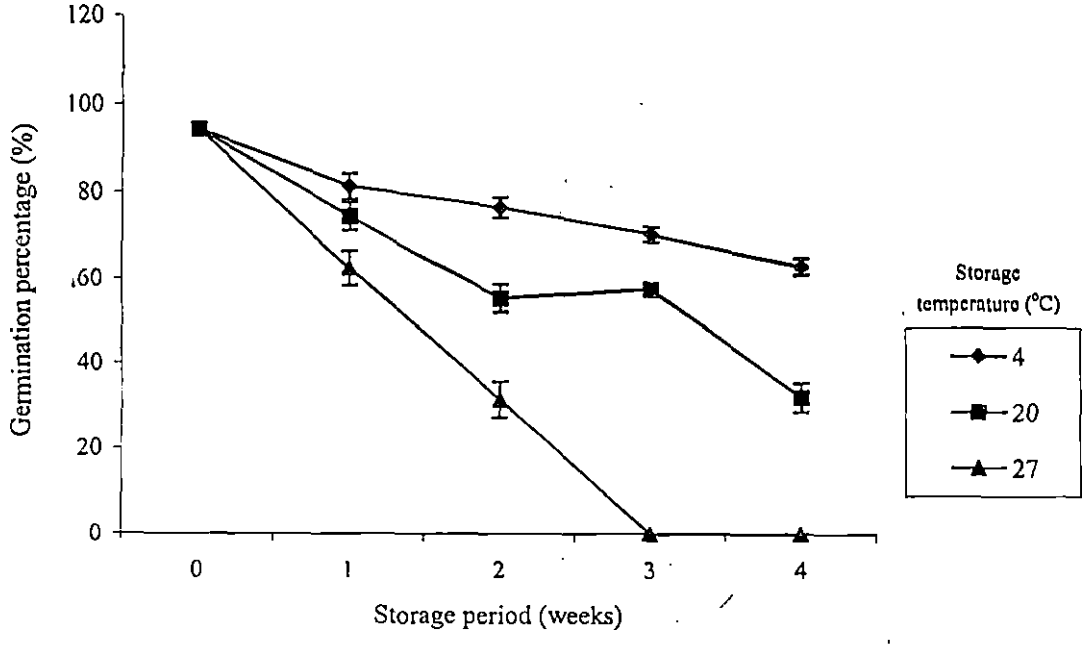


Fig. 6. Cumulative germination percentage of synthetic seeds at seven weeks after anthesis of *Hopea parviflora* as affected by their storage at different temperatures for different periods

at 27°C resulted in complete failure of germination due to three weeks of storage itself.

4.6.2 Germination Value (GV)

Significant differences were observed in the GV of synthetic seeds due to the effect of maturity levels ($P<0.01$) of the embryonic axes, storage of the synthetic seeds at different temperatures ($P<0.01$) for different periods ($P<0.01$) and their interaction ($P<0.01$). The data pertaining to this are as shown in Fig. 7 and 8. GV of synthetic seeds at six weeks after anthesis was 87.4, which was significantly higher than the GV (68.6) of synthetic seeds at seven weeks after anthesis.

Fresh synthetic seeds had a GV of 137.3 at six weeks after anthesis. When stored at 4°C, the GV decreased gradually and significantly after two weeks of storage. Further storage also reduced the GV significantly and the largest reduction was observed after four weeks of storage. In this case, 64 per cent reduction was observed, compared to the control. Storage at 20°C and 27°C resulted in a significant increase in GV after one week of storage. However, this reduced with increasing periods of storage. In both cases, largest reduction was observed due to four weeks of storage. At 20°C, this reduction was equal to 85 per cent, compared to the control. As none of the synthetic seeds stored at 27°C germinated after four weeks of storage, the GV was zero.

At seven weeks after anthesis, fresh synthetic seeds had a GV of 143.1. At all temperatures, storage for one resulted in significant reduction in GV of

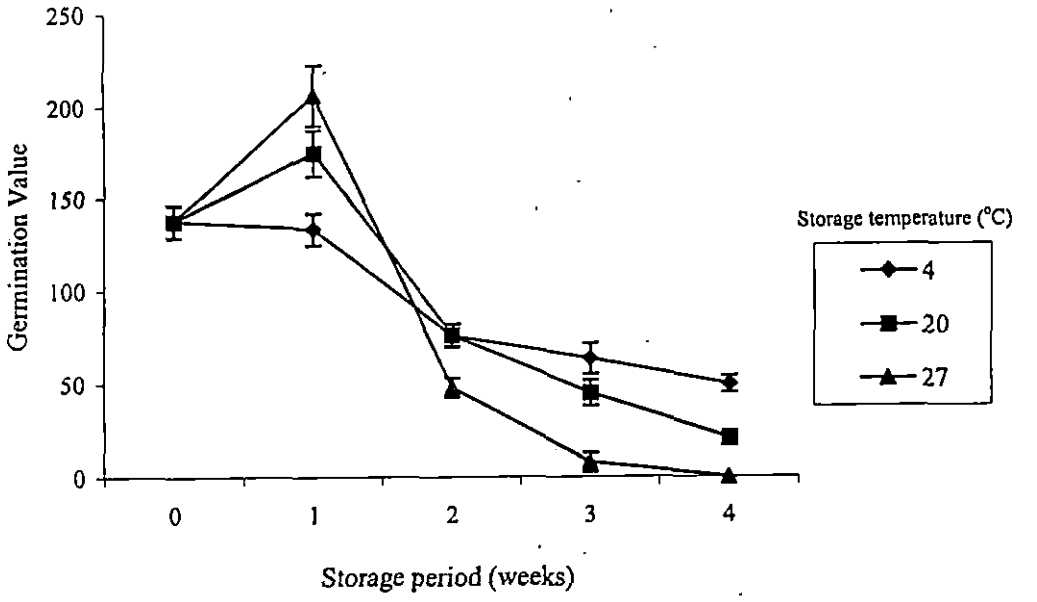


Fig. 7. Germination Value of synthetic seeds at six weeks after anthesis of *Hopea parviflora* as affected by their storage at different temperatures for different periods

