Introduction
Water has physical properties that make it an ideal biological solvent; thus plays many roles in the process of life. Because it is an incompressible fluid, it can fill cells and organelles, giving them structure. The fluid environment provided by water allows the diffusion of substrates to the active sites of enzymes. Hydrophilic and hydrophobic interactions stabilize macromolecular confirmations and allow for the sequestering of cellular constituents. Water is a reactant or product in many important reactions. Evidence suggests that water may inhibit deleterious reactions by preventing molecules from interacting; in this sense, water also serve as a protectant of macromolecular structure. Because of its myriad roles, water controls the level of metabolic activity in plants (Clegg, 1978; Adams and Rinne, 1980; McIntyre, 1987; Leopold and Vertucci, 1989). Thus the loss of water will profoundly affect the nature of physical and biochemical reactions (Karel, 1975; Leopold and Vertucci, 1989; Bryant and Wolfe, 1992; Crowe et al., 1992).

**WATER & METABOLISM**

As water is removed from the cell, the concentration of solutes is increased, and eventually the fluidity of the aqueous medium declines. These changes affect the metabolic status of the cell. The changes in metabolic activity are believed to occur at specific moisture levels (Leopold and Vertucci, 1989). Critical moisture levels have been postulated for germination metabolism (Palit, 1987), germination (McIntyre, 1987; Palit, 1987) continued embryogenesis (Xu et al., 1990; Galau et al., 1991; Morris et al., 1991), the cessation of growth (Adams and Rinne, 1980; Saab and Obendorf, 1989) and cell division (Adams and Rinne, 1980; Myers et al., 1992). Below a moisture level of about -1.5 MPa, tissues no longer grow or expand (Hegarty, 1978; McIntyre, 1987) and protein and nucleic acid synthesis pattern change (Dhindsa and Cleland, 1975; Skriver and Mundy, 1990). This slight desiccation may induce production of protectants (Dure et al., 1989; Close and Chandler, 1990; Skriver and Mundy, 1990;
Blackman et al., 1991). Greater levels of desiccation can result in metabolic imbalances. At about 0.45 g H₂O/g dm or about -3 MPa (Dell’Aquila, 1992) protein synthesis ceases and repair processes become inoperative (Dhindsa and Cleland, 1975; Clegg, 1978; Osborne, 1983). Respiratory activity continues until tissues are dried below about 0.25 g/g or -11 MPa (Leopold and Vertucci, 1989; Vertucci, 1989). At moisture levels between -3 and -11 MPa (about 0.45 to 0.25 g H₂O/g dm), catabolic activities continue unabated and processes utilizing the high-energy intermediates are impaired (Leopold and Vertucci, 1989; Vertucci, 1992). These cells may utilize food reserves and accumulate toxins, such as free radicals (Leprince et al., 1992; Hendry et al., 1992). Desiccation-tolerant organisms must be able to withstand such changes in metabolism (Rogerson and Matthews, 1977; Levitt, 1980; Leprince et al., 1992). Cells may be more susceptible to the rate of desiccation rather than to loss of water per se (Pritchard, 1991; Pammenter et al., 1991; Berjak et al., 1993). When held at moisture levels where only catabolic activities occur, cells that are innately desiccation-tolerant may suffer damage because they have been subjected to the stress of an unregulated mechanism for a longer time period than if dried rapidly.

**Storage Physiology: Orthodox / Recalcitrant / Intermediate**

The ability to withstand complete loss of cellular water is an unusual factor in life; yet it is an adaptive feature of seeds of many species. These seeds are termed "orthodox" because they can be stored for years under cold dry conditions. Generally, such seeds undergo a period of drying during their maturation and are shed at low water contents which are in equilibrium with the prevailing relative humidity. While seed composition determines the equilibrium with the prevailing relative humidity. While seed composition determines the equilibrium water content at any particular relative humidity, all orthodox seeds can withstand dehydration to around 2.5% (wet basis), even in those instances where
maturation drying is not completed prior to shedding. Furthermore, unless debilitated by xero-tolerant fungi, dry orthodox seeds should retain viability for relatively protracted periods (at least from harvest until the next spring, extending to years, depending on the species) at moderate temperature (Berjak et al., 1989) or for many decades at -18°C (IBPGR, 1976). Any seeds that do not behave this way, are not orthodox and, infact, the seeds of a great number of species may accordingly be non-orthodox.

