

Loss of desiccation tolerance in *Copaifera langsdorffii* Desf. seeds during germination

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(With 3 figures)

Abstract

This study evaluated the loss of desiccation tolerance in *C. langsdorffii* seeds during the germination process. Seeds were imbibed for 24, 48, 72, 96, 120 and 144 hours and dried to the initial moisture content, kept in this state for 3 days after which they were submitted to pre-humidification and rehydration. Ultrastructural evaluations were done aiming to observe the cell damage caused by the dry process. Desiccation tolerance was evaluated in terms of the percentage of normal seedlings. Seeds not submitted to the drying process presented 61% of normal seedlings, and after 24 hours of imbibition, followed by drying, the seeds presented the same percentage of survival. However, after 48 hours of imbibition, seeds started to lose the desiccation tolerance. There was twenty six percent of normal seedlings formed from seeds imbibed for 96 hours and later dried and rehydrated. Only 5% of seeds imbibed for 144 hours, dried and rehydrated formed normal seedlings. At 144 hours of imbibition followed the dry process, there was damage into the cell structure, indicating that the seeds were unable to keep the cell structure during the drying process. *Copaifera langsdorffii* seeds loses the desiccation tolerance at the start of Phase 2 of imbibition.

Keywords: Copaíba, desiccation sensitivity, imbibition.

Perda da tolerância à dessecação em sementes de *Copaifera langsdorffii* durante a germinação

Resumo

Este estudo avaliou a perda da tolerância à dessecação em sementes de *C. langsdorffii* durante o processo germinativo. Sementes foram embebidas por 24, 48, 72, 96, 120 e 144 horas e depois secas até a umidade inicial, sendo mantidas neste estado durante 3 dias, quando então foram submetidas a pré-umidificação e reidratação. Avaliações ultraestruturais foram realizadas objetivando observar danos nas células causados pelo processo de secagem. A tolerância à dessecação foi avaliada pelo percentual de plântulas normais. Sementes não submetidas ao processo de secagem apresentaram 61% de plântulas normais, sendo que após 24 horas de embebição seguida de secagem, houve o mesmo percentual de sobrevivência. Contudo, após 48 horas de embebição, as sementes começaram a perder a tolerância à dessecação. Vinte e seis por cento de sementes formaram plântulas normais após embeberem por 96 horas e secas. Apenas 5% de sementes embebidas por 144 horas e secas formaram plântulas normais. Após 144 horas de embebição, seguida de secagem, verificou-se danos na estrutura celular, o que indica que as sementes não são capazes de manter a estrutura celular durante o processo de secagem. Verificou-se no presente estudo que sementes de *C. langsdorffii* perdem a tolerância à dessecação no início da fase 2 da embebição.

Palavras-chave: Copaíba, sensibilidade à dessecação, embebição.

1. Introduction

The term seed desiccation tolerance, is defined as the tolerance to conditions of low water availability (Marcos Filho, 2005) or ability to germinate after fast drying (Golovina et al., 2001). The capacity to tolerate drying occurs in the most varied types of organisms, and in the plant species it is more common in seeds, pollen grains

and spores, being able to happen in some plants in the vegetative state (Alpert, 2005). Seeds tolerant to desiccation (orthodox) acquire that capacity during their maturation process (Bewley and Black, 1994), the moment of the loss of this capacity being quite variable among the species or even among different lots of a same species.

The desiccation tolerance mechanisms are, in general, related to the maintenance of the cell structure integrity, not only during drying, but also during imbibition, because the water uptake can also cause damage to the cell structure (Corbineau et al., 2004; Pammenter and Berjak, 1999). The mechanisms are usually related to protein and oligosaccharide synthesis that maintains the cellular structure during the dehydrated state (Barbedo and Bilia, 1998) such as the LEA proteins (*late embryogenesis abundant*), synthesised at the end of seed development, and suggested to be related not only to desiccation tolerance, but also the other stress types (Alpert, 2005; Bartels, 2005; Black et al., 1999; Hoekstra et al., 2001; Tunacliffe and Wise, 2007). In desiccation-sensitive seeds, these mechanisms are absent or appear incomplete, resulting in an insufficient accumulation of protective molecules, leading to cell susceptibility to damage from drying (Kermode and Finch-Savage, 2002; Pammenter and Berjak, 1999).

