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## Original Paper

# Anatomical and histochemical characterization of seeds of *Cattleya intermedia* subjected to different storage conditions

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**Abstract**

Studies involving morphological and anatomical changes resulting from seed storage are rare, but relevant to ensure information related to the quality of seeds and seedlings originated from these seeds. The objectives of this work as to carry out the anatomical and histochemical characterization of seeds from the *Cattleya intermedia* orchid, verifying the occurrence of possible alterations coming from different temperatures and storage periods. The treatments consisted of temperatures of: 25 (± 2 °C) (room), -20 °C (freezer), -80 °C (ultra freezer) and - 196 °C (cryopreservation); and storage periods: two, four and six months. For the anatomical and histochemical analyses, scanning electron microscopy and light microscopy were performed. The following stains were employed: toluidine blue (TBO), Coomassie brilliant blue (CBB), Sudan IV and periodic acid-Schiff (PAS). The dehydration observed in the seeds was considered the main damage resulting from storage. Changes in the internal structures of the seeds were also noted, such as degeneration, mainly in seeds kept at room temperature 25 (± 2 °C), which caused a reduction in the main components of reserves. The -80 °C (ultra freezer) temperature during the two and four months period was efficient in the conservation seed of the tissues and cells, confirming the possibility of using this condition for better conservation of the seeds of this species.

**Key words**: cold storage, Orchidaceae, seed reserves, storage damage.

**Resumo**

Estudos que envolvem alterações morfológicas e anatômicas decorrentes do armazenamento de sementes são escassos, porém relevantes para garantir informações relacionadas á qualidade de sementes e plântulas. O objetivo desse trabalho foi realizar a caracterização anatômica e histoquímica de sementes da orquídea *Cattleya intermedia*, verificando a ocorrência de possíveis alterações decorrentes de diferentes temperaturas e períodos de armazenamento. Os tratamentos consistiram das temperaturas de: 25 (± 2 °C) (ambiente), -20°C (freezer), -80 °C (ultra freezer) e -196 °C (criopreservação) e períodos de armazenamento: dois, quatro e seis meses. Para as análises anatômicas e histoquímicas foram empregadas a microscopia eletrônica de varredura e a microscopia de luz com colorações de azul de toluidina (ATO), azul brilhante de Coomassie (CBB), Sudan IV e ácido periódico de Schiff (PAS). A desidratação observada nas sementes foi considerada o principal dano decorrente do armazenamento. Também foram observadas alterações nas estruturas internas das sementes, como a degeneração dos principais componentes de reserva, principalmente em sementes mantidas na temperatura ambiente de 25 (± 2 °C). A temperatura de -80 °C (ultra freezer) durante os períodos de dois e quatro meses foi eficiente na conservação dos tecidos e células, confirmando a possibilidade do uso desta condição para a melhor conservação de sementes desta espécie.

**Palavras-chave**: armazenamento a frio, Orchidaceae, reservas das sementes, danos de armazenamento.

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## Introduction

Orchidaceae is the largest and oldest botanical described family among the higher plants, with 899 era and 27,801 representatives distributed across the planet (The Plant List 2021). In Brazil, 251 genus and 2,692 species are described, and these have different morphological, anatomical and physiological adaptations, thus having extremely specialized leaves, stems and roots, giving the species the ability to occupy many different habitats (Silva *et al.* 2006; Flora do Brasil 2020, continuously updated).

Despite this fact, many orchid species are alarmingly disappearing from their natural environment, putting them at risk of extinction or near extinction, due to the destruction of their habitats, extraction and illegal exploitation of environmental resources. Among these species, *Cattleya intermedia* Graham *ex* Hook., is included in the vulnerable category of the Red List of Flora of Brazil (CNC Flora 2012).

Given the importance of orchids to nature, many studies aim the conservation and elaboration of methods for the preservation of these species being considered essential and necessary. Among conservation strategies, seed storage is an important tool of the *ex situ* conservation. However, the conservation success depends on the capacity to keep the seeds alive and with quality during the storage (Barbedo & Santos Júnior 2018).

The knowledge of the physiological and possible anatomical alterations during the seed storage is necessary, due to the particular requirements of each species to ensure better conditions, to maintain the longevity and preserve to some possible damage that may happen during storage (Nery *et al.* 2014). It is suggested that orchid seeds show an orthodox behavior, in that case, they are tolerant to freezing at extremely low temperatures, including the cryopreservation method (Pritchard *et al.* 1999; Hay & Probert 2013).

For seed storage to be successful, the seeds must be put under adequate conditions (Becerra-Vázquez *et al*. 2018). Yet, even under ideal conditions the seeds may undergo a deterioration process, depending on environmental conditions and the characteristics of the seed itself (Felix *et al.* 2017). Thus studies that reduce the deterioration effects during the storage stage are particularly relevant (Terskikh *et al.* 2008). The use of low temperatures promotes the reduction of chemical reactions, preserving the physiological quality for a longer period and reducing the deterioration process of the seeds (Gonçalves *et al.* 2018).

The success and longevity of the seed storage are also influenced by the reserve components present in them, which may vary between different species (Carvalho & Nakagawa 2000). McDonald (1999) states that seeds with a high oil content are more favorable to deterioration, since lipids have less chemical stability. Orchid seeds are classified as oily and rich in lipids reserves, which might facilitate deterioration, especially at higher temperatures (Arditti 1992; Fanan *et al.* 2009; Colville *et al.* 2016).

Knowing the morphology and anatomy of seeds, before and after storage, is also important for *ex situ* conservation, especially of native species. So far, there are few records of morphological and anatomical aspects of orchid seeds, because they are considered very small and their embryo contains few reserve cells (Koopowitz 2001). Given the above, works related to morphoanatomical aspects of seeds of this family have become extremely relevant for the elucidation of new data related to their preservation (Molvray & Kores 1995; Ferreira 2000).

Changes caused in seeds resulting from storage conditions may occur and need to be studied (Silva *et al.* 2011). Therefore, the use of histochemical techniques associated with anatomy studies are important tools for better visualization and comprehension of the changes resulting from the freezing of the seeds during storage (Gallão *et al.* 2006; Bewley *et al.* 2013; Lima *et al.* 2018).

Morphological and anatomical investigations in orchid seeds of the genera *Cattleya*, subjected to storage are considered incipient. Little is known about the changes that occur in seeds during the storage stage, therefore, the objectives of the present work as to carry out the anatomical and histochemical characterization of the seeds of the species *C. intermedia*, verifying the occurrence of possible changes originated from low temperatures and storage periods.

## Materials and Methods

### Plant material and experiment site

Seeds of the *Cattleya intermedia* were removed from mature capsules at beginning of dehiscence, from the Orquidário Carlos Gomes, located in the neighborhood of Ribeirão da Ilha, in the city of Florianópolis (-27°35’48’’S,

-48°32'57''W, altitude 3 m) in Santa Catarina. The capsules were obtained from four plant specimens in March 2019. After gathering, the capsules were transported in plastic bags to the Lab for Research in Biotechnology and Plant Development (NPBV) from the Department of Plant Science at the Federal University of Santa Catarina (UFSC), Florianópolis/SC campus to carry out surface disinfestation in a solution of water, commercial sodium hypochlorite (Q-Boa®) at 0.4% and neutral detergent (0.5 mL). The capsules were dried at room temperature (25 °C) for 4 hours (adapted from Alvarez-Pardo *et al.* 2006).

The capsules were opened using a stylet and the seeds were removed. By the method proposed in the Seed Analysis Rules manual (Brasil 2009) the initial moisture content was determined. Later, these seeds were subjected to different storage treatments at the Laboratory of Development Physiology and Plant Genetics (LFDGV/CCA/UFSC).

## Experimental design and seed storage conditions

A completely randomized design was used, in a 4 × 3 factorial arrangement (temperatures × storage periods) with twelve treatments, four replications and a total of 48 plots. The experimental unit was represented by a microtube (2 mL) containing the seeds. The treatments consisted of four temperature conditions: 25 (± 2°C) (room), -20 °C (freezer), -80 °C (ultra freezer) and -196 °C (cryopreservation) and three storage periods (two, four and six months), containing 100 mg of seeds each. A portion of seeds referring to storage time zero were evaluated.

Under the conditions of room temperature 25 (± 2 °C), -20 °C (freezer) and -80 °C (ultra freezer) the seeds were stored in sealed plastic microtubes (2 mL) and wrapped in aluminum foil. For cryopreservation (-196 °C), 2 mL cryotubes were directly immersed in liquid nitrogen.

