CHAPTER I

Germination, Moisture & Seed Vigour
Introduction

Moisture content of seeds play a critical role in the determination of seed longevity during storage (Roberts, 1973; Roberts et al., 1984; Ellis et al., 1991; Hong and Ellis, 1992; Oliveira and Valio, 1992; Chaitanya and Naithani, 1994). Moisture content is simply a measure of the concentration of water in the seed (Vertucci and Roos, 1993). Roberts in 1973 introduced the term orthodox and recalcitrant to define the dichotomy in seed storage physiology mainly on the basis of desiccation-tolerance/sensitivity of the seeds. Orthodox seeds are desiccation-tolerant as they can be stored for long periods, if their moisture contents are reduced to below 1-5% (fresh weight basis) (Ellis and Roberts, 1980; Roberts and Ellis, 1989). The longevity of the seeds is increased by decreasing moisture content and temperature (Roberts, 1973; Ellis et al., 1990). Recalcitrant seeds, in contrast, are highly desiccation-sensitive (Chaitanya and Naithani, 1994; Fu et al., 1994), therefore, they are killed when dehydrated below very high moisture content (20-50% fresh weight basis). Most of the recalcitrant seeds are also chilling-sensitive (Chin, 1988; Berjak et al., 1992). For these reasons, such seeds cannot be stored under the conventional low-moisture, low-temperature storage conditions commonly used for orthodox seeds (Roberts and King, 1980). Several researchers have reported the loss of viability in recalcitrant seeds as soon as the moisture content is reduced below 40% to 28% (Probert and Longley, 1989; Roberts and Ellis, 1989; Fritchard, 1991) which is relatively very high as compared to orthodox seeds (Roberts and Ellis, 1989). Seeds of many tropical species including Shorea robusta (Chaitanya, 1997; Chaitanya and Naithani, 1999), Avicennia marina (Berjak et al., 1989), Quercus robur (Finch-Savage, 1992), Landolphia kirkii (Farrant et al., 1989), Pometia pinnata (Soetisna, 1997) and Hopea odorata (Corbineau and Come, 1989) belong to this category and have been reported to
possess "recalcitrant storage physiology". Recalcitrant seeds are characteristically shed from the parent plant at high moisture contents (King and Roberts, 1980; Berjak et al., 1984) as they lack maturation drying (Berjak et al., 1990), they are desiccation-sensitive and therefore lose viability at relatively high moisture contents (Probert and Longley, 1989; Tompsett, 1992; Chaitanya and Naithani, 1994; Chaitanya and Naithani, 1999). In recent years, Ellis and coworkers (1990, 1991a, 1991b and 1991c) have suggested that there is a third category of seed storage behaviour intermediate between those defined by Roberts (1973). Seeds with "intermediate storage physiology" can be desiccated safely to critical moisture contents ranging from 9 - 13%, but further dehydration reduces the viability of the surviving seeds sharply. They are damaged at freezing and subzero (Stanwood, 1985) temperatures also. Several seeds like Fagus (Suszka, 1975) Citrus (King et al., 1981), Papaya (Ellis et al., 1991b), Coffee (Ellis et al., 1990), oil palm (Grout et al., 1983; Ellis et al., 1991c) once labelled as recalcitrant, are now considered as intermediate in storage behaviour.

Various methods have been used to quantify desiccation-tolerance/sensitivity so that comparisons between seed lots of various provenances and differences in storage behaviour can be made out. Sasaki (1980) used the phrase "critical moisture content" to describe the moisture content at which all seeds were dead. Whilst Tompsett (1986) proposed the term "lowest safe moisture content" (LSMC) defined as the moisture content below which freshly collected seeds died. In addition, a linear relationship between seed viability and moisture content has been demonstrated for a range of recalcitrant forest tree species, viz., wide range of tropical timber species in Dipterocarpaceae (Tompsett, 1985), Araucariaceae (Tompsett, 1982; 1983), Zizania palustris, Spartina anglica and Porteresia coarctata (Probert and Longley, 1989), Quercus rubra (Pritchard, 1991), Quercus robur (Finch-Savage, 1992) etc.
Gosling (1989), Finch-Savage (1992) and Grange and Finch-Savage (1992) demonstrated that in the intact seed, loss of viability appeared to be determined by critical moisture content in the cotyledons. Their data suggests that the rate of desiccation of axes attached to cotyledons, does not determine survival during desiccation, as viability loss is due to an event related to moisture content in the cotyledons. Different levels of desiccation-sensitivity was recorded in cotyledon and axis of temperate recalcitrant species (Berjak et al., 1990; Pritchard, 1991; Grange and Finch-Savage 1992). Comparatively, the rate of desiccation was rapid in cotyledonary tissue than the axes (Finch-Savage, 1992).