The desiccation sensitivity is associated with high metabolic rate, that is linked to the consumption of reservations and the cell cycle (Farrant et al, 1988). During the germinative process many physiological, cellular and molecular processes occur in the orthodox cell seeds that are similar those is linked to the desiccation sensitivity in recalcitrant seeds (Castro et al, 2004; Bruggink and Van Der Toorn, 1995). The injuries caused by drying (both in recalcitrant and orthodox seeds) is related to free radicals generating oxidative damage. Both recalcitrant and orthodox germinated seeds presents subcellular development and vacuolation (linked to the active metabolism), and the damage caused by drying is similar in recalcitrant and germinated orthodox seeds (Farrant et al, 1988).

A considerable number of plant species of interest bear desiccation sensitive seeds (Berjak and Pammenter, 2001; Hong and Ellis, 1996; Tweddle et al., 2003), so the understanding of the desiccation sensitivity is of great importance the establishment of seed conservation strategies (Bovi et al., 2004). However, desiccation sensitive (recalcitrant) seeds have low availability throughout the year and cannot be stored, which hinders the study of the desiccation sensitivity in these species. Thus, considering that germinating orthodox seeds presents a behaviour comparable with recalcitrant seeds, Sun (1999) proposed that germinating orthodox seeds can be useful in desiccation sensitivity studies, and the availability and storability enables its use.

Copaifera langsdorffii Desf. is the most important representative of the *Copaifera* genus in Brazil, given its wide geographical distribution (Andrade Junior and Ferraz, 2000; Lorenzi, 2000). The species raises interest for the medicinal application of the oil extracted from its trunk (Veiga Junior et al., 2007), besides the use of its wood in building, furniture making, lathed pieces, floorboards and other items such as tool and broom handles and weapon gunstocks (Guerra et al., 2006; Lorenzi, 2000; Veiga Junior and Pinto, 2002). Furthermore, the species is widely used in rural and urban forestation projects (Jeller and Perez, 1997) and in the recovery of degraded areas (Lorenzi, 2000).

C. langsdorffii seeds present physiological dormancy caused by the presence of germination inhibitors (Lima et al., 2006), besides coat imposed dormancy (Fowler and Bianchetti, 2000). The most common treatments for germination are the immersion in water for 96 hours, stratification for 15 days (Borges et al., 1982; Fowler and Bianchetti, 2000), immersion in ether for 20 minutes (Perez and Prado, 1993) and mechanical scarification (Fowler and Bianchetti, 2000).

C. langsdorffii seeds present orthodox behaviour, which makes them useful for studies related to desiccation tolerance and sensitivity, and it is expected that these seeds lose the desiccation tolerance around the start of phase 3 of imbibition (visible radicle protrusion). Therefore, the objective of this research was to characterise the loss of desiccation tolerance in *C. langsdorffii* seeds during the germinative process.

2. Material and Methods

The experiments were carried out at the Laboratory of Tree Seeds of the Federal University of Lavras, MG, with seeds collected in August 2009 and stored until February 2010. Seeds were collected in a native population of *Copaifera langsdorffii* near Lavras, MG (21°16'S; 45°02'W, altitude 911 m). The initial seed moisture content was 17% and after drying the seeds in an acclimatised room (20°C 50% UR), it dropped to 11.27%. Seeds were stored in a cold chamber at 5°C and 40% RH until the beginning of the experiments and showed no variation in moisture content during the storage.

In all tests, the seeds were submitted to mechanical scarification with sandpaper. The imbibition curve of the seeds was established using 20 repetitions of 1 seed that were maintained in paper rolls and conditioned in an incubator at 25°C under constant light, weighing at regular intervals for up to 3 days after the protrusion of the radicle (visible germination).