For all storage periods, the seeds kept at temperatures of -20 °C (freezer), -80 °C (ultra freezer) and -196 °C (cryopreservation), underwent fast thawing, according to the method by Santos & Salomão (2010). The thawing consisted of keeping the microtubes with the seeds under water bath conditions, at 40 °C for 2 minutes. Subsequently, the preparation of seeds for the anatomical and histochemical analyses, described below, was performed.

## Analysis under scanning electron microscopy (SEM)

Seeds from the *Cattleya intermedia* were submitted to surface analysis by scanning electron microscopy. For this, the seeds of each storage treatment were fixed in a solution of glutaraldehyde (2.5%), sucrose (2%) and a sodium cacodylate buffer (0.1M) under vacuum for a week. Then, washing and dehydration were performed, in series of ethanolic gradient (30%, 50%, 70%, 90% and 100%), for 30 minutes at each concentration, except for 100% ethanol, in which two dehydrations were performed, 30 minutes each (adapted from Schmidt *et al.* 2012).

After dehydration, the samples were subjected to hexamethyldisilazane solvent (HMDS)(Silveira 1989) and adhered to aluminum holders (stubs), using double-sided carbon tape. Later, the samples were coated with 20 nm gold in a metallizer (Baltec, CED 030) (Schmidt *et al.* 2012), observed and photographed in a scanning electron microscope (Jeol JSM-6390LV), from the Central Laboratory of Electron Microscopy (LCME/UFSC).

## Analysis under light microscopy (LM)

The seeds of *Cattleya intermedia* were submitted to anatomical analysis by light microscopy. Seed samples from each storage treatment were fixed in a solution of glutaraldehyde (2.5%) and sodium phosphate buffer (0.1M), in a 1:1 ratio, under vacuum for a week. Then, they were washed in a phosphate buffer three times and dehydrated in series of ethanolic gradient (30%, 50%, 70%, 90% and 100%) for 40 minutes. After dehydration, the samples were embedded in Leica™ historesin with 100% PA ethyl alcohol at a ratio of 1:1, for 72 hours and then in Leica™ historesin, according to the manufacturer's instructions. The embedded material was kept in an oven at 35 ºC for 3 days. 5 µm-thick sections were obtained by rotational microtome (microTec, CUT 4055).

For histochemical analyses, samples were treated with toluidine blue (TBO) for detection of acid polysaccharide, Coomassie brilliant blue (CBB) for protein, Sudan IV for lipids and periodic acid-Schiff (PAS) for neutral polysaccharides (starch and cellulose) (Ventrella *et al.* 2013).

The materials were analyzed under a light microscope (Olympus BX-40), with records made by a high-resolution digital color camera (Olympus DP71) and Image Q Capture Pro 5.1 Software, at the Laboratory of Developmental Physiology and Plant Genetics (LFDGV/ CCA/UFSC).

## Results

### Characterization of seeds not submitted to storage

Aspects of the flower, fruit and seed of *Cattleya intermedia* can be observed in Figure 1a-b. Thousands of seeds contained within the fruits are small and resemble a powder (Fig. 1c). These seeds might be viable (tetrazolium-marked embryos) or unviable (seeds without embryo) (Fig. 1d). The seeds presented a threadlike shape, showing the testa, the chalazal and micropylar ends and the swollen region of the seed, where the embryo is located, which is covered by the testa (Fig. 1e). The cells forming the testa are smooth to linear and do not have ornamentation between the walls (Fig. 1f). The ellipsoid-shaped embryo is located in the center of the seed, with larger cells in the micropylar region and smaller cells in the chalazal region, which characterizes the bipolar embryo which also contains the main reserve cells (Fig. 1g). The main reserves present in the embryonic cells of seeds were proteins (Fig. 1h), lipids in the lining cells, characterizing a differentiated cuticle (Fig. 1i), in addition to the accumulation of starch reserves (Fig. 1j). The initial moisture contente of these seeds was above 30%.

### Seed analysis under scanning electron microscopy (SEM)

Viable and non-viable seeds submitted to different temperatures and storage periods can be seen in the figures below (Fig. 2) and described in the (Tab. 1).

### Histochemical analysis with toluidine blue (TBO)

Anatomical characteristics seeds subjected to different temperatures and storage periods can be observed by light microscopy and histochemical analysis with TBO in Figure 3 and described in the Table 2. The observed metachromatic reactions show purple coloration on the cell walls indicating the pectic nature.

### Histochemical analysis with Coomassie Brilliant Blue (CBB)

Anatomical characteristics of seeds subjected to different temperatures and storage periods can be observed by light microscopy and histochemical analysis with CBB in Figure 4 and described in the Table 3.

### Histochemical analysis with Sudan IV

Anatomical characteristics of seeds subjected to different temperatures and storage periods can be observed by light microscopy and histochemical analysis with Sudan IV in Figure 5 and described in the Table 4.

### Histochemical analysis with periodic acid-Schiff (PAS)

Anatomical characteristics of seeds subjected to different temperatures and storage periods can be observed by light microscopy and histochemical analysis with PAS in Figure 6 and described in the Table 5.

## Discussion

### Characterization of seeds not submitted to storage

Structures verified in recently benefited seeds of *Cattleya intermedia* are considered essential to guarantee the conservation and survival of the species. Seeds from this family are known by its tiny size, which ranges from 0.05 to 6 mm, and usually have a testa, which is considered a coating structure and which has the function of protecting the embryo (Ziegler 1981). In the embryo of *C. intermedia seeds*, a suspender attached to it was noted. This structure may be present in some families of orchids and when it exists, it plays a fundamental role for the embryo development, facilitating the translocation of nutrients present in the maternal tissues to the embryo (Yeung 2017). According to this author, mature orchid embryos have protein and lipid reserves in their cells, but these are considered minimal to promote the germination of these seeds in a natural environment. Yeung (2017) emphasizes that the presence of starch grains may eventually occur, but as of now, it is unknown how these products influence asymbiotic germination. All of these reserves were observed in the embryo of *C. intermedia* freshly benefited, in addition, histochemistry with PAS detected the presence of cellulose in the cell wall of these seeds and according to Mateu *et al.* (2014) the cell wall is based on cellulose microfibril nanocompounds.

The main function of the proteic reserve is the storage of nutrients such as nitrogen and sulfur that are essential for the synthesis of new proteins, nucleic acids and secondary compounds, vital for the growth of seedlings (Lima *et al.* 2008). Pavithra *et al.* (2014) state that proteins

