INNSPUB

International Journal of Agronomy and Agricultural Research (IJAAR)
ISSN: 2223-7054 (Print) 2225-3610 (Online)
http://www.innspub.net
Vol. 5, No. 6, p. 103-115, 2014

RESEARCH PAPER OPEN ACCESS

Ultrastructural changes during cryopreservation of plumules and embryos of coconut (*Cocos nucifera* L.)

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Article published on December 29, 2014

Key words: Coconut, cryopreservation, plumule, ultrastructural, zygotic embryo.

Abstract

This article aims to show the changes occurring during cryopreservation of embryos and plumules of coconut which are responsible of their death or survival. Embryos have been cryopreserved by preculture-dehydration for 24h, 28h, 30h, 34h, 38h and 48h on agar medium containing 600g/L of glucose combined with silica gel. The plumules were cryopreserved by encapsulation dehydration on solid medium containing 0.5 M, 0.75 M and 1M followed by dehydration with 40g of silica gel for different durations before rapid freezing. This study indicates that the damages undergone by seed samples can be divided into three types. The first stage of changes concerned the plasmolysis of cells with small vacuoles, condensation of chromatin, changing in the conformation of the DNA and the nucleus and stopping of mitosis. These types of changes are described in general in the context of a desiccation tolerance. The second degree of the changes was the retraction of the cytoplasm inside the cell, the increase in the periplasmic volume. The third degree of modification concerned the deformation of the walls, the invagination or the lysis of the plasma membrane resulting in the observation of distorted cells and and the bursting of the nucleus. These two types of modifications are irreversible and correspond to an absence of regrowth of the samples. Understanding the damage or changes that occur in cryopreserved cells is an important part of understanding how dehydration and frozen affect the viability of recalcitrant plants cells. These changes are made by dehydration and accentuated by freezing.

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Introduction

The coconut palm is a perennial plant which genetic resource conservation is actually assured through the maintenance of field coconut trees (collections) because of the seed characteristics. The coconut seeds are recalcitrant; they cannot stand either dehydration or low temperatures. However, this storage method poses significant problems (Engelmann, 1997; Withers and Engels, 1990). Several studies have been conducted through the world to overcome these difficulties (Bajaj, 1884; Assy Bah *et al.*, 1987, Chin and Krishnapillay, 1989, Assy and Engelmann, 1992, Karun *et al.*, 2005; Sajini *et al.*, 2006; N'nan *et al.*, 2008; 2012). *In vitro* conservation for short-term conservation and long-term conservation (cryopreservation), have been studied as complementary conservation strategies of coconut genetic resources. Conservation of zygotic embryos in *in vitro* culture for 12 months (Assy Bah, 1992) has been developed for short-term storage.

For long-term conservation such as cryopreservation, studies started in 1984 with the works of Bajaj, followed by the works of Chin and Krishnapilla in 1989 with zygotic embryo. However it was only in 1992, that these successive studies resulted in a success with the work of Assy Bah and Engelmann (1992). These work conducted on 4 accessions, allowed recovery rates of 30 to 80% depending on the accession. The cryopreservation of ten accessions by N'Nan *et al.* (2012) showed that this technique can be considered under control although some accessions still seem recalcitrant. The results obtained by Assy Bah and Engelmann (1992) on the whole zygotic embryo also showed the destruction of the majority of the cells of haustoria after freezing. This finding allowed further studies that revealed that the plumule (shoot meristem surrounded by leaf primordia), once excised from zygotic embryo can restore an entire plant (Chan *et al.*, 1998). Cryopreservation of excised plumule of the embryo by encapsulation dehydration was carried out by several studies (Malaurie *et al.*, 2006; N'Nan *et al.*, 2008; Bandupriya *et al.*, 2010). These studies showed that during cryopreservation, the response of the sample is based on the type of accession and also on damage that occurred during dehydration and freezing.

The aim of this study is to describe the structural and ultrastructural changes that occur during cryopreservation and which are responsible for the death or survival of the embryos and the plumules.

Material and methods

Plant material

The plant material consisted of whole zygotic embryos extracted from mature seeds (10-12 months) and plumules excised from zygotic embryos. The nuts, consisting of the autogamous accession Malayan Yellow Dwarf (MYD), were supplied by the Marc Delorme Research station in CNRA, Côte d'Ivoire, in the form of endosperm cylinders containing zygotic embryos. The harvest of nuts, the extracted endosperm cylinders, their disinfection and their conditioning in plastics bag were performed following the protocol described by Assy Bah *et al.* (1987) in Côte d'Ivoire. Endosperm cylinders containing embryos were then immediately airmailed. After receiving the plant material as endosperm cylinder within his embryos; their disinfection, the isolation of embryos and the excise of plumules were performed according to the process described by N 'Nan *et al.* (2008, 2012).

Methods

Cryopreservation of plumules

The plumules were cryopreserved by encapsulation dehydration. Several concentrations of sucrose, 0.5 M; 0.75 M and 1 M were used for pretreatment combined with dehydration with 40 g of silica gel for different durations: 6 h, 8 h, 14 h and 16 h before rapid freezing (N'Nan *et al.*, 2008). Three samples were used for each concentration of sucrose after dehydration and freezing.

Cryopreservation of whole zygotic embryos

The embryos were cryopreserved by preculture dehydration following N'Nan *et al.* (2012). The plant material after cryopreservation undergoing various treatments (without freezing i.e only

dehydration or dehydration followed by freezing) was cultured for 3 days in culture media before being used for histological studies. One sample was used for each treatment.