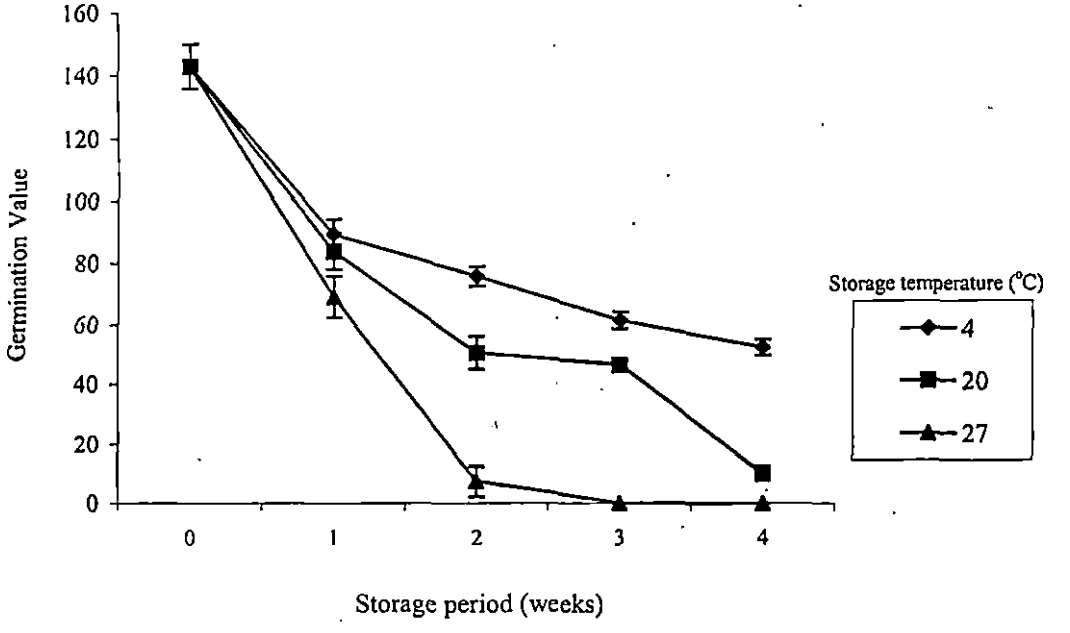


Fig. 8. Germination Value of synthetic seeds at seven weeks after anthesis of *Hopea parviflora* as affected by their storage at different temperatures for different periods

synthetic seeds. In this case also, largest reduction was observed due to four weeks of storage at all temperatures. But, when stored at 27°C for three weeks, the GV came down to zero as all the synthetic seeds stored at this temperature failed to germinate.

4.7 Germination parameters as affected by cryopreservation

Cryopreservation studies were carried out using intact seeds, seeds without seed coat and excised embryonic axes of *Hopea parviflora* at six and seven weeks after anthesis after subjecting them to different levels and duration of desiccation. The synthetic seeds developed from the embryonic axes subjected to the best desiccation treatment were also cryopreserved. Both direct plunging of the propagules into liquid nitrogen and slow freezing them in a programmable biofreezer were tried. The propagules were pretreated with DMSO (Di-Methyl Sulfoxide) prior to subjecting them to cryopreservation.

After cryopreservation and thawing, the propagules were inoculated on to the recovery medium to study their regeneration potential. But seeds, seeds without seed coat, embryonic axes and synthetic seeds of *Hopea parviflora*, failed to regenerate after cryopreservation. However, the intact seeds and seeds without seed coat retained their green colour for two to three days after which they turned brown, showing no signs of viability.

Discussion

DISCUSSION

The present investigation was carried out to find out the possibility of storing the intact seeds, seeds without seed coat or excised embryonic axes of *Hopea parviflora* Bedd. using cryopreservation techniques. As a prelude, attempt was made to find out the best desiccation treatment by applying different levels of desiccation for different duration on intact seeds, seeds without seed coat and excised embryonic axes at six and seven weeks after anthesis. The study also aimed at developing synthetic seeds, finding out their best storage temperature and exploring the possibilities of their cryopreservation. The results obtained are discussed in this chapter.

5.1 Flowering and fruiting

The year 1999 was not at all a seed year as far as *Hopea* trees were concerned. The unavailability of seeds during this year made it impossible to do the research work and hence the completion of the work was delayed by one year. The year 1998 was a good seed year in this species. Troup (1921) observed that in *Hopea parviflora*, intensive seeding occurs once in four to six years and such years are followed by one or two years of comparative sterility and same number of moderate seed years. The periodicity of flowering was not the same in 2000 and 2001. Sunilkumar and Sudhakara (1998) also noticed irregularity in the periodicity of flowering in *Hopea parviflora* trees.

5.2 Effect of maturity levels and levels of desiccation for various duration on germination parameters of seeds, seeds without seed coat and embryonic axes of *Hopea parviflora* Bedd.

The initial moisture content of intact seeds collected from *Hopea parviflora* trees was 49.8 per cent. There was no significant difference in moisture content due to the maturity levels of the seeds. Sunilkumar and Sudhakara (1998) observed 40.8 per cent moisture content in the seeds of *Hopea parviflora* after natural shedding. They also noticed that the manually shed seeds had slightly higher moisture content (41.7%). The initial moisture content of seeds without seed coat at six and seven weeks after anthesis was 47.4 and 55.4 per cent respectively. Excised embryonic axes had initial moisture content of 48.4 per cent. But, Sunilkumar and Sudhakara (1998) observed highest moisture content of 68.5 per cent in the embryonic axis of *Hopea parviflora* seeds. The reduced moisture content observed in the present study compared to the previous studies may be due to seasonal variations. Variation in axis water content has been recorded both intra- and inter-seasonally (Berjak *et al.*, 1989; Berjak *et al.*, 1993; Finch-Savage and Blake, 1994; Finch-Savage, 1996). Higher moisture content of embryonic axes has also been reported in other recalcitrant seeds viz., *Avicennia marina*: 63 per cent (Berjak *et al.*, 1984), *Azadirachta indica*: 91 per cent (Maithani *et al.*, 1989) and *Artocarpus heterophyllus*: 68 per cent (Fu *et al.*, 1993) also. Chin (1991) reported that initial moisture content of recalcitrant seeds might range from 30 to 70 per cent (on wet weight basis).

The moisture content of intact seeds and excised embryonic axes decreased significantly due to desiccation at 46.6 per cent relative humidity. The decreasing trend was further magnified at 30 and 20 per cent relative humidities. The moisture content did not show any significant change when kept at 100, 85.3 and 75.6 per cent relative humidities. The moisture content of the seeds and embryonic axes also decreased due to desiccation for six hours and beyond. Largest reduction was observed due to 24 hours of desiccation. At both maturity levels, when the seeds without seed coat were desiccated at 20 per cent relative humidity, a highly significant reduction in moisture content was observed. At seven weeks maturity, desiccation at 30 per cent relative humidity also resulted in significant reduction in moisture content. Irrespective of maturity levels, the moisture content of seeds without seed coat decreased due to 24 hours of desiccation. Seed moisture content has been considered as the most important factor controlling physiological reactions in seeds (Fang and Moore, 1998). They also reported that seed moisture content increased with increase in relative humidity and decreased with increase in time; but relative humidity had the greater influence

Critical moisture content levels vary with the method of drying (Farrant *et al.*, 1985; Pritchard, 1991). The values of the lowest safe moisture content vary between extremes of 23 per cent for cocoa (*Theobroma cacao*) (Mumford and Brett, 1982) and 61.4 per cent for *Avicennia marina* (Farrant *et al.*, 1993a). Critical moisture levels have been postulated for germination metabolism, germination and continued embryogenesis and the cessation of growth and cell division (Palit, 1987; Mc Intyre, 1987; Adams and Rinne, 1980). In the present