Germination tests were done with 4 replicates of 25 seeds. For evaluation of the desiccation tolerance, seeds previously submitted to the mechanical scarification treatment were maintained in a paper roll in an incubator at 25°C under constant light. After 24, 48, 72, 96, 120 and 144 hours of imbibition, the seed moisture content was evaluated, by drying them in an oven at 103°C for 17 h (Brasil, 2009). For each imbibition time, using the formula proposed by Hong and Ellis (1996), the target weight was determined, until the seeds reached the initial moisture content (11.27%, see Table 1). After this, seeds were dried in a gerbox containing 120 ml of silica gel until the target weight was reached. Moisture content was determined to confirm if the initial moisture content was reached.

After the initial moisture content had been reached, seeds remained in the gerbox for 72 hours. After this, seeds were pre-humidified at 100% RH and constant light for 24 hours, and returned to the same initial germination conditions. As control, a sample of seeds of the same mechanically scarified lot (sandpaper) not submitted to the

Table 1. Germination speed index of *Copaifera langsdorffii* seeds submitted to drying.

Time	Moisture (%)**	Germination Speed Index	
		Average	Deviation
0*	11.27	1.53a	0.09
24	32.85	1.78a	0.11
48	49.07	2.21a	0.11
72	49.64	1.69a	0.02
96	53.50	1.18ab	0.12
120	54.64	0.36b	0.07
144	56.77	0.20b	0.03
p		8.17×10 ⁻⁷	

Same letters indicate no differences between means by Tukey test at 5% probability. *Values for initial germination test (control). **Moisture content evaluated after the time of imbibition.

desiccation/rehydration process previously mentioned was used. Germination (1 mm protruded radicle) was scored daily for the calculation of germination speed index using the method proposed by Maguire (1962). Together with final germination and normal seedlings percentage, this data was used to evaluate the loss of desiccation tolerance in seeds.

Ultrastructural analyses were carried out seeking to verify possible damage caused by the desiccation/rehydration process in *C. langsdorffii* seeds. The material for analysis was collected starting from fresh seeds at 24, 48, 72, 96, 120 and 144 hours after imbibition. These points were appraised after the imbibition, drying back to the initial moisture content and after pre-humidification. The ultrastructural evaluations were conducted at the Electron Microscopy Laboratory of the Department of Phytopathology of UFLA using the laboratory methods (Alves, 2004).

The evaluations were carried out on the embryonic axis, using the scanning electronic microscope (SEM LEO EVO 40 XVP), preparing the samples according to Alves (2004). The samples were fixed in Karnovsky fixative (2.5% glutaraldehyde; 2.5% formaldehyde and sodium cacodylate buffer 0.05M, pH 7.2, CaCl₂ 0.001M) for 24 hours. After that, the samples were immersed in 30% glycerol for 30 min, later washing in distilled water (two times of 15 min). Cryofracture consisted of immersion of the samples in liquid nitrogen and cutting using a scalpel blade. Later, samples were dehydrated in acetone at a concentration gradient (25, 50, 75 and 100% 3 times). The dehydrated samples were submitted to drying in a Bal-Tec critical point apparatus.

The samples were finally mounted on stubs, conducting the metallisation (gold) and their subsequent visualisation. Images were taken and used for the comparison of the results.

The percentage data (germination and normal seedlings) were submitted to the Shapiro-Wilk test of normality. When non-normal data distribution was verified ($p < 0.05$), it were converted to the $\arcsin\sqrt{x}/100$. After that, the data (when normalised) was submitted to the variance analysis (ANOVA) and, when difference among the treatments was verified by the F test, Tukey tests to 5% of probability were

done. Data that did not present normal distribution, even after conversion, was analysed by the GLM (Generalised Linear Models) method through binomial distribution and, when difference among the treatments by the Chi-squared test was verified, the Tukey at 5% of probability was used. The fresh mass values of the seeds obtained in the imbibition curve evaluations were used for calculation of the mass of imbibed water over time, for making the imbibition curve of the seeds. Also a curve of desiccation tolerance loss was established during the imbibition time based on the seed survival values (expressed by germination and the percentage of normal seedlings). Using that data, the calculation of the explanatory equation for the data was made.

All analyses were conducted through the software R for Windows version 2.12.0 (R Foundation for Statistical Computing, 2010), using Microsoft Office Excel® version 2007 for graph preparation.