The aforementioned literature indicates that the important relationship exists between moisture and viability and it is linearly expressed by probit analysis in recalcitrant seeds. Using desiccation-sensitive seeds of neem, we have shown here the strong correlation between moisture content and viability. Also an attempt has been made to demonstrate the contribution of moisture of axes and cotyledons individually in loss of viability. Moreover, characterization of germination response may also be valuable in identifying optimal conditions for the storage of desiccation-sensitive seeds (Pritchard, 1996). Often, such seeds progress from development to germination without the post-maturation drying and quiescent phase, and has been hypothesized that the rate of germinative metabolism is linked to storage life (Pammenter et al., 1994). There is usually a strong correlation between germination and vigour in both orthodox (Ellis and Roberts, 1980) and desiccating recalcitrant seeds (Pritchard et al., 1995). The distinct way in which poor seed vigour could influence the yield is by reduction in potential field emergence (Perry and Harrison, 1977). Thus the study of various parameters depicting seed vigour becomes important. Any biosynthetic and metabolic change which precedes loss in germination could serve as a vigour test in providing biochemical indices to be correlated with corresponding physiological parameters of vigour (Woodstock, 1973; McDonald, 1980).
The triphenyl tetrazolium (TZ) test is one of the most valuable techniques and biochemical approaches for analyzing seed quality (Enescu, 1991). The TZ test, which uses a pale yellow-coloured solution that is reduced within viable tissues to a bright red coloured compound has received increased attention and the test has been prescribed for assessment of viability in many forest tree seeds (Enescu, 1991). The seeds are subjectively placed into vigour categories ranging from strong to weak after performing the TZ test. The test has been successfully used as efficiently in the estimation of viability in many seed species including *Fraxinus mandshurica*, *Juglans regia* and *Pinus fungeana* etc. (Yu and Wang, 1994). TZ viability test was included as a measure of maximum possible germination for comparative purposes (Allen and Meyer, 1990). This parameter of biochemical integrity of the seed have shown to correlate strongly with desiccation injury in recalcitrant seeds like mango (Fu et al., 1990) and *Camellia sinensis* (Berjak et al., 1993).

Speed of germination is one of the oldest seed vigour concepts to establish the vigour status of a seed. Many methods for determining germination rate have been employed (Tucker and Wright, 1965; Nichols and Heydecker, 1968). Hence, an important component of seed vigour is the rate of germination following imbibition (Tarquis and Bradford, 1992), an effective index of germination rate (Mauromicale and Cavallaro, 1996). If the deterioration within a seed due to desiccation/ageing continues far enough, then it dies, but before this catastrophic end-point is reached, many sub-cellular changes occur (Roberts and Ellis, 1982), giving rise to slower germination and many other performance symptoms (Ellis and Roberts, 1980) collectively described as poor vigour (Perry, 1978). It has been argued that most measurable attributes of seed vigour are closely correlated, with the results of the standard germination test (Ellis and Roberts, 1980; Khah et al., 1986; Roberts, 1986). Decline in germination index precedes the loss in germination percentage was reported in *Shorea robusta*
in this laboratory (unpublished). It confirms to the findings that the time to germination increases logarithmically as viability declines during ageing (Ellis and Roberts, 1981; Argerich et al., 1989). Thus there is a definite quantifiable relationship between germination rate, seed viability and seed deterioration during storage. It is generally considered that the delay in germination of aged seeds is related to damage accumulated during storage at moisture contents too low for repair processes to occur (Priestley, 1986). The delay in germination upon imbibition must be presumably due to the repair before germination could commence and there are considerable evidences that repair processes occurs soon after the imbibition of aged seeds (Argerich and Bradford, 1989).

Mean germination time (MGT), a measure of time required for germination, was also related to viability during ageing of lettuce seeds (Tarquis and Bradford, 1992). It is also used to compare seeds with impermeable and hard endocarp. Increasing MGT has been observed in lettuce seeds quite before the viability began to decline. As viability was lost, the mean germination time of the remaining viable seeds continued to increase (Tarquis and Bradford, 1992). It has been previously observed for lettuce (Kraak and Vos, 1987) and tomato (Argerich et al., 1989) seeds that viability was maintained but germination rate decreased. MGT was reported to be very low in seeds of herbage grasses (Mauromicale and Cavallaro, 1996) and lowered with priming. Germination rate may provide the first indication of desiccation stress in recalcitrant seeds (Pritchard, 1996) thus mango seeds exhibited a reduced vigour index before there was any noticeable fall in germination per se (Fu et al., 1990).

Other than the germination index and mean germination time, the seedling vigour index also yields information regarding seed vigour as vigour index is an accurate index for testing seed quality. This parameter becomes important under circumstances when the stored
seeds exhibit 100% viability even after slight or large desiccation, especially in the intermediate seeds. For example, after slight desiccation, the percentage germination of the wampee Clausena lansium (Lour) Skeels seeds at 67-88 days after anthesis was maintained 100%, but their vigour index decreased considerably (Fu et al., 1994). With desiccation, vigour index decreased and when the desiccation became more severe, the per cent germination also decreased. Thus, the aim of the present study was to understand the storage physiology of neem seeds by identifying the relationship of moisture content and storage period with seed viability and vigour.

