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Physiological behavior of *Cryptocarya aschersoniana* seeds subjected to drying

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ABSTRACT. Seeds of *Cryptocarya aschersoniana*, as well as other species of the Lauraceae family, have frequently been reported to be sensitive to desiccation, which hinders their *ex situ* conservation. This study investigated the changes that occur during the drying of these seeds. Seeds harvested over 3 years were processed and dried at 20°C in boxes containing silica gel (with relative humidity ranging from 13.5 to 40%) to achieve a target water content of 40, 35, 30, 25, and 20%. For freshly harvested seeds, at each target water content, samples were taken, and germination tests were performed. Cell analyses were performed by using scanning electron and light microscopy (with reactions for starch and lipids). In addition, the sugars and lipid contents were determined. The results indicated that *C. aschersoniana* seeds are sensitive to desiccation and that under the conditions tested, the critical water content is approximately 30%, and the lethal water content is less than 18%. The seeds are dispersed while dormant, and this dormancy is partially overcome by partial drying. These seeds have some protection systems against desiccation, such as increased sucrose concentrations, during artificial drying; however, these systems are not efficient at protecting the seeds from damage caused by more intense desiccation. The images obtained allowed the verification of changes only at the point where the seeds were already completely unviable.

Keywords: Lauraceae; desiccation tolerance.

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Introduction

The Lauraceae family appears in most floristic surveys of forest remnants in southern Minas Gerais, which emphasizes its ecological importance. Among the many species of this family, *Cryptocarya aschersoniana* (popularly known as *canela-batalha* or *canela-fogo* in Brazil) is a tree climax species in the southern and southeastern states of the Atlantic forest (Fontana et al., 2016). Seeds of this species, as well as others of the Lauraceae family, have often been classified as desiccation sensitive (Jaganathan et al., 2019; Vicente et al., 2016). This behavior makes their handling difficult and compromises the conduct of research on the species, their storage, and, consequently, the availability of seeds, which leads to a bottleneck in the production of quality seedlings.

In general, desiccation-sensitive seeds are common in species typical of wet tropical regions because the characteristics of the original environment do not require them to tolerate desiccation. In temperate regions, the occurrence of species with recalcitrant seeds is common; however, these species are less sensitive to water loss (Wyse & Dickie, 2017). Additionally, according to Pritchard et al. (2022), approximately 10% of species that produce desiccation-sensitive seeds also present some type of dormancy, the majority of which are classified as physiological dormancy.

The acquisition of desiccation tolerance by orthodox seeds occurs at the end of their development. This phase is characterized by the accumulation of reserves and the activation of protective mechanisms, such as the production of osmoprotectants, carbohydrates, antioxidants, late embryogenesis abundant (LEA) proteins, and heat shock proteins (Saha et al., 2022), which prepare the seed for maturation drying and enable its survival in the dry state. As desiccation-sensitive seeds do not go through a drying phase, these

mechanisms may not be activated or may often be incomplete, which leads to an insufficient accumulation of protective molecules and increases susceptibility to desiccation injuries (Marques et al., 2019).

The seeds of *C. aschersoniana* are sensitive to desiccation. In the study region, flowering begins in August, with fruit ripening, and seeds are dispersed at the end of the rainy season (February-April; Carvalho, 2006). This is an unusual situation because after dispersal, the seeds are exposed for a long time to water-deficit conditions (March) in the soil, until September, the beginning of the next rainy season (Tonetti et al., 2016). To cope with these adverse conditions, species have developed several mechanisms that regulate their reproductive strategy, which may include anatomical adaptations, as investigated by Vaz et al. (2016) in *Swartzia langsdorffii* seeds. The objective of this study was to evaluate the behavior of *C. aschersoniana* seeds during drying and associate it to the physiological and biochemical modifications.

Material and methods

Plant material characterization

Ripe, yellow *C. aschersoniana* fruits was harvested from five trees - with a good phytosanitary status and without apparent damage - located on the campus of the Federal University of Lavras (21°13' S, 44°58' W) in the region of Lavras, state of Minas Gerais, Brazil, over 3 years. The processing was performed by rubbing the fruit over a sieve under running water until the pulp was completely removed; the dispersion structures formed by the seed within the woody endocarp were considered the "seeds." The seeds were spread in a single layer in open air until the loss of surface water; these specimens were called freshly harvested seeds. Samples of these seeds were taken for initial characterization of the lot through moisture and germination tests (for the three collections), histological analysis (scanning electron microscopy and light microscopy), and extraction and quantification of lipids and sugars (only for seeds harvested in the last year).