study, rapid drying did not have much deleterious effect on the germination parameters of seeds/ seeds without seed coat. There are many reports on the advantage of rapid drying over slow drying which would help surpass desiccation injuries if any, inflicted due to the loss of structural water from the cells. Bonner (1996) suggested that the rate of desiccation could influence the expression of recalcitrant behaviour in seeds. But in the case of embryonic axes, reduction in moisture content as evidenced from the results of our study had a deleterious effect on the germination parameters, especially when they were subjected to rapid drying. It was also observed that the moisture content of the embryonic axes desiccated at 20 per cent relative humidity for 24 hours decreased from 38.7 per cent to 22.8 per cent. At this stage, germination percentage reduced from 85.5 per cent to 10.1 per cent. This indicates that the embryonic axes could still survive some more degree of desiccation for longer duration, as the lowest safe moisture content has not yet been reached. However, results were not significantly different. Chandel *et al.* (1995) reported that in recalcitrant seeds of tea (*Camellia sinensis* L.), cocoa (*Theobroma cacao* L.) and jackfruit (*Artocarpus heterophyllus* L.), fully mature seeds survived desiccation to 24, 35 and 31 per cent moisture content respectively. Lin-Tsan and Wu (1995) reported that *Michelia compressa* seeds could survive desiccation to moisture content as low as 10 per cent (fresh weight basis). Recalcitrant seed species will, sooner or later, die under storage conditions that do not permit any significant degree of water loss (Pammenter *et al.*, 1994). When the seeds of *Shorea almon*, *S. robusta* and *S. roxburghii* were stored above 40 per cent moisture content, problem in germination was encountered (Tompsett, 1985). The exact causes of recalcitrant seed death could be due either to the moisture content falling below a certain critical value or simply

a general physiological deterioration with time and that a number of factors may contribute to viability loss.

In the present study, drying rate was found to have little influence on the germination parameters of intact seeds and seeds without seed coat as compared to embryonic axes. In the case of embryonic axes, the germination reduced by 88 per cent due to rapid drying at 20 per cent relative humidity. But intact seeds and seeds without seed coat maintained more than 90 per cent germination even when rapidly desiccated at 20 per cent relative humidity. The effect of drying rate on intact seeds and seeds without seed coat was less marked than for excised axes. This may be simply because intact seeds and seeds without seed coat are too large to dry sufficiently rapidly for the effect to become pronounced, but Finch-Savage (1992a) has pointed out the possibility that the removal of the axis from cotyledonary material may influence the response of the isolated axis to dehydration.

Leachate conductivity was not significantly different at six and seven weeks after anthesis in the case of intact seeds and excised embryonic axes. However, the seeds without seed coat showed significant difference in leachate conductivity due to maturity levels. The initial leachate conductivity of seeds without seed coat at six weeks after anthesis was 0.045 mhos cm^{-1} and that at seven weeks after anthesis was 0.085 mhos cm^{-1}.

Intact seeds showed significant difference in leachate conductivity due to duration of desiccation only. A significant increase was observed only after 24 hours of desiccation. Six weeks mature seeds without seed coat also registered

significant difference in leachate conductivity after 24 hours of desiccation. But seven weeks mature seeds without seed coat showed significant increase in leachate conductivity after 12 hours of desiccation itself. Leachate conductivity of excised axes also increased after 12 hours of desiccation. Keeping in different relative humidities did not show any significant increase in leachate conductivity of six weeks mature seeds without seed coat. However, the leachate conductivity of seven weeks mature seeds without seed coat increased when desiccated at 46.6 per cent relative humidity. At both maturity levels, leachate conductivity of seeds without seed coat desiccated at 30 and 20 per cent relative humidities was lowest when compared to that at other desiccation levels. The leachate conductivity of seeds/seeds without seed coat/embryonic axes increased significantly after 24 hours of desiccation. Although this did not affect the germination of intact seeds and seeds without seed coat, a drastic reduction in germination was found in the case of embryonic axes. However, Bonner (1996) reported that leachate conductivity was not at all a sensitive indicator of loss of viability. The presence of soluble sugars does not adequately explain the nature of desiccation tolerance in recalcitrant seeds (Sun *et al.*, 1994). Hence the increase in leachate conductivity, at least in seeds without seed coat when kept at relative humidities from 100 to 46.6 per cent, i.e., before effecting significant desiccation may not be connected to loss of viability at all.

Germination parameters of the embryonic axes were significantly reduced due to different levels of desiccation for various duration. When the seeds/ seeds without seed coat were rehydrated at this stage to study germination, the membrane repair mechanisms might have been fully activated to ward off

deleterious effects, if any, inflicted by the water stress due to desiccation. Vertucci *et al.* (1994) reported that detrimental effects during storage under dry conditions were not initially evident, but became more apparent with time. So it may be assumed here that cellular membrane disruption might have occurred with increasing duration of desiccation levels in embryonic axes unlike in the case of seeds/ seeds without seed coat. This may be the reason behind the low germination percentage of embryonic axes as against the comparatively good germination percentage obtained in the case of seeds/ seeds without seed coat. Song *et al.* (1984) reported in *Hopea hainanensis* that desiccation up to 31 per cent moisture content disturbed the ribosomes and endoplasmic reticulum that were reversed on hydration. As per the present study, moisture content of the seeds/ seeds without seed coat/ embryonic axes act as an indicator of seed germination rather than leachate conductivity. The inverse relation between leachate conductivity and viability has proven useful with a number of agricultural and tree species (Perry, 1981; Bonner and Agmata-Paliwal, 1992), but all were orthodox in storage behaviour. Desiccation tolerance as per them is a complex phenomenon, possibly requiring the interaction of several biochemical processes.

Germination percentage of intact seeds collected at six and seven weeks after anthesis was 96.0. Seeds without seed coat and embryonic axes did not show any difference in germination percentage due to maturity levels and had a germination of 96.0 and 86.9 per cent respectively. Germination percentage of intact seeds and seeds without seed coat exhibited decreased significantly when they were desiccated at higher desiccation levels of 30 and 20 per cent relative humidities. Germination percentage of intact seeds at six weeks after anthesis

decreased significantly due to desiccation at 30 and 20 per cent relative humidities. However, intact seeds and seeds without seed coat at seven weeks after anthesis showed significant reduction due to desiccation at 20 per cent relative humidity only. In the present study, germination percentage of six weeks mature intact seeds decreased significantly after six hours of desiccation itself. However, desiccation for 24 hours only reduced the germination percentage of seven weeks mature seeds and seeds without seed coat significantly. Sunilkumar (1996) reported that the moisture content at which seeds are stored is an important factor that determines the viability of stored seeds. Storing seeds below 30 per cent moisture content either under different temperatures or in different media did not enhance the seed longevity whereas the seeds collected before the initiation of any germination related events retained higher germination percentage and seed vigour when they were dried to 37 per cent moisture content and stored under low temperature.