**Materials and Methods**

**Site Of Seed Collection**

The neem seeds were collected from plus trees growing in Pt. Ravishankar Shukla University campus, Raipur (21°14'14" N latitude and 81°38'55" E longitude; elevation: 298 meters above sea level). Nearly 20-25 plus trees were marked in the area which were used for tagging of flowers (to identify the duration for seed maturation) and collection of mature fruits.

**Neem Fruit**

Flowering in neem is spread over January to May depending on the latitude, though occasional flowering during October to November has also been reported in neem trees from some other parts of the country (Guhabakshi, 1984). In Raipur, the flower buds appear by late February to mid March. The flowers arise acropetally (Garudamma, 1956). The inflorescence is long and slender, axillary or terminal panicle with abundant flowers. The flowers, white or pale yellow with a sweet aroma, bloom in large bunches and they
cover up the trees. The fruit matures 12-13 weeks after anthesis (Maithani et al., 1989). One of the six ovules develop into a seed. Two or three layers of nucellar cells persist in the seeds as a perisperm. Both the integuments contribute to the formation of the seed coat. The fruit is an ovoid drupe (Randhawa and Parmar, 1996). Neem fruits are green when young and turn yellow to brown when ripe. The epicarp is thin and the endocarp is hard and bony whereas the mesocarp is pulpy. The fruits in Raipur generally ripen in early June to late June, and fall as soon as they ripen. The fruits are oblong, around 1.17 cm in length and 1.22 cm wide. Mature fruits are firm, thinly milked and with hard endocarp.

Neem Seed And Its Collection

The seed is ovoid or spherical, pointed above and has a thin testa. It is exarillate with a small adaxial sacrotesta (Pennigton and Styles, 1975). The mature fruit occasionally comprises of two to three seeds also. The mature, yellow fruits were harvested (84 days after anthesis) from trees either by hand plucking or collecting the freshly dropped fruits over a cloth sheet spread on the ground; and then brought to the laboratory within an hour of harvesting. In order to have sufficient fruits with morphological similarity, the fruits of almost identical size and colour were sorted out. A few seeds from the lot were depulped manually to determine the initial seed moisture content (by hot air oven method) at the time of harvest. The seeds were extracted from the fruits by soaking them in 20% \( \text{H}_2\text{SO}_4 \) (Merck, India) for 30 min. followed by macerating them manually. Acid treatment (McDonald and Copeland, 1992) to the fruits rendered easy depulping of the fruit tissue and extraction of the seeds. The seeds with hard endocarp were rubbed gently over a wire mesh and was then thoroughly washed under running water to remove the traces of pulp adhering to the endocarp (Pritchard and Daws, 1997). The seeds, with endocarp, were dried to their initial moisture content under shade for 12 hours (Pukittayacamee et al., 1995) and stored in perforated
trays at ambient conditions (27-32°C and 35-45% RH). The seeds were ready for analyses within 18 hours (time required for depulping, extraction and drying to initial moisture content) of harvest. Seeds were harvested at desired intervals for various analyses from zero day after harvest (dah) to 160 dah. The neem seeds were harvested consecutively for 3 years (from 1996 to 1998) and all the experiments except cryopreservation (conducted only in 1998-1999) were performed three times. For convenience, the data of 1998-1999 has been reported.

**Percentage Germination**

The hard bony endocarp of the seeds were removed by slight hammering and breaking of the seed coat, slight enough to avert any damage to the seed kernel. Excised seeds (seeds without endocarp) were surface sterilized with 1% sodium hypochlorite solution (BDH, India) for 15 minutes (Motete *et al*., 1997). The seeds were then thoroughly washed 4-5 times with glass distilled water. These seeds were placed between two layers of filter paper towels (Sonar, India) saturated with distilled water and placed in Petri dish (Chaitanya and Naithani, 1994). The Petri dishes were kept in dark at 27-32°C and germination was scored every 24 hours as the radicle emergence to 5mm in length. Distilled water was supplied to the germinating seeds as and when necessary. The experiment was terminated when the seeds showed partial or complete decay with severe fungal infestation and/or no germination, even after a week since the last seed germinated. Germination test was performed with 15 seeds in four replicates.

**Moisture Content**

The % moisture content of the intact seed with endocarp was determined on fresh weight basis following the method given by International Seed Testing Association (ISTA, 1985). Four replicates each of 3g were used for the test. Seeds were weighed before and after drying
in a hot air oven (Tempo, India) at 103°C for 17 hours and percentage moisture content of the seed was calculated as percentages water of fresh weight. The seed moisture content was determined by the following formula:

\[
\text{% Seed Moisture Content} = \frac{\text{Seed Fresh Weight} - \text{Seed Dry Weight}}{\text{Seed Fresh Weight}} \times 100
\]

Similarly, the moisture content of the embryonic axis and the cotyledon was also determined on fresh weight basis. Four replicates each of 50 mg embryonic axis and 500 mg cotyledon were used for the determination of moisture content. The procedure of hot air oven method, as described for seeds, was used for estimation of moisture content in the embryonic axes and cotyledon.