3. Results

The variation in the germination values is presented as a quadratic equation. An increase was observed in the germination percentage in seeds that were imbibed for 48 hours, being dried soon afterwards (Figure 1). The same was observed for GSI, increasing to 48 hours, declining afterwards (Table 1). Significant differences among the averages were observed, having higher germination values for seeds dried after 48 hours of imbibition (Figure 1). As for the GSI, a reduction in the values was observed when seeds with 120 and 144 hours of imbibition are dried to the initial moisture.

The incidence of normal seedlings was reduced as the imbibition time of the seeds before dehydration and rehydration increased (Figure 1). There was reduction in the percentage of normal seedlings from 61.25% (control and seeds after 24 hours of imbibition) to 5% (after 144 hours of imbibition).

Possible variations of the embryonic axis cell volume were observed during the imbibition process (Figure 2). Dry seeds (not put for imbibition) presented compact cytoplasm, with no vacuoles being observed. After 24 hours

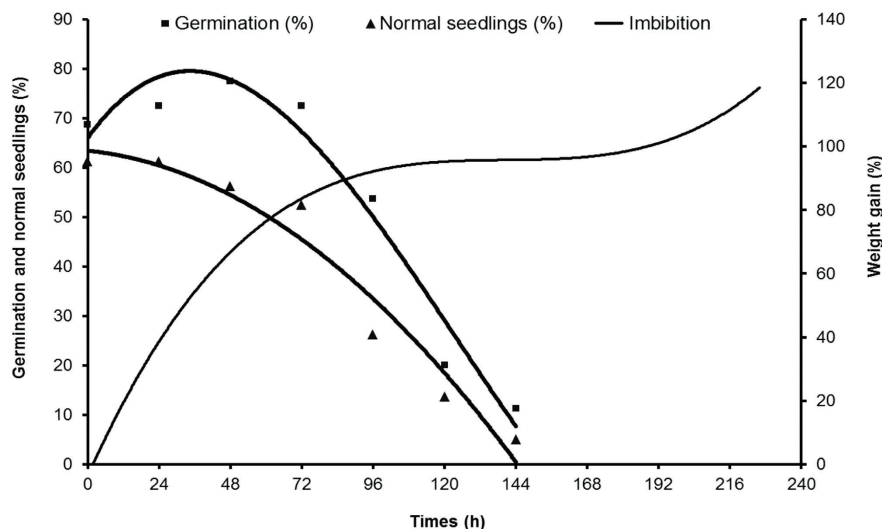


Figure 1. Loss of desiccation tolerance in *Copaifera langsdorffii* seeds expressed by the germination percentage and normal seedling formation during the imbibition process. Equations: Germination: $y=69.1369+0.0792x-0.001173x^2$. Normal Seedlings: $y=69.136905+0.396205x-0.005865x^2$.

of imbibition (2B), there still were no considerable increases in the cell volume. After 120 and 144 hours of imbibition (2F,G), besides an increase in cell volume, we verified changes in cell morphology, comparing with the previous points, as the formation of vacuoles.

Seeds imbibed for 24 hours and dehydrated did not present damage to their cell structure (Figure 3A). Through the evaluation of the obtained images, apparent cell damage after drying and pre-humidification was not verified (Figures 3C, E).

After 144 hours of imbibition, the seeds that were submitted to drying presented damage to their cell structure and collapse, with apparent detachment of the plasmalemma from the cell wall (Figure 3B) and damage to the cell wall (Figure 3C). Furthermore, in seeds with 144 hours of imbibition submitted to drying and subsequent pre-humidification, the occurrence of cell lysis was observed (Figure 3F).

4. Discussion

In general, there is no occurrence of metabolic events in Phase 1 of the imbibition (Bewley and Black, 1994), and usually seeds that are at this point are capable of tolerating desiccation (Castro et al., 2004). The *C. langsdorffii* seeds, however, appeared to start to lose desiccation tolerance already in Phase 1 of imbibition. The accentuated decrease of the capacity to tolerate desiccation happened between 72 hours (52.25%) and 96 hours (26.25%). This reduction of the normal seedling percentage occurs in the beginning of Phase 2 of imbibition (96 hours), the stage where, in general, the re-activation of the cell metabolism starts.