Two types of histological treatments were performed, ultrastructural and structural studies.

Histological studies

Light microscopy

Embryos and plumules at the different steps of the cryopreservation process were collected for fixation after three days of culturing in culture media. Untreated plumules (control) and plumules sampled at different steps of the cryopreservation process were fixed for three days in 10% (v/v) phosphatebuffered paraformaldehyde and 1% caffeine. The 10% (v/v) phosphatebuffered paraformaldehyde was composed of 4 ml of glutaraldehyde solution at 25% (v/v) to a final volume of 100 ml, and 1% caffeine. After fixation, samples were dehydrated through an ethanol series (30, 50, 60, 70, 80, 90, and 100%) for one hour each. Samples were then embedded in resin. Polymerisation was carried out for 12 hours at room temperature. Sections (3 µm thick) were obtained using a microtome with steel blades and mounted on glass slides (Verdeil*et al.*, 1994; 2001). Sections were double stained with Periodic Acid Schiff (PAS) reagent combined with protein specific Naphthol Blue-Black (NBB) (Fischer, 1968). PAS stains starch reserves and cell walls pink and NBB specifically stains soluble or stored proteins dark blue (Buffard-Morel *et al.*, 1992).

Electron microscopy

At different steps of cryopreservation process, the samples were fixed in a mixture of glutaraldehyde - sodium cacodylate buffer (pH 7) and distilled water for 2h at room temperature. The fixation was followed by three buffer rinses (30 mn each) and post fixed for 1 h in 1% osmium tetroxide buffer (0.1 M) (Spurr, 1969). The samples were dehydrated in ethanol series (30, 40, 50, 60, 70, 80, 90, and 99.8 %). The inclusion of samples was performed following the process described by Verdeil *et al.* (2001). Ultrathin sections were cut using glass knives on an UltracutUltratome (LKB Ultratome) and collected on formvar coated copper grids, contrasted with alcoholic uranyl acetate and lead citrate Reynolds (1963). The sections of samples were examined and photographed under Joel 100 transmission electron microscope.

Results

Embryos and plumules survival and regrowth after cryopreservation

The survival and recovery of plumules ranged from 0 to 30% (N'Nan *et al.*, 2008). Those of embryos ranged from 20 to 80% (N'Nan *et al.*, 2012).

Histological studies

Histological studies on light microscopy

Effect of dehydration on plumules and embryos structure

Embryos and plumules were cryopreserved respectively by preculture-dehydration and by encapsulation-dehydration. Three samples were used for each concentration of sucrose before dehydration (control), after only dehydration and after dehydration and freezing for histological studies. The embryo is composed of two parts: the haustorium and the gemmule. Only cells of the gemmule particularly, the plumule are involved in shoots regeneration. The figures 1a, b, c and d showed a section of gemmule showing an apical dome surrounded by the primordia leaf. The haustorium is composed of large sizes cells which were totally destroyed after cryopreservation. Plumule consists of apical dome surrounded by two or four primordia leaf (Figure 2a). As the apical dome surrounded by primordia is the zone involved in shoot regeneration, most of the results have focused on it. The apical dome is composed of meristematic cells. These cells were isodiametric in control samples and presented many mitotic figures (Figure 2b). They were turgid and contained large highly stained spherical nuclei located in central position inside the cell compartment. The nuclei presented dark blue areas which were proteins condensation zones and corresponded to heterochromatin. Some cells had many nucleoli (Figure 2b) and many starch grains.

Procambium cells were linear with well-rounded nuclei.

After dehydration, the cells showed various changes whose intensity varied from one sample to another. It has been difficult to associate each morphology change to a particular treatment because of the random selection of samples. Samples used in this study provided from uncontrolled fertilization plants. Depending on the changes undergone by the cells, the samples were classified into three groups.

