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Ultrastructural Changes of *Hevea brasiliensis* Muell.-Arg. Seeds during Imbibed Storage

M.N. NORMAH* and H.F. CHIN
Agronomy Department
Faculty of Agriculture
Universiti Pertanian Malaysia
43400 UPM Serdang, Selangor Darul Ehsan, Malaysia

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ABSTRAK

Pertukaran ultrastruktur dicerap pada bijih benih Hevea yang disimpan di dalam suhu 10°C, 22°C dan 27°C. Satu ciri kerosakan yang paling biasa ialah kesusutan membran. Pada semua suhu penyimpanan, didapati plasmalemma terlipat, tersepai dan tertarik daripada dinding sel. Dalam kajan ini pelarutan tonoplas juga dicerap.

ABSTRACT

Ultrastructural changes were observed in Hevea seeds stored at 10°C, 22°C and 27°C. Membrane degeneration appeared to be the most common feature of deterioration. At all storage temperatures, the plasmalemma was observed to be increasingly folded, disintegrated or withdrawn from the cell wall. The dissolution of the tonoplast was also widely observed.

INTRODUCTION

Seeds that have been classified as recalcitrant by Roberts (1973) are those that are highly susceptible to desiccation injury and not storable under conditions suitable for orthodox seeds i.e. at low moisture content and at low temperature. *Hevea* seeds are included in this group of seeds.

Changes during deterioration in seeds have been suggested to be due to several factors. According to Delouche (1969), delayed seed germination is one of the earliest physiological signs of deterioration. The decline in respiratory rate has also been reported for various seeds such as in barley and corn under different conditions of deterioration (Woodstock and Grabe 1967). Reduction in levels of enzymes such as cytochrome oxidase, malic and alcohol dehydrogenase has been reported in non-viable maize seeds (Throneberry and Smith 1955). Impairment of carbohydrate, protein and nucleic acid synthesis are among the biocnemical events that takes place during deterioration (French 1959; Abdul-Baki 1971; Abdul-Baki 1980). Harrington (1973) believed strongly that a major cause of ageing is protein denaturation.

The diverse metabolic changes associated with seed deterioration and viability loss could result in a greater or lesser extent from disruption of membrane systems (Bewley and Black 1982). Berjak and Villiers (1970) reported that membrane aberrations increased with increasing age of maize embryos. Disintegration of cell membranes was also observed in seeds of *Hevea brasiliensis* (Chin *et al.* 1981) and *Theobroma cacao* (Hor 1984) killed by dehydration. Berjak (1968) and Hallam (1973) observed that mitocondria in aged corn seeds and in non-viable rye seeds were markedly swollen, had little development of cristae and there was a decrease in matric density.

* Present address: Botany Department, Faculty of Life Sciences, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor D.E., Malaysia

Chin *et al.*, (1981) have shown that *Hevea* seeds are killed by dehydration, high temperature or freezing. Since temperature is one of the important factors in determining the viability of seeds, this study was undertaken to examine the ultrastructural changes that occur in *Hevea* seeds during storage at different temperatures. The imbibed storage method was employed as it was the conventional method used for storing *Hevea* seeds at the time this study was carried out. It is hoped that by understanding the changes that occur in the seeds during their deterioration, an improved method of storage may be devised by delaying their deterioration.

MATERIAL AND METHODS

Seeds of clone RRIM 600 were obtained from Prang Besar Seed Gardens located in Telok Intan, Perak, Malaysia. Sawdust used in the study was cleaned by soaking in water for three days and changing the water everyday to remove toxic substances that might be present. It was then sterilised in an oven at 100°C for 48 hours and cooled before use. The sawdust was then mixed with distilled water to make up to 20% moisture content. Freshly harvested seeds were mixed in equal volume with moistened sawdust and seeds were spread in shallow layers inside perforated black polythene bags (30cm × 36cm) (ten holes of approximately 0.5cm in diameter). The seeds were then stored at 10°C (cold room), 22°C (air-conditioned room) and 27°C (ambient room). The storage duration was three months. After each month of storage, the ultrastructure of seeds was examined. Fresh seeds were used as a control.