For most of the species studied so far, desiccation tolerance in seeds is retained until slightly before or after radicle protrusion. Faria et al. (2005) verified that the

desiccation tolerance in seeds of *Medicago truncatula* is lost only after germination. Masetto (2008) observed that *Sesbania virgata* germinated seeds with a 1 mm radicle still retained desiccation tolerance and the total loss of this capacity took place when radicles reached 2 mm. The loss of desiccation tolerance only after radicle protrusion was also observed in *Peltophorum dubium* (Guimarães et al., 2011), *Solanum lycopersicum* and *Abelmoschus esculentus* (Lin et al., 1998). Oliveira (2009) observed that scarified seeds of *Leucaena leucocephala* lost desiccation tolerance only after radicle protrusion, differently to this study. There are no reports of seeds presenting loss of desiccation tolerance between imbibition Phases 1 and 2, whether seeds were scarified or not.

Dehydration of desiccation-sensitive tissues causes extensive damage to cell structure, among them, solute crystallisation, protein denaturation and membrane damage (Black and Pritchard, 2002). The maintenance of the cell integrity or the capacity to repair the damage caused by drying is indispensable to enable the seed to tolerate desiccation (Pammenter and Berjak, 1999). With the onset of imbibition Phase 2, when the seed water content passed 50% (Table 1), it was possible to observe a large increase in the cell volume and occurrence of vacuoles that is linked to the increase of the metabolism storage of metabolites and water and (Figures 2D-G).

After 144 hours of imbibition and subsequent drying, it was possible to observe damage to the cell structure of the seeds, which indicates that at this point the cells lose the capacity to maintain their integrity during the dehydration. These seeds presented collapse of the cells, detachment of the cell wall and cell lysis. (Figure 3B-C). These occurrences are indications that the cell structure is quite damaged due to the drying and that the seeds do not possess effective prevention or repair mechanisms