Non-orthodox or recalcitrant seeds are generally understood to be 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). Such seeds are shed in an hydrated condition, but the water content can be anywhere in a wide range—generally from 0.43 to 4.0 g g⁻¹ which is 30-80% on a wet mass basis. Shedding water content is partly 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). This reveals that recalcitrant seeds are not equally desiccation-sensitive, but that variable degrees of dehydration are tolerated, depending on the species. This, in turn, indicates that the processes or mechanisms that confer desiccation tolerance are variably developed or expressed, in non-orthodox seeds that are described as "RECALCITRANT". As several mechanisms have been suggested to be involved in the acquisition of desiccation tolerance and maintenance of the integrity of seeds in the dehydrated state, it should be appreciated that any one of these may be absent, or present but ineffective, in recalcitrant seeds. It should also be stressed that desiccation tolerance is probably controlled by the interplay of mechanisms or processes, and not by any one, acting in isolation: thus the
absence or incomplete expression of any proposed dehydration-tolerance mechanism could have profound consequences on the ability of a seed species to withstand a measure of dehydration below a particular level of hydration.

The phenomenon of differential desiccation sensitivity means that recalcitrant seeds of various species do not all respond similarly when subjected to the same drying regime. Those of some species will tolerate only a slight degree of dehydration, while others will survive to far lower water contents. Related to this is the fact that 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 seeds, e.g. developmental status (Berjak et al., 1992; Berjak et al., 1993; Finch-Savage and Blake, 1994; Finch-Savage, 1996; Berjak and Pammenter, 1997) and chilling sensitivity (Berjak and Pammenter, 1997).

It is thus not merely a matter that a seed species is recalcitrant, but rather, how recalcitrant it is. This led to the proposal made originally some twelve years ago, of a continuum of recalcitrant seed behaviour, from species that are highly desiccation- and probably also chilling-sensitive, to those that will tolerate drying to the lowest water contents still commensurate with recalcitrant seed behaviour, and will also tolerate relatively low temperatures (Farrant et al., 1988).

The concept of a continuum of post-harvest seed behaviour (that is dependent on pre-shedding developmental events) extends beyond the category of recalcitrant seeds. The continuum grades from extreme desiccation-sensitivity through the minimally-recalcitrant types to intermediate seed species that do not react adversely to low temperatures, through
those that are chilling-sensitive when dehydrated (Hong and Ellis, 1996), to orthodox seeds that will tolerate less or more extreme dehydration (Vertucci and Roos, 1990). The important concept underlying the continuum is that there are seed species that behave in a manner that characterises them as lying **between** the hitherto (loosely) defined categories, recalcitrant and orthodox. The idea of an extended continuum of seed behaviour from the most desiccation-tolerant of orthodox species, to the recalcitrant species that are most sensitive to even slight water loss, embodies many properties of seeds and their responses (Berjak and Pammenter, 1994; 1997). It has its foundations in an appreciation of the physiological status of seeds at various water potentials (Vertucci and Roos, 1990; 1993; Vertucci, 1992; Vertucci and Farrant, 1995) and the properties of water at the various hydration levels corresponding to specified water potential ranges (Vertucci, 1992; Vertucci and Farrant, 1995).

In continuation to the concept of continuum, Ellis and coworkers (1990, 1991a, 1991b, 1991c) have suggested that there is a third category of seed storage behaviour intermediate between those define by Roberts (1973). The phenomenon of intermediate storage behaviour has only recently been defined (Ellis et al., 1990). Embryos that cannot withstand the synergistic effects of low moisture levels and low temperatures fall into this category [e.g., papaya (Ellis et al., 1991, *Elaeis* (Ellis et al.,1991c), *Zizania* (Kovach and Bradford, 1992) and also partially germinated seeds (Hong and Ellis, 1992). Vertucci and Farrant (1995) have suggested that seeds with intermediate storage characteristics lose viability rapidly when stored at moisture levels less than -150 MPa, while seeds with orthodox characteristics lose viability slowly (i.e., they age) at moisture levels below this value (Vertucci and Roos, 1990; 1993). The considerable desiccation-tolerance of seeds with intermediate storage behaviour may be a result of completed PVS stage (as has been documented for *Zizania* using protein markers) (Bradford and Chandler, 1992). Since tolerance of virtually complete removal of water is enhanced during
the very final stages of seed development (Hong and Ellis, 1992), one may speculate that seeds with intermediate storage characteristics lack the pre-desiccation stage described by Galau and coworkers (1991). It is intriguing to think that seeds can survive absolute dehydration only if the entire embryogenic program is completed (Vertucci and Farrant, 1995). It is plausible that seeds adapted for very harsh environments (e.g., desert species) have stringent regulation of the latter stages of embryogenesis to ensure their completion (Vertucci and Farrant, 1995).