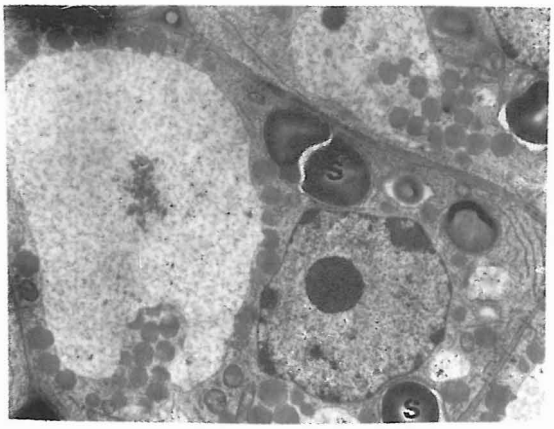
Electron Microscopy

Approximately 1 mm of the radicle of the embryonic axis about 1 mm from the tip was dissected with new razor blades while submerged under 4% glutaraldehyde solution. The tissues were then transferred immediately to fresh 4% glutaraldehyde solution and fixed for 4 hours at 4°C. The tissues were then dehydrated in acetone and embedded in resin (Epon 812-Agar 100, Araldite 212). Ultra thin sections were stained for 15-20 minutes in saturated uranyl acetate and rinsed in 50% ethanol twice and with double distilled water twice. They were further stained for 15-20 minutes in lead citrate and washed with double distilled water twice. The sections were then examined using a Phillips 400 transmission electron microscope.

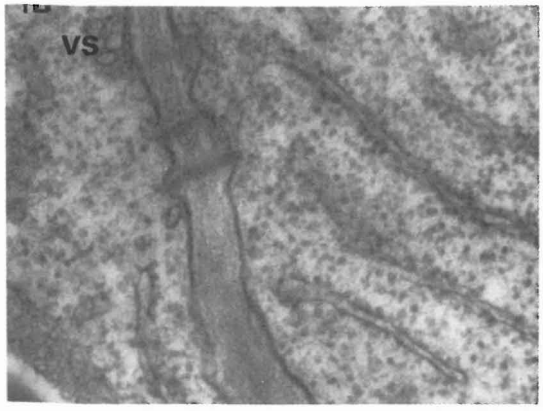
RESULTS

Cells from fresh seeds had clearly defined cell walls (*Plate 1A*). The plasmalemma adhered closely to the cell wall. The nucleus was clearly delimited by a double membrane nuclear envelope and a spherical nucleolus was located in the nucleoplasm. Within the cytoplasm, endoplasmic reticulum and mitochondria were present and appeared intact. Ribosomes were present attached to the endoplasmic reticulum and were present also in great numbers apparently free in the cytoplasm (*Plate 1B*). Numer-

Plate 1 Ultrastructure of radicle cells from fresh Hevea seeds



A. Typical cell with prominent starch grains (S). (×4,600)



B. Well-defined plasmalemma and cytoplasmic layer with endoplasmic reticulum and numerous ribosomes. Note small vesicles (VS) close to the plasmalemma. (×46,000)

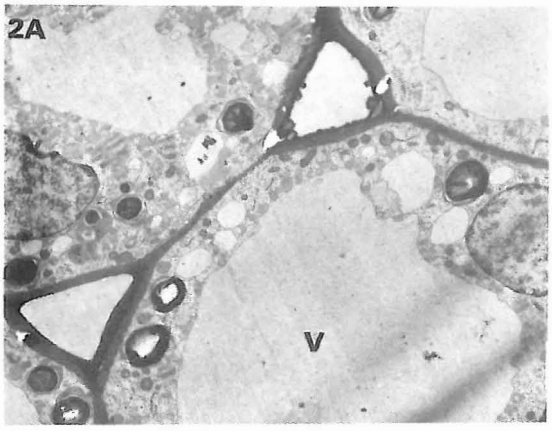
ous starch grains and lipid bodies were present. Dictyosomes were absent but small vesicles were present close to the plasmalemma.

After one month of storage at 10°C the cells were filled with large vacuoles with cytoplasm lying close to the cell wall (*Plate 2A*). However, in some cells, the tonoplast separating the vacuole and the cytoplasm was not clearly visible. Invagination of the plasmalemma was observed indicating derangement of the membrane (*Plate 2B*). Mithochondria were not well defined. Endoplasmic reticulum was present and numerous dictyosomes and small vesicles were present. Starch grains and lipid bodies were also observed.