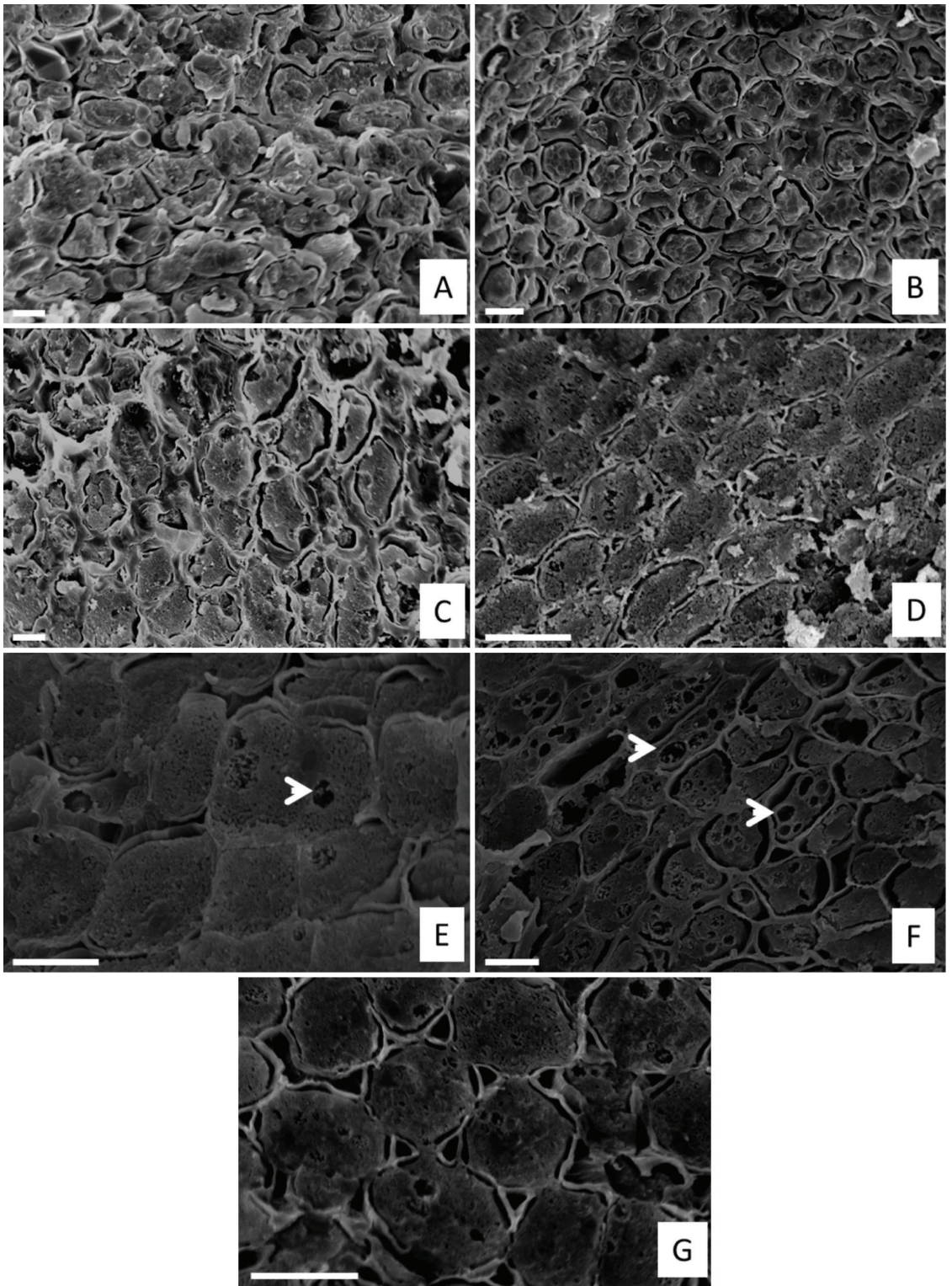


Figure 2. Scanning electron micrographs of *Copaifera langsdorffii* seeds during the imbibition process. (A) Fresh seed. B-G: Seeds after imbibition. (B) 24 h (C) 48 hours (D) 72 hours (E) 96 hours (F) 120 hours (G) 144 hours. Bars in A, C, E and F = 10mm. Bars in B, D and G = 20mm. Arrows indicate cell vacuoles.

