CHAPTER II
Membrane Damage
&
Leakage Loss
Introduction

Membrane deterioration has been revealed to play an exceptionally important role during seed deterioration and ageing (Koostra and Harrington, 1969; Simon and Raja Harun, 1972; Harman and Maltick, 1976). Adverse storage conditions like desiccation or water loss from the tissues induce cellular membrane damage and increased leakage loss (McKersie and Stinson, 1980; Senaratna and Mkersie, 1983). Damage to cellular membrane resulting in the enhanced efflux of cellular constituents in the seed leachates, was considered to be one of the earliest events associated with loss of seed viability (Delouche, 1969; Roberts, 1979).

The diverse metabolic changes and failures associated with seed deterioration and viability loss could result into a greater or lesser extent from disruption of membranes (Bewley, 1986). On imbibition, inorganic ions, sugars, amino acids, proteins and organic acids leak out (Larson, 1968; Simon and Raja Harun, 1972; Duke and Kakefuda, 1981) from the seeds. Leakage has been found to vary for different solutes, different species and different seeds (McKersie and Stinson, 1980; Lott et al., 1991). Attempts have been made to correlate this leakage with the extent of seed viability (Bewley, 1986; Chaitanya and Naithani, 1994). Excess leakage from deteriorated seeds may represent loss of respirable substrates from some seed species, whereas others may lose more amino acids than sugars (Bewley, 1986). Koostra and Harrington (1969) have shown that a decline in polar lipids, chiefly owing to their oxidation, may be the immediate cause of leaky membrane in seeds. McKersie and Tomes (1980) recorded an increase in cytoplasmic leakage from damaged seeds of Lotus corniculatus, which they presumed to reflect a loss in membrane integrity as a result of dehydration contributing to reduced germination.
and vigour of the seeds. In many recalcitrant seeds a good correlation has been established between dehydration and electrolyte leakage (Fu et al., 1990; Chaitanya and Naithani, 1994).

There is a plethora of literature indicating that during imbibition, seeds leach out various metabolites, viz., K, Ca, Mg, Mn (Simon, and Raja Harun, 1972; Simon, 1974; Loomis and Smith, 1980; Lott et al., 1991; Beecroft and Lott, 1993; Chaitanya, 1997), amino acids and proteins (Simon and Raja Harun, 1972; McKersie and Tomes, 1980; Nautiyal and Purohit, 1985) and other solutes. Potassium loss from seeds is substantial and is a general indicator of electrolyte release (Mullett and Considine, 1980; Beecroft and Lott, 1993). Increased K leakage in aged pea seeds depicts the well-established fact that imbibitional damage increases with age and damage to the seed (Loomis and Smith, 1980; Schoettle and Leopold, 1984; Bruggink et al., 1991). It is possible that some mechanism involving the interaction of oxygen free radical with some membrane K carrier or pathway mediate the K loss. Moreover, K is the dominant cation in the mature plant cells (Gorham and Wyn Jones, 1983; Hajibagheri et al., 1988) (distributed in the vacuole and cytoplasm) and its functions in each of these components are well known (Leigh and Wyn Jones, 1984). It has a purely osmotic role in the vacuole, whereas in the cytoplasm, it is involved in several biochemical reactions. Also many enzymes are K-dependent (Evans and Sorger, 1966).

Calcium (Ca) is another important element especially in the structural maintenance of membrane and influences the changes in electrical conductivity (Cheour et al., 1990). The structure and functions of cell walls (Glenn et al., 1988) and certain aspects of cell metabolism are also affected by Ca concentration (Cheour et al., 1992). Ca has been reported to be functional in delaying the senescence in leaves also (Pooviah and Leopold, 1973). It binds extracellularly to the membrane phospholipids and thus maintains membrane integrity and controls membrane associated functions (Glenn et al., 1988). However an increase in cytosolic
Ca may stimulate lipolytic enzyme activity and accelerate membrane deterioration (Thompson, 1988). The protection of cell membrane integrity by Ca during ageing (Thompson, 1988), has been explained by the ability of Ca to bind membrane phospholipid and thereby stabilize the membrane and control membrane associated functions (Pooviah et al., 1988).