MEMBRANE PERTURBATIONS: LIPID PEROXIDATION

When moisture levels are considerably reduced, the structure of nucleic acids, proteins, and polar lipids can be altered because the hydrophilic and hydrophobic interactions that stabilize confirmations are weakened (Leopold, 1986; Crowe et al., 1987; Carpenter and Crowe, 1988). Ultrastructural studies of tissues after desiccation have revealed that cellular membranes are one of the primary sites of injury. Desiccation imposed on developing immature *Phaseolus vulgaris* embryos at the desiccation-intolerant stage induced a general collapse of membranes, in contrast to cells from embryos dried at the tolerant stage, whose membranes retained their integrity (Dasgupta et al., 1982). In germinating maize seeds, desiccation was associated with an irreversible breakdown of nuclear and plasma membranes in desiccation-sensitive radicals, whereas those of tolerant radicles remained intact (Crevecœur et al., 1976; Sargent et al., 1981). An early indicator of desiccation-induced damage to membranes is leakage of various cytoplasmic solutions (ions, sugars and proteins) that occurs upon rehydration of desiccated seed tissues (Senaratna and McKersie, 1983; Crowe et al., 1989). The rate and extent of cytoplasmic leakage is positively correlated with the degree of desiccation-sensitivity (Senaratna and McKersie, 1983; 1986). Experiments on kinetics of leakage and nature of leaked substances showed that leakage reflects a partial loss of membrane semi-permeability,
suggesting that desiccation-injury is closely associated with membrane dysfunction (Senaratna and McKersie, 1986). The effect of desiccation on the physical organization of the lipid bilayer has been investigated in several experimental systems including model membranes isolated either from living tissues (Crowe et al., 1986; Senaratna and McKersie, 1986) or prepared from pure lipid mixtures (Crowe et al., 1988; McKersie et al., 1990).

Functional aspects of cellular membrane changes as a consequence of the unregulated chemical reactions that occur when desiccation-sensitive seeds are dehydrated below critical moisture levels (Chaitanya and Naithani, 1994; 1998). The reactions are believed to be peroxidative and result in lower levels of fatty acids unsaturation, lipid hydroperoxides and their by-products, and free fatty acids (Chan, 1987; Priestley, 1986; Leopold and Vertucci, 1989). Accumulation of peroxidised lipids is considered as a molecular indicator of both membrane injury and degradation of unsaturation lipids (Leprince et al., 1993). In desiccated maize radicles, loss of desiccation-tolerance during germination was accompanied by a 4 fold increase in the accumulation of malondialdehyde (MDA), a measure of peroxidative damage to lipid acyl chains (Leprince et al., 1990). On restoration of water supply, lipid peroxidation was increased in intolerant material resulting in 15 fold rise in MDA (Leprince et al., 1990). In contrast, tolerant radicles did not accumulate lipid peroxides. Similar injury symptoms were associated with loss of viability in recalcitrant acorn embryos (Hendry et al., 1992) and sal seeds (Chaitanya and Naithani, 1994; 1998; 1999) desiccated during drying.

Oxidative Stress

All of these alterations in chemical composition, which have been correlated with damage to membranes and impaired regrowth following rehydration, are probably of a common origin. The presence of highly reactive free radicals, probably activated oxygen, has
been suggested by several lines of evidence (Chaitanya and Naithani, 1994; 1998; 1999). Oxidative attack resulting in phospholipid de-esterification and alterations in membrane physico-chemical properties was conducted in vitro by exposing microsomal membranes to activated oxygen generated by xanthine oxidase. Microsomes from soybean axes which still retained some tolerance of desiccation were less susceptible to damage from activated oxygen (Senaratna et al., 1987). In conclusion, alteration of membrane physico-chemical properties after lethal desiccation may be the consequence of an oxidative attack by free radicals (Chaitanya et al., 2000). Direct evidence for the involvement of free radicals in loss of viability of seeds is accumulating although it is difficult to detect, identify and quantify free-radical species. Furthermore, free-radical processes differ quantitatively and qualitatively between dead and living tissues; the longer the seed has been dead, the more difficult it becomes to correlate radical processes with loss of viability (Hendry, 1993). Using EPR techniques, free radicals in various seed tissues including the cotyledon and axes of soybean, as well as the endosperm and embryo of maize (Buchvarov and Gantcheff, 1984; Priestley et al., 1985), increased during natural and accelerated ageing and loss of viability. However, Hepburn et al. (1986) were unable to correlate the free radical concentrations of different cultivars of Brassica and legume species with seed viability and seedling vigour. Recently Hendry et al. (1992) found that EPR-detected stable free radicals accumulated in the embryonic axes from acorns of Quercus robur (showing recalcitrant storage behaviour) coincidentally with loss of moisture and viability. One of the two radical species detected was indistinguishable from a stable free radical formed in intolerant desiccated mosses (Seel et al., 1991). In maize, an EPR response similar to that of mosses and acorn seeds was obtained in desiccated, germinating radicles (Leprince et al., 1990). In addition, a significant increase in free radicals also occurred in desiccated, intolerant tissues following 8 h of rehydration (Leprince, 1992).
ANTIOXIDANT ENZYMES