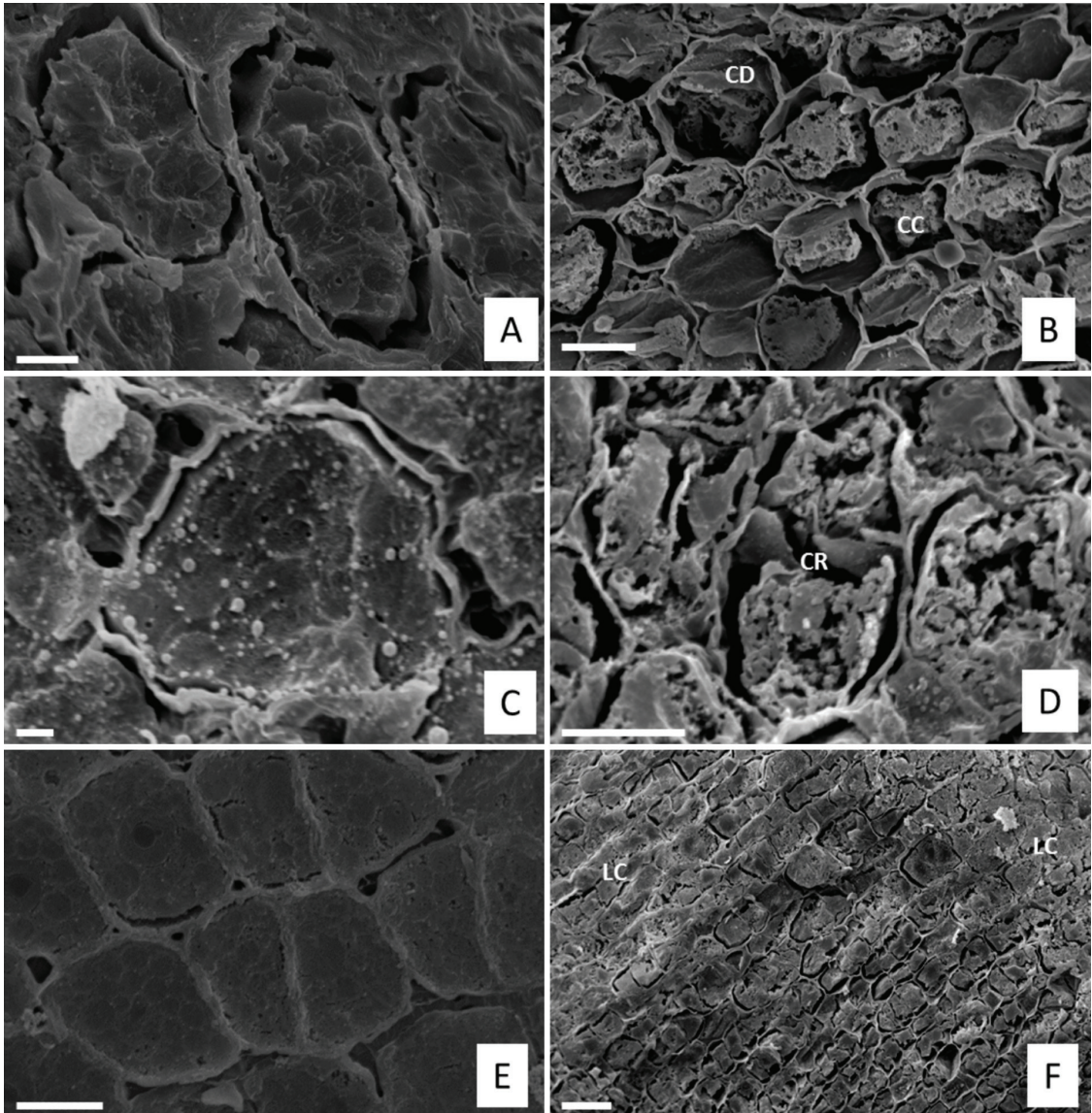


Figure 3. Scanning electron micrographs of *Copaifera langsdorffii* seeds. (A) After 24 h of imbibition. (B) After 144 hours of imbibition. (C) After 24 h of imbibition and drying until the initial moisture. (D) Cell wall damaged after 144 hours of imbibition and drying until the initial moisture content. (E) After 24 h of imbibition and drying until initial moisture remaining as is for 72 hours and pre-humidification for 24 hours. (F) After 144 hours of imbibition, drying until initial moisture remaining as is for 72 hours and pre-humidification for 24 hours. Bars in A, C, D, F =20 μ m, Bars in B, =10mm, Bar in E =1mm. CD = Cell damage; CC = Cell collapse; CR = Cell wall rupture; LC = cell lysis.

for that damage. This can be one of the factors linked to the loss of desiccation tolerance in *C. langsdorffii* seeds during the germinative process.

Various authors have verified that the cell structure is irreversibly damaged with the drying of the seed during the germinative process. Guimarães et al. (2011) verified that the drying of in *Peltophorum dubium* germinated seeds causes complete disorganisation of the cell structure, with organelle membrane degradation and plasma membrane damage. Damage to the cell structure has also been reported in other species such as recalcitrant embryonic axes of

Trichilia emetica (Kioko et al., 2006) and orthodox seeds of *Leucaena leucocephala* (Oliveira, 2009). Koster and Leopold (1988) observed that one of the factors related to cell damage, especially in the membrane, is related to the reduction of the sucrose content in the seed.

5. Conclusions

The loss of desiccation tolerance in *C. langsdorffii* seeds begins in Phase 1 of imbibition, being totally lost in the middle of Phase 2.

The cell structure of the germinating seed is damaged by desiccation.

The loss of desiccation tolerance in seeds of *C. langsdorffii*, at the onset of imbibition Phase 2 is an indication that seed metabolism is re-activated at the beginning of Phase 1 of imbibition.

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