Similarly Zinc is considered to play a critical role in the structure and function of biomembranes. It has number of structural functions, viz., (a) it is bound with high affinity to amino acids - cysteine, histidine, glutamate and/or aspartate, in number of metallo-proteins (Vallee, 1990); (b) a stabilizer of supramolecular structures - membranes/membrane skeleton (Bashford et al., 1988; Mahadevan et al., 1990), organelles (Bettger and O'Dell, 1993). Zn has important catalytic functions too - (a) it is an effector molecule of enzyme; transporter and has a role in membrane channel activity - allosteric and protein translocation; (b) an effector of gene expression (Bettger and O'Dell, 1993). Besides, Zn deficiency has been associated with altered lipid metabolism, altered susceptibility to lipid peroxidation (Bray and Bettger, 1990; Bettger, 1993) and altered membrane structures and functions (Bettger and O'Dell, 1981) in many tissues. Zn functions as a site-specific antioxidant and renders protection against the disrupting effects of lipid peroxidation and protein oxidation (Wilson, 1989; Bray and Bettger, 1990). Above all, Zn has a role in the direct alteration of the physical state of the membrane lipids (Gordyziel et al., 1982; Bevon et al., 1983; Barfield and Bevon, 1985, Deleers et al., 1986; Hauser, 1991).

Thus to assess the membrane integrity during desiccation-induced loss of viability in neem seeds, an attempt was made to study the electrolyte leakage loss and the changes in leachate constituents, like sugar, protein, phenol, and ions especially K, Ca and Zn, the status of which is yet to be reported in case of neem seeds.
**Materials and Methods**

Collection of Seed Leachates

The seed steep water from the seeds kept for germination was used for the estimation of leachate conductivity. The seed leachate after 24 hours of seed imbibition was collected and the seeds were washed repeatedly with distilled water so as to collect the last traces of exudate. Thirty millilitre of seed leachates was collected and used for the determination of specific conductance (Chaitanya and Naithani, 1994). Ten millilitre of the collected leachates was stored in plastic vials (Laxbro, India) at 4°C and later used for various analyses (Simon and Raja Harun, 1972). Four replicates of seed leachates were used for estimating following parameters of leachate analyses.

Leachate Conductivity

Leachate conductivity was determined following the method described by Nautiyal and Purohit (1985) using Digital Direct Reading Conductivity Meter 304 (Systronics, India). Leachate conductivity was expressed in mMhos/ml.

Element Analysis in Leachates

The levels of potassium, calcium and zinc ions were analysed in the seed leachates. Zn^{++} was determined on Atomic Absorption Spectrophotometer (Camag, United Kingdom) at School of Studies in Chemistry, Pt. Ravishankar Shukla University, Raipur (courtesy: Dr. K.S. Patel). Amounts of other ions (K^{+} and Ca^{++}) levels were determined on the Systronics Flame Photometer (at School of Studies in Chemistry, Pt. Ravishankar Shukla University, Raipur; courtesy: Dr. Kallol K. Ghosh). All the ion levels were expressed in mg/ml leachate.
The standard-curve for these ions were prepared using salts of potassium (BDH, India), calcium (Qualigens, India) and Zinc (Qualigens, India)

**Leachate Sugar Content**

Sugar content in the leachates was quantified by the method of McCready *et al.* (1950). In a glass tube known volume of the leachate solution was mixed with anthrone reagent {0.2% anthrone [CDH, India] dissolved in Conc. H₂SO₄ [Qualigens, India]}. The whole mixture was shaken and boiled for 10min in a boiling water bath. Then, it was allowed to stand in dark till the cooling of the mixture. Finally, absorbance of the dark brown mixture was recorded at 620nm using a UV-Vis spectrophotometer. The final concentration of sugar in the leachate was determined by preparing calibration curve of glucose (Qualigens, India) solution and expressed in terms of mg sugar/ml leachate sample.

**Leachate Protein Content**

Protein content in the leachate of seeds was determined colorimetrically by following the method of Lowry *et al.* (1951) on a UV-Vis spectrophotometer (Unicam UV2, UK). Leachate-protein content was expressed as mg protein/ml leachate against a calibration curve of Bovine Serum Albumin (Sigma, USA).