Drying

After initial characterization, the remaining seeds were placed in plastic boxes containing silica gel at the bottom inside an air-conditioned room (20°C). The silica gel was changed whenever the color of the humidity indicator (blue) became pale to maintain conditions of 20°C and a relative humidity of 13.5-40% inside the boxes. After reaching a target water content of 40, 35, 30, 25, and 20%, seed samples were taken, and all tests performed with the freshly harvested seeds were repeated.

Tests

Water content determination

The seed water content was determined by cutting the seeds and placing them in an oven at 101-105°C for 17 hours; four replicates of five seeds each were analyzed (Brasil, 2009). The results are expressed as percentages on a wet weight basis.

Germination test

The germination test was performed with four replicates of 25 seeds previously washed in 1% sodium hypochlorite for 10 minutes. The germination test was performed in plastic trays (27 × 40 cm) containing autoclaved sand in a Mangelsdorf-type germinator set at 25°C with continuous light. The seeds that produced normal seedlings (those that presented a well-structured primary root and a shoot at least 2 cm long formed by epicotyl and the first visible pair of leaves) were considered germinated seeds, and the test ended when seedling formation stabilized. At the end of the test, the seeds that did not germinate were cut, immersed in 0.5% 2,3,5 triphenyltetrazoliun chloride for 24 hours at 25°C, and subsequently evaluated. The seeds that were pinkish/red, which indicates respiratory activity, were considered viable and scored as dormant seeds. Those that did not develop this color were classified as dead.

Ultrastructural analysis

The ultrastructure of the samples was analyzed by scanning electron microscopy at the Laboratory of Electron Microscopy of the Federal University of Lavras (UFLA), according to the following protocol. The samples comprised five seeds that were cut cross-sectionally in the region of the cotyledons, close to the embryonic axis, and fixed in modified Karnovsky solution (2.5% glutaraldehyde and 2.5% formaldehyde in

0.05 M sodium cacodylate buffer, pH 7.2, 0.001 M $CaCl_2$) for at least 24 hours. The specimens were cut with a razor blade to form the observation surface; then, they were washed in distilled water and dehydrated in a series of increasing concentrations of acetone (25, 50, 75, 90, and 100%) for 10 minutes at each concentration. The samples were dehydrated three times in 100% acetone and transferred to a Bal-Tec critical point drier for evaporation of acetone without loss of tissue conformation.

The dried samples were glued onto carbon tape stubs covered with aluminum foil, subjected to metallization in an SCB 050 sputter coater, and examined with a scanning electron microscope (LEO EVO 40 XVP). The images were obtained in the transition region from the embryonic axis to the cotyledons and were recorded at an acceleration voltage of 9 V. The characteristics found at each point in all five analyzed samples were considered standard.

Histochemical tests

Analyses were carried out according to the protocols proposed by Ventrella et al. (2013), and described below, performed in five seeds manually cut cross-sectionally with a razor blade. The sections of each seed were kept separate; the samples were stored in distilled water and subsequently transferred to specific solutions according to the molecule to be identified, as described below.

For starch localization, the sections were placed in Lugol solution (0.02% free iodine) for 30 seconds and transferred directly to a slide containing glycerin water; then, they were covered with a coverslip and analyzed under a microscope. Areas rich in starch were identified by a purple/black color in the images.

For lipid localization, the sections were incubated in a Sudan solution (3% in 70% ethanol) for 10 minutes. Then, the sections were washed in 70% ethyl alcohol, transferred to a slide containing glycerin water, covered with a coverslip, and observed under a microscope. Samples from different seeds were placed on separate slides. Areas rich in lipids were identified by an orange color.

The analyses were performed with an Olympus BX 51 microscope, and the images were recorded with an Olympus C 5060-ADU camera using the Image-Pro Plus 5.1 image analysis system. As a control, sections without any solution treatment were analyzed to verify the appearance of the tissues.

Quantification of sugars and lipids

For the extraction and quantification of lipids and sugars, three replicates with 20 seeds per replicate were used. The seeds were dried in an oven at 40°C for 48 hours, macerated, and frozen (-20°C) until the time of testing. The samples were placed in filter paper cartridges, weighed, and transferred to a Soxhlet extractor for 24 hours. The extraction was performed cold with petroleum ether, and the result is expressed as the percentage of lipids (oils) extracted (Silva, 1990).