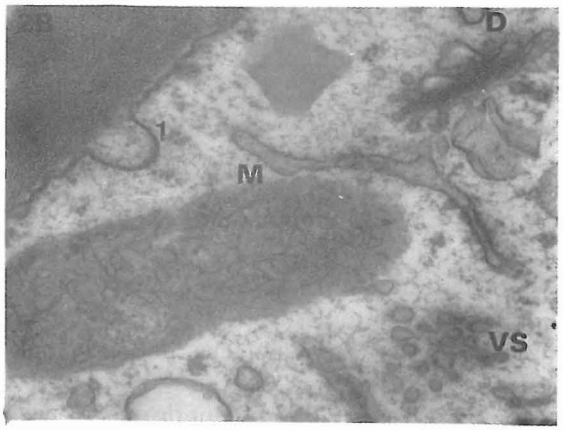
After two months of storage at 10°C the nuclei of the root cells became lobed and irregular (*Plate 2C*). Endoplasmic reticulum were in short profiles and mitochondria were seen to contain fewer cristae or were more electron dense but with electron transparent areas. Lipid bodies were present but starch grains were rare. DIctyosomes were present. Cell walls were constricted at certain places especially at the plasmodesmata, and the plasmalemma appeared to be absent in certain places and in some cells, withdrawn from the cell wall.

After three months of storage at 10°C, the irregular shape of the nucleus persisted. The double membrane nuclear envelope appeared to be breaking down. Mithochondria contained fewer cristae or were more electron dense and the mithochondrial membrane was not clearly discernible. Endoplasmic reticulum was pres-

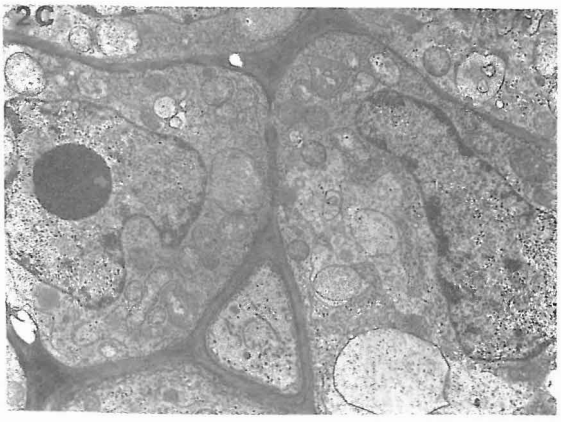
Plate 2. Ultrastucture of radicle cells from Hevea seeds stored at 10°C after one month (A and B), two months (C) and three months (D).



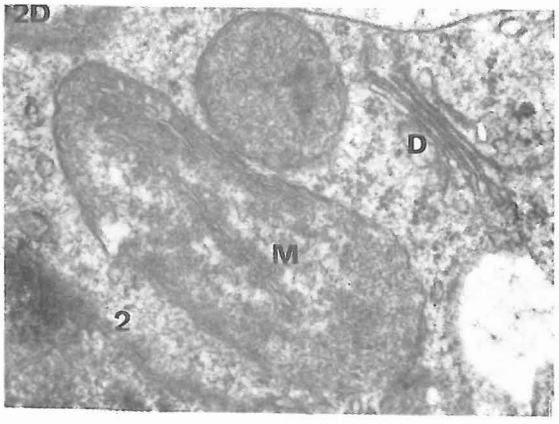
A. Typical cells with large vacuoles (V). (× 2,150)



B. Cytoplasm with dictyosomes (D), small vesicles (VS) and mitochondria (M). Note the invagination of plasmalemma (1). (× 46,000)



C. Typical cells with lobed nuclei. (× 4,600)



D. Cytoplasma with dictyosome (D) and degenerated mitochondria (M). Note nuclear membrane dissolution (2). (× 27,500)

ent in short profiles and dictyosomes were present. Invagination of the plasmalemma indicated that some deterioration had occured (*Plate 2D*).

After one month of storage at 22°C, cytoplasm appeared more electron dense with one large vacuole and many small secondary vacuoles. Nuclei were well defined while numerous starch grains and lipid bodies were still present as in the control. Endoplasmic reticulum was present in short profiles.