**Leachate Phenol Content**

The leachate-phenol content was estimated under UV-range. A 0.5ml aliquot of the leachate was diluted to 5ml with distilled water and the absorbance of the same was read at 260 nm (Biggrass and Calme, 1993). The leachate-phenol content was expressed as A₂₆₀/ml leachate.
Results

Leachate Conductivity

The changes in the specific conductivity of the seed leachates obtained from imbibing the naturally desiccating stored seeds from 0dah (fresh seeds) to 160dah (non-viable seeds), is depicted in Fig. 2.1. Very gradual increase in the specific conductivity of the seed leachates was observed during the initial stages of seed desiccation, (from 0.139mMhos/ml in 42.2% moisture content seeds on 0dah to 0.386mMhos/ml in 13.4% moisture content seeds on 12 dah). Thereafter, a steep and gradual increase was discernible in the conductivity of the leachate.

Fig. 2.1 Showing the affect of desiccation of neem seeds on electrolyte leakage after imbibition (expressed as specific conductance of leachates). Vertical bar represent maximum ± SD. Least : showing negative correlation between loss of germination and specific conductivity of leachates. The regression line plotted was plotted using a sixth order polynomial equation and the coefficient of determination was $R^2 = 0.878$.
as the seeds were desiccated below LSMC (10.9\% moisture content on 15 dah). Nearly 4-fold escalation in the leachate conductivity was calculated thereafter (i.e., from 0.386 mMhos on 12 dah to 1.315 mMhos on 160 dah). Besides this, a very strong negative correlation was established between leachate conductivity and the desiccation of seeds with coefficient of determination $R^2 = -0.989; P=0.01$; $y = 6E-06x^4 - 0.0007x^3 + 0.0306x^2 - 0.539x + 3.7086$). Moreover, the specific conductivity of the leachates expressed a close and very high negative correlation with loss of viability (% germination) ($R^2 = -0.978; P=0.01$) also. The regression line was plotted using a sixth order polynomial equation, $y = -8E-11x^6 + 2E-08x^5 - 3E-06x^4 + 0.0001x^3 - 0.0035x^2 + 0.0324x + 1.3149$).

**Calcium**

The pattern of calcium levels in the seed leachates is shown in Fig.2.2. The calcium loss was minimum in the 100\% viable seeds (0 dah) and increased gradually with the loss of viability in desiccating neem seeds showing a maximum loss in seeds with 8.3\% moisture content on 70 dah (7.66 mg/ml leachate). No leakage of calcium was discernible in the seeds desiccated up to LSMC and thereafter, there was an almost 8 fold increase in the calcium ion leakage from seeds desiccated from 10.9\% moisture (15 dah) to 70 dah old seeds with 8.3\% seed moisture content (Fig. 2.2). The calcium levels in the leachates of the further desiccated seeds (7.6\% to 6.2\% moisture content) fell down sharply. A concentration of 0.66 mg/ml leachate was recorded in the seed leachates of the 160 dah non-viable seeds.

**Potassium**

The levels of potassium in the leachates from ageing seeds were determined in mg and plotted. The pattern of potassium ion loss in the seed leachates were similar to that of the calcium loss. Initially, a gradual increase in potassium levels were registered in fresh seeds
with 42.2% moisture content on 0 dah (1.33 mg/ml leachate) to seeds desiccated to 10.8% (Fig. 2.2). A steep increase (3 fold) in the potassium ion levels were discernible in the seeds desiccated below LSMC (from 10 mg/ml leachate in 10.9% moisture seeds to 30.73 mg/ml leachate in 7.4% moisture seeds). Thereafter, decline in K levels in the leachates was observed up to 160 dah (6.2% moisture content). Maximum levels of K in leachates were obtained in 7.4 % moisture content seeds on 100 dah (30.73 mg/ml leachate).

**Zinc**

The zinc concentration in the seed leachates was estimated from deteriorating neem seeds during storage (Fig. 2.3). Zinc levels registered a very slow and gradual increase in
Fig. 2.3 Showing the leakage of zinc ions from neem seeds on imbibition with respect to changes in seed moisture content (% f.w.). Vertical bar represent maximum ± SD.

Seed leachates from 0.0mg/ml leachate on Odah (42.2% moisture) to maximum 0.122mg/ml leachate on 70dah (8.3% moisture content). After 70dah of storage, a steep decline in zinc concentration was recorded in the seed leachates and fell to as low as 0.0025mg/ml leachate in the seeds desiccated to 6.2% moisture content.