In the case of embryonic axes, significant reduction in germination percentage was observed when kept at 100 and 85.3 per cent relative humidities for 24 hours duration. But at lower relative humidities, significant decrease in germination percentage was observed after three hours of desiccation itself. The decrease was tremendous beyond this. Embryonic axes are delicate structures and more sensitive to desiccation than other propagules. At partially dehydrated state, seeds may exhibit mild water stress upsetting the relative rate of individual reactions associated with germination (Vertucci, 1993). However, the membrane disruption would not have been so drastic as to cause severe viability loss. The propagules might have been subjected to rapid drying while being kept at lower

levels of relative humidities like 30 and 20 percent. There are several reports that rapid drying will not reduce germination parameters. Due to the very rapid drying of excised axes the duration of aqueous-based deleterious reactions is minimized (Pammenter *et al.*, 1991; Berjak and Pammenter, 1997; Pritchard, 1991; Pammenter and Berjak, 1999). But in the present study, it was seen that rapid drying at 20 per cent relative humidity for 12 hours drastically reduced the germination percentage of excised axes by about 88 per cent. The moisture content of these excised axes was just below 23 per cent. No matter how rapidly desiccation-sensitive tissue is dried, there is a lower limit, below which it cannot survive, which is always higher than the water content to which orthodox and intermediate seeds can be dried. In many instances it is close to, but not lower than, the water content at which the remaining water is non-freezable (Pammenter *et al.*, 1991; Berjak *et al.*, 1993; Pammenter *et al.*, 1993; Pritchard, 1991), which led to the suggestion that truly recalcitrant seeds cannot survive the removal of any structure-associated water. However, there are instances of axes from recalcitrant seeds surviving to water contents in the range 0.11 to 0.16 g g^{-1}, (Fu *et al.*, 1990; Chaudhury *et al.*, 1991; Kioko *et al.*, 1998), which are probably lower than the level of non-freezable water. Several scientists have proposed rapid drying as one of the methods to reduce desiccation injury. The more rapidly the dehydration can be achieved, the lower is the water content to which seeds or axes can be dried, without damage accumulation that culminates in viability loss. This is particularly the case when excised axes are dried (Normah *et al.*, 1986; Berjak *et al.*, 1989). The rate of drying also depends on the amount of seed (particularly the depth of the layer of seeds), the circulation of dry air within the drying cabinet and the species (Hong and Ellis, 1996). Because embryos from recalcitrant seeds are always

metabolically active and never achieve the competence for complete desiccation, this type of damage probably occurs during dehydration unless drying is extremely rapid (Pammenter *et al.*, 1991). Hunter (1959) found that a rate of drying of 5 per cent loss in moisture content per day is apparently no more deleterious than a rate of 1.9 per cent per day, and the viability of seed dried to 16.7 per cent moisture content was the same for both treatments. But in the present study, about 16 per cent loss in moisture content was observed after 24 hours of rapid drying in the case of embryonic axes. This might have resulted in severe desiccation injuries to the embryos. Bonner (1996) suggested that the rate of desiccation could influence the expression of recalcitrant behaviour in seeds.

In the case of embryonic axes, rapid decline in germination percentage might have been caused by the progressive cellular deterioration when kept at decreasing relative humidity levels with increasing duration. The deteriorative changes due to water stress have been reported by Pammenter *et al.* (1994), the possible process being: reduced rates of protein synthesis, increased proteolysis and variable effects on the catabolic activity of different enzymes. Removal of structural water from the cells in embryos might have happened on dehydration to a greater magnitude than that happened in seeds/ seeds without seed coat. This might have resulted in minimum values as shown in vigour parameters of embryonic axes especially at 12 and 24 hours duration at all relative humidities. Drying embryos may result in the interruption of metabolism, which becomes lethal, possibly because of the build up of high energy intermediates of metabolism that have toxic effects (Mc Kersie *et al.*, 1988; Le Prince *et al.*, 1992). The result obtained in the case of embryonic axes may bank on this finding also.

In terms of vigour parameters of propagules, after being kept at different relative humidities, trend was similar to the germination percentage. Mean daily germination (MDG) of intact seeds showed significant difference due to maturity levels and was affected by duration of desiccation also. The initial MDG of intact seeds at six and seven weeks after anthesis was 19.5 and 23.8 respectively. MDG of seven weeks mature seeds reduced significantly due to desiccation for 24 hours only. But in the case of six weeks mature seeds, the MDG exhibited significant decrease even after 3 hours of desiccation. The reduction in MDG was more in the case of six weeks mature seeds compared to that of seven weeks mature seeds. Seeds without seed coat at six and seven weeks after anthesis had an initial MDG of 40.7 and 41.1 respectively. In this case, significant reduction in MDG was observed after three hours of desiccation itself and decreased with increasing duration at both maturity levels. Peak values of germination of intact seeds and seeds without seed coat did not show any significant difference due to maturity levels. In general, highest peak values of germination were observed in seeds without seed coat at both weeks and the lowest in embryonic axes. Drying/ desiccation to extremely low water contents may shorten seed longevity (Walters and Engels, 1998). More over, the embryonic axes are more delicate structures and are sensitive to desiccation. This may be the reason for the lowest peak values of germination recorded in the case of embryonic axes rapidly dried at 30 and 20 per cent relative humidities for maximum durations of time.

Germination value (GV) of intact seeds/ seeds without seed coat/ embryonic axes did not show any significant difference due to maturity levels.

Intact seeds had an initial GV of 832.5. Only duration of desiccation significantly affected the GV of intact seeds. Significant reduction in GV was observed after three hours of desiccation itself. In the case of seeds without seed coat, the initial GV was 2406.3. In this case, a significant reduction was observed after six hours of desiccation only. The lower GV observed in the case of intact seeds might be due to the presence of impermeable seed coat. There are reports that the testa may delay germination in dried seeds. Cohen (1958) and Mumford and Grout (1979) suggested that the germination of lemon seed (*Citrus limon* L.) was improved when the testa was removed and they produced evidence to prove that the testa of lemon seeds has a marked deleterious effect on the seed's response to desiccation. Hong and Ellis (1996) suggested that removal of seed covering structures, filing or chipping seeds with a scalpel, or nicking with a needle might help to promote germination during prolonged tests. When kept at 100 per cent relative humidity, the embryonic axes showed significant reduction in GV after 12 hours and when desiccated at 85.3 per cent relative humidity, the reduction was significant after six hours of desiccation itself. At relative humidities ranging from 75.6 to 20 per cent, the GV registered significant reduction after three hours of desiccation itself. Higher values were recorded in the case of seeds without seed coat and lower in the case of embryonic axes. The embryos might have aged more rapidly under extremely dry conditions. This may be a probable reason for the decrease in vigour parameters when desiccated for longer duration under higher desiccation levels.

Seeds/ seeds without seed coat collected at seven weeks after anthesis registered higher germination parameters by and large compared to that obtained at

six weeks after anthesis in the whole study. But the trend was not much evident in the case of embryonic axes. Reports are available about higher germination vigour parameters in the case of seeds, which are more mature. Complete maturation is required for *Acer platanoides* to survive complete desiccation (Hong and Ellis, 1992). Recalcitrant seeds may become increasingly tolerant of drying as maturation proceeds (Berjak *et al.*, 1992; Finch-Savage, 1992b), even if they remain hydrated and metabolically active throughout development (Berjak *et al.*, 1992; Farrant *et al.*, 1992). The performance of the propagules of seventh week over that of sixth week, even though of lesser magnitude may bank on this finding. Recalcitrant seeds appear to initiate germination-related metabolism shortly after shedding (Farrant *et al.*, 1988; Berjak *et al.*, 1989). Sunilkumar and Sudhakara (1998) also observed vivipary in *Hopea parviflora* on natural shedding which coincides with the monsoon showers. At the early stages of development, embryos are extremely sensitive to dehydration stress (Rogerson and Matthews, 1977).