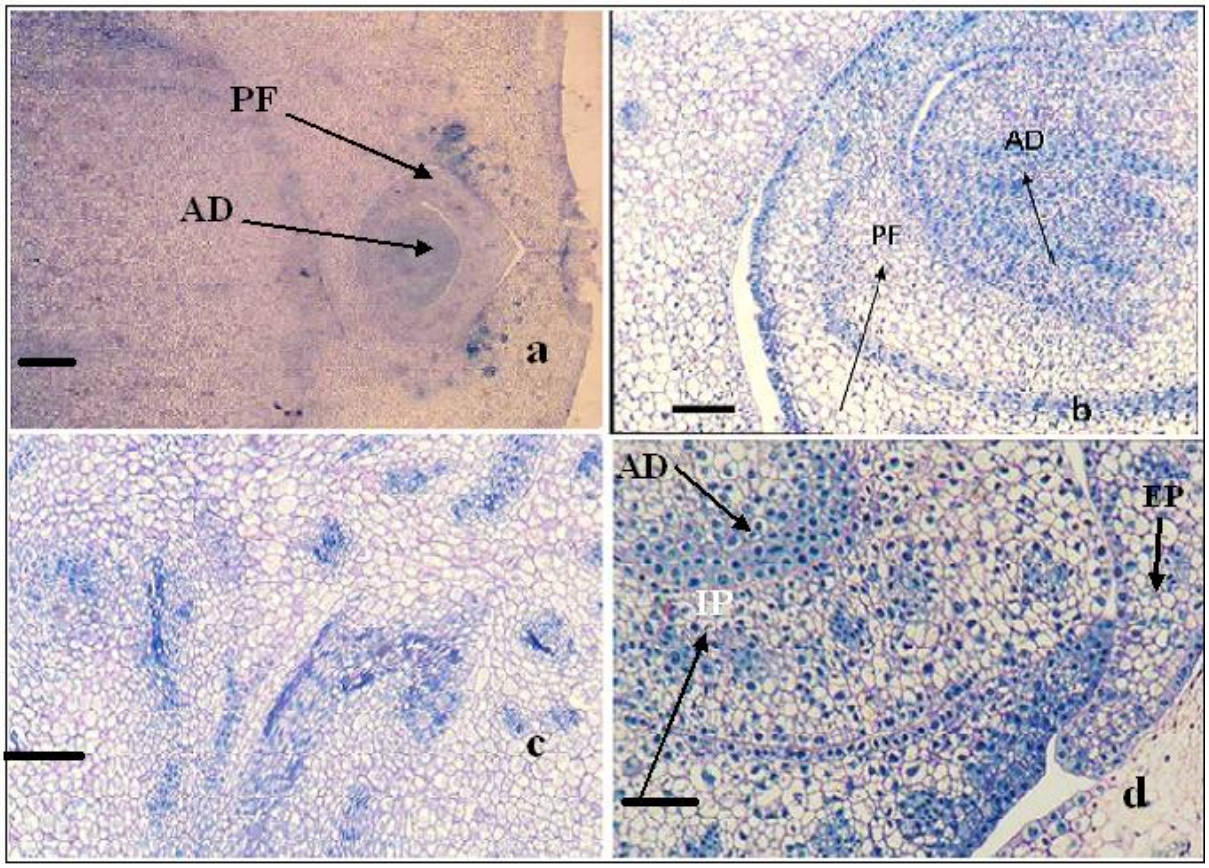

Fig. 1. Histological sections of embryos used as controls as viewed by light microscopy: **a**: general appearance of the embryo showing the part containing the plumule which is consisted of apical dame and primordial leaf. **b** and **d:**primordial leaf surrounding the apical dome. **c**: cells at the base of the dome showing elongated cells of procambium. **AD**: apical dome. **PF**: primordia leaf. **EP**: External primordia leaf. **IP**: internal primordia leaf (Bars = 156 µm).

The first group consists of samples with turgid cells at the apical dome (Figure 2c) and at young primordia leaf, leaf near the apical dome (Figure 2d). These cells had the same appearance as in control samples (Figure 2a). However, they showed little mitotic figures. Outside the dome, some cells (external primordia leaf) are slightly plasmolysed. The nuclei were deformed and retracted within the cells.

The second group consists of samples whose cells were plasmolysed (Figure 2e, f), with very deformed and retracted nuclei (oval). The retraction of the nuclei was too pronounced and made observation of the nucleoli impossible (Figure 2f). The nuclei took strongly dyes. In most cases, the cytoplasm is retracted inside the cell with the lysis of plasmodesmata in some cells (Figure 2e).

The third group consists of samples whose cells undergone great changes. Cells without nuclei were observed (Figure 3a). Damages of the wall and the plasma membrane characterized by the observation

of none individual cells or none distinguishable cells with different appearances (Figure 3c, d) were identified. These damages concern limited cells or all the cells of the sample.

Effect of freezing on embryos and plumules structure

After freezing, two groups of samples were identified. The first group consists of samples whose cells were completely destroyed causing a change of the morphology of cells (Figure 3d). It was difficult in this case to clearly distinguish cells individually. Non-observation of starch granules and procambium was possible on these samples.