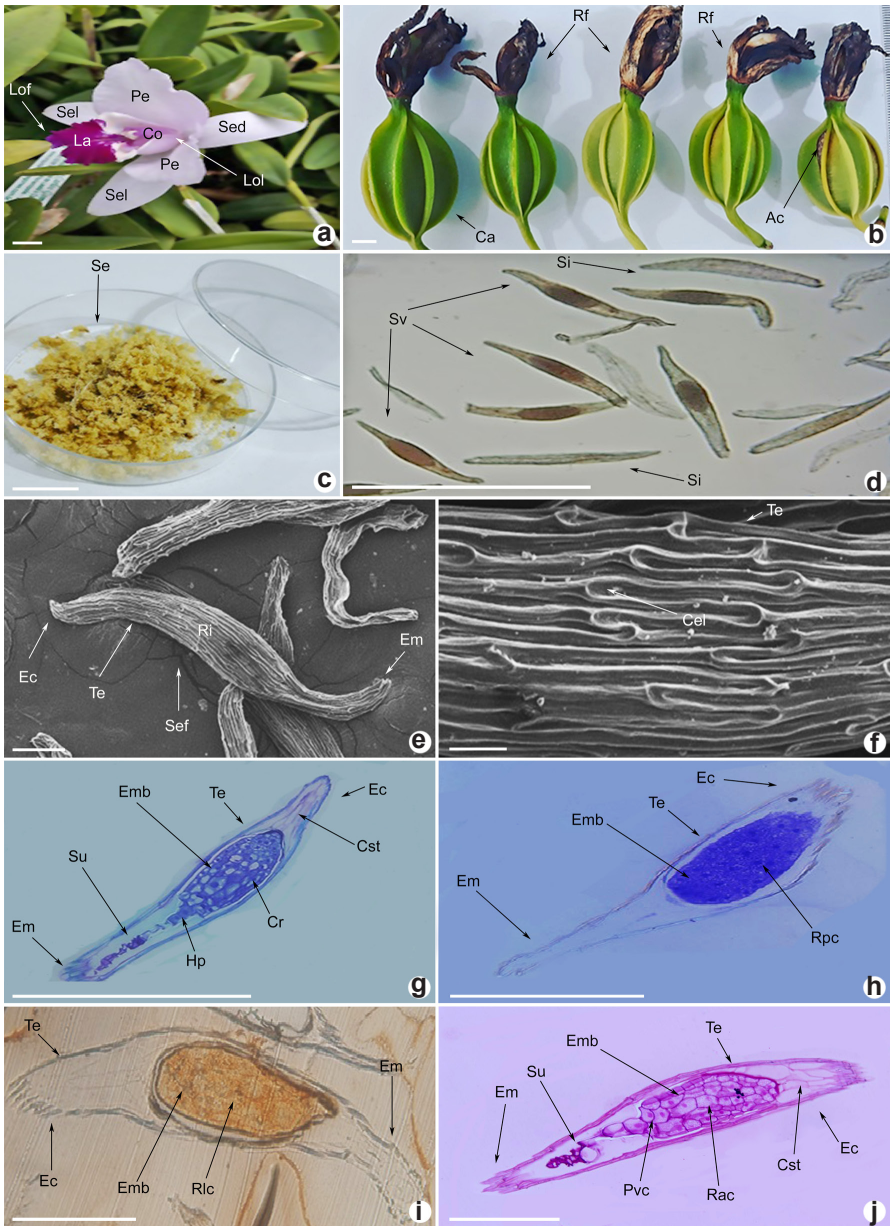


**Figure 1** – a-j. Scanning electron microscopy and light microscopy of the flower, fruit and seed of *Cattleya intermedia* – a. Floral pieces; b. Fruits with floral remains. It is possible to identify the beginning of the opening to release the seeds; c. Seeds extracted from fruits; d. Seeds submitted to the tetrazolium test, showing viable embryos (stained by tetrazolium) and non-viable seeds (without the presence of the embryo); e. Thready seed, showing the testa, the chalazal and micropylar ends and the swollen region (key) where the embryo is located; f. Details of the testa; g. TBO: seed with cells from the testa, embryo and suspender, evidenced by the action of the reagent with cellulosic compounds and pectins; h. CBB: seed showing protein reserve in embryonic cells; i. Sudan IV: seed with embryonic cells evidenced by the action of the reagent with lipid reserves; j. PAS: seed with cells from the testa, embryo and suspender marked by the action of the reagent with starch and cellulose. Afru = opening of the fruits; TBO = toluidine blue; CBB = Coomassie brilliant blue; Cel = testa forming cell; Co = spine; Cr = reserve cell; Cst = superficial testa cell; Ec = chalazal end; Em = micropylar end; Emb = ellipsoid embryo; Fru = fruits; La = lip; Lof = frontal lobes; Lol = side lobes; PAS = periodic acid Schiff; Pe = petal; Pvc = cellulosic vegetable wall; Rac = starch reserve in embryonic cells; Re = floral remains; Ri = swollen region of the seed; Rlc = lipid reserve in embryonic cells; Rpc = protein reserve in embryonic cells; Se = seeds; Sed = dorsal sepal; Sef = seed with a threadlike shape; Sel = lateral sepal; Si = unviable seed; Sus = suspender; Sv = viable seed; Te = testa of the seed. Scale bar: a- c = 1 cm; d = 1 mm; e = 100 µm; f = 20 µm; g = 500 µm; h-j = 200 µm.

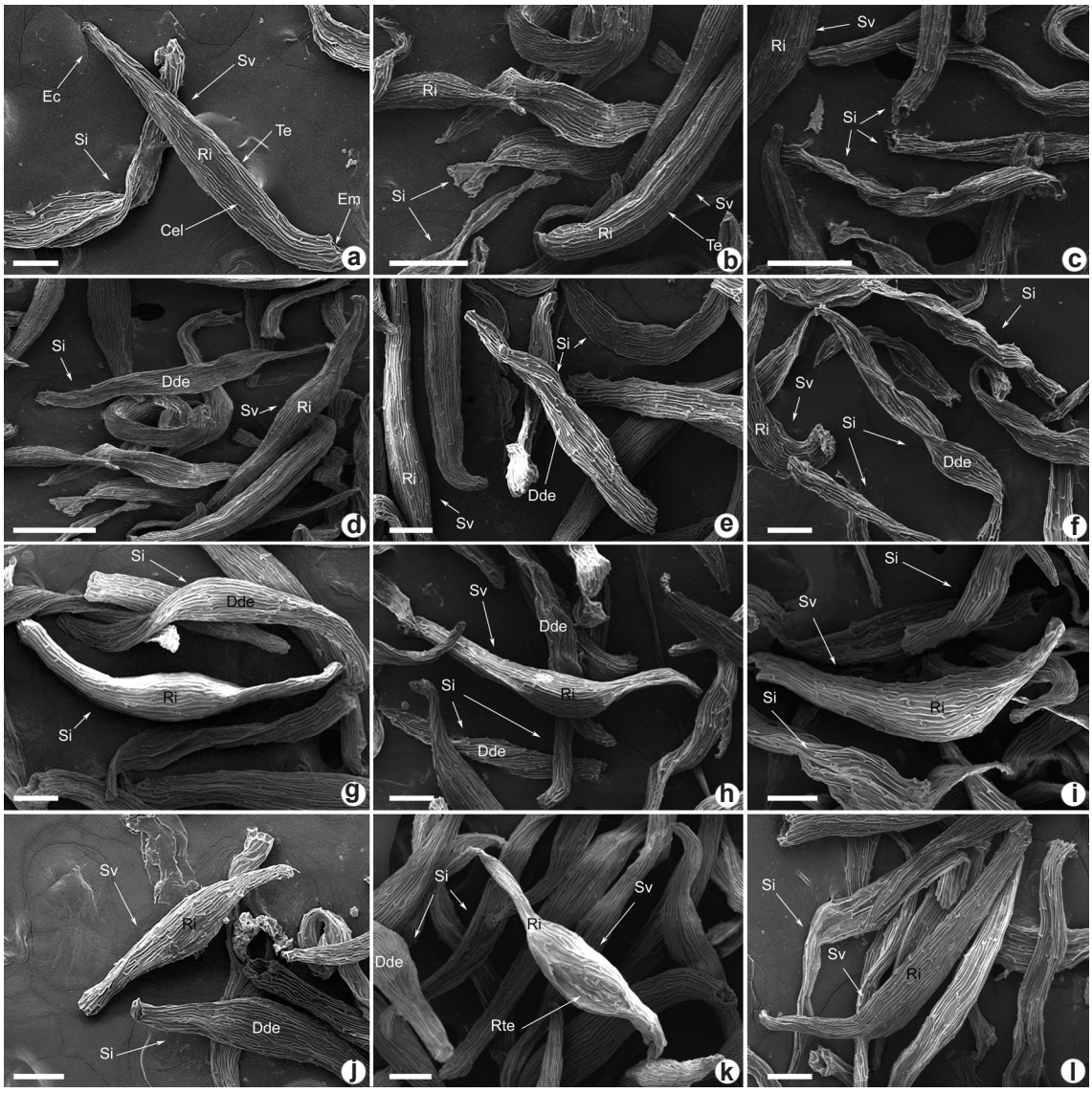


**Figure 2** – a-l. Scanning electron microscopy of seeds of *Cattleya intermedia* subjected to different temperatures and storage periods – a-c. Temperature of 25 (± 2 °C) – a. (two months) Viable seeds, evidencing the testa, the chalazal and micropylar ends and the swollen region (key) where the embryo is located. Non-viable seeds are noted, with damage by dehydration; b. (four months) Viable seeds, evidencing the testa and swollen region (key). Predominance of dehydrated seeds; c. (six months) Viable seeds evidencing the swollen region, where the embryo is located. Predominance of dehydrated and non-viable seeds; d-f. Temperature of -20 °C – d. (two months) Viable seeds, evidencing the swollen region (key) and non-viable seeds, showing damage by dehydration (key); e. (four months) Viable seed, evidencing the swollen region (key) and non-viable seeds, dehydrated (key); f. (six months) Viable seed, evidencing the swollen region (key). Predominance of non-viable and dehydrated seeds (key); g-i. Temperature of -80 °C – g. (two months) Viable seed, evidencing the swollen region (key), where the embryo and un-viable seed are located, showing damage by dehydration (key); h. (four months) Viable seed, evidencing the swollen region (key), where the embryo and non-viable seeds are located, dehydrated (key); i. (six months) Viable seed, evidencing the swollen region (key), where the embryo is located. Predominance of non-viable seeds, showing damage by dehydration (key); j-l. Temperature of -196 °C – j. (two months) Viable seed, evidencing the swollen region (key), where the embryo and non-viable seed are located, showing damage by dehydration (key); k (four months) Viable seeds, evidencing the swollen region (key); l. (six months) Viable seeds, evidencing the swollen region (key), where the embryo and non-viable seed are located. Cel = testa forming cell; Dde = damage by dehydration; Ec = chalazal end; Em = micropylar end; Rte = rupture of the integument of testa; Si = non-viable seed; Sv = viable seed; Te = testa. Scale bar: a, e-l = 100 µm; b-d = 200 µm.