**Leachate Proteins and Sugar**

The protein levels in the stored seed leachates from imbibing seeds determined by Folin Lowry method of protein concentration determination is displayed as in Fig.2.4. There was just a marginal increase in the protein levels in the leachates despite rapid decline in the moisture content of the seeds in first two weeks of storage. The leakage loss of proteins
increased substantially (9 fold) on further desiccation of the seeds below LSMC (0.0369 mg/ml leachate in 10.9% moisture seeds on 15 dah to 0.313 mg/ml leachate in 6.2% moisture seeds on 160 dah) (Fig. 2.4). Increase in leakage of proteins in the seed leachates exhibited a strong negative correlation (coefficient of determination, $R^2 = -0.993$, $p = 0.01$; $y = -3E-08x^4 + 6E-06x^3 - 0.0004x^2 + 0.0067x + 0.313$) with desiccation (loss of seed moisture content).

![Graph demonstrating the effect of desiccation of neem seeds on sugar and protein leakage on imbibition. Vertical bars represent maximum ± SD.](image)

Similarly, the loss of sugar in the seed leachates of dehydrating neem seeds also showed a similar trend as expressed by the leachate proteins. A gradual increase in the sugar levels was observed in the desiccating seeds during initial days of storage. Thereafter, a steep increase (5 fold) in sugar loss was registered in the seeds desiccated below LSMC (from 0.0217 mg sugar/ml leachate in 10.9% moisture seeds to 0.113 mg sugar/ml leachate in seeds...
6.2% seed moisture content). Increase in leakage of sugar in the seed leachates also exhibited a strong negative correlation with desiccation with coefficient of determination $R^2 = -0.983$. The regression line was plotted using a quartic polynomial equation, $y = -1E-08x^4 + 2E-06x^3 - 0.0001x^2 + 0.0019x + 0.1151$.

![Graph](image_url)

**Fig. 2.6** Displaying the effect of desiccation of neem seeds on phenol leakage (recorded in the leachates on imbibition). Vertical bar represent maximum ± SD.

**Leachate Phenol**

The loss of phenol as measured at 260 nm in the collected seed leachates also exhibited a trend similar to that of the loss of leachate protein and sugar (Fig. 2.5). The increase in leachate phenol was gradual in the initial stages of desiccation (from 42.2% to 10.9% moisture content). About 10 fold increase was registered in the desiccating seeds (from 0.029 A260/ml leachate in fresh seeds to 0.28 A260/ml leachate in non-viable seeds).
Discussion

The loss of viability in neem seeds (reported in Chapter 1), seems to be closely influenced by its membrane permeability. As suggested by Roberts (1979) that loss of membrane integrity results in the enhanced efflux of cellular constituents into the seed leachates, and is one of the earliest events associated with loss of seed viability, the desiccating neem seeds too registered a severe leakage loss and concomitant increase in specific conductance of leachates (Fig. 2.1). The severe leakage loss was manifested by the increased conductivity of the seed leachates continuously through 0dah to 160dah. Though a regular and gradual increase in specific conductance of the leachates was registered, as long as the seeds exhibited 100% viability (i.e., from 0dah to 15dah), relatively low leachate conductivity was observed (0.139 to 0.58 mMhos/ml). With the commencement of loss of viability on 20dah when the seeds were desiccated to 10.8% seed moisture content, there was greater increase in leachate conductivity and the leakage loss continued up to the end of the study. It indicates that the deterioration and viability loss in neem seeds resulted due to disruption of membranes (McKersie and Stinson, 1980; Senaratna and McKersie, 1983; Bewley, 1986). More importantly, in these seeds, a very close correlation was established between leachate conductivity (coefficient of determination, $R^2 = -0.989$), the leachate proteins ($R^2 = -0.996$) and leachate sugar ($R^2 = -0.983$) with desiccation of the seeds. Both the parameters exhibited a downturn in their magnitudes only after the desiccation of seeds below LSMC (10.9% seed moisture content) on 15 dahn, which perhaps indicates that the membrane damage/leakage loss observed in intermediate neem seeds is an outcome of the desiccation of the seeds and is analogous to the observation of McKersie and Tomes, (1980), Nautiyal and Purohit (1985) and Chaitanya (1997) recorded in recalcitrant seeds. The severe
leakage loss in neem seeds during loss of viability may be a resultant of the architectural impairment of membrane lipids due to desiccation/low water content, as suggested by others (Koostra and Harrington, 1969; Simon, 1974; McKersie and Tomas, 1980; Nautiyal and Purohit, 1985).