**Triphenyl Tetrazolium Chloride Test**

The tetrazolium test (TZ) was performed according to International Seed Testing Association (ISTA, 1996). The test is based on the ability of dehydrogenase enzymes (activated by presoaking and incubation) in the actively respiring areas of the living tissues in embryo and endosperm (Enescu, 1991) to produce hydrogen ions, which in turn react with the colourless solution of tetrazolium salt to form a stable red-coloured triphenyl formazan which is insoluble in water (Yu and Wang, 1994). The dead tissues, on the other hand, did not stain.

The TZ test is recommended for testing seed viability. The test was performed by imbibing the excised seeds in dark for 18 hours in water at 27-32°C as per the details described in the germination test. After soaking, the papery brown seed cover was removed.
carefully using a scalpel and forceps (Kuo et al., 1996). De-coated seeds were immersed in 1% solution of 2,3,5 Triphenyl Tetrazolium Chloride (Sigma, USA) (Moore, 1985) and incubated in dark at 27-32°C for 12 hours. The red -coloured formazan, formed in seed tissues, was extracted in ethanol and the absorbance of the ethanolic solution was read at 520nm (Rudrapal and Basu, 1979). Formation of formazan, was correlated with viability and expressed as $A_{520}/g$ fresh mass of seed tissue (cotyledon or embryonic axis). The experiment was performed in three replicates of weighed cotyledons and embryonic axes.

**Germination Index**

To evaluate the speed and efficiency of germination, the germination index (GI) of the seed was estimated by the method given by Czabator (1962). The data obtained in the germination test was used for estimating germination index in the following manner:

\[
GI = MDG \times PV
\]

where,

- \(GI\) = Germination Index
- \(MDG\) = Mean Daily Germination, and
- \(PV\) = Peak Value

\(MDG\), is a measure of the totality of germination and is calculated as the final percentage germination achieved in the test divided by the duration of the test. \(PV\), was calculated as maximum value of percentage germination on any day divided by the number of days taken to achieve that percentage germination.

**Mean Germination Time**

To determine the rate of germination, the mean germination time (MGT) was calculated according to Ellis and Roberts (1981) and expressed as:
$MGT(h) = \frac{\Sigma (hn)}{\Sigma n}$

where, $h =$ the number of hours from the beginning of germination test

$n =$ the number of seeds germinating in hours $h$.

The termination of germination test was considered to be the time when the last observation was recorded, whereafter, no seed germinated.

**Vigour Index**

The vigour index (VI) of desiccating or ageing seed was determined by the method given by Fu et al. (1994). The root length of all the germinated seeds during germination test was measured after three days, counted from the day it exhibited germination (5mm radicle length). Vigour index was computed as follows:

$VI = \text{Germination (\%)} \times \text{Root Length (cm)}$

**Results**

**Percentage Germination**

The neem seeds showed absolute loss in viability within 160 days of maturation (Fig. 1.1). The freshly harvested seeds exhibited 100% viability up to 15 dah. An effective decline in per cent germination was discernible as early as 40 dah (10% seed moisture content) when the seed lot showed 76% germination. Thereafter, a gradual and steady fall in percentage germination was registered (60% on 70 dah and 30% germination on 120 dah) until all the seeds were non-viable on 160 dah (6.2% seed moisture content). The per cent germination of the seeds displayed a strong negative correlation with duration of
Fig. 1.1 The decline in percentage germination (●) and moisture content (% fw) (○) with age of mature neem seeds during storage under ambient conditions. Each value of germination is mean of 60 observations. Inset: showing the rapid decline in percent moisture content as compared to 100% germination throughout the first 15 days of storage. Vertical bar represent maximum ± SD.

Storage of the seeds with coefficient of determination, $R^2 = -0.989$, $p = 0.01$ ($y = -0.6353x + 103.2$). On the contrary, a strong positive correlation $R^2 = 0.981$, $p = 0.01$ was expressed between seed moisture content and percentage germination of the seeds as rapid fall in germination was recorded with the loss in moisture content. The regression line was plotted using a quintic polynomial equation, $y = 4E-05x^5 - 0.0054x^4 + 0.2964x^3 - 7.8333x^2 + 98.614x - 367.85$. On desiccation up to 10.9% seed moisture content, the seeds retained 100% viability. But on further desiccation, a gradual loss in the percentage germination was registered (Fig. 1.2). Fig. 1.1 (inset) exhibits no fall in per cent germination despite rapid decline in seed moisture content during the first 15 days of storage.
Seed Moisture Content