The lipid-free extract was frozen and sent to Bio Agro, located at the Federal University of Viçosa (UFV). Glucose, fructose, raffinose, and stachyose were quantified in a Shimadzu high-performance liquid chromatography (HPLC) in a Dextrapak cartridge column (8 x 100 mm; Waters Corp, Milford, MA) and analyzed in a Waters 410 refraction index detector, sensitivity 32 x 20, positive polarity, in 20-µL aliquots according to Bernal-Lugo and Leopold (1992).

Data analysis

All experiments were performed in accordance with a completely randomized design (CRD). The data were analyzed by analysis of variance (ANOVA). When there were statistical differences between treatments, they were compared with Tukey's test at a 5% probability level.

Results and discussion

Under the drying conditions mentioned, it took 38 days for the seeds from years 1 and 3 to reach close to a 20% water content, and around 50 days for the seeds from year 2 to reach this water content (Figure 1).

In general, freshly harvested seeds with a water content above 45% showed low germination values, ranging from 6 to 28%. Drying to around a 30% water content did not negatively affect viability (Table 1); furthermore, most viable seeds were still dormant after 120 days under the germination conditions (Figure 2A, C, and E). The germination values for the seeds from years 2 and 3 increased as drying progressed, up to the limit of a 28-30% water content (Table 1). Simultaneously, the mean germination time (MGT) decreased (Table 1) and the germination speed increased, as evidenced by the slopes of the cumulative germination

curves (Figure 2B, D, and F). The percentage of viable seeds (germinated + dormant) remained constant during desiccation up to the limit of a 28-30% water content, while the percentage of dormant seeds tended to decrease, especially for the seeds from year 3 (Table 1). With further drying to a water content of less than 28-30%, the viability (considering germinated and dormant seeds) decreased rapidly; it was close to zero when the water content was 15-21%.

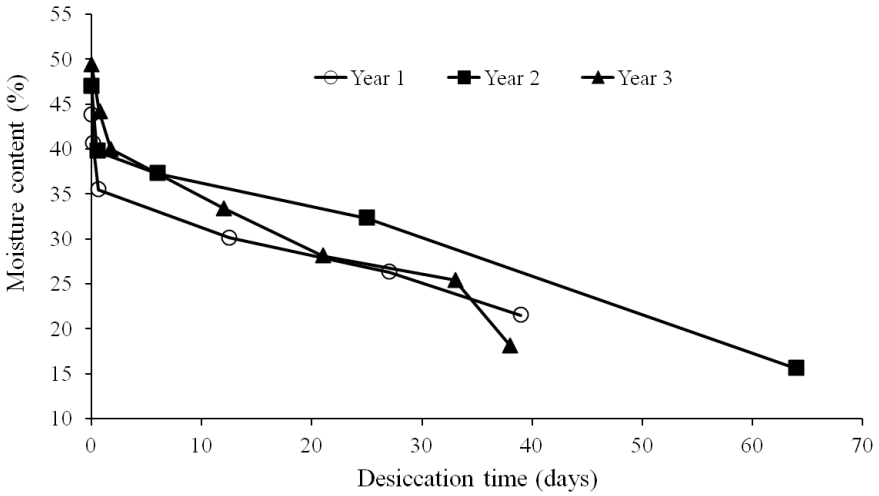

Figure 1. Drying curves of *Cryptocarya aschersoniana* seeds in silica gel collected in three years.

Table 1. The mean number of germinated, dormant, viable, and dead *Cryptocarya aschersoniana* seeds, and the mean germination time (MGT), after 120 days under the germination test conditions.

Year	Water content (%)	Seed condition after 120 days (%)								MGT (days)	
		Germinated (G)		Dormant (D)		Viable (G+D)		Dead			
1	43.8	6	a	48	a	62	a	18	b	146	a
	40.6	11	a	48	a	59	a	21	b	152	a
	35.5	7	a	59	a	66	a	14	b	126	a
	30.1	27	a	33	a	60	a	20	b	67	b
	26.3	22	a	0	b	22	b	58	a	44	b
	21.5	5	a	0	b	5	b	75	a	56	b
2	46.8	26	ab	51	ab	77	a	23	b	116	ab
	39.9	34	ab	41	b	75	a	25	b	77	b
	37.4	16	bc	66	a	82	a	17	b	152	a
	32.3	37	a	40	b	77	a	23	b	79	b
	15.6	0	c	0	c	0	b	100	a	0	c
3	49.4	28	b	27	a	55	a	18	b	64	a
	44.2	17	b	27	a	44	a	21	b	69	a
	40.0	29	b	14	b	43	a	14	b	67	a
	33.4	46	a	12	b	58	a	20	b	49	b
	28.1	59	a	3	bc	62	a	58	a	50	b
	25.4	3	c	14	b	17	b	75	a	46	b
	18.1	3	c	0	c	3	b	77	ab	47	c