Since susceptibility to peroxidation may increase with drying (Bewley, 1979; McKersie et al., 1988; Leprince et al., 1990; Hendry et al., 1992) one may reason that free radical processing systems are an important part of desiccation-tolerance. Vegetative tissues with greater drought or desiccation-tolerance appear to have more efficient antioxidative enzyme systems (Hendry et al., 1992; Pastori and Trippi, 1993). Antioxidant systems in developing embryos depend on the species and tissue (embryonic axis versus storage tissues) as well as the developmental status of the embryo (Arrigoni et al., 1992; Hendry et al., 1992; Cakmak et al., 1993). Ascorbate and ascorbate oxidising enzymes are plentiful during the early stages of embryogenesis, are less significant during the maturation stages, and then become increasingly important again with germination (Pantarulo et al., 1988; Arrigoni et al., 1992; Chaitanya et al., 2000). Changes in activity and importance of catalase, superoxide dismutase and glutathione reductase with embryogenesis and germination are variable (Leprince et al., 1990; Arrigoni et al., 1992; Cakmak et al., 1993), but there appears to be a general trend toward increasing activity of enzyme scavenging systems with increasing activity of enzyme scavenging systems with increasing mitochondrial activity. This is consistent with the primary function of these enzyme systems: to metabolize peroxides produced from about 1% of the oxygen consumed from unstressed mitochondria (Pantarulo et al., 1988). Thus as mitochondrial activity declines with drying, the requirement for these enzyme systems may be alleviated.

Enzyme systems that process peroxides leaked from mitochondria may be inefficient at processing other sources of free radicals. Perhaps this is the reason for their decline during the latter stages of embryo maturation when enhanced desiccation-tolerance is acquired but respiratory activity declines. In this case, antioxidants such as tocopherol (Senaratna et al.,
1985; McKersie et al., 1988; Leprince et al., 1990), sucrose (Smirnoff and Cumbes, 1989) or phytate (Graf et al., 1987) may be more effective. In orthodox seeds, free radical scavengers accumulate during maturation and are lost during germination (Murray, 1984; Senaratna et al., 1985; Koster and Leopold, 1988). Under stressed conditions, the protective mechanisms, viz., antioxidants (tocopherol, ascorbate, glutathione) and antioxidant enzymes (SOD/CAT/POD) (Dhindsa and Matowe, 1981; Leprince et al., 1990; Dhindsa, 1991; Price and Hendry, 1991), may be impaired, leading to oxidative damage (Hendry and Brocklebank, 1985; Price and Hendry, 1989).

PROTEIN METABOLISM

Apart from alteration in lipid membranes, qualitative and quantitative changes in intramembranous proteins have also been associated with desiccation-induced injury, although data are scant. Following desiccation, a decrease in protein content of desiccation-sensitive soybean axes (Senaratna et al., 1987) and sal axes and cotyledon (Chaitanya et al., 2000) was observed. In addition qualitative changes, detected using a thiol-specific fluorescent probe indicated that, lethal desiccation resulted in a loss of thiol groups of membrane proteins from microsomal fractions. As a consequence of drying, a marked change in protein profile and a suppression of the synthesis (more or less permanently) of developmental proteins has been reported in Ricinus communis and Phaseolus vulgaris seeds (Bewley et al., 1989). A similar switch in the direction of protein synthesis occurs in the embryonic axes and cotyledons following premature drying (Kermode and Bewley, 1986). As proteins can also act indirectly in controlling the water-binding characteristics of the seeds, they are considered to have an ability to protect the intracellular components during desiccation (Blackman et al., 1991). Decline in protein content may
be a cumulative effect of reduced protein synthesis and enhanced proteolytic activity (Bewley and Black, 1985). Enhanced proteolytic activity with ageing (natural or accelerated) of seeds has been well documented (Chin and Schoolcraft, 1968; Perl et al., 1978; Agarwal and Kharlukhi, 1987). Besides desiccation-induced inhibition of protein synthesis and hydrolytic damage to the proteins, free radical-mediated damage to protein is also important (Prasad, 1996).

**CRYOSTORAGE**

Today it is a well known fact that little attention as been given to the problems of storage of seeds of tropical forest tree species (Chin, 1988). One of the difficulties in *ex situ* storage of germplasm conservation is the fact that more than 70% of the tree species of tropical forests produce desiccation-sensitive seeds (Gunn, 1991) which are non-storable by methods commonly used for orthodox seeds. Reports from the past half century revealed that recalcitrant seeds can only be stored for short periods of a few months to a year. Although long-term storage is presently not possible, any improvement of short-term storage will be appreciated as improvements in conventional methods. Recent literature suggests that cryostorage could be an alternative method for the *ex situ* conservation of desiccation-sensitive seeds (Roberts et al., 1984; Farrant et al., 1988; Bonner, 1990; FAO, 1993).