The neem seeds with relatively high initial moisture content (42.2\% f.wt. basis) recorded a rapid decline in moisture content within 15 days after harvest (dah) (Fig. 1.1). The seed moisture content reduced to almost half (22.4\%) by 8 dah and nearly 74\% loss in moisture was recorded by 15 dah. Thereafter, a gradual but steady decline in the seed moisture content was recorded. The lowest safe moisture content (LSMC) was recorded to be 10.9\% on 15 dah (Fig. 1.2). Further desiccation resulted in loss of viability. The seeds became non-viable when desiccated to 6.2\% moisture content after 160 dah. The decline in seed moisture content exhibited a strong negative correlation with seed storage. The coefficient of determination calculated was $R^2 = 0.983$, $p = 0.01$. The regression line was plotted using a sixth order polynomial equation, $y = 3E-10x^6 - 1E-07x^5 + 3E-05x^4 - 0.003x^3 + 0.1561x^2 + 3.8976x + 45.138$.

Fig. 1.2 The relationship between seed moisture content (%fw) and viability during storage and illustrating the lowest safe moisture content (LSMC) of neem seeds. The value of coefficient of determination obtained between germination and seed moisture content was $R^2 = 0.981$. 

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LSMC = 10.9\%
Embryonic Axes Moisture Content

Estimation of moisture content in one of the seed components, the embryonic axes exhibited a gradual decline (Fig. 1.3), although the initial moisture content of embryonic axes was substantially higher than the cotyledon as well as the total seed moisture content. The moisture content of embryonic axes of the freshly harvested seed lot was 56.5% (0 dah) which reduced sharply to 20.2% on 20 dah and to 10.3% on 50 dah.

Fig. 1.3 Decline in moisture content (% fw) in embryonic axes of neem seeds during storage. Inset: showing correlation between % moisture content of the embryonic axes and % germination with coefficient of determination $R^2 = 0.99$. Vertical bar represent maximum ± SD.

Thereafter, decline in moisture content of the embryonic axes too was gradual, as in case of the seeds. The moisture content of the axes of non-viable seeds (160 dah) was 6.2%. At LSMC of the seed on 15 dah, the moisture content of the embryonic axes was calculated to be 22.7% (fresh weight basis). The moisture content of the embryonic axes documented a
strong negative correlation with duration of storage \((R^2 = -0.982, \ p = 0.01; \ y = 1E-06x^4 - 0.0004x^3 + 0.049x^2 - 2.5734x + 54.549)\) and on the other hand expressed an impressive positive correlation with coefficient of determination, \(R^2 = 0.991, \ p = 0.01\), with the loss of viability (Fig. 1.3: inset). The regression line was plotted using a sixth order polynomial equation, \(y = -5E-07x^6 + 0.0001x^5 - 0.0093x^4 + 0.3874x^3 - 8.6457x^2 + 98.332x - 355.29\).

**Cotyledon Moisture Content**

The cotyledon moisture content also showed an identical pattern to that of the changes in the embryonic axes and seed moisture contents during storage. It declined from 46.5% on 0 dah to 9.7% on 15 dah (Fig. 1.4). Approximately 80% moisture content decline in

![Fig. 1.4](image-url)  
*Fig. 1.4* Changes in moisture content (% fw) in cotyledon of neem seeds during storage. Inset: showing the regression line plotted between % moisture content of cotyledon and % germination using a sixth order polynomial equation. The coefficient of determination calculated was \(R^2 = 0.962\). Vertical bar represent maximum ± SD.
the cotyledon was recorded within the first 15 days of storage. The moisture content declined to 6.7% on 50 dah and to as low as 4.9% on 160 dah (non-viable seeds). The cotyledon moisture content of the seed at LSMC was 9.7% (fresh weight basis). The cotyledon moisture content expressed a strong negative correlation with the time of storage with coefficient of determination, $R^2 = 0.984$, $p = 0.01$; $y = 3E-10x^6 - 2E-07x^5 + 3E-05x^4 - 0.0035x^3 + 0.1816x^2 - 4.5284x + 48.282$ and on the other hand registered an outstanding positive correlation ($R^2 = 0.962$, $p = 0.01$; $y = -5E-06x^6 + 0.0008x^5 - 0.0488x^4 + 1.5431x^3 - 25.506x^2 + 207.45x - 552.07$) with the loss of viability (Fig. 1.4: inset).