With the loss of membrane integrity, an enhanced leakage loss of various metabolites has been extensively reported (Larson, 1968; Simon and Raja Harun, 1972; Simon, 1974; Loomis and Smith, 1980; McKersie and Tomas, 1980; Nautiyal and Purohit, 1985; Lott et al., 1991; Beecroft and Lott, 1993). Most important of all, the sugar, protein (Fig. 2.4) and phenol (Fig. 2.5) have been recorded in good amounts in the seed leachates, which indicates that the membrane deterioration was severe after 15dah, when the seeds desiccated below 10.9% moisture content whereby considerable amounts of sugar, protein and phenol were lost into the seed steep water. Besides this, a very strong negative correlation has been recorded between desiccation of neem seeds and the upsurge in leachate sugar, protein and phenol levels, which indicates that the membrane deterioration as well as the leakage of these macromolecules into the leachates are two inter-related phenomenon. Besides the large molecules, the efflux of vital inorganic moieties too plays a major role in the maintenance of membrane integrity and thus the normal functioning of the cell. Potassium (K) for example, which is considered to be an index of electrolyte release (Mullett and Considine, 1980; Beecroft and Lott, 1993), has been recorded in substantial amounts in leachates of neem seeds (Fig. 2.2). This increased leakage of K supports the well-established fact that leakage loss during imbibition increased with desiccation and damage to the seed (Loomis and Smith, 1980; Schoettle and Leopold, 1984).
Calcium (Ca), besides Zinc, is another important inorganic moiety in the maintenance of membrane integrity, and controls membrane associated functions (Glenn et al., 1988; Cheour et al., 1992). Ca levels exhibited a pattern similar to Zn loss (Fig. 2.2). Ca loss was lower in seeds desiccated from 42.2 to 10.9% moisture content, but in seeds desiccated beyond LSMC, there was an abrupt increase in its levels in leachates of these seeds. This implies that the membrane deterioration, which initiated in the seeds with desiccation to 10.9% moisture due to loss of other ions and organic compounds, was further intensified by the Ca loss from the seeds. Besides, Ca is considered to protect cell membrane during ageing (Thompson, 1988) by its ability to bind membrane phospholipid and render stability (Pooviah et al., 1988; Cheour et al., 1992).

The altered membrane structure and function associated with altered lipid metabolism and susceptibility to lipid peroxidation have been shown to be affected by lowering of Zn levels especially from the specific membrane proteins (Bray and Betger, 1990; Betger, 1993). It may be noted that loss of Zn (Fig. 2.3) increased from 0dah (42.2%) to 15dah (10.9% mc) and during this period the leakage of proteins from the seeds was marginal (Fig. 2.4). Thereafter, when the Zn levels in the seed leachates increased, there was a parallel upsurge in the protein and sugar loss into the leachates. The source of leakage of protein however was not characterized, whether they are released from membrane or cytoplasm fractions. The loss of Zn may also aggravate the membrane deterioration and enhance the oxidative stress in the tissues, because Zn is a potent site-specific-antioxidant, which renders protection against lipid peroxidation and protein oxidation (Wilson, 1989; Bray and Betger, 1990). The severity of leakage may majorly be attributed to Zn loss, as Zn has a direct implication in the alteration of the physical state of membrane lipids (Gordziel et al., 1982; Bevon et al., 1983;
Barfield and Bevon, 1985; Deleers et al., 1986; Hauser, 1991). The increased loss of Zn from the seeds was registered even in the initial stages (0dah to 15dah), when the seeds exhibited 100% viability though with a lower magnitude. This indicates that the membrane integrity was hampered right at the initial stages (from 0dah), although with relatively less magnitude which was perhaps repairable (reversible) and was severely perturbed (i.e., irreparable or irreversible) in the later stages of desiccating neem seeds. Limited amounts of solute leakage was observed in various absolutely viable seeds and therefore it is perhaps the threshold magnitude of leachates which is critical and considered to be important index in the loss of viability. The threshold value may vary from seed of one species to another.

Thus, from the above discussed observations, it can be forthrightly stated that the membrane deterioration as manifested by leakage loss of protein, sugar, phenols and vital inorganic ions, viz., K, Ca and Zn has the foremost and prominent role in the loss of viability in intermediate neem seeds.