Means followed by the same letter in the same column and same year do not differ significantly among themselves based on Tukey's test at a 5% probability level.

These observations indicate that within a defined water content range, dormancy is overcome to a certain degree (Figure 2A, 2C, and 2E). The concomitant occurrence of desiccation sensitivity and dormancy in seeds is a rare phenomenon: It is observed in less than 1.0% of the seed-plant flora (Pritchard et al., 2022; Wyse & Dickie, 2017), mainly in climax species from temperate regions (Jaganathan, 2021) and from subtropical regions at relatively high altitudes (Obroucheva et al., 2016). Seeds with such characteristics have already been reported in the species under study, in other species of the same family (Jaganathan et al., 2019; Tonetti et al., 2021; Vicente et al., 2016), and in other families (Viana et al., 2020; Wyse & Dickie, 2017). Considering that the seeds of *C. aschersoniana* are sensitive to desiccation and are dispersed at the end of the rainy season, the presence of dormancy is an important ecological strategy, as it guarantees their permanence in the soil seed bank until the beginning of the next rainy season, as shown by Tonetti et al. (2016).

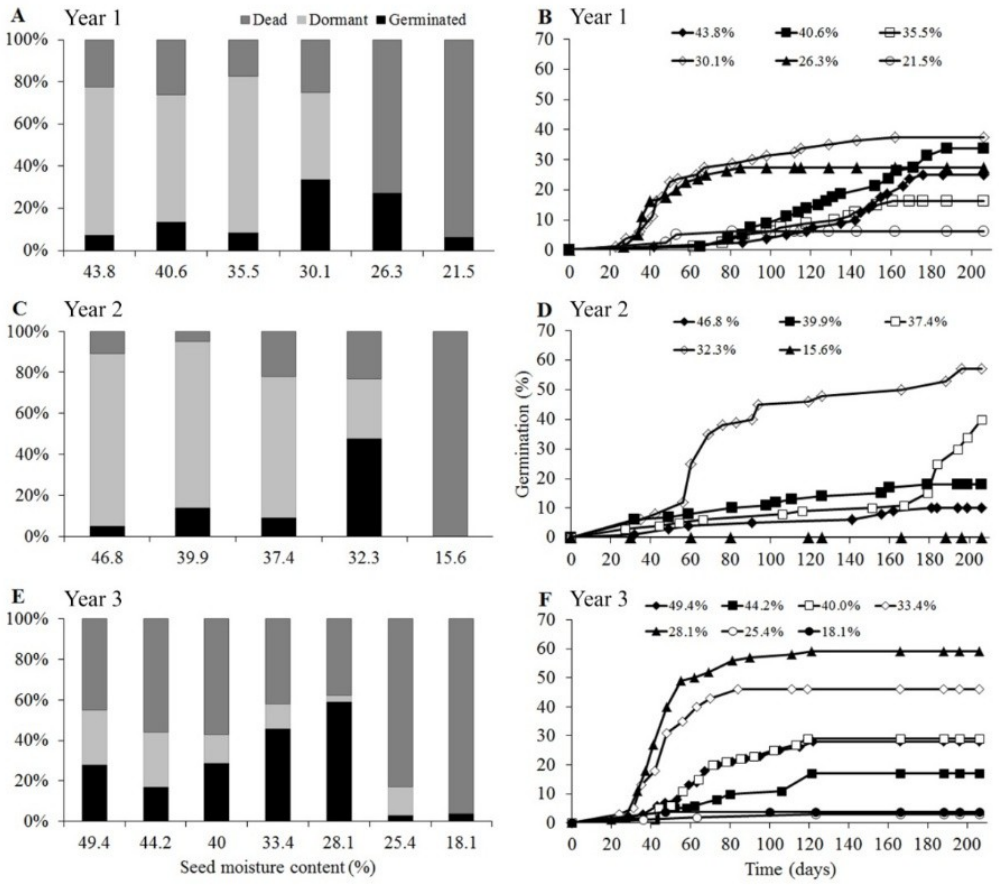

Figure 2. Behavior of *Cryptocarya aschersoniana* seeds harvested in three years, according to the degree of moisture. A, C, and E: The final percentages obtained after 120 days under the germination conditions. B, D, and F: The percentage of cumulative germination.