**Triphenyl Tetrazolium Chloride Test**

The triphenyl tetrazolium (TZ) test, showed a steady and gradual decline both in the embryonic axes (Fig. 1.5) and cotyledon (Fig. 1.6) of neem seeds concomitant to the decline in the percent seed moisture content and percentage germination during storage at ambient conditions. Very high values (13 fold) in the embryonic axes compared to cotyledon were registered. At six dah when the seed moisture content was 28.6%, the TZ colouration dropped by 4.4 and 12% in the embryonic axes and cotyledon respectively. The TZ colouration further declined gradually both in the embryonic axes and cotyledons of the desiccating seeds. These values declined sharply in the embryonic axes of seeds desiccated below LSMC from $1074.52 \text{ A} \_520 / \text{g f.wt.}$ on 15 dah to $369.88 \text{ A} \_520 / \text{g f.wt.}$ in seeds with 8.9% moisture on 60 dah and $137.05 \text{ A} \_520 / \text{g f.wt.}$ in seeds of 100 dah (Fig. 1.5) with 7.4% moisture content. Similarly the TZ colouration levels in the cotyledons also declined from $66.11 \text{ A} \_520 / \text{g f.wt.}$ on 15 dah in seeds at LSMC (10.9%) to as low as $4.05 \text{ A} \_520 / \text{g f.wt.}$ in seeds with 7.4% moisture content.
Fig. 1.5 The relationship between seed moisture content (% fw) and tetrazolium staining of embryonic axes of neem seeds during storage. Values are mean of 3 replicates. Vertical bar represent maximum ± SD. Inset: showing strong positive correlation ($R^2 = 0.988$) using a fourth order polynomial equation between tetrazolium staining of the embryonic axes and % germination.

(Fig. 1.6). Higher rates of loss of dehydrogenase activity in the cotyledon continued at the later stages than in the embryonic axes. Negligible staining in the embryonic axes and cotyledon of seed was recorded in the non-viable and seeds with lower viability in the later stages of the study. The TZ colouration in the embryonic axes registered a highly positive correlation with loss of seed moisture with coefficient of determination, $R^2 = 0.967$, $p = 0.01$ ($y = 8E-05x^4 + 0.0116x^3 + 0.6668x^2 - 18.721x + 257.44x - 1472x + 2784.4$) and loss in viability ($R^2 = 0.988$, $p = 0.01$ from a quartic polynomial equation, $y = -2E-10x^4 + 6E-07x^3 + 0.0006x^2 + 0.3103x + 4.5531$). Similarly, the cotyledon tissue of the seeds also evidenced a strong positive correlation with decline in seed moisture content (coefficient of determination, $R^2 = 0.981$, $p = 0.01$; $y = 5E-06x^5 - 0.0007x^4 + 0.0415x^3 - 1.1373x^2 + 15.208x^1 - 82.649x + 141.05$).
Fig. 1.6  The relationship between seed moisture content (% fw) and tetrazolium staining of cotyledon during storage of neem seeds. Values are mean of 3 replicates. Vertical bar represent maximum ± SD.

Inset: showing positive correlation between tetrazolium staining of the cotyledon and % germination with the coefficient of determination $R^2 = 0.988$.

and loss in viability ($R^2 = 0.988$, $p = 0.01$). The regression line was plotted from a sixth order polynomial equation, $y = -1E-08x^6 + 4E-06x^5 - 0.0005x^4 + 0.0299x^3 - 0.8616x^2 + 11.764x - 3.9036$).

Germination Index

Unlike percentage germination, the germination index was maintained at maximum only for four days of storage when the seed moisture content decreased from 42.2% to 34.6% (fresh weight basis) (Fig. 1.7). Further dehydration to 28.6% (six dah) resulted in an abrupt loss of GI from 10,000 to 2,500 which subsequently declined to 1110.88 at 10.9% seed moisture content (LSMC on 15 dah). Though 100% germination was exhibited by the seeds
up till 15 dah, the GI of the seeds had decreased considerably (75%) during this period. The GI values further continued to decrease gradually with increased desiccation to 254.42 in 8.9% moisture content seeds (60 dah) and to a very low of 35.36 on 100th day of storage (7.4% seed moisture content). Except for the very high values of GI in the first 8 days of seed storage, these values were comparatively very low in the seeds recorded in the seeds with moisture content below 22.4% (Fig. 1.7). The germination index values exhibited a strong positive correlation and the coefficient of determination calculated was $R^2 = 0.977, p = 0.01$ using a sixth order polynomial equation, $y = 0.0004x^6 - 0.0573x^5 + 3.492x^4 - 103.3x^3 + 1544.4x^2 - 10807x + 27883$ with desiccation.
Mean Germination Time

The rate and speed of germination, calculated as mean germination time (MGT) showed a gradual and consistent increase throughout the period of analyses. The time required for the germination of the seeds increased with increasing desiccation of the seeds. When in the freshly harvested seeds the MGT was 24 h, this value increased gradually up to 38.06 h in the seeds at LSMC (10.9%) and these values increased to a high of 152 h on 140 dah in the seeds with 6.5% moisture content (Fig. 1.8). No values of MGT could be recorded on 160 dah as the seeds did not exhibit germination. The mean germination time showed a close correlation with seed moisture content \( R^2 = -0.99; p = 0.01 \) and the regression line was plotted using quintic polynomial equation. 

\[
y = -6E-05x^5 + 0.0078x^4 - 0.4207x^3 + 10.844x^2 - 133.7x + 659.78
\]
The relationship between seed moisture content (% f.w) and seedling vigour index (SVI) of neem seeds during storage.