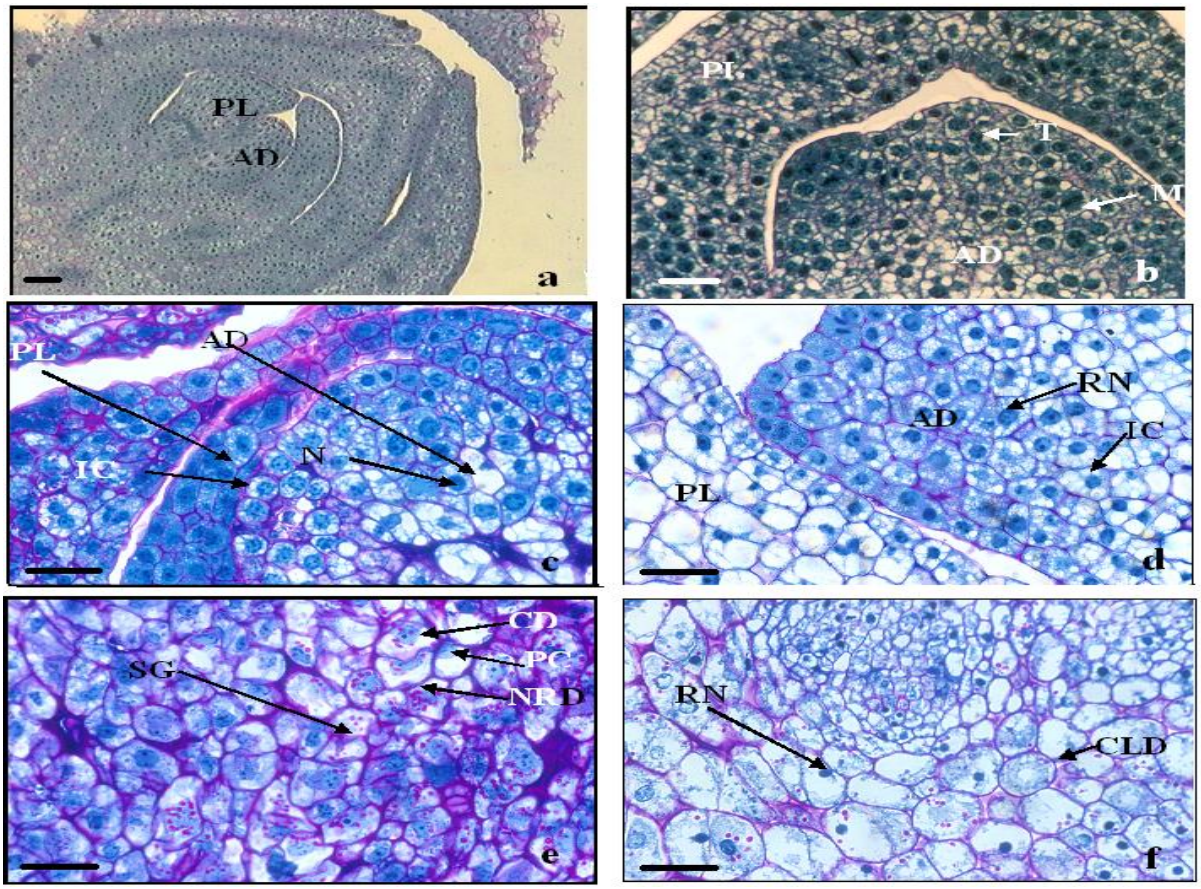

Fig. 2. Histological sections of controls, dehydrated and dehydrated and frozen plumules viewed by light microscopy showing cells at the first and the second step of changes. **a**: general structure of the coconut plumule. **b**: apical dome and primordial leaf of a control sample showing different cells with mitotic figures. **c**: sample dehydrated without freezing showing isodiametric cells with round nuclei. **d**: sample dehydrated without freezing. **e:** dehydrated sample with freezing: cells at the base of the apical dome. **f**: dehydrated and frozen sample. **CD** : Distorted cell; **IC** : isodiametric cell; **CLD**: cytoplasms lightly deformed ; **PC** : plasmolysed cell; **AD**: apical dome ; **PL** : primordial leaf ; **SG** : starch grains ; **M**: metaphase ; **RN**: round nuclei ; **DN**: deformed nucleus ; **NR** : retracted nucleus; **T**: telophase (Bars = 51µm).

The second group consists of samples whose cells had the same characteristics as the samples of the first, second and third group after dehydration. However, it has been generally difficult to observe an intact procambium in most of the cells.

Histological studies on electron microscopy

No dehydrated (control) samples had cells with walls electron opaque at the apical dome. The plasma membrane is contiguous to the wall in all cells (Figure 4a). The cells had large nuclei with rounded nucleoli (Figure 4b) and large vacuoles. Some mitochondria, endoplasmic reticulum, Golgi apparatus and

amyloplasts with many starch grains were observed. All organelles cells were present in all cells.

After dehydration and freezing, samples exhibited a change in morphology of the cells up to damage. The intensity of damages varied to one sample to another. These differences concerned the morphology of cells, vacuoles, nuclei, nucleoli, of different organelles, of the wall, the plasma membrane and nuclear membrane. However three steps of changes were identified.