5.3 Effect of desiccation of embryonic axes on germination parameters of synthetic seeds

Synthetic seeds developed from fresh embryonic axes had an initial germination of 70.2 per cent. However, they did not show any significant difference in germination percentage due to maturity levels of the embryonic axes. When the embryos were kept at 100 per cent relative humidity prior to encapsulation, synthetic seeds had a germination of 66.1 percentage. Significant reduction was observed when the embryos were desiccated at 46.6 per cent relative humidity. Desiccation of embryos for three hours also resulted in significant

reduction in germination percentage of the synthetic seeds. Desiccated polyox-encapsulated somatic embryos of carrot showed a one to four per cent survival rate, but hardening treatments increased mean survival of desiccated, encapsulated embryos to 20 per cent (Kitto and Janick, 1985).

Vigour parameters showed similar trend as that of germination percentage. Maturity levels of the embryonic axes affected the GV significantly and a GV of 79.8 and 55.0 were observed at six and seven weeks after anthesis respectively. The GV showed significant reduction due to levels of desiccation at six weeks after anthesis only. Keeping the embryos at 46.6 per cent relative humidity only significantly reduced the GV. Synthetic seeds developed from fresh embryos at six and seven weeks after anthesis had GV of 114.4 and 84.1 respectively. GV of synthetic seeds developed from six weeks mature embryos decreased due to six hours of desiccation only. But the GV of synthetic seeds developed from seven weeks mature embryos decreased due to desiccation for three hours of itself.

5.4 Effect of storage on germination parameters of synthetic seeds

Results of the present study indicated that synthetic seeds produced by encapsulation of excised embryos retained their viability to a great extent and was dependent on storage temperature. Synthetic seeds developed from excised embryos at six and seven weeks after anthesis and stored at 4°C retained 54.3 and 62.3 per cent viability respectively after four weeks of storage. This is against an initial viability of 87.4 and 94.3 per cent respectively exhibited by fresh synthetic seeds. Sunilkumar (1996) found that the synthetic seeds had high

germination percentage compared to that of fresh intact seeds. Similar high germination percentage of synthetic seeds was also reported in cocoa when they were stored at 10°C (Sudhakara *et al.*, 2000). Sunilkumar *et al.* (2000) reported that synthetic seeds retained 100 per cent germination when stored at 10°C up to two weeks and 90 per cent germination when stored up to four weeks. They also found that synthetic seeds stored at 4°C had lower germination percentage than those stored at 10°C. Storage at room temperature (27±2°C) for one week drastically reduced the germination percentage; beyond this period, complete mortality was observed. But in the present study, the synthetic seeds stored at 27°C lost their viability completely after three weeks of storage only. The desiccation of the embryos to lower moisture contents before encapsulation might have helped the synthetic seeds retain their viability for more time.

Synthetic seeds obtained from embryos at six and seven weeks after anthesis which were stored at 4°C showed a significant reduction in germination percentage after two and three weeks respectively. The germination percentage of synthetic seeds developed from six week mature embryos decreased significantly after two weeks of storage at all the temperatures. But in the case of synthetic seeds of seven weeks mature embryos, storage at 20°C and 27°C resulted in significant reduction in germination percentage after one week itself. The germination percentage decreased drastically with increasing periods of storage. The reduced germination percentage may be associated with the delayed initiation of germination showing overall decline in vigour. The reduction in germination percentage indicates that low temperature around and below 4°C will cause

damages to synthetic seeds in long run. Such low temperature dehydration has been reported to cause rapid decline in viability of excised embryos of *Zizania palustris* (Berjak *et al.*, 1993). Recalcitrant seeds are generally sensitive to low temperature storage at higher moisture content (Hendry, 1993). Sunilkumar *et al.* (2000) achieved successful production of synthetic seed with high germination percentage in comparison to that of fresh intact seeds. The failure in further development of plantlets from synthetic seed may be due to the problems associated with storage or growth conditions. Gupta and Durzan (1987) encapsulated somatic embryos of loblolly pine in alginate. But conversion of embryo into seedling was not achieved after storing at low temperature (4°C) for four months. However, successful development of plantlets was reported by Redenbaugh *et al.* (1986) with encapsulated alfalfa and celery somatic embryos using sodium alginate. This technique was successfully applied for the encapsulation of axillary and apical shoot buds of *Valeriana wallichii* and *Picorrhiza kurroa*. Occurrence of germination-associated events during storage indicates the necessity of making the embryonic axes dormant for long term storage. Dormancy and desiccation tolerance may be imparted to the embryos by treating them with appropriate concentration of ABA or high sucrose concentrations (Anandarajah and Mc Kersie, 1990; Liu *et al.*, 1992). So the production of synthetic seeds by the method adopted in this study can be used for exploiting the possibilities of long term storage of synthetic seeds but the storage techniques and growth conditions have to be perfected.

5.5 Effect of cryopreservation on the viability of intact seeds, seeds without seed coat, embryonic axes and synthetic seeds

Successful cryopreservation requires the optimization of numerous variables including the size of the specimen, the correct type and concentration of cryoprotectant, sample water content and rate of freezing and thawing (Normah *et al.*, 1986). There are many reports on the advantage of rapid drying over slow drying which would help surpass desiccation injuries if any, inflicted due to the loss of structural water from the cells. Several scientists have proposed rapid drying as one of the methods to reduce desiccation injury. Bonner (1996) suggested that the rate of desiccation could influence the expression of recalcitrant behaviour in seeds. So rapid drying and ultra rapid drying can be used as a pre-treatment for the cryopreservation techniques. Results of the present study showed that the seeds/ seeds without seed coat/ embryonic axes/ synthetic seeds of *Hopea parviflora* at six and seven weeks after anthesis failed miserably to regenerate following cryopreservation techniques. Berjak *et al.* (1995) reported that in hydrated seeds of *Azadirachta indica*, in response to chilling, a decline in viability was accompanied by structural degeneration: mitochondria and plastids in axis cells lost internal organization and vacuoles generally collapsed. Areas of advanced degradation also occurred, comprising cells in which the plasmalemma was discontinuous and vesiculated.

Immature embryos are said to be more adaptable to manipulation than mature embryos or embryo axes. This is because of their smaller size than mature embryos that allows more uniform cryoprotection, cooling, and thawing in hydrated freezing procedures and more uniform desiccation in dry/ freezing

procedures (Withers, 1979). Immature embryos are also highly embryogenic than mature embryos (Pence, 1991). But in the present study, both six and seven weeks mature embryos behaved the same way after storing them in liquid nitrogen.

Cryopreservation techniques provide the potential for indefinite preservation by reducing metabolism to such a low level (Ashwood-Smith and Farrant, 1980) that all biochemical processes are significantly reduced and biological deterioration virtually stopped. The longevity of seeds or the maintenance of seed viability is a balance between extrinsic and intrinsic deleterious factors and repair or protective mechanisms. Deleterious factors may include depletion of essential metabolites, denaturation of macromolecules, accumulation of toxic metabolites, attack by microorganisms and insects and effects of ionizing radiation (Osborn, 1977, 1980).