Seedling Vigour Index

Monitoring the rate of root growth for the first three days after germination exhibited a decline in the vigour of the seeds with the degree of desiccation during storage of neem seeds. The seedling vigour index was recorded 288 on zero day, which gradually declined to almost half (138.46) with a loss of about 75% moisture by 30 days (Fig. 1.9). A sharp decline in the seedling vigour index values were recorded in the later period of the study (30.8 at 7.6% moisture content and 9.5 at 7.1% moisture content). The decline in seedling vigour index value was gradual throughout the period of the study and very low values of 1.3 was obtained for desiccated seeds with 6.5% moisture content on 140 days. The SVI values showed a close positive correlation (Fig. 1.9) with decline in seed moisture content during storage ($R^2 = 0.976$, $p = 0.01$; $y = 2E-05x^4 + 0.0032x^3 + 0.1813x^2 - 5.0446x + 69.625x^2 - 419.38x + 878.91$).
Discussion

*Intermediate storage behaviour* of neem seeds was evident from our results. Initially, the freshly harvested mature neem seeds appeared tolerant to desiccation as they exhibited 100% viability inspite of massive reduction (70%) in moisture content from 42.2 to 10.9% (Fig. 1.1: inset). Slight dehydration of seed below the lowest safe moisture content (LSMC) 10.9%, a relatively desiccated state compared to recalcitrant seeds, resulted in a considerable loss of viability (Fig. 1.2). For example, marginal desiccation (1.4%) from 10.9% (15 dah) to 9.5% (50 dah) leads to almost 30% loss in germination whereas, further desiccation to 6.2% moisture content, with advance in age (160 dah) of stored seeds, resulted in complete loss of germination (Fig.1.1). The data on % germination clearly indicates the phenomenon of rapid loss of viability in these seeds. In fact, a close correlation with coefficient of determination \( R^2 = 0.981 \), was established between the % germination and % seed moisture (Fig.1.1). The present observations on % moisture content and % germination strengthen conclusions drawn about the desiccation-sensitivity at a critical moisture content i.e., LSMC. Most of the intermediate seeds, which are shed at high moisture content, like coffee (Ellis *et al.*, 1990), papaya (Ellis *et al.*, 1991b), *Elaeis guineensis* (Ellis *et al.*, 1991c) including neem (Gamene *et al.*, 1996; Sacande *et al.*, 1996; DFSC, 1997), have been shown to survive with high percentage of viability when desiccated to "*intermediate moisture content*" [9-13%]. They showed rapid loss of viability when dried below intermediate moisture content. Desiccation of neem seeds from 42.2 to 6.2% moisture content (at ambient conditions), over a span of 160 dah of storage, was relatively slow in comparison to rapid drying (using silica gel), wherein the seed moisture content was reduced to 5.8% (DFSC, 1997) and 2.9% (Pukittayacamee, 1997) in 16 and 31 days respectively. Irrespective of the *modus operandi* of drying, neem seeds exhibit similar value of LSMC, 9-12% (Gamene *et al.*, 1996; Pritchard and Daws, 1997).
In addition, rapid loss of viability in seeds dehydrated below LSMC clearly indicated that desiccation is the detrimental factor in deterioration of desiccation-sensitive neem seeds during storage.

Thus it is concluded that the neem seeds, in principle, are desiccation-sensitive but unlike recalcitrant seeds which do not survive desiccation below very high critical moisture content (>30%) (Tompsett, 1992; Chaitanya and Naithani, 1994; 1999), they can be desiccated safely up to intermediate moisture content. Neem seeds (Sacande, 1998) like most of the recalcitrant seeds (Farrant et al., 1986; Finch-Savage, 1992; Chaitanya and Naithani, 1998) do not undergo maturation drying, i.e. desiccation which is important in the transition from the developmental to germination mode for most of the orthodox seeds (Kermode and Bewley, 1986), and thus exhibits desiccation-sensitivity. In the absence of maturation-drying phase during development, the neem seeds are shed at high moisture content at about 42.2%. Therefore, increasing desiccation-sensitivity in neem or the intermediate seeds in general during storage may also be due to the initiation of germination associated events and may, therefore, be analogous to the sensitivity of recalcitrant seed and or germinating seeds of orthodox species (Berjak et al., 1984). Hence short-term storage with optimum longevity of these seeds is recommended at intermediate moisture content at temperature 27 - 32°C (Gamene et al., 1996). It is clear from the reports of Farrant et al. (1988) and Finch-Savage (1992) that loss of bulk-water from desiccation-sensitive seeds has little effect on metabolism other than the rate of reactions, but the loss of “structured water” may result in the disorganization of metabolism, leading to loss of stability of subcellular structures, including membranes and ultimately loss of viability.
Relatively lower moisture content was observed in the cotyledons (Fig. 1.4) as compared to the embryonic axes (Fig. 1.3) of neem seeds. On zero day, the %moisture content of the embryonic axes was 56.5% (Fig. 1.3), which was far higher than the cotyledon moisture, which was 46.5% (Fig. 1.4). The lower moisture content in the cotyledon as compared to embryonic axes has been reported in other recalcitrant seeds, viz., Avicennia marina (Berjak et al., 1990), Quercus robur (Finch-Savage, 1992). They showed different levels of matrix-bound water in the cotyledons and in the axis, and hypothesized that, if cells within recalcitrant seeds cannot survive the loss of all free cell water, this would result in different levels of desiccation-sensitivity between these tissues. Loss of viability in desiccation-sensitive seeds appeared to be determined by a critical moisture content in the cotyledons which subsequently caused loss of viability in the axis (Finch-Savage, 1992). The %moisture content of both the embryonic axes and cotyledons exhibited significant correlation with ageing (coefficient of determination $R^2 = -0.982$ and $R^2 = -0.984$) respectively, as well as with loss of germination ($R^2 = 0.99$ and $R^2 = 0.962$). This further supports the view that loss of viability in desiccating neem seeds is contributed by rapid desiccation of both the tissues, viz., embryonic axes and cotyledons, almost at same rates.