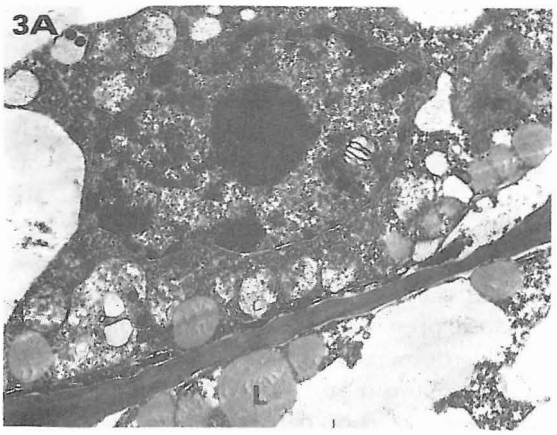
After two months of storage at 22°C withdrawal of the cytoplasm occurred at various places (*Plate 3A*). In some cells the cytoplasm appeared to be close to the cell wall and plasmalemma appeared to be absent or might have been severely broken down. Fusion of the vacuoles and breaking down of the tonoplast occurred resulting in the encroachment of the vacuolar matrix into the cytoplasm. Nuclei were not well defined. The presence of lipid bodies was evident. Starch grains, mitochondria and endoplasmic reticulum were rare.

After three months of storage at 22°C, severe collapse of the cytoplasm was evident (*Plate 3B*). The plasmalemma was breaking down at various places. The dissolution of the tonoplast resulted in no clear definition between cytoplasm and vacuoles. The nucleus was electron dense and not well defined, as the nuclear envelope appeared to be breaking down causing the nucleoplasm to merge with the cytoplasm (*Plate 3C*). Mitochondria appeared to be present but not clearly discernible. Other organelles were rare.

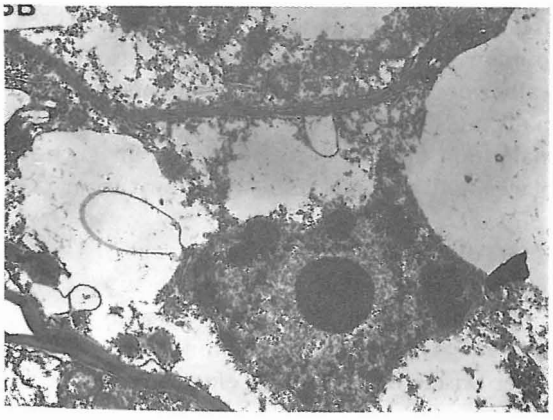
After one month of storage at 27°C, the cells were vacuolated while the nucleus was well defined with spherical nucleolus in the nucleoplasm. Well defined mitochondria with cristae were numerous. Endoplasmic reticulum were of short profiles with ribosomes associated with them. Ribosomes were also present free in the cytoplasm. Lipid bodies and starch grains were present. However, certain parts of the plasmalemma and tonoplast appeared to be breaking down.

After two months of storage at 27°C, what appeared to be tonoplast dissolution could be observed. Plasmalemma disintegration and invagination appeared at various places. Some

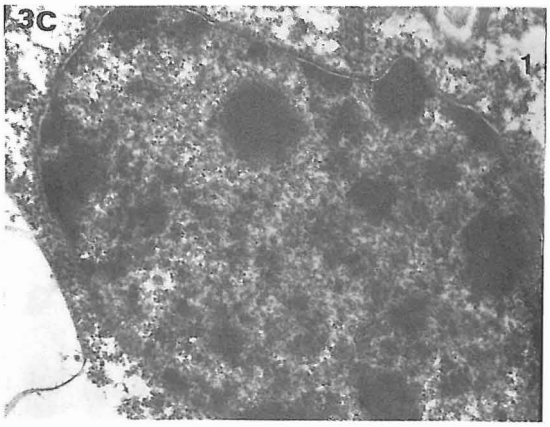
Plate 3. Ultrastructure of radicle cells from Hevea seeds stored at 22°C after two months (A) and three months (B and C).



A. Cell with withdrawn plasmalemma and dissolved tonoplast. Note lipid bodies (L) lying close to plasmalemma. (× 7,750)



B. Typical cell showing dissolution of tonoplast (× 6,000)



C. Nucleus with incomplete outer membrane. Note dissolution of tonoplast (1). (16,500).

parts of the nuclear membranes were not clearly defined and mitochondria were with less cristae or with electron dense deposits. No endoplasmic reticulum was observed but ribosomes were present in the cytoplasm. Starch grains and lipid bodies were also present.

The cells at one and two months of storage were thus not very discernibly different than the control.