**Table 1** – Description of viable and non-viable seeds of *Cattleya intermedia* submitted to different storage conditions observed through scanning electron microscopy.

| Temperature | Months | Observed description |
|---|---|---|
| 25 (± 2 °C) (room) | two | Seed dehydration (Fig. 2a) |
| 25 (± 2 °C) (room) | four | Seed dehydration (Fig. 2b) |
| 25 (± 2 °C) (room) | six | Dehydration was more intense (Fig. 2c) |
| -20 °C (freezer) | two | The viable seeds were characterized by having the region close to the swollen embryo, while the non-viable seeds showed damage by dehydration (Fig. 2d-f) |
| -20 °C (freezer) | four | |
| -20 °C (freezer) | six | |
| -80 °C (ultra freezer) | two | The viable seeds were characterized by having a swollen area, close to the embryo, and the non-viable seeds showed damage by dehydration (Fig. 2g-i) |
| -80 °C (ultra freezer) | four | |
| -80 °C (ultra freezer) | six | |
| -196 °C (cryopreservation) | two | Viable seeds, with the region of the embryo swollen and non-viable showing damage by dehydration (Fig. 2j-k) |
| -196 °C (cryopreservation) | four | |
| -196 °C (cryopreservation) | six | In the viable seed, a rupture in the integument was observed (Fig. 2l) |

are linked to lipid reserves in order to prevent the coalescence caused by the action of hydrolytic enzymes. Lipid reserves are accumulated in bodies and are mainly used as a source of energy during the initial process of germination and embryo growth (Somerville *et al.* 2000; Graham 2008).

## Seed dehydration during storage

The damage caused by dehydration observed in seeds of *Cattleya intermedia* stored at different temperatures throughout the different storage periods make the seeds non-viable. Berjak & Pammenter (2003) describe that the loss of viability is one of the main consequences in regard to this damage, that happens because of the unbalanced metabolism and the multiple injuries that are noted in cell membranes due to dehydration. Oliveira & Valio (1994), when studying the storage of *Hancornia speciosa* seeds, found a loss of viability as a consequence of evidenced damage to cell membranes caused mainly by dehydration in the seeds.

The dehydration present in the seeds also provokes a series of alterations in the reserve components, and one of the first symptoms observed is the occurrence of variations in the lipid reserves (Navari-Izzo *et al.* 1989, 1995).

## Alterations in seed protein reserves

In the embryo of the *Cattleya intermedia* seeds, it was also noted variations in the protein reserves, which were observed mostly when the embryo showed signs of degeneration in their cells. Abbot *et al.* (2014), when studying the storage of *Tabebuia roseoalba* seeds for different storage periods, found a reduction in the protein content, which was also observed in the present study for *C. intermedia* seeds and are in accordance with these authors. However, in our study, the changes that occur in these compounds could be related to the degenerative process that was taking place in the embryo. A possible explanation may be related to the high moisture content that these seeds were stored, causing them to have a higher incidence of metabolic activity, which contributes to the depletion of accumulated reserves. This event can be explained for all the reserves found in these seeds, as in the other cases detailed below.

## Alterations in seed lipid reserves

Variations in the lipid reserves present in the embryo of *Cattleya intermedia* seeds were evidenced. For the seeds kept at the temperature of 25 (± 2 °C) (room) there was no reaction with the Sudan IV reagent, showing a broader degradation of the lipid reserve when subjected to

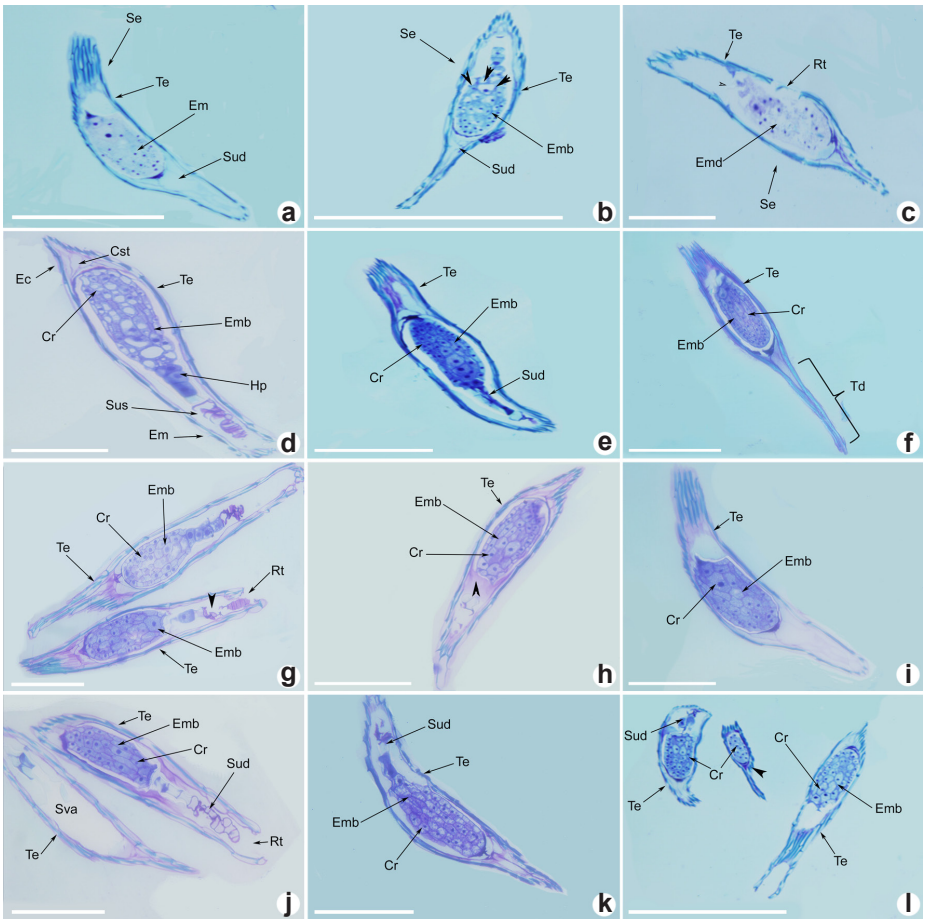


**Figure 3** – a-l. Light microscopy and histochemical analysis with TBO of seeds of *Cattleya intermedia* subjected to different temperatures and storage periods – a-c. Temperature of 25 (± 2 °C) – a. (two months) Seed highlighting reactive integument and embryo cells poorly reactive to TBO. The vacuolization of the suspender cells (arrowhead) can be noted by the lack of reaction with this dye; b. (four months) Seed showing embryo with cells poorly reactive to TBO, also evidencing some apparent vacuolization (arrowheads); c. (six months) Seed showing little reaction from the embryonic cells with TBO, and the lack of reaction with the suspender cells (arrowhead); d-f. Temperature of -20 °C – d. (two months) Seeds showing the chalazal end with superficial cells of testa highlighted by the reaction with TBO. The embryo reserve cells show evidence of vacuolization by the lack of reaction with the dye. The suspender and other cells with different intensities of reaction with TBO can be noted; e. (four months) Seed showing reactive embryonic cells and suspender with some apparent degeneration due to lesser reaction with TBO; f. (six months) Seed showing the dehydrated testa (key), embryonic cells reactive to TBO and the absence of suspender, by the lack of reaction with the dye (arrowhead); g-i. Temperature of -80 °C – g. (two months) Seeds showing different intensities of reaction from the embryonic cells with TBO, damage to the testa and apparent degeneration from the suspender cells due to the lack of reaction with TBO (arrowhead); h. (four months) Seed showing embryonic cells reactive to TBO and the lack of reaction with suspender cells (arrowhead); i. (six months) Seeds showing different reactions from the embryonic cells with TBO and apparent vacuolization. The absence of reaction with the suspender cells can be noted in one of the seeds, evidencing its degeneration (arrowheads); j-l. Temperature of -196 °C – j. (two months) Empty seed can be noted due to absence of reaction of internal structures with TBO. In the preserved seed the dye-reactive embryonic cells can be seen. The suspender cells, on the other hand, present different intensities of reaction, showing degeneration of cell walls; k. (four months) Seed showing embryonic cells with vacuolization evidenced by the lack of reaction with TBO. Degeneration process of the suspender, showing cells less reactive to the dye; l. (six months) Seeds showing different intensities of reaction of the embryonic cells with TBO. Degeneration of absence of the suspender can be observed, due to the less intensity of lack of reaction of the cells with TBO (arrowhead). Cr = reserve cell; Cst = superficial cell of the testa; Ec = chalazal end; Em = micropylar end; Emb = ellipsoid embryo; Emd = degenerating embryo; Rt = testa rupture; Se = seed; Sud = degenerating suspender; Sus = suspender; Sva = empty seed; Td = dehydrated testa; Te = testa. Scale bar: a-b, e-f, h, l = 500 µm; c-d, g, i-k = 200 µm.