Another subtle change during seed deterioration which is related to loss of vigour is the loss of dehydrogenase activity as indicated by the inability of the embryo to reduce TZ to their coloured insoluble formazan salt (Lakon, 1949; Yu and Wang, 1994). Higher dehydrogenase activity is reported to be associated with higher viability and vigour (Throneberry and Smith, 1955; Nautiyal and Joshi, 1991). TZ activity, estimated in cotyledon and axis, declined sharply as the seed moisture decreased to 28.6% on six day of storage. It continued to decrease almost by 9.3% and 28% in axis and cotyledons respectively in the
seeds of 15 dah with 10.9% moisture content. The dehydrogenase activity declined gradually as the desiccation of seed progressed during storage thus demonstrating positive relationship with dehydrating embryonic axis (Fig. 1.5) and cotyledon (Fig. 1.6). TZ test has been extensively used for testing viability and vigour of seeds of several tree species (Bodhipuks et al., 1992; Espindola et al., 1994; Yu and Wang, 1994). Extraordinarily high (13 fold) TZ activity in axis than in cotyledon may be due to the location of all the major deterioration related changes occurring in the axes (Bewley, 1986). Similarly higher rates of loss of TZ activity in axis than the cotyledon can be explained on the basis of their differential desiccation-sensitivity during loss of viability as also reported in Quercus robur (Finch-Savage, 1992) and Araucaria hunsteinii (Pritchard and Prendergast, 1986). Finch-Savage (1992) concluded that in whole seeds, loss of viability appeared to be determined by critical moisture in the cotyledon, which subsequently caused loss of viability in the axis.

The simple analysis of the percentage germination obscures a documented fact that seeds go through various stages of deterioration during storage before they become incapable of germinating (Roberts, 1973). As the neem seeds are moderately desiccation-tolerant absolutely no loss in seed vigour was observed when the freshly mature seeds with 42.2% moisture were desiccated to 34.6% in four days after harvest during storage. Various physiological tests revealed highest germination index (GI), seedling vigour index (SVI) and lowest MGT for freshly mature seeds and were maintained high even when the moisture content of the seeds dropped to 34.6%. Vigour declined however, when the seeds were desiccated to 28.6% (six dah) as substantial loss in GI (75%) (Fig. 1.7), an increase in MGT (Fig. 1.8) and marginal loss in SVI (20%) (Fig. 1.9) was observed. But later on, declining
pattern, with higher magnitude even in SVI was discernible in seeds desiccated to 13.4% (GI = 89% and SVI = 31%) and 10.9% (GI = 89% and SVI = 34%) moisture content. It clearly points out significant vigour loss in the desiccated seeds when the viability was still 100%. Similar correlation was made to describe loss of vigour in deteriorating seeds by measuring GI (Farrant et al., 1986) and SVI (Fu et al., 1994) in other seeds. Loss of GI was also observed during loss of viability in seeds of Avicennia marina (Farrant et al., 1993). The decline in GI in these seeds appeared to result from a decline in germination vigour due to increase in lag period for germination and finally reducing the germination ability (Farrant et al., 1993). The suggestion was supported by the fact that ultrastructure of embryos exhibiting loss of viability in storage showed evidence of subcellular damage (Farrant et al., 1986).

Our data therefore strongly indicate that germination percentage declines only after significant loss of seed vigour. Hence, it is recommended that seed vigour should be essentially considered along with percentage germination as one of the important criterion for storage evaluations of neem as well as other seeds exhibiting intermediate storage behaviour.