Cryopreservation refers to the use of ultra-low temperatures (-80°C to -196°C) for the preservation of biological materials (Stanwood and Roos, 1979; Stanwood, 1985; Towill, 1985). Cryo, a derivative of cryogenic, defined as very low temperature is the essence of cryopreservation (FAO, 1993). Cryopreservation is defined as "the preservation or storage in very cold temperatures, usually in liquid nitrogen at temperatures close to -196°C (IBPGR, 1982;
Pritchard, 1995). The potential advantages of cryopreservation over conventional techniques are an absence of complicated temperature and humidity controls, the freedom from damage by pests and diseases, and indefinite longevity with little or no genetic damage (Styles et al., 1982). Steps involved in cryopreservation includes desiccation under sterile air flow current, direct fast freezing in liquid nitrogen and thawing during retrieval.

Though cryopreservation offers promise for the storage of desiccation-sensitive seeds, not all the seed species can be stored at cryogenic temperatures because of differences in chemical composition of the seeds and storage physiology (Jorgensen, 1990; FAO, 1993). The ideal candidates for cryopreservation must be species with inherently short-lived seeds and also species which are endangered with critical small population sizes; both types of material would benefit from the unlimited storage potential that liquid nitrogen storage appears to offer (Berjak et al., 1990). For successful cryopreservation an optimal range of moisture content is critical for cooling and rewarming process. Stanwood (1985) developed a high moisture freezing limit (HMFL), the threshold moisture content above which loss of seed viability occurs during the cooling-rewarming process. Stanwood (1985) classified seeds of different species into three categories according to their response to LN2 exposure. (1) Desiccation tolerant and LN2 tolerant seeds which includes most of the agricultural and horticultural species, conifers and small seed hardwoods, and they can be generally characterised by their storage behaviour as truly orthodox or sub-orthodox seeds. There has been considerable success in cooling these seeds to LN2 temperatures and rewarming them to an ambient temperature of 25 - 30°C without any loss of viability (Stanwood, 1985; Pence, 1991; Gonzalez-Rio et al., 1994). True orthodox seeds of valuable woody species can be desiccated to low moisture content (Sasaki, 1980) and stored in
liquid nitrogen for an indefinite period of time beyond the rotation age with little or no genetic change (Salomao, 1995). (2) *Desiccation tolerant and LN2 sensitive seeds* which are exemplified by forest trees such as *Fagus sylvatica*, *Gmelina arborea*, *Populus* spp. They can be characterized by desiccation tolerance to intermediate moisture content (Varghese and Naithani, 2000) below 10% (% fresh wt. basis) and a storability of less than five years (Stanwood, 1985). They are however sensitive to temperatures lower than 40°C. These seeds are rich in storage lipids which may be as high as 60 to 70% in some species (Bewley and Black, 1978), e.g., *Prunus* spp, *Gmelina arborea*, *Fagus sylvatica*. (3) *Desiccation sensitive and LN2 sensitive seeds* which are represented by the seeds of both temperate and tropical recalcitrant seeds, including those of many economically important trees such as rubber (*Hevea brasiliensis*), Cocoa (*Theobroma cacao*), Mango (*Mangifera indica*) and many timber species of the families *Diptocarpaceae* and *Araucariaceae*. These are characterised by their large size, short life span and sensitivity to desiccation and freezing temperature (Chin, 1988). Together with this, the major problem with the storage of recalcitrant seeds is their sensitivity to desiccation (Farrant et al., 1986; Berjak et al., 1990; Tompsett, 1992; Fu et al., 1993; Chaitanya and Naithani, 1994).