In the present study, the propagules, especially seeds and seeds without seed coat at six and seven weeks after anthesis survived various levels of desiccation for different durations but they altogether failed to overcome cryopreservation both in terms of direct plunging and slow freezing techniques. Chandel *et al.* (1995) studied the desiccation and freezing sensitivity in recalcitrant seeds of tea (*Camellia sinensis* L.), cocoa (*Theobroma cacao* L.) and jackfruit (*Artocarpus heterophyllus* L.). Fully mature seeds of tea, cocoa and jack fruit survived desiccation to 24, 35 and 31 per cent moisture content respectively, but at these moisture levels seeds were not able to tolerate freezing in liquid nitrogen. This finding is having a bearing on the result obtained in the present study. Biochemical investigations on fully mature axes after freezing showed that the decline in viability with moisture level was associated with increased leachate

conductivity, lipid peroxidation products and/or soluble carbohydrates (Chandel *et. al* 1995). Seeds/ seeds without seed coat were able to retain their green colour for two to three days after cryopreservation; there exists a ray of hope in achieving success in long term storage of *Hopea parviflora* seeds using this highly promising treatment. But very soon, the whole tissues turned dark brown due to oxidation of phenolics released from the cells. In this context, use of antioxidants during thawing and regeneration stage assumes significance. The type and concentration of cryoprotectant and freezing rate are also of great significance. In general, a fixed concentration of 5 to 10 per cent DMSO appears to be optimal for the tissue survival of low temperatures. But for the maintenance of large structures, it may be necessary to alter the concentration of the cryoprotectant as the temperature is reduced (Chin and Roberts, 1980). Different protocols may be formulated and tried for cryopreservation studies to overcome chilling injury. Different types of propagules of *Hopea parviflora* may also be tried. Low relative humidities and different rates of drying under extreme aseptic conditions should be tried as drying rate is having a pivotal role in enabling the tissues to stand reduced moisture contents and subsequent shocks due to water stress.

Summary

SUMMARY

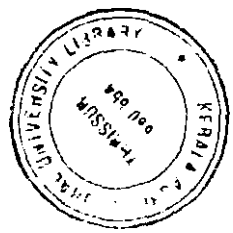
The present investigations were undertaken at the College of Forestry, Vellanikkara to develop storage techniques for *Hopea parviflora* Bedd. species using intact seeds/ seeds without seed coat/ excised embryonic axes/ synthetic seeds through cryopreservation. The salient findings are summarized below.

1. Moisture content of the propagules decreased with increase in intensity and duration of desiccation levels. Moisture content of intact seeds and excised embryonic axes was not significantly different at six and seven weeks after anthesis. However, the seeds without seed coat showed significant difference in moisture content due to maturity levels.
2. Leachate conductivity was not significantly affected due to maturity levels in the case of intact seeds and excised embryonic axes. But seeds without seed coat showed significant difference in leachate conductivity due to maturity levels and it was higher at seven weeks after anthesis. Increasing the duration of desiccation significantly increased the leachate conductivity of intact seeds/ seeds without seed coat/ embryonic axes. The leachate conductivity of the propagules was higher at lower desiccation levels. But when rapidly dried at 30 and 20 per cent relative humidities it finally decreased.
3. Germination percentage of the intact seeds was significantly higher at seven weeks after anthesis. But seeds without seed coat and embryonic axes did not show any significant difference due to maturity levels. Germination parameters of the seeds and seeds without seed coat was not affected much when kept at different relative humidity levels for different duration in both weeks' study. But in the case of embryonic axes germination parameters were

significantly reduced when rapidly dried at 30 and 20 per cent relative humidities for different duration.

4. Rapid drying had little influence on the germination parameters of intact seeds and seeds without seed coat. However, the germination percentage of embryonic axes was drastically reduced due to rapid drying at 30 and 20 per cent relative humidities.
5. Synthetic seeds stored at 4°C retained higher germination percentage compared to those stored at 20 and 27°C after four weeks. The viability of synthetic seeds stored at 27°C declined rapidly and none of them retained their viability after 3 weeks of storage.
6. After the cryopreservation studies i.e., both direct plunging and slow freezing techniques, the propagules invariably failed to regenerate. Chilling injury of the propagules on exposure to liquid nitrogen might have led to the loss of viability. However, the seeds and seeds without seed coat retained their green colour for two to three days before turning to dark brown. Implicit in this is that different rate of drying, different protocols of cryopreservation, concentration of cryoprotectant and freezing rate might help to achieve success in the long run.
7. Reduction in moisture content observed due to different treatments could be correlated to the loss of viability whereas the leachate conductivity was found to be a poor indicator. Desiccating the seeds and seeds without seed coat at different relative humidities for different durations did not reduce the germination parameters by and large. However, germination parameters of embryonic axes were declined drastically due to rapid drying at 30 and 20 per

cent relative humidities. Different rates of drying, different protocols of cryopreservation, concentration of cryoprotectant and freezing rate may be tried to achieve success in the case of cryopreservation.



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* Originals not seen

Plates

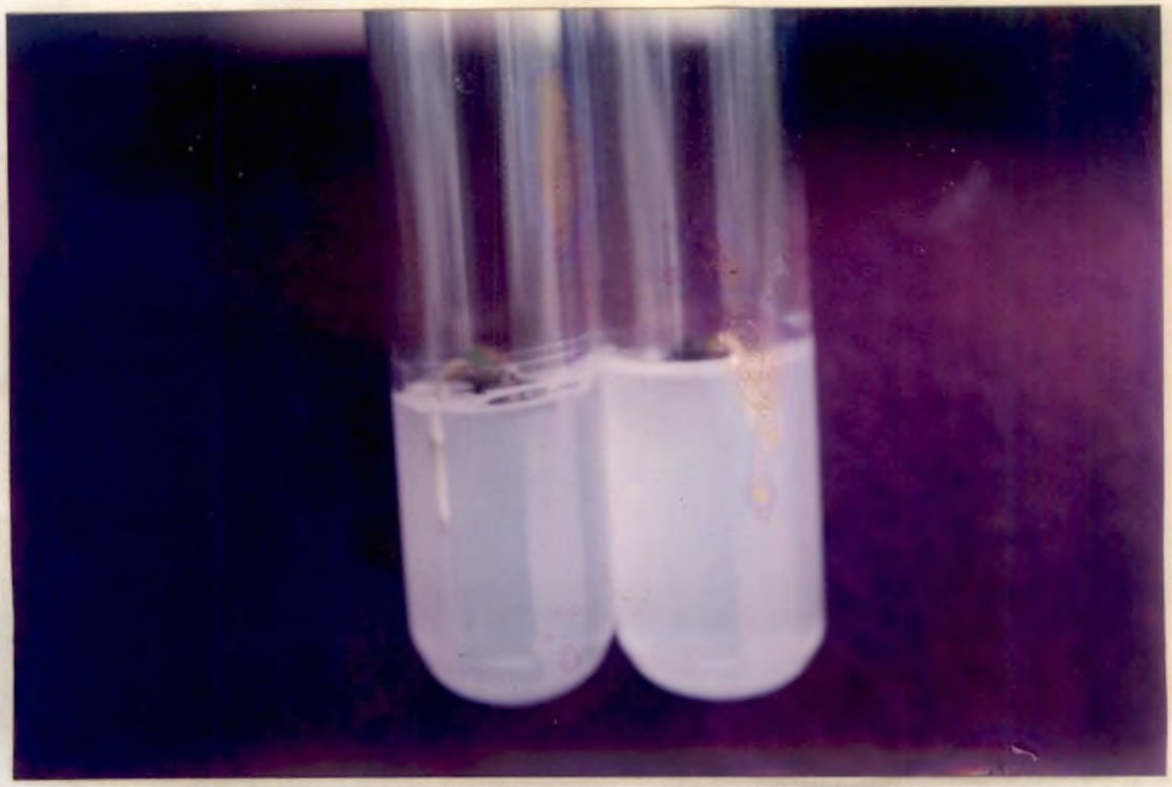

Plate 1. Intact seeds of *Hopea parviflora* at six weeks after anthesis germinating in MS medium - 2 DAS