**Table 2** – Description of light microscopy and histochemical analysis with TBO of *Cattleya intermedia* seeds submitted to different temperatures and storage periods.

| Temperature | Months | Observed description |
|---|---|---|
| 25 (± 2 °C) (room) | two | Seed showed the integument and embryo cells, but little reactive to TBO vacuolization of the suspender cells is also observed, due to the lack of reaction with the dye (Fig. 3a) |
| 25 (± 2 °C) (room) | four | Seeds showed little reactive embryo, with apparent vacuolization in the embryo cells, due to the degeneration process (Fig. 3b) |
| 25 (± 2 °C) (room) | six | It can be noted little reaction from the embryo cells with the TBO, in addition to an absence of reaction in the suspender cells, due to the ir degeneration (Fig. 3c) |
| -20 °C (freezer) | two | Seeds with the chalazal end and the superficial cells of the testa evidenced by reaction with TBO. There is evidence of vacuolization in the embryo cells, due to the lack of reaction with the dye, in addition to the suspender and the other cells with different intensities of reaction with the TBO (Fig. 3d) |
| -20 °C (freezer) | four | Seeds show reactive embryo cells and the suspender with apparent degeneration, due to less reaction with the reagent (Fig. 3e) |
| -20 °C (freezer) | six | Seed evidenced the dehydrated testa, and the embryo cells reactive to TBO. The absence of the suspender is observed, due to the lack of reaction with the TBO (Fig. 3f) |
| -80 °C (ultra freezer) | two | Embryo cells with different intensities of reaction with TBO. Damage to the testa structure and apparent degeneration of the suspender cells were also observed, due to the lack of reaction with TBO (Fig. 3g) |
| -80 °C (ultra freezer) | four | Embryo cells reactive to TBO and the absence of reaction in the suspender cells (Fig. 3h) |
| -80 °C (ultra freezer) | six | Seeds presented different reactions with TBO in the embryonic cells and their apparent vacuolization. Absence and degeneration of the suspender structure is observed, due to the lack of reaction with the suspender cells in one of the seeds (Fig. 3i) |
| -196 °C (cryopreservation) | two | It is possible to notice empty seeds, due to the lack of reaction with the TBO in the internal structures of the seeds, while in the the preserved seed, it is noted reaction of the embryo cells with the TBO and the suspender cells show different intensities of reaction, evidencing degeneration of the cell walls (Fig. 3j) |
| -196 °C (cryopreservation) | four | Vacuolization in the embryo cells is observed, they are evidenced by the lack of reaction with the TBO and the suspender in degeneration, showing cells less reactive to the dye used (Fig. 3k) |
| -196 °C (cryopreservation) | six | Embryo cells with different intensities of reaction with the TBO, in addition to seeds in degeneration or lack of suspender, due the less intensity or lack of reaction of the cells with the dye used (Fig. 3l) |

this condition. On the other hand, the seeds kept at the temperatures of -20 °C (freezer), -80 °C (ultra freezer) and -196 °C (cryopreservation) showed reactive embryos to Sudan IV, however, over the different periods lower intensity or absence of cell reaction with this dye was observed, due to the occurrence of degeneration in the structure of the embryo and this, consequently, led to alterations in the lipid reserves of these seeds. Abreu *et al.* (2012), when studying the storage of sunflower seeds, also observed reductions in the levels of lipid reserves, as in the present work.

Koutroubas *et al.* (2000) state that lipid reserves are the ones that suffer most from variations in their content when seeds are stored at room temperature conditions, with the lipid being