The major problem associated with the cryostorage of recalcitrant seeds is their sensitivity to desiccation. Moreover, injury is hypothesized to be due either to the loss of membrane integrity and the entry of extracellular ice into the cell on freezing or to irretrievable loss of membrane material causing cell membrane rupture upon rehydration (Harrington, 1972; Meryman and Williams, 1985). On the contrary, the intermediate seeds can be dried safely to lower moisture contents (see chapter 1) and this improves chances of its survival under LN2 temperatures.
Cryopreservation is considered to be the most stabilising method of germ plasm conservation, as all metabolic activity is thought to cease at -196°C (Stanwood and Bass, 1981; Stanwood, 1985), the temperature of liquid nitrogen. The pre and post-storage treatments of cryopreservation incorporate many stressful components (e.g. chemical toxicity, mechanical and freezing damage, dehydration and osmotic injury). Thus, of all the approaches to germplasm conservation, cryopreservation is probably the most challenging in terms of biochemical stress physiology (Benson, 1990). Of all the manifestations of freezing stress, membrane damage is probably the best documented. Indeed, disruption of the plasma membrane is considered a primary cause of freezing injury, an observation supported by the fact that cold acclimation is thought to involve alterations in membrane structure (Steponkus, 1984). Although most of the work relates primarily to the physical stresses imposed on the membrane during freezing, several observations show that biochemical factors may be important (Benson, 1990). One indication of this is the flow of membrane material to and from the plasma membrane during the freezing cycle. In some respects, this phenomenon has much in common with the membrane repair mechanisms described in the seeds. Some studies (Steponkus, 1984; Williams et al., 1975) suggest that this is a protective measure which accommodates the vast physical changes in cell dimensions by loss of membrane from the plasmalemma during osmotic contraction and re-incorporation during expansion. Morris et al. (1975) suggested that membrane damage may be due to a fundamental biochemical lesion. They noted an increase in saturation of fatty acids after freeze/thawing and also a release of free fatty acids which did not occur in the unfrozen cells. However, many reports have evidenced the damage to membranes (Senaratna and McKersie, 1983; Hoekstra et al., 1989) during the storage of seeds intern, leading to loss of viability.
Status of Neem and Research on Neem Seed Storage

Neem (*Azadirachta indica* A. Juss; synonymous: *Melia indica* Brandis, *Melia azadirachta* Linn.), a native of the Indian subcontinent, is a highly esteemed tree not only for the people of this region but also throughout the globe (Randhawa and Parmar, 1996). The species belongs to the family *Meliaceae* and has been well known since the ancient times by several other common names such as nim, Indian lilac, margosa tree etc. The commercial use of neem has been known since the Vedic period in India over 4000 years B.C. For centuries, its derivatives have found use in agriculture, toiletries, cosmetics, live stock production, insecticide, timber, shade, fodder, public health, contraceptives, oil etc. (Randhawa and Parmar, 1996; Maithani *et al.*, 1989). The tree has aroused considerable interest due to the many biologically active compounds in its tissues, including the seeds (National Research Council, 1992). Extracts from the tree are known for their high effective medicinal and pesticidal properties (Von Maydell, 1983) and their effects on a wide array of organisms including pests, molluscs, fungi and viruses is well documented (Koul *et al.*, 1990; Schmutterer, 1995) and hence, it has been rightly projected as a tree for solving global problems (National Research Council, 1992). Probably no other tree yields as many strange and varied products, or has as many exploitable by-products. Neem is also used as a multipurpose tree species in agroforestry systems especially in reforestation programmes (Pliske, 1984). The tree has been considered so miraculous and invaluable that it has become a major inseparable component of the Indian ecosystem. There is a worldwide focus on the species. In 1993, the International Neem Network was initiated and 22 countries began collaborating on improving the genetic quality of neem (Thomsen, 1999). FAO panel of experts on Forest Gene Resources regarding collection, evaluation, and conservation of forest species have selected neem for urgent action in India (Chaudhury and Chandel, 1991). Efforts have been made in this direction by
various institutions and agencies including the International Plant Genetic Resources Institute, Rome; Indian Council of Forestry Research and Education, Dehradun, India and Danida Forest Seed Centre, Denmark etc.

Neem is mostly regenerated through seeds which are reputed to have short span of viability (Raynor, 1940; Dent, 1948; Ezumah, 1986; Maithani et al., 1989). This has restricted the progress in germplasm collection, ex-situ conservation, establishment of plantation, species trials and tree improvement etc. (Gamene et al., 1996). To maintain the biological diversity of neem, some sort of germplasm conservation will be essential (Bhardwaj and Chand, 1995). Moreover, gap encountered between seed collection and sowing and also transporting the seed to long distances becomes a limiting factor (Maithani et al., 1989) in its plantation and species trials. Since neem tree like other trees produce abundant fruits much emphasis was not given in the past on storage aspects of these seeds (Ezumah, 1986). But the short viability of these seeds remained a major problem in propagating neem for various purposes (National Research Council, 1992). Several studies indicate orthodox to recalcitrant storage behaviour and very few individuals have yet succeeded in storing the neem seeds for more than 6-12 months (Thomsen, 1999). This has led the researchers to work on the storage of neem seed. Efforts are being made to understand the storage physiology of neem seed i.e., their ability to tolerate desiccation during storage at various temperatures.