Plate 2. Excised embryonic axes of *Hopea parviflora* at six weeks after anthesis germinating in MS medium - 21 DAS

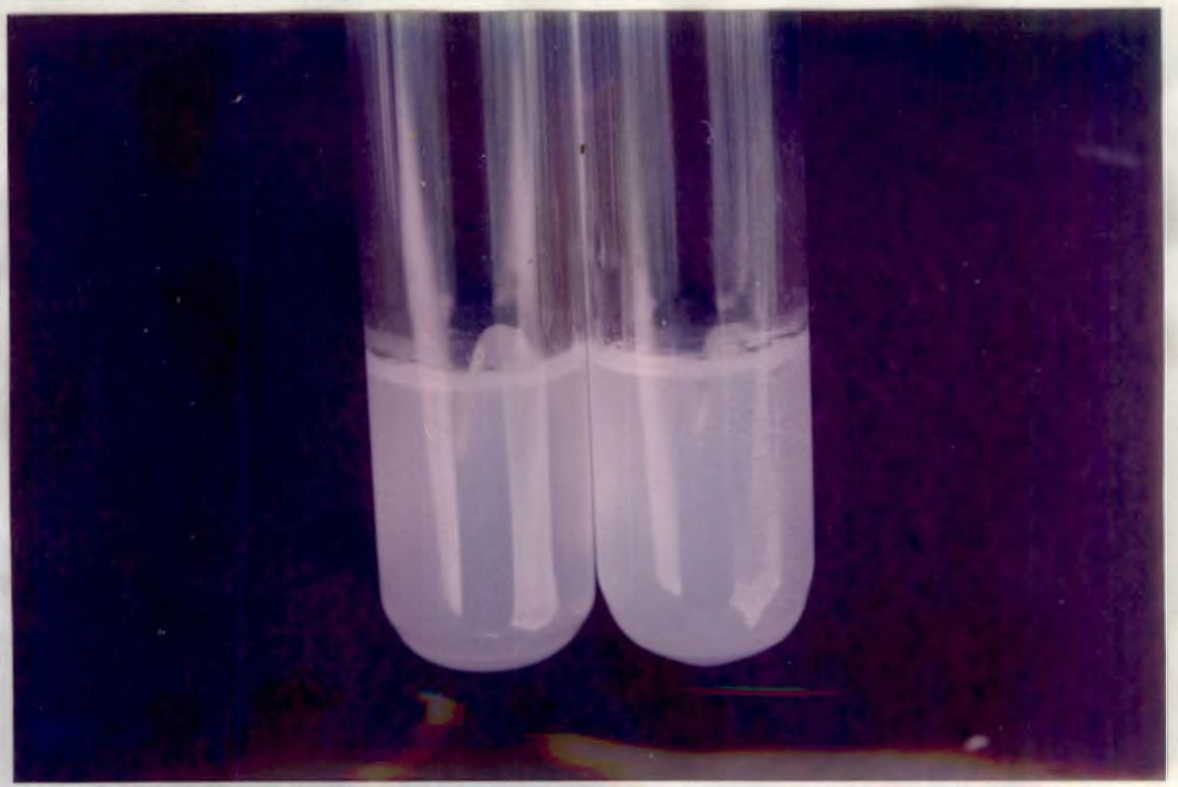

Plate 3. Synthetic seeds developed from excised embryonic axes of *Hopea parviflora* at six weeks after anthesis germinating in MS medium - 21 DAS

Plate 4. Synthetic seeds developed from six weeks mature excised embryonic axes of *Hopea parviflora* kept at 100 per cent relative humidity for six hours germinating in MS medium - 7 DAS

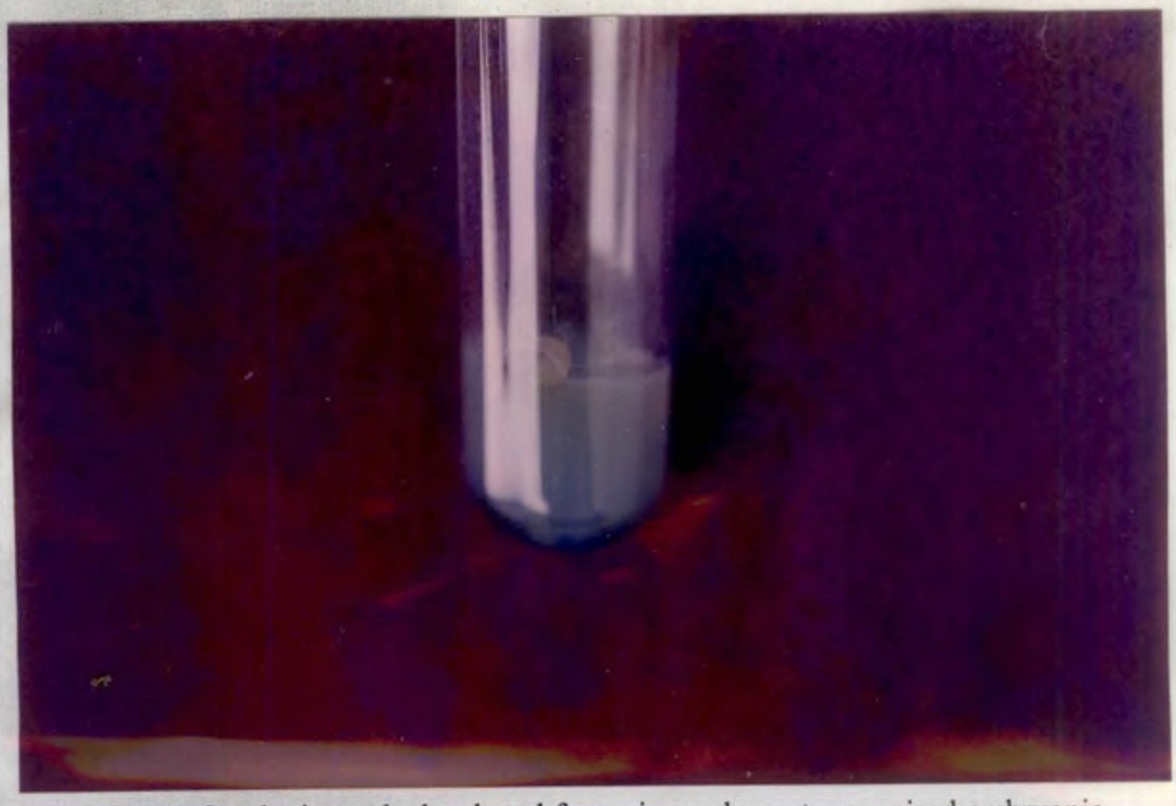

Plate 5. Synthetic seeds developed from six weeks mature excised embryonic axes of *Hopea parviflora* subjected to desiccation at 46.6 per cent relative humidity for six hours germinating in MS medium - 7 DAS