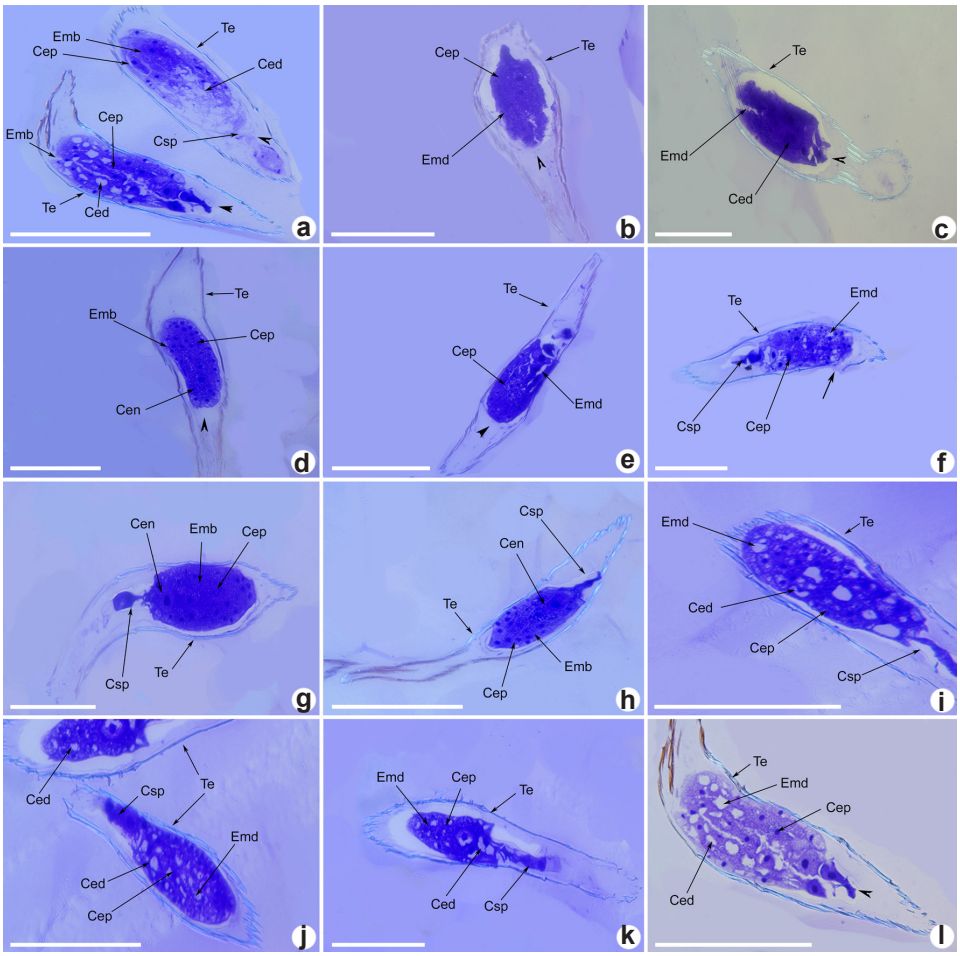


**Figure 4** – a-l. Light microscopy and histochemical analysis with CBB of seeds of *Cattleya intermedia* subjected to different temperatures and storage periods – a-c. Temperature of 25 (± 2 °C) – a. (two months) Seeds showing embryos and embryonic cells poorly reactive to proteins. Arrowhead points to an absence of suspender; b. (four months) Seed evidencing testa, embryo and embryonic cells reactive to proteins. Degeneration of the embryo and absence of the suspender can be noticed (arrowhead); c. (six months) Seed with embryo and embryonic cells reactive to proteins. Degeneration of the embryo and absence of suspender can be noted (arrowhead); d-f. Temperature of -20 °C – d. (two months) Seeds showing embryo and embryonic cells with prominent nuclei evidenced by the reaction with CBB. Arrowhead indicates the absence of the suspender by the lack of reaction with this dye; e. (four months) Seed showing embryo and embryonic cells reactive to proteins. Degenerating embryo and absence of the suspender are observed (arrowhead); f. (six months) Seed with degenerating embryo, embryonic and suspender cells poorly reactive to proteins can be noted. Rupture of the testa is noticed (arrow); g-i. Temperature of -80 °C – g. (two months) Seed evidencing embryo, embryonic and suspender cells reactive to proteins. Embryonic cells with prominent nuclei and suspender cells reactive to proteins are observed. Embryonic cells with prominent nuclei are evidenced by the reaction with CBB; h. (four months) Seed showing embryo, embryonic and suspender cells reactive to proteins. Embryonic cells with prominent nuclei evidenced by the reaction with CBB can be noted; i. (six months) Seeds showing embryo, embryonic and suspender cells reactive to proteins. Degeneration of the embryo and embryonic cells, evidenced by the lack of reaction with the chemical is observed. j-l. Temperature of -196 °C – j. (two months) Seeds showing embryo, embryonic and suspender cells reactive to proteins. Degeneration of the embryo and embryonic cells, due to the lack of reaction with CBB are perceived; k. (four months) Seed evidencing embryo, embryonic and suspender cells reactive to proteins. Degeneration of the embryo and embryonic cells, due to the lack of reaction with the dye is observed; l. (six months) Seed showing embryo and embryonic cells reactive to proteins. Degeneration of the embryo and embryonic cells, evidenced by the lack of reaction with CBB are noted. Arrowhead indicates the absence of the suspender. Ced = embryonic cells in degeneration; Cen = embryonic cells with prominent nuclei; Cep = embryonic cells reactive to proteins; Csp = suspender cells reactive to proteins; Emb = embryo; Emd = degenerating embryo; Te = testa. Scale bar: 200 µm.

**Table 3** – Description of light microscopy and histochemical analysis with CBB of *Cattleya intermedia* seeds submitted to different temperatures and storage periods.

| Temperature | Months | Observed description |
|---|---|---|
| 25 (± 2 °C) (room) | two | Embryos poorly reactive to proteins, due to the degeneration of these structures. Absence of suspender can be observed, due to the lack of reaction with the dye (Fig. 4a) |
| 25 (± 2 °C) (room) | four | Seeds show the testa, and embryo cells reactive to proteins. It also can be noted the degeneration of the embryo and suspender structure, due to the lack of reaction with CBB (Fig. 4b) |
| 25 (± 2 °C) (room) | six | Seed with embryo cells reactive to proteins. Again, embryo degeneration and absence of suspender can be noted, due to the less intensity or lack of reaction of the cells with the dye (Fig. 4c) |
| -20 °C (freezer) | two | Embryo cells with prominent nuclei were evidenced by the reaction with CBB, as well as the absence of the suspender structure due to the lack of reaction with CBB (Fig. 4d) |
| -20 °C (freezer) | four | Embryo cells reactive to proteins. An embryo in degeneration and absence of suspender can be noted, due to the lower intensity or lack of reaction of the cells with the dye (Fig. 4e) |
| -20 °C (freezer) | six | Seed with embryo in degeneration can be observed, while the embryon and cells appear to be less reactive to CBB. A rupture of the testa is also noticed (Fig. 4f) |
| -80 °C (ultra freezer) | two | Structures of the embryo, are observed by the reaction with CBB. The embryo cells show ed prominent nuclei due to the intense reaction with this dye (Fig. 4g-h) |
| -80 °C (ultra freezer) | four | |
| -80 °C (ultra freezer) | six | Degeneration of the embryo evidenced by the lack of reaction with CBB (Fig. 4i) |
| -196 °C (cryopreservation) | two | Embryo reactive to proteins. Degeneration of the embryo cells can be noted, due to the lack of reaction with CBB (Fig. 4j-k) |
| -196 °C (cryopreservation) | four | |
| -196 °C (cryopreservation) | six | Degeneration of the embryo is noticed, as well as the absence of the suspender, due to the lower intensity or lack of reaction of the cells with this dye (Fig. 4l) |

considered the the most susceptible constituent to the degenerative process during the storage stage. This occurrence was also observed in *C. intermedia* seeds kept at 25 (± 2 °C).

## Alterations in carbohydrates present in seeds

Another way of survival related to the seed exposure to low temperatures is correlated with the reduction of starch content contained in the seeds (Kaplan *et al*. 2006). For the freshly benefited seeds of *Cattleya intermedia* starch reserves were found in the interior of the embryo, however, after the storage at different temperatures and storage periods, no starch was observed any more. According to Yeung *et al*. (2018) the starch grains that occur in the seeds are replaced for protein reserves and lipid bodies, which is why these grains are found to be rare in the embryo of the orchid seeds. It is noteworthy that this reserve was present in newly benefited seeds.

Another modification observed in seeds is associated with the cell wall, which may present several alterations in the polysaccharides. However, there are still few studies related to this topic in literature, and according to Vicré *et al*. (2004) and Moore *et al.* (2006) the main consequence of dehydration is the unfolding between the cell walls, resulting in cell compaction.

## Modifications in embryo and suspender forming compounds and their effects on the ability to give rise to new plantlets after storage

The *Cattleya intermedia* seeds stored at the temperature of -80 °C (ultra freezer) for the period of two and four months showed to be the most effective regarding the conservation of the reserves