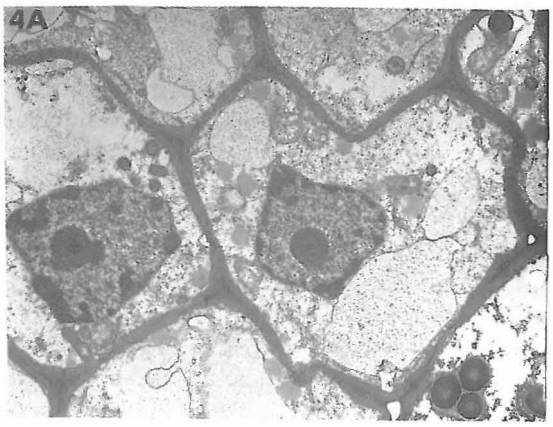
After three months of storage at 27°C, increased vacuolation was evident (*Plate 4A*). Dissolution of the tonoplast was quite severe at various places causing the vacuolar content to merge into the cytoplasm. The nucleus was still intact though some parts of the envelope were discontinuous (*Plate 4B*). Invagination of the plasmalemma occured at various places and often the plasmalemma was discontinuous or not observed. Mithochondria contained electron dense deposits or had less cristae and appeared disintegrated (*Plate 4C*). Endoplasmic reticulum was rarely observed and ribosomes were scattered throughout the cytoplasm. Lipid bodies were present but appeared fewer compared with those in the cells of the control embryos. Starch grains were rare.

DISCUSSION

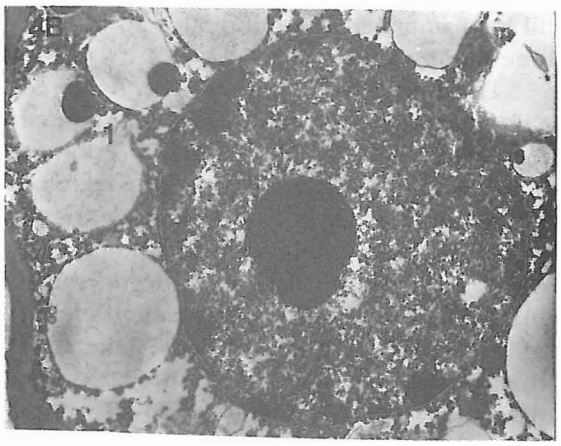
The withdrawal of the plasmalemma observed in cells from seeds stored at 22°C and aslo from some seeds stored at 10°C is believed not due to desiccation as the seeds were stored imbibed. The moisture contents of seeds after two months of storage at 10°C and 22°C were 48.56% and 33.93% (fresh weight basis) respectively (Normah, 1987). Withdrawal of plasmalemma has been observed by Hor (1984) in cocoa seeds that had been given chilling treatment. At 10°C the temperature might be too low for *Hevea* seeds, while at 22°C other factors might be involved to cause the withdrawal of plasmalemma.

In air-conditioned (22°C) storage, dissolution of the tonoplast resulted in the absence of compartmentalisation of the vacuolar matrix. The nuclear envelope was also dissolved causing the nuclear content to merge with the cytoplasm. Hence, it is suggested that destruction of the membrane system contributed significantly to seed death.

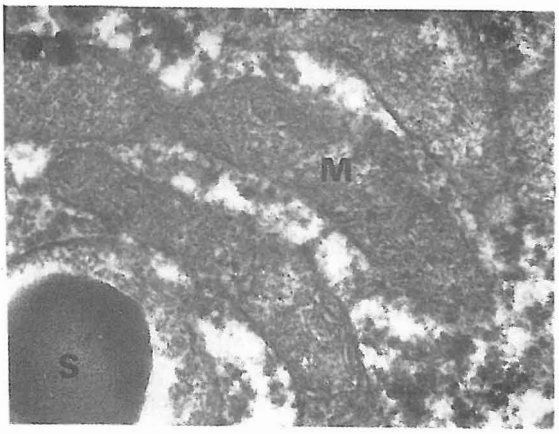
Plate 4. Ultrastructure of radicle cells from Hevea seeds stored at 27°C after three months.