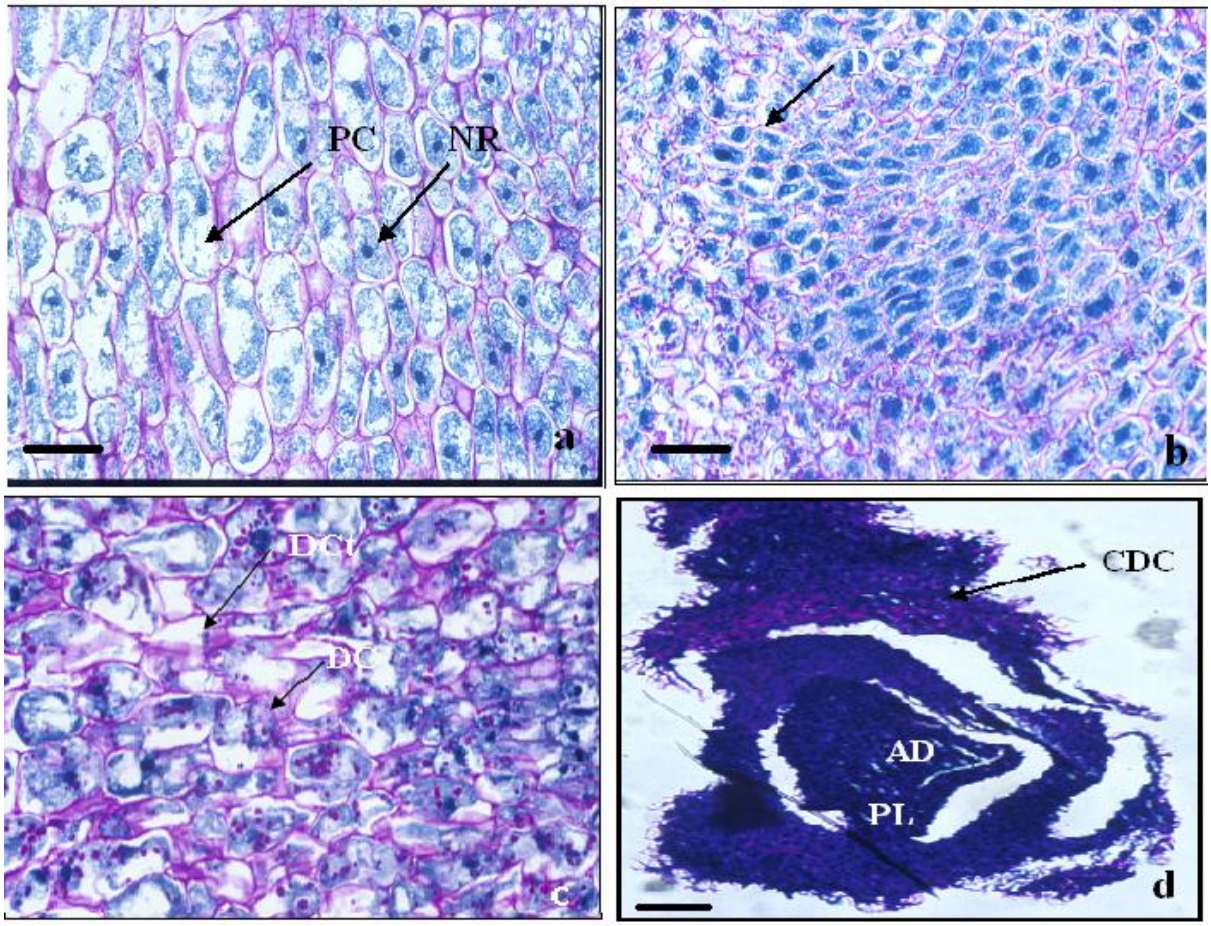

Fig. 3. Histological sections of dehydrated and dehydrated and frozen samples as viewed by light microscopy, showing cells at the second and third steps of changes. **a**: dehydrated and frozen samples :palsmolysed cells with retracted nuclei. **b**: dehydrated and frozen sample :deformed cells with irregular walls. **c**: dehydrated sample without freezing: the presence of cells without a nucleus, distorted cells. **d**: dehydrated and frozen sample: cells virtually indistinguishable individually. **CDC**: completely destroyed cells; **DC**: Distorted cell; **DCt**: very distorted cells without nucleus; **PC**: plasmolysed cell; **AD**: apical dome; **PL**: Primordial leaf; **NR**: retracted nucleus (Bars = 47 µm).

The first level consists of samples whose cells had an appearance similar to those of cells in the control samples. The cells had electron dense walls (Figure 4c). A plasma membrane fastened the wall, large nuclei with rounded nucleoli (Figure 4d) and several large vacuoles (Figure 4c) were observed. Some organelles, such as the endoplasmic reticulum, mitochondria and plastids (Figure 5, b) were identified. Mitochondria identified by the presence of the double membrane showed no crests. It is the same of plastids (Figure 5a). The stacking of endoplasmic reticulum devoid of ribosome with a particular organization (3 to 5 parallel endoplasmic reticulum) was observed (Figure 5b). The endoplasmic reticulum is also presented in coiled form (Figure 5c).

The second group comprises samples whose cells undergone theses changes. Cells from these samples showed less electron opaque. They were electron transparent (5c, d). Some cells are very plasmolysed with a lysis of plasmodesmata. The cytoplasm is retracted inside the cells (Figure 5d). Cells contained small vacuoles which became from the split of the initial large vacuoles (Figure 5c), a round retracted electron dense nuclei, which made the dyes. The nuclei size decreased (Figure 5c). Cellular organelles were absents or very unrecognizable in most cells.