Limited work done so far in India on storage behaviour of neem seeds reveal contradictory reports. Surendran et al. (1993) considered neem seeds to be generally recalcitrant. Similarly, neem seeds collected from Northern part of India (Dehradun) were classified as recalcitrant seeds as these seeds were shed at high moisture content and showed limited extended longevity at 15°C but sharp loss of viability at ambient and 5°C (Maithani
et al., 1989). This group working at Forest Research Institute (FRI), Dehradun tried to store neem seeds at different conditions of storage and temperature. Their results show that seeds collected 8 to 10 weeks after flowering could be stored for six months with a very low (10%) viability when stored in perforated polythene bags at room temperature. When stored in sealed polythene bags, these seeds lost their viability within one month at room temperature. Though slight extension in viability period was recorded for seeds stored at 15°C, the results were not significant in any of the storage conditions including storage over silica gel in desiccators and card boxes. In contrast, the neem seeds reported from Southern part of India exhibited desiccation-tolerance with improved longevity when dehydrated to 6% moisture content (sun and shade drying) and were thus classified as orthodox seeds (Singh et al., 1997). Attempts were also made to store the seeds in wet storage conditions, to retain high moisture content (Ponnuswamy et al., 1989; Surendran et al., 1993). Even reports of Chaudhury and Chandel (1991) have also categorised neem as desiccation-tolerant or orthodox on the basis of desiccation and freezing sensitivity. Neem seeds have been categorised as orthodox based on electrical conductivity measurements of fresh seeds (Khare et al., 1989).

The international scenario regarding the storage behaviour of neem seeds is also far from clear. It has been variously described as desiccation-tolerant (Dickie and Smith, 1992), moderately desiccation-tolerant but chilling sensitive (Sacande et al., 1996), desiccation-sensitive (Gamene et al., 1996; Msanga, 1996) and desiccation as well as chilling sensitive (Ezumah, 1986). Roberts (1983) had earlier designated neem as recalcitrant. In a later report (Holden and Williams, 1984) it was found that viability of seeds is drastically reduced on desiccating the intact neem seeds. The storage behaviour of neem seeds from African countries have been investigated only scarcely until the recent past and the information available is equivocal (Gamene et al., 1996). Ezumah (1986) reported recalcitrant storage
physiology in several Nigerian seed lots: the viability of these seeds, with moisture content ranging from 11 to 18%, decreased more rapidly at 6-7°C than at 26-28°C, and at both temperatures the germination capacity was less than 10% after 20 weeks of storage. Similarly, Berjak et al. (1995) noted that the ultrastructural damage that developed during storage of hydrated Kenyan neem seeds at both 24°C and at 4°C was indicative of recalcitrant behaviour. They also commented that seeds that had been air dried retained the germination only for a few weeks.

In sheer contrast, Bellefontaine and Audinet (1993) stored seeds from several West-African countries and concluded that neem is potentially orthodox. Seeds with low moisture contents 6-7% maintained high germination capacity for many years, while seeds with 11% moisture content lost their viability comparatively very rapidly. Moreover, they reported that seeds maintained better viability at 4°C than at ambient temperatures irrespective of the moisture content. Similarly, Roederer and Bellefontaine (1989) have reported that seeds with endocarp stored at the Centre Technique Forestier Tropical showed 42% germination capacity even after more than 5 years of storage. Above all, Dickie and Smith (1992) reported that a Nigerian seed lot still had high viability after 12 years of storage at -20°C and 4% moisture. Even Eeswara et al. (1998) concluded that the neem seeds from fruits harvested at ripe stage retained the ability to germinate when dried to 7.1% moisture content and were not sensitive to 3°C storage and suggested that they might be considered orthodox in nature. The apparent orthodox behaviour of Asian neem seeds is synonymous to the findings of Gamene et al. (1994). Bellefontaine and Audinet (1993) concluded that the storage behaviour of neem seeds should be classified as orthodox. These authors observed that the germination capacity of a seed lot with moisture content of 6.7% (fresh wt. basis) was 21% after two years at 4°C, while that of a different lot with seed moisture content of 6.2%
was still 59% after eight months at 4°C. In addition, two other lots (moisture content unknown) stored for four years maintained their germination capacity better at 4°C than at ambient conditions (Bellefontaine and Audinet, 1993). Nayal et al. (1998) working at FRI, Dehradun have also reported extended storage of these seeds. Seeds dried to 7% when stored at 15°C showed 85.3% germination even after 293 days of storage. They have suggested that though neem seeds are short-lived, but being desiccation-tolerant, they could be stored for longer periods, if stored well and proper drying techniques are adopted.