Germination was higher and faster for the seeds collected in year 3 (Figure 2; Table 1), with a lower percentage of dormant seeds at the end of the germination tests. In terms of variation in climatological parameters between the years of collection, precipitation stands out: It was higher during the seed formation period of year 3 (Figure 3). The seeds collected in that year showed faster germination (Figure 2; Table 1), which suggests that seeds formed in wetter periods develop dormancy that is not as deep.

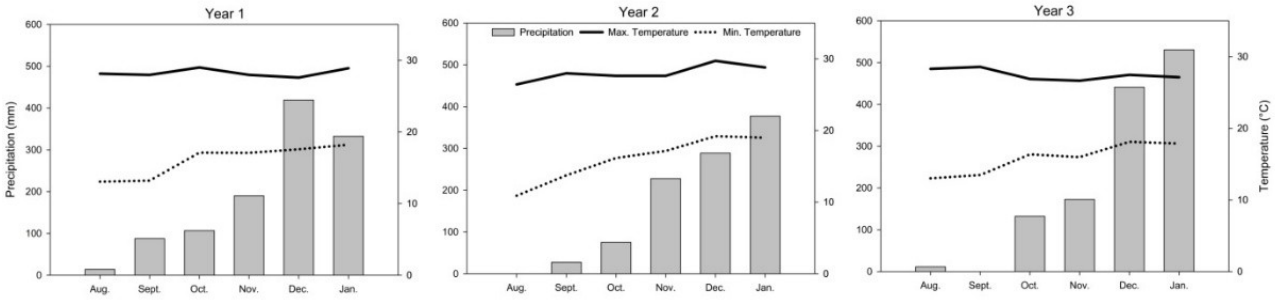

Figure 3. Precipitation and maximum and minimum temperatures during the period of seed formation of *Cryptocarya aschersoniana* for the three years of collection.

These results agree with those previously obtained for the same species by Muxfeldt et al. (2012). Research on seeds of several species of the Lauraceae family, including the present study, has classified them as desiccation sensitive (Jaganathan et al., 2019; Muxfeldt et al., 2012; Thapliyal et al., 2004). Species whose seeds are sensitive to desiccation are typical of tropical regions, but they also occur in temperate regions or regions with higher altitudes where dispersal occurs in autumn-winter (Obroucheva et al., 2016). In these cases, there is also less water loss at low temperatures, which reduces the risk of death by desiccation. Seeds from temperate regions are generally more tolerant to desiccation and have greater longevity than those of tropical origin (Wyse & Dickie, 2017).

Based on the scanning electron micrographs, the seeds have cells filled with starch granules (Figure 4). According to Obroucheva et al. (2016), for most desiccation-sensitive seeds, the majority of the reserve is composed of carbohydrates. Only dead seeds displayed apparent cell damage (Figure 4B). At this point, the cells lost their normal configuration and exhibited folds in the cell walls, as well as a reduction in cell content (Figure 4B). According to Zhang et al. (2023), only after reaching the critical water content does the tissue begin to show apparent cell damage.