A. Typical cells with disintegrating cytoplasma. (× 4,600)



B. Intact nucleus with part of the membrane disintegrating. Note the dissolution of tonoplast (1). (7,700).



C. Disintegrating mitochondria (M) and a starch grain (S). (35,500).

Endoplasmic reticulum has been suggested to be associated with synthesis of membranes for the cells and organelles. Hence, the synthesis of membranes might be affected by deterioration. Berjak (1968) observed that with ageing, short profiles of endoplasmic reticulum remained. The nucleus and nucleolus, however, appeared intact. Having an intact nucleus and possibly still functional mitochondria might have resulted in the viability of the seeds stored at 27°C (Normah 1987). Normah (1987), showed that the viability of *Hevea* seeds was maintained for the longest period when the seeds were stored at 27°C, follwed by 22°C and 10°C respectively. It may also be speculated that repair mechanisms similar to that suggested by Villiers and Edgcumbe (1975) were operative to a certain extent so that germination could still be maintained at around 50 percent after three months of storage at 27°C.

Deterioration in the cells of seeds stored at 27°C was not uniform; some cells deteriorated more than others. With the other two storage temperatures, deterioration was observed to be quite uniform in all cells. Berjak *et al.* (1984) reported that uniformity of cell deterioration in *Avicennia* propagules and the degenerative situation became equalised on further deterioration. Thus, it might be that the degenerative situation of *Hevea* seeds after three months storage at ambient temperature was not critical, so the repair mechanism could still be operative. Also Withers (1978) reported that damage to the endoplasmic reticulum and derived structures would appear to be less prejudicial to survival than damage to self-replicating organelles such as mitochondria, plastids and nucleus.

Features of cellular deterioration such as breaks in the structure of the plasmalemma and its withdrawal away from the cell wall; disintegrating plastids and mitochondria; condensed chromatin in the nucleus, which itself might be lobed; are among the features of aged or deteriorated orthodox seeds, including maize (Berjak and Villiers 1970), wheat (Anderson *et al.* 1970), rye (Hallam 1973), and also in deteriorated recalcitrant seeds such as *Avicennia marina* (Berjak *et al.* 1984) and cocoa (Hor 1984). These include ultrastructural changes associated with natural ageing, storage fungi and dehydration.

From this study, it appeared that *Hevea* seeds also showed similar features of deterioration, even though the methods of storage and the cause of deterioration were different. Osborne (1980) concluded that orthodox seeds that are stored imbibed maintained their viability by maintaining metabolic activities in the cells and loss of viability is due to loss of DNA integrity. With *Hevea*, loss of viability might not only be due to the loss of DNA integrity as has been shown by other degenerative characteristics of the cells.

Recalcitrant seeds do not undergo maturation drying on parent plants and are shed at relatively high moisture content. The seeds are ready to germinate after shedding if given the right conditions for germination as has been mentioned by Berjak *et al.* (1984) for *Avicennia* propagules. The same process might occur in *Hevea* seeds. Hence, when *Hevea* seeds were stored imbibed, they were given sufficient moisture and if stored at ambient temperature, they were ready to germinate. According to Berjak (1989), when *Hevea* seeds are stored under conditions maintaining their moisture content, they will initiate subcellular germination associated events. Though it was not quantified, the loss of stored seeds through germination was also observed in this study especially for those seeds stored at 27°C. However, at 10°C, the seeds were given the moisture but not the right temperature for germination. It appeared that at such low temperature the seeds still imbibed water and were ready to germinate as shown by the presence of dictyosomes. Dictyosomes appeared after 24 hours of imbibition and produced numerous vesicles (Berjak and Villiers 1970). When the optimum temperature was not given for a certain time, the seeds were unable to germinate and thus deteriorated. It might be that at 27°C and 22°C the appearance of dictyosomes was faster to the extent that by the end of one month, no further dictyosomes could be observed as a result of their degeneration. Berjak (1968) reported that the early degeneration of dictyosomes involves 'unstacking' of the cisternae followed by their apparent loss.

Nevertheless, the cause of deterioration might not be due to a single factor for all the three temperatures of storage. At low tempera-

ture such as 10°C, to a certain extent, deterioration might have been due to disintegration of the organelles. It might have been also because of plasmalemmal invagination and other factors involved that were not included in this study. For 22°C and 27°C storage, the dissoluion of plasmalemma and tonoplast causing vacuolar materials to encroach into the cytoplasm and cell organelles, might have resulted in the eventually loss of organelles and cell function and finally death. The deterioration at 27°C storage was less than that of 22°C storage.

However, it should be emphasised that due to complexity of the cell, there may have been other factors involved in seed deterioration and loss of viability which were not tested in the present study.

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