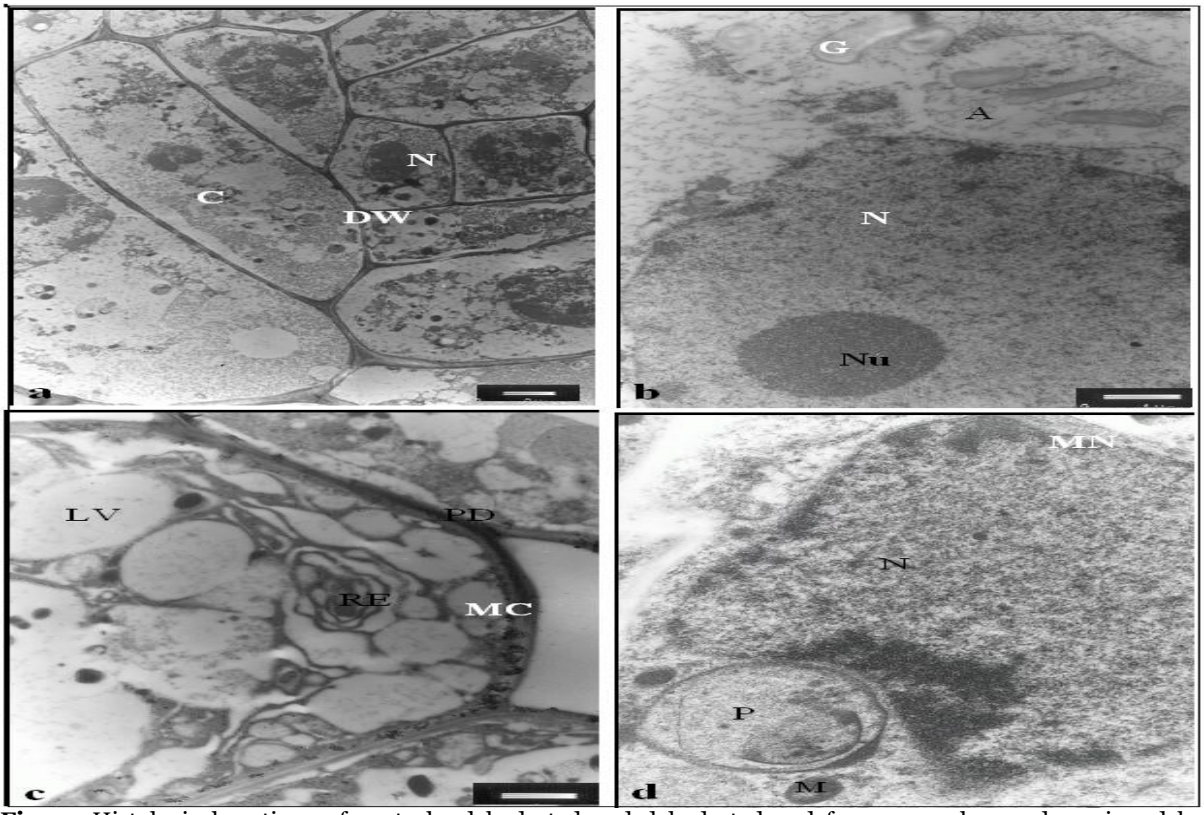

Fig. 4. Histological sections of controls, dehydrated and dehydrated and frozen samples used as viewed by electron microscopy showing cells at the first degree of changes. **a**: Overview of the cells of a control sample with turgid cells. **b**: the nucleus of a cell of a control sample. **c**: the cell of treated sample showing very little change after treatment. **d**: the nucleus of a cell that has undergone little change after treatment. **A**: amyloplasts; **C**: cytoplasm; **G**: grain starch; **LV**: large vacuole; **M**: mitochondria; **MC**: normal plasma membrane; **MN:** nuclear membrane; **Nu**: nucleolus slightly electron-dense; **P**: plastid; **DW**: dense electron wall (Bars = 1µm).

The third group consists of samples whose cells undergone great changes. They were damaged. The wall is fewer electrons dense. It was deformed at some places (Figure 6a). The plasma membrane presented some lysis or was an accordion shape (Figure 6b). Some invaginations and fragments of the plasma membrane were observed (Figure 6d). The cells exhibited disruption of the nuclear membrane in some places (Figure 6a) with a completely unstructured content and a non-homogeneous nuclei. The cytoplasm presented dense places that might correspond to residues from the nuclei (Figure 6b, c). Vacuoles in regression were observed. The majority of organelles cells have disappeared. Leftover of cytoplasm were observed in some cells (Figure 6c).

Discussion

The structural and ultrastructural studies have provided an understanding of the impact of cryopreservation on cells. Through observations, it was found that dehydration is in most cases responsible of the changes and damage that occurred during cryopreservation of samples. No significant difference was observed between the change of

morphology of dehydrated samples and dehydrated and frozen samples. Xu *et al.* (2009) have observed the same morphology changes with banana cells during the cryopreservation, showing the detrimental effect of dehydration. Histology studies revealed cells with large vacuoles occupying most of the cell size, the presence of different mitotic figures, and some cytoplasm with many proteins. The coconut is a recalcitrant seed in which the embryo remains active at maturity. There is no stopping in development and no metabolic dehydration takes place during the maturation. To freeze the seed, it is necessary to dehydrate it. Like all recalcitrant seeds, coconut embryo is sensitive to desiccation. Histology studies allowed describing the structural and ultrastructural change that accompanies this desiccation sensitivity (Wesley *et al.*, 1995; 2001; Pammeter *et al.*, 1999).