On the other hand, seeds from a Kenyan lot showed intermediate storage behaviour (Ellis et al., 1990; Dickie and Smith, 1992). In an investigation on neem seeds from Burkina Faso (Gamene et al., 1996), it was observed that the seeds showed intermediate storage behaviour. The seeds tolerated desiccation to about 9 to 13% (drying at 55 and 75% relative humidity, respectively) and maintained high viability for at least nine weeks of storage. However, drying these seeds to still lower moisture resulted in loss of germination. The storage trials by Sacande et al. (1996), Pritchard and Daws (1997), Omondi (1997) and Pukittayacamme (1997) also resulted in concluding the intermediate storage physiology of neem seeds. Chaisurisri et al. (1986) obtained success in storing neem seeds to a certain extent in a cotton bag at 15°C. Seeds dried to 48.16% moisture before storage retained viability (62%) for more than 4 months. Sacande et al. (1996) desiccated seeds to 15% moisture content and still obtained 91% germination. Marambe and Gunasena (1995) also obtained good results with desiccation to 7% moisture content. The seed lot from Gede region of Mombasa, Kenya, was earlier classified as recalcitrant by Berjak et al., 1995 but the response to dehydration presently described suggests that they show elements of intermediate storage behaviour as well. This emphasises the view that seed behaviour should be viewed as constituting a continuum on the basis of dehydration and temperature responses, rather
than the seed being categorised into close-ended classes (viz., orthodox, intermediate and recalcitrant) on the basis of post-harvest responses. Moreover, variation in the storage physiology may be attributed to various reasons that include differences in seed maturation (Gamene et al., 1996; Sacande, 1998), local harvest and drying conditions (Ellis et al., 1990; Ellis et al., 1991a; Poulsen, 1995) or due to provenancial differences (Ellis et al., 1991a; Berjak and Dumet, 1996).

The fact that neem seeds are sensitive to low temperatures (between 6 and -20°C) has been demonstrated by Aziz (1985), Chaisurisi et al. (1986), Ezumah (1986), Maithani et al. (1989), Berjak et al. (1995), Elteraify (1996) and Sacande et al. (1996). Aziz (1985) found a slight tendency for seeds stored at 0°C at 50% RH to survive longer than the seeds stored at the same temperature but at 80% RH, though the viability was low in both the cases. However, Gamene et al. (1996) found contradictory results in one experiment: seeds stored for two weeks at 3°C with a moisture content of 9% displayed the same high germination per cent as at 20°C and 30°C. Marambe and Gunasena (1995) found that storage at 10°C gave results similar to storage at 28-30°C. Yet, it is generally recommended to avoid storing these seeds at temperatures below 15-20°C until it has been proved that the seed lot tolerates low temperature (Thomsen, 1999).

Attempts have also been made for long term germplasm conservation of neem seeds by cryopreservation, as cryopreservation is considered as a promising approach for the storage of recalcitrant and intermediate seeds (FAO, 1993). The non-desiccated neem seeds with a moisture content of above 20% (fresh wt basis) lost viability after 1 h at -196°C (Berjak and Dumet, 1996). However, the same seeds were stored successfully in liquid nitrogen for 24 h after desiccation to between 18.5% and 4% moisture (Chaudhury and Chandel, 1991).
and for four months when dried to between 0.09 and 0.06 g g\(^{-1}\) (dry mass basis) (Berjak and Dumet, 1996). Inspite of all these efforts and research world wide, the storage behaviour of neem seeds still remains enigmatic and systematic efforts are desirable in this direction, especially storage of the seeds without decline in vigour, so that availability and conservation of quality neem seeds can be possible.

The OBJECTIVES of the present study were to set the Storage Physiology of neem seeds and understand the physiological and biochemical mechanism underlying the desiccation-induced loss of viability. The cryostorage potential of neem seeds was also evaluated by monitoring physiological and biochemical changes. Following parameters were studied to accomplish the objectives:

1. INTERMEDIATE STORAGE PHYSIOLOGY
   a. \% Moisture Content, \% Germination, Viability Assessment by Triphenyl Tetrazolium Test, Seed Vigour Index, Germination Index and Mean Germination Time
   b. Membrane Perturbation: Leachate Conductivity and Leakage Loss of Ions (K\(^{+}\), Ca\(^{++}\) and Zn\(^{++}\)) Protein and Sugar
   c. Superoxide Radical and Lipid Peroxidation
   d. Antioxidant Enzymes: Superoxide Dismutase (SOD), Catalase (CAT), Ascorbate Peroxidase (AsPOD) and Guaiacol Peroxidase (GPOD)
   e. Soluble Protein, Total Protein and Protease

2. CRYO STORAGE
   a. Standardization of Drying Procedures for Cryostorage
   b. \% Germination and Seed Vigour in LN2 Exposed Neem Seeds, Leachate Conductivity, Superoxide Radical and Lipid Peroxidation, Antioxidant Enzymes: Superoxide Dismutase, Catalase & Peroxidase