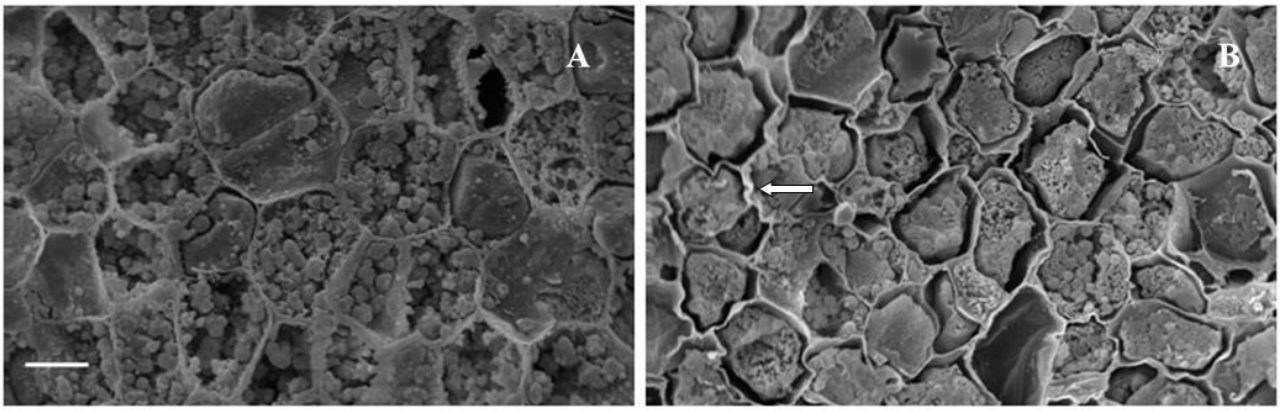

Figure 4. Scanning electron micrographs of cotyledons of *Cryptocarya aschersoniana* seeds. A: Newly harvested seed with a 49.4% water content; B: dried seed with an 18.4% water content (scale bar = 20 µm). The arrow indicates cell wall folding.

During drying, there was an increase in the sucrose and glucose contents, while the raffinose content remained stable and close to what has been found in desiccation-sensitive seeds of other species (Hell et al., 2019) (Figure 5). Desiccation-sensitive seeds of some species display mechanisms of desiccation tolerance, such as cell vacuolization, accumulation of protective molecules, activation of antioxidant systems, and repair mechanisms during rehydration (Tsou et al., 2022). However, the presence of an isolated mechanism is not enough for the seeds to develop desiccation tolerance.

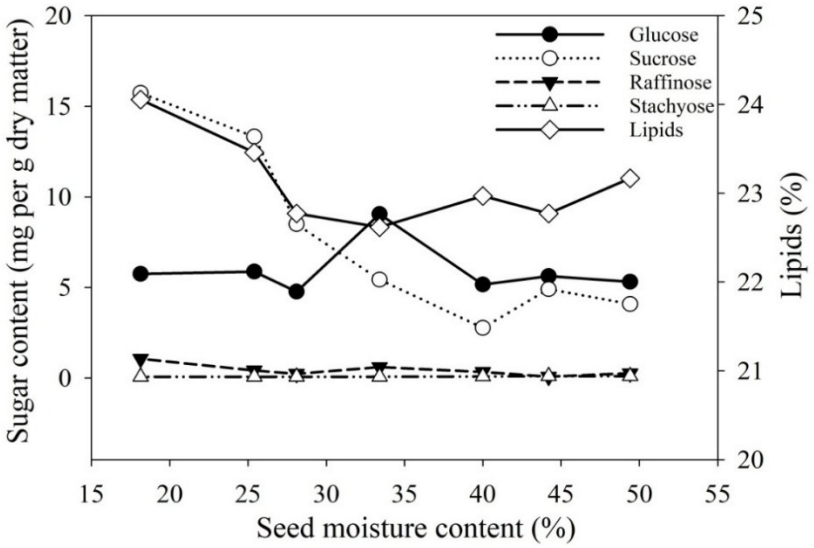

Figure 5. Quantification of lipids and sugars in *Cryptocarya aschersoniana* seeds with different water contents.

According to Obroucheva et al. (2016), it is common to find high amounts of sucrose and raffinose in the cotyledons of desiccation-sensitive seeds. Sucrose is related to the availability of reserves and is produced by plants during photosynthesis. As photosynthesis does not occur in seeds, the increase in the amount of sucrose may have originated from the beta oxidation of lipids (glycogenic pathway) or the degradation of starch, which is very abundant in cells, as evidenced by the reaction with Lugol solution (Figure 6A and B).

Sucrose is a carbohydrate used by cells as an energy source for immediate consumption and is quite common in seeds that are tolerant to desiccation. When combined with raffinose, sucrose also appears to promote membrane protection or the formation of the vitreous state of cells, which contributes to a reduction

in chemical reactions, thus reducing the risk of deleterious reactions in seeds (Marcos Filho, 2016). These processes can occur in desiccation-sensitive seeds (Marques et al., 2019), in which some protection mechanisms are present but are not sufficient to protect them from the damage caused by desiccation, as evidenced by Barbedo (2018). It is also possible that in recalcitrant seeds, there is a silencing of desiccation tolerance inducers, despite the presence of some protective mechanisms.