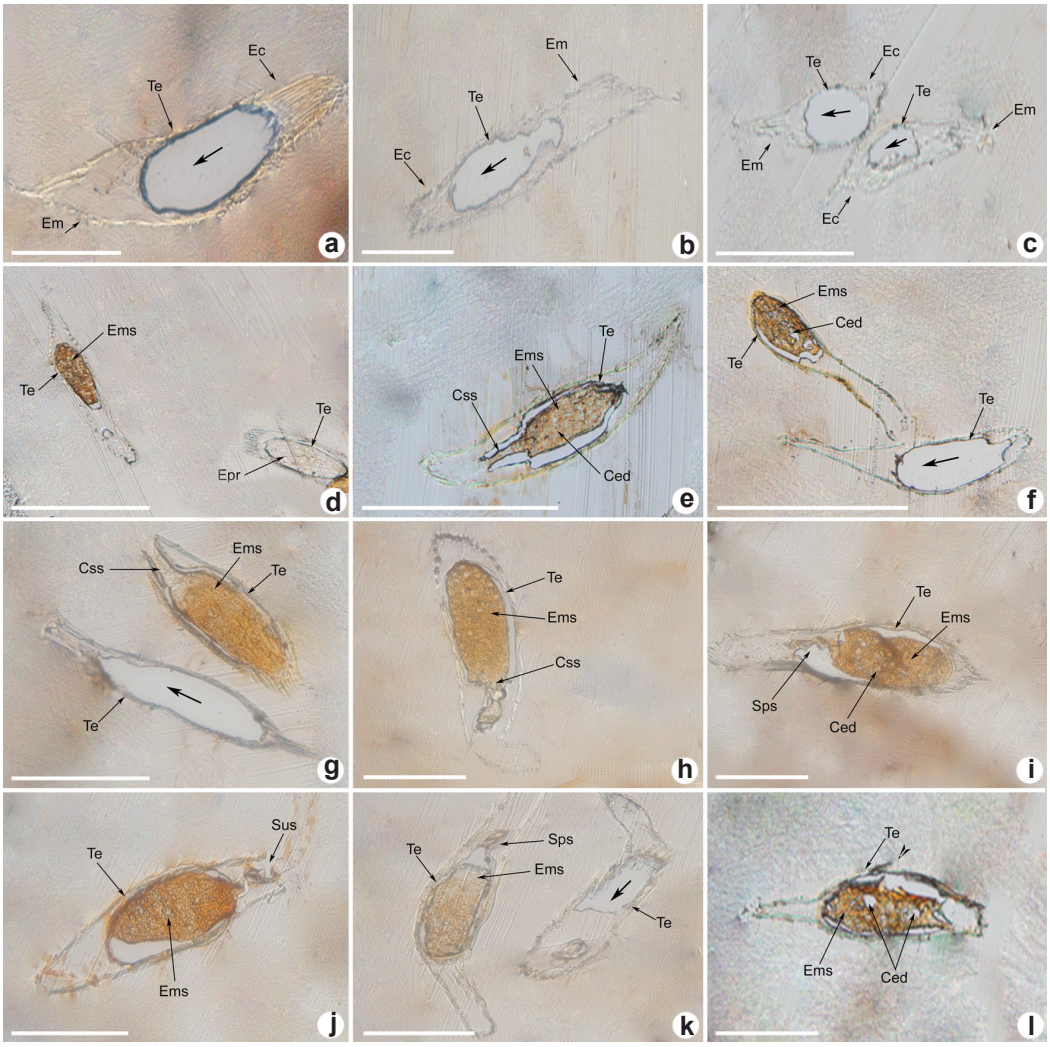


**Figure 5** – a-l. Light microscopy and histochemical analysis with Sudan IV of seeds of *Cattleya intermedia* subjected to different temperatures and storage periods – a-c. Temperature of 25 (± 2 °C) – a. (two months) Seeds showing testa, micropylar and chalazal ends. Arrow points to a lack of reaction to Sudan IV; b. (four months) Seed showing the testa, micropylar and chalazal end, lack of reaction to Sudan IV is noted (arrow); c. (six months) Seeds showing testa, micropylar and chalazal ends. Arrows indicate lack of reaction to Sudan IV; d-f. Temperature of -20 °C – d. (two months) Seed evidenced embryo reactive to Sudan IV is seen on the left. Absence of the suspender is perceived (arrowhead). On the right, a seed with an embryo poorly reactive to Sudan IV is noted, due the lack of reaction with the dye; e. (four months) Seed showing embryo and suspender cells reactive to Sudan IV. It can be seen embryonic cells in degeneration, due to the lack of reaction with the dye; f. (six months) Seed with reactive embryo for Sudan IV is seen on the left. Embryonic cells show degeneration caused by the lack of reaction with Sudan IV. On the right, a seed can be seen with no reaction to Sudan IV; g-i. Temperature of -80 °C – g. (two months) On the left, a seed with no reaction to Sudan IV is observed. On the right, it can be seen a seed showing embryo and suspender cells reactive to Sudan IV; h. (four months) Seed showing reaction to Sudan IV in the embryo and suspender cells; i. (six months) Seed showing embryo and suspender reactive to Sudan IV. Degeneration of the embryonic cells is perceived, due to the lack of reaction with the dye; j-l. Temperature of -196 °C – j. (two months) Seed showing embryo and suspender reactive to Sudan IV; k. (four months) On the left, seed showing embryo reactive to Sudan IV and suspender poorly reactive to Sudan IV. On the right, seed with no reaction to Sudan IV is noted; l. (six months) Seed with embryo reactive to Sudan IV and embryonic cells in degeneration can be observed, due to the lack of reaction with the dye. A damaged testa structure is noted (arrowhead). Ced = degenerating embryonic cells; Css = suspender cells reactive to Sudan IV; Ec = chalazal end; Em = micropylar end; Ems = embryo reactive to Sudan IV; Epr = embryo poorly reactive to Sudan IV; Sps = suspender poorly reactive to Sudan IV; Sus = suspender; Te = testa. Scale bar: a-b, g-j, k = 200 µm; c-f, l = 500 µm.

**Table 4** – Description of light microscopy and histochemical analysis with Sudan IV of *Cattleya intermedia* seeds submitted to different temperatures and storage periods.

| Temperature | Months | Observed description |
|---|---|---|
| 25 (± 2 °C) (room) | two | Seeds show the testa, micropylar and chalazal end, however, there is no reaction with Sudan IV in the embryo (Fig. 5a-c) |
| 25 (± 2 °C) (room) | four | |
| 25 (± 2 °C) (room) | six | |
| -20 °C (freezer) | two | Seeds with the embryo reactive to Sudan IV and the absence of the suspender can be observed, due to the lack of reaction with this dye. A seed with the embryo poorly reactive to Sudan IV can be noted (Fig. 5d) |
| -20 °C (freezer) | four | Seed shows the embryo cells reactive to Sudan IV, however, degeneration of these structures is seen, due to the lack of reaction with the chemical (Fig. 5e) |
| -20 °C (freezer) | six | Seed with the embryo reactive to the product can be noted, however, the embryo cells show degeneration, due to the lack of reaction with Sudan IV. Also, a seed with total lack of reaction with Sudan IV in the embryonic area is observed (Fig. 5f) |
| -80 °C (ultra freezer) | two | Seed with lack of reaction to Sudan IV and a seed with the embryo and the suspender cells reactive to the dye (Fig. 5g) |
| -80 °C (ultra freezer) | four | The reaction to Sudan IV in the embryo cells is evidenced (Fig. 5h) |
| -80 °C (ultra freezer) | six | Degeneration of the embryo cells is noted, due to the lack of reaction with the dye (Fig. 5i) |
| -196 °C (cryopreservation) | two | Embryo structure and the suspender reactive to Sudan IV (Fig. 5j) |
| -196 °C (cryopreservation) | four | Seeds with the embryo reactive to Sudan IV and the suspender poorly reactive to the dye can be observed, evidencing the degeneration. Yet, it is noticed a seed with a complete lack of reaction to Sudan IV in the embryo (Fig. 5k) |
| -196 °C (cryopreservation) | six | Seed with the embryo reactive to Sudan IV is perceived, however, the embryo cells are in degeneration, due to the lack of reaction to the dye (Fig. 5l) |

in the interior of the embryo. According to Shibata *et al.* (2012), seeds that have higher protein content in their interior are considered more vigorous to endure the storage period. This case was observed in the present study, since the seeds kept at a temperature of -80 ºC showed better efficiency in relation to the conservation of the protein reserve.

Seeds of *C. intermedia* from storage at different conditions show alterations in the structures that are considered to be vital to the occurence of the germination process and the formation of new plantlets. Vieira *et al.* (1994) state that an analogous process happens to the aging of the seeds during the storage, which causes degenerative changes, especially in the internal structures that form the seeds, this contributes to a lack of metabolic control, preventing the exchange of water and solutes between the cells and the external environment, as consequence these seeds end up losing germination.

During the different treatments of storage, it was verified that the seeds of *C. intermedia* showed degeneration of the structures of the embryo. Oliveira *et al.* (2011) suggest the importance of studying these modifications right after the storage, as these data can provide essential infos to ensure better conservation of seed quality and seedling production. Histochemical-related research has proven the importance of the reserve substances in the embryo, especially, for the germination and formation of new seedling phases, because the greater the content of existing reserves in the seeds, the more vigorous the plantlet originated from it will be (Carvalho & Nakagawa 2000). But it is worth remembering that orchid seeds do not germinate only with the reserves contained inside the embryo, which are considered minimal. Therefore, they make mycorrhizal associations with fungi to promote the germination process