Plate 6. Synthetic seeds developed from six weeks mature excised embryonic axes of *Hopea parviflora* subjected to desiccation at 46.6 per cent relative humidity for three hours germinating in MS medium - 7 DAS

Appendices

APPENDIX - I

Abstracts of ANOVA tables of germination characteristics of intact seeds of *Hopea parviflora* as affected by maturity, levels and duration of desiccation

Source	df	Mean square					
		GP	LNGP	MDG	PV	GV	SQRTGV
Age (A)	1	268.177**	0.0399**	3976.804**	2.659	218868.883	203.589
Desiccation (B)	5	38.123	0.0056	124.202	252.927**	244790.994	98.628
Time (C)	5	16.311	0.0025	71.549	724.473**	1273809.363**	584.305**
A x B	5	670.261*	0.0732*	94.133	140.166	281599.805	111.646
A x C	5	761.296**	0.0943**	171.348**	85.161	327936.899	77.786
B x C	24	31.270	0.0046	42.889	93.962*	162449.235	64.648
A x B x C	24	41.637	0.0060	40.396	53.531	97546.046	41.563
Error	320	30.958	0.0040	63.786	52.877	134256.500	45.269

* Significant at 5% level
** Significant at 1% level

APPENDIX - II

Abstracts of ANOVA tables of germination characteristics of seeds without seed coat of *Hopea parviflora* as affected by maturity, levels and duration of desiccation

Source	df	Mean square					
		GP	LNGP	MDG	PV	GV	SQRTGV
Age (A)	1	60.470	0.01890	580.672**	167.601	1289469.456	420.057
Desiccation (B)	5	134.694*	0.0187*	41.613	324.924**	1046208.774*	268.738*
Time (C)	5	318.070**	0.0403**	220.362**	3257.449**	14841358.280**	2830.880**
A x B	5	79.788	0.01546	99.486	166.967	669202.674	146.087
A x C	5	89.318	0.01243	2362.358**	174.470	195659.592	84.422
B x C	24	120.039**	0.0237**	78.142	128.466	434655.103	101.560
A x B x C	24	76.186	0.01236	68.655	117.383	424727.646	79.849
Error	320	48.969	0.01153	71.066	92.713	390393.774	74.133

* Significant at 5% level
** Significant at 1% level

APPENDIX - III

Abstracts of ANOVA tables of germination characteristics of excised embryonic axes of *Hopea parviflora* as affected by maturity, levels and duration of desiccation

Source	df	Mean square					
		GP	LNGP	MDG	PV	GV	SQRTGV
Age (A)	1	109.671	0.128	3.148	1.169	668.964	2.853
Desiccation (B)	5	6738.176**	9.953**	202.989**	195.525**	32316.905**	202.628**
Time (C)	5	35740.004**	22.873**	792.210**	726.329**	183356.950**	758.989**
A x B	5	87.397	0.162	1.028	4.858	183.211	1.080
A x C	5	351.030	0.104	20.535	23.649	1936.406	24.626
B x C	24	760.441*	0.882*	23.102*	25.261*	4653.645*	22.932*
A x B x C	24	161.171	0.109	3.600	4.136	538.404	3.384
Error	320	437.023	0.239	11.676	13.537	2825.913	12.093

* Significant at 5% level
** Significant at 1% level

APPENDIX - IV

Abstracts of ANOVA tables of germination characteristics of synthetic seeds of *Hopea parviflora* as affected by maturity, levels and duration of desiccation of the embryonic axes

Source	df	Mean square					
		GP	LNGP	MDG	PV	GV	SQRTGV
Age (A)	1	2613.333	0.655	540.035**	547.841**	167773.916**	543.948**
Desiccation (B)	5	7282.469**	1.385**	3.627	3.848	1864.729	3.739
Time (C)	5	55600.741**	7.560**	72.821*	72.822*	190971.655**	540.392**
A x B	5	148.148	0.0222	115.241**	116.943**	31138.263**	116.094**
A x C	5	1623.333	0.550	539.498**	541.560**	27910.320**	72.840*
B x C	24	2023.58	0.414	31.657	32.168	8667.945*	31.911
A x B x C	24	121.111	0.0242	2.214	2.356	854.409	2.286
Error	320	1079.646	0.194	17.924	17.987	4104.120	17.952

* Significant at 5% level
** Significant at 1% level

APPENDIX - V

Abstracts of ANOVA tables of germination characteristics of synthetic seeds of *Hopea parviflora* as affected by maturity levels of embryonic axes, storage periods and storage temperatures

Source	df	Mean square					
		GP	LNGP	MDG	PV	GV	SQRTGV
Age (A)	1	2613.333**	2.214**	540.035**	547.841**	167773.916**	543.948**
Storage temperature (B)	5	108615.926**	12.682**	1037.694**	1038.105**	52932.927**	1038.234**
Storage period (C)	5	149693.889**	21.462**	2680.165**	2691.630**	616228.853**	2685.693**
A x B	5	4503.333**	0.811**	106.763**	107.369**	16244.350**	107.040**
A x C	5	2836.481**	0.945**	150.299**	155.051*	93761.999**	152.619**
B x C	24	15740.000**	2.803**	250.552**	250.887**	27368.650**	250.819**
A x B x C	24	1184.815**	0.574**	25.426**	25.456**	7485.989**	25.451**
Error	320	249.862	1.059	4.661	4.671	1629.241	4.661

* Significant at 5% level
** Significant at 1% level

DEVELOPMENT OF STORAGE TECHNIQUES FOR THAMPAKAM *(Hopea parviflora* Bedd.) SEEDS

By

SHAJI, M.

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Forestry

Faculty of Agriculture

KERALA AGRICULTURAL UNIVERSITY

DEPARTMENT OF SILVICULTURE AND AGROFORESTRY

COLLEGE OF FORESTRY

VELLANIKKARA, THRISSUR - 680656

KERALA, INDIA

2002

Abstract

A detailed study was conducted at College of Forestry, Kerala Agricultural University, Vellanikkara, Thrissur, Kerala during 1999-2001 to develop a suitable storage technique for the seeds of *Hopea parviflora* Bedd. species through synthetic seed and cryopreservation technology. Moisture content of intact seeds/ seeds without seed coat/ excised embryonic axes decreased with increase in intensity and duration of desiccation levels and only the seeds without seed coat showed significant difference in moisture content due to maturity levels. Leachate conductivity was not significantly affected due to maturity levels of intact seeds and excised embryonic axes. But seeds without seed coat showed significant difference in leachate conductivity due to maturity levels and it was higher at seven weeks after anthesis. Increasing the duration of desiccation significantly increased the leachate conductivity of intact seeds/ seeds without seed coat/ embryonic axes. Germination parameters of intact seeds and seeds without seed coat were not affected significantly by increased desiccation. However, excised embryonic axes showed significant difference in germination parameters when the intensity and duration of desiccation was increased. The synthetic seeds developed from these desiccated axes also showed significant difference in their germination parameters. Synthetic seeds stored at 4°C retained maximum viability after four weeks, compared to those stored at 20°C and 27°C. When stored at 27°C, the synthetic seeds developed from six weeks mature embryos retained viability up to three weeks whereas those at seven weeks after anthesis retained viability up to two weeks only. After cryopreservation, the propagules invariably failed to regenerate but could retain green colour for two to three days.