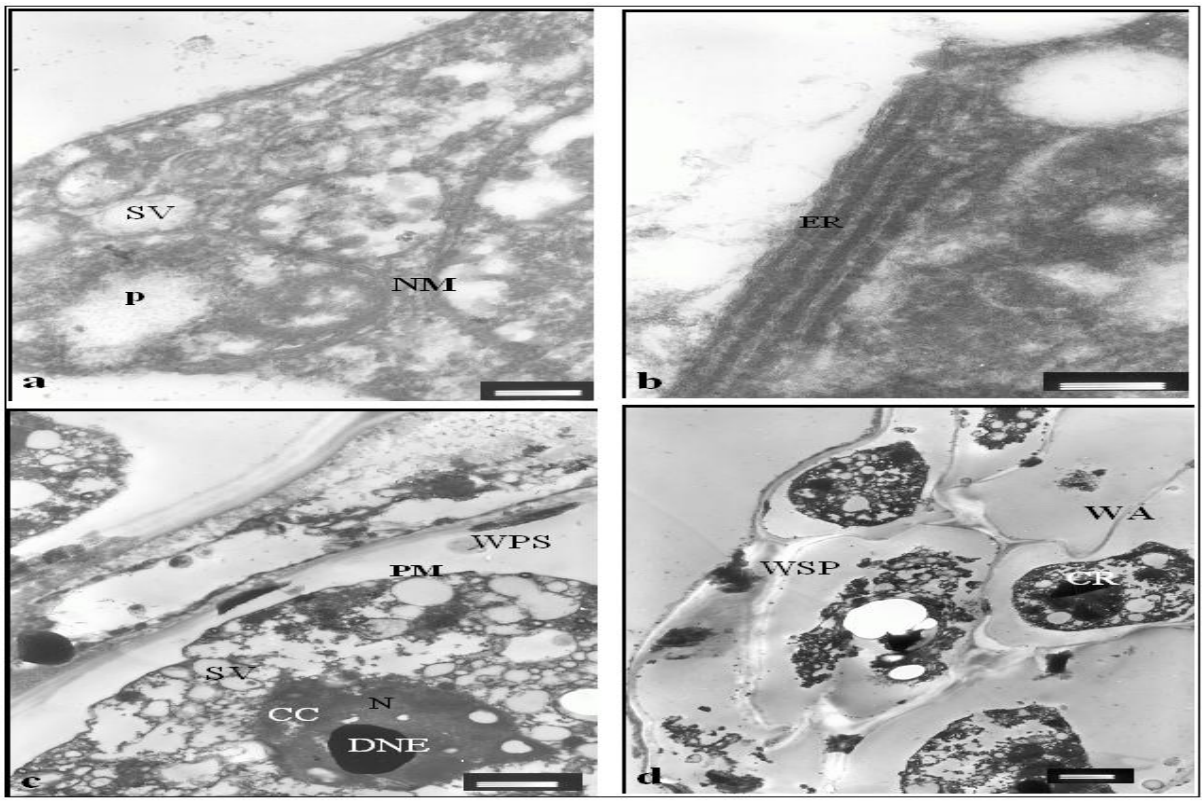

Fig. 5. Histological sections dehydrated and dehydrated and frozen samples used as viewed by electron microscopy showing cells at the first and the second steps of change **a**:Cells presenting some organelles such as plastids, vacuole and a small part of the nucleus. **b**: stacking of the endoplasmic reticulum. **c**: plasmolysed cells with several small vacuoles from the split of the initial vacuoles, a nucleus with condensed chromatin and a nucleolus that takes the dye. **d**: very plasmolysed cells with lysis plasmodesmata and retraction of the cytoplasm inside the cell. **CC**: condensed chromatin; **WPS**: wide periplasmic space; **PM:** plasma membrane; **NM**: nuclear membrane; **N**: nucleus; **DNE**: dense nucleolus electrons; **P**: plastid; **WA:** wall distorted accordion; **SV**: small vacuole; **ER**: endoplasmic reticulum (Bars = 1µm).

These studies have defined three increasing degrees of changes. The first step of change concerned the plamolysis of cells, chromatin condensation, changes in the conformation of the DNA, and in the form of the nuclei, without any change of the walls, the stopping of mitosis, stacking of the endoplasmic reticulum, the presence of all organelles. These types of changes correspond to the changes described in the general context of desiccation tolerance. Cells have the structural and ultrastructural characteristics of cells alive according to several authors (Dereuddre, 1981; Helliot *et al.*, 2003; Kusharenko *et al.*, 2010; Kaczmarczyk *et al.*, 2011). Changing in the shape of the nuclei, reducing of the size of the nuclei and

making the dye by nuclei suggest proteins condensation. Proteins are associated with the DNA to form chromatin. Changes in the conformation suggest chromatin condensation and a lack of nuclear activity. According to Cottignies (1981), during the slowed life, the activity of DNA synthesis is stopped; there is no division of nuclei which remain in the G1 phase. This condensation has been clearly identified during ultrastructural studies. Several authors have observed similar effects after dehydration among different species (Mari *et al.*, 1995; Pammenter and Berjak, 1999; Wesley *et al.*, 2001; Wilkinson *et al.*, 2003; Kusharenko *et al.*, 2010, Kaczmarczyk *et al.*, 2011). Stacking of endoplasmic reticulum has been associated to a decrease of cellular activity (Dereuddre, 1981). According to Catesson (1981) stacking is caused by the split in the initial endoplasmic reticulum into several endoplamic that pile up on top of each other, following stress caused by cold. In this study, the stacking was also observed among both samples: only dehydrated and dehydrated and frozen samples. The cold is therefore not only responsible for this change. They are caused by dehydration and accentuated by freezing. For Kusharenko *et al.* (2010) the nuclear activity is correlated with the presence of linear chromatin filament. All these change are compatible with the survival of the samples after dehydration and after freezing. This group corresponds to the samples dried sufficiently, but with water contents higher than the threshold allowed for their survival after drying and allow for a better freezing.