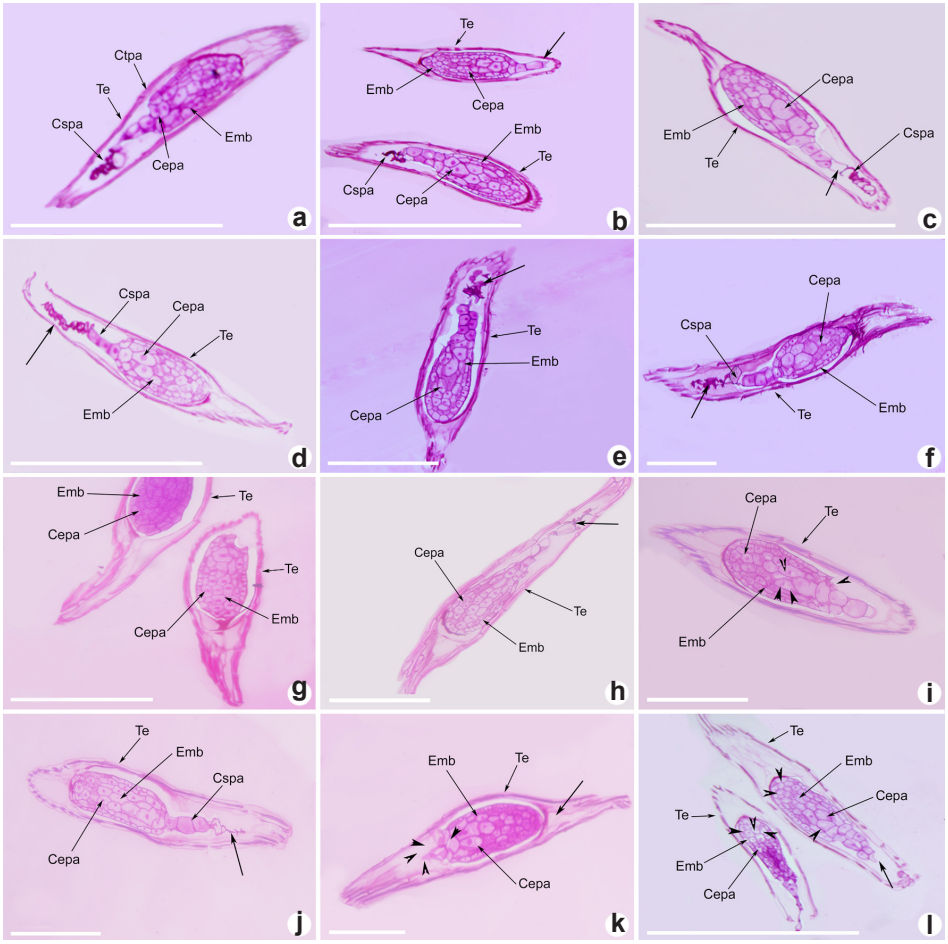


**Figure 6** – a-l. Light microscopy and histochemical analysis with PAS of the seeds of *Cattleya intermedia* subjected to different temperatures and storage periods – a-c. Temperature of 25 (± 2 °C) – a. (two months) Seed showing reaction to PAS in the testa structures, testa cells, embryo, embryonic and suspender cells; b. (four months) Seeds showing testa, embryo, embryonic and suspender cells evidenced by reaction to PAS are observed. Arrow indicates the lack of reaction with PAS in the suspender; c. (six months) Seed showing testa, embryo, embryonic and suspender cells reactive to PAS. Lack of reaction with the suspender cells can be perceived, pointing to its degeneration (arrow); d-f. Temperature of -20 °C – d. (two months) Seeds showing testa, embryo, embryonic and suspender cells reactive to PAS. Suspender cells show changes evidenced by the reaction to PAS (arrow); e. (four months) Seed showing reactive testa and embryo and embryonic cells poorly reactive to PAS. Alterations in the suspender cells can be noted evidenced by the reaction with the dye (arrow); f. (six months) Seed showing different intensities of reaction to PAS, reactive testa and embryo and embryonic and suspender cells poorly reactive to PAS can be observed. Changes in the suspender cells reactive to PAS are noted (arrow); g-i. Temperature of -80 °C – g. (two months) Seed showing different intensities of reaction in the embryo and embryonic cells with PAS; h. (four months) Seed evidencing embryo and embryonic cells poorly reactive to PAS. Alterations in the suspender cells evidenced by the reaction with the dye are noted (arrow); i. (six months) Seed showing different intensities of reaction in the embryo and embryonic cells with PAS. Changes can be seen in the cells of the embryo, due to less reaction with PAS (arrowheads); j-l. Temperature of -196 °C – j. (two months) Seed showing embryo, embryonic and suspender cells reactive to PAS. Alterations in the suspender cells reactive to the dye are noted (arrow); k. (four months) Seed showing testa, embryo and embryonic cells reactive to PAS. Changes in the embryonic cells (arrowhead), due to the lack of reaction with the dye and absence of reaction to PAS in the suspender (arrow) can be observed; l. (six months) Seeds showing different intensities of reaction to PAS in the embryo and embryonic cells, evidencing their degeneration. Changes in the embryonic cells (arrowhead), due to the poor reaction with the dye and seed with absence of reaction to PAS in the suspender (arrow) can be noted. Cepa = embryonic cells reactive to PAS; Cspa = suspender cells reactive to PAS; Ctpa = testa cell reactive to PAS; Emb = embryo; PAS = periodic acid-Schiff; Te = testa. Scale bar: a-d, l = 500 µm; e-k = 200 µm.

**Table 5** – Description of light microscopy and histochemical analysis with PAS of *Cattleya intermedia* seeds submitted to different temperatures and storage periods.

| Temperature | Months | Observed description |
|---|---|---|
| 25 (± 2 °C) (room) | two | Reaction to PAS in the structures of the testa cells, embryo, embryo cells and suspender cells (Fig. 6a) |
| 25 (± 2 °C) (room) | four | Absence of the suspender, due to the lack of reaction with PAS (Fig. 6b) |
| 25 (± 2 °C) (room) | six | Degeneration in the suspender cells can be seen, due to the lack of reaction with the dye (Fig. 6c) |
| -20 °C (freezer) | two | Seeds show testa, embryo, and suspender cells, due to the reaction with the dye. Changes in embryo cells can be seen, evidenced by the reaction with PAS (Fig. 6d) |
| -20 °C (freezer) | four | Seed with a reactive testa can be observed, while the embryo cells seem to be poorly reactive to PAS, alterations in the suspender cells evidenced by the reaction with the dye can also be noted (Fig. 6e) |
| -20 °C (freezer) | six | Seeds show different intensities of reaction with PAS, reactive testa and embryonic and suspender cells poorly reactive to the chemical are noted, evidencing these structures degeneration. The suspender cells show changes evidenced by the reaction to PAS (Fig. 6f) |
| -80 °C (ultra freezer) | two | Different intensities of reaction with PAS in the embryo cells (Fig. 6g) |
| -80 °C (ultra freezer) | four | Seeds showed to be poorly reactive to PAS in the embryo structure and embryonic cells. It was possible to note changes in the suspender cells, evidenced by the reaction to PAS (Fig. 6h) |
| -80 °C (ultra freezer) | six | Seeds showed different intensities of reaction to PAS in the embryo cells, as well as alterations in the embryonic cells, caused by the less reaction with the dye, that way pointing to degeneration of these cells (Fig, 6i) |
| -196 °C (cryopreservation) | two | Embryo and suspender cells reactive to PAS, some changes in the suspender cells are observed (Fig. 6j) |
| -196 °C (cryopreservation) | four | Seeds show reaction to PAS in the embryo and suspender cells, also showing alterations in the embryonic cells and absence of the suspender, due to the less or lack of reaction with PAS (Fig. 6k) |
| -196 °C (cryopreservation) | six | Seeds show different intensities of reaction to PAS for the embryo cells, which indicates their degeneration. Changes in the embryo cells and absence of the suspender are noted, due to the lack or less reaction with the dye (Fig. 6l) |

and establishment of the protocorm in nature (Dearnaley 2007).

The data presented in this work show that the high temperature alongside the high humidity of seeds during the storage stage causes degenerative changes in the internal structures of the seeds. Seeds of *C. intermedia* were stored with initial humidity over 30%, and in this case, this process occurs more intensely, and the first signs observed are related to the loss of cell membrane integrity. It also can be observed the depletion of reserves, alterations in the chemical composition, lipid peroxidation, and cellular damage (Delouche & Baskin 1973; Vieira *et al.* 1994; Zonta *et al.* 2014).

Storck *et al.* (2005) state that the chemical composition in the embryo is compromised when the seeds are submitted to different storage conditions. This was observed in this work for the seeds of *C. intermedia*, where alterations in the accumulation of reserves were observed in different storage conditions, varying according to the environment conditions, the humidity level and the characteristics of the seed itself, it can occur quicker or slower, affecting the seedling

production process (Vieira *et al.* 2001; Walters *et al.* 2010; Souza *et al.* 2011).

Works related to orthodox seeds reveal that reductions in starch and protein content are linked to lower seed germination and vigor (Henning *et al.* 2010). Among the consequences involved in the depletion of reserves is the increase in the number of seedlings considered abnormal (Nedel 2006). Strenske *et al.* (2017) when studying the storage of quinoa seeds, observed an increase in the occurrence of seedlings considered abnormal, due to the occurrence of alterations in the reserve composts, especially those related to proteins, once this reserve is essential for the growth of seedlings.

In conclusion, after the process of seed storage under different temperatures and storage periods it was evidenced by damage dehydration, responsible for the loss of viability of the seeds. In addition, it causes alterations in the main reserves, due to the degeneration of structures considered essential to ensure the quality of the seeds and seedlings originated from them.

The storage under the temperature of -80 °C (ultra freezer) during the period of two and four months has proven to be the most effective for the conservation of the main internal structures of the seeds, these results confirm the possibility of usage of this condition for the better conservation of seeds of this species.

This work is one of the pioneers in reporting the morphoanatomical alterations resulting from the storage of orchid seeds of the species *C. intermedia* and provides a basis for further studies related to this topic to be carried out.

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