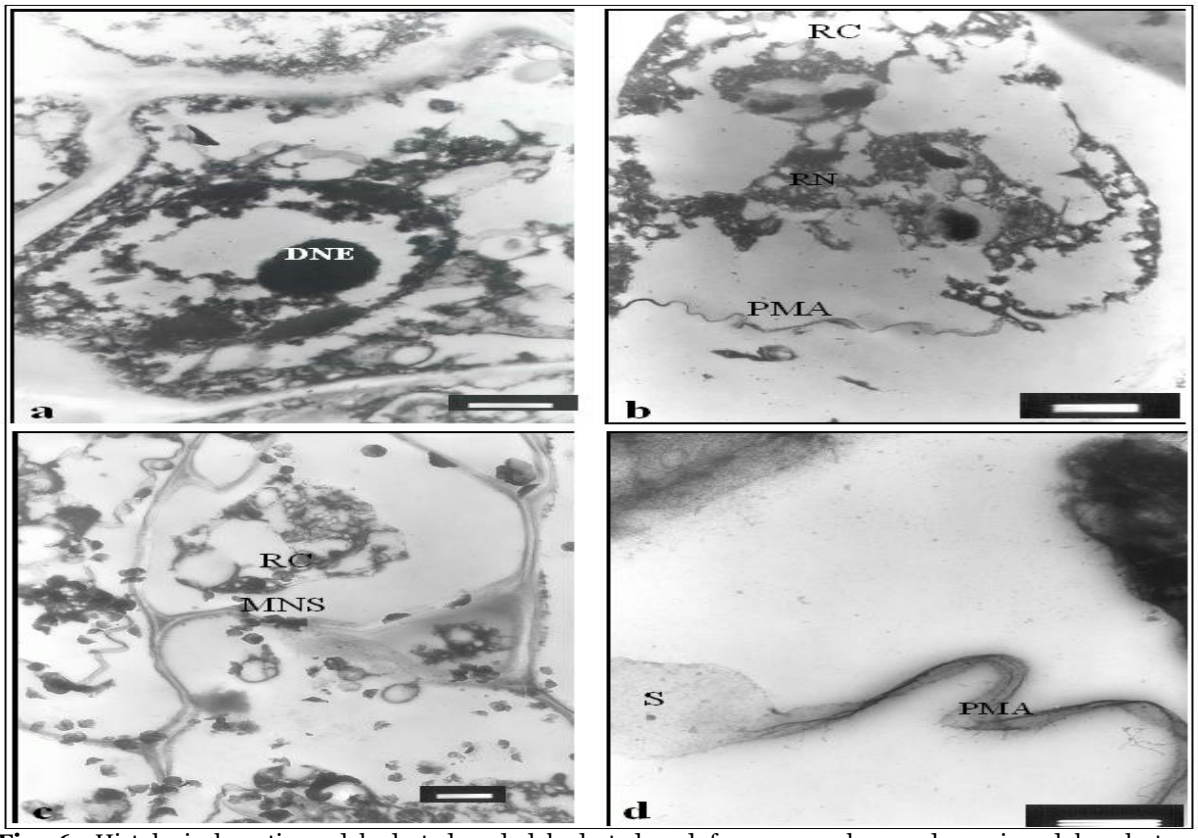

Fig. 6. Histological sections dehydrated and dehydrated and frozen samples used as viewed by electron microscopy showing cells at the third degree of changes. **a**: cell with a damaged nucleus, a distorted wall and a dense nucleolus electrons. **b**: cell showing irregular plasma membrane with interruptions in some place. **c**: cells with cytoplasm completely destroyed and with low electron-dense walls. **d**: membrane fragment showing a total destruction of the cell, little electron-dense wall. **S**: spacing between woodlark layers of the plasma membrane; **PMA**: plasma membrane in accordion; **NMS**: nuclear membrane with dark layers separated; **RC**: the rest of cytoplasm; **RN**: rest of the nucleus, **DNE**: dense nucleolus electron (Bars = 500 nm).

The second step of changes corresponds to the stop of mitosis, condensation of chromatin, the lysis of plasmodesmata, the retraction of the cytoplasm inside the cell, the increase in the periplasmic space. These cytoplasmic characteristics are consistent with very hard dehydration. Such observations were made by Kaczmarczyk *et al.* (2010) on the apex of potato after freezing and warming but not in the cells of shoot regeneration area. These damages could be considered as irreversible damage. According to Wilkinson *et al.* (2003), the lysis of plasmodesmata and retraction of the cytoplasm within the cell were responsible for the necrosis and malformation in samples regeneration. However it is possible in this case to obtain a hardware development which would be much slower than that obtained in the case where the cells undergo less change. According for these authors, the regeneration of a plant come from several areas located between the dome and leaf primordia. To survive to cryopreservation, the areas implied in regeneration have to be less damaged. Depending on the damage of this area after dehydration and freezing, samples will suffer a particular harm in its regeneration. Drew *et al.* (2000) also showed that dehydration of recalcitrant seeds from species to low water levels is a source of disruption of cellular components causing a drop in viability of about 96%.

The third degree of change is deformation of the walls concerned, the lysis of the plasma membrane resulting in the observation of heap cells hardly deformed. These types of changes are irreversible and correspond to no regeneration of samples as shown by Pammenter *et al.* (1999). These irreversible changes are responsible for the death of samples. According to Golovina *et al.* (2001), the acquisition of desiccation tolerance, estimated by regrowth is associated with viscous cytoplasm with accumulation of lipid bodies and protein, with the preservation of membrane integrity after dehydration and rehydration. This destruction may leak out of solutes. The ability to repair membrane damage and bearing leaking fluids after dehydration can be possible for some orthodox species (Bewley and Krochko 1982; Chandel *et al.*, 1995), but not for recalcitrant seeds for which the integrity of membrane is a prerequisite for the acquisition of tolerance to dehydration.

The samples were conserved in culture media 3 days before being fixed for histological studies. Three days culture on a medium can be considered as sufficient for samples to be turgid. There are some materials for which, a few hours of rehydration are sufficient to enable them to return to their initial state. These are the axes of *Pisumsativum* for which, only 24 hours are sufficient (Wesley *et al.*, 1995), the meristem of Isoetes that after two days of rehydration return to their original state from life slowed to an active lifestyle (Michaux-Ferriere, 1981).

Conclusion

The aim of this study is to demonstrate the structural and ultrastructural changes occurring during cryopreservation and which are responsible of the death or the survival of the samples. It showed that dehydration is in most cases responsible for damages suffered by cells. Freezing only accentuate them. The recovery of the material after drying and freeze request the integrity of the structure of the different cells and organelles in the apical dome and in leaf primordia. Irreversible damage such as the lysis of the plasma membrane, the DNA damage is associated with the death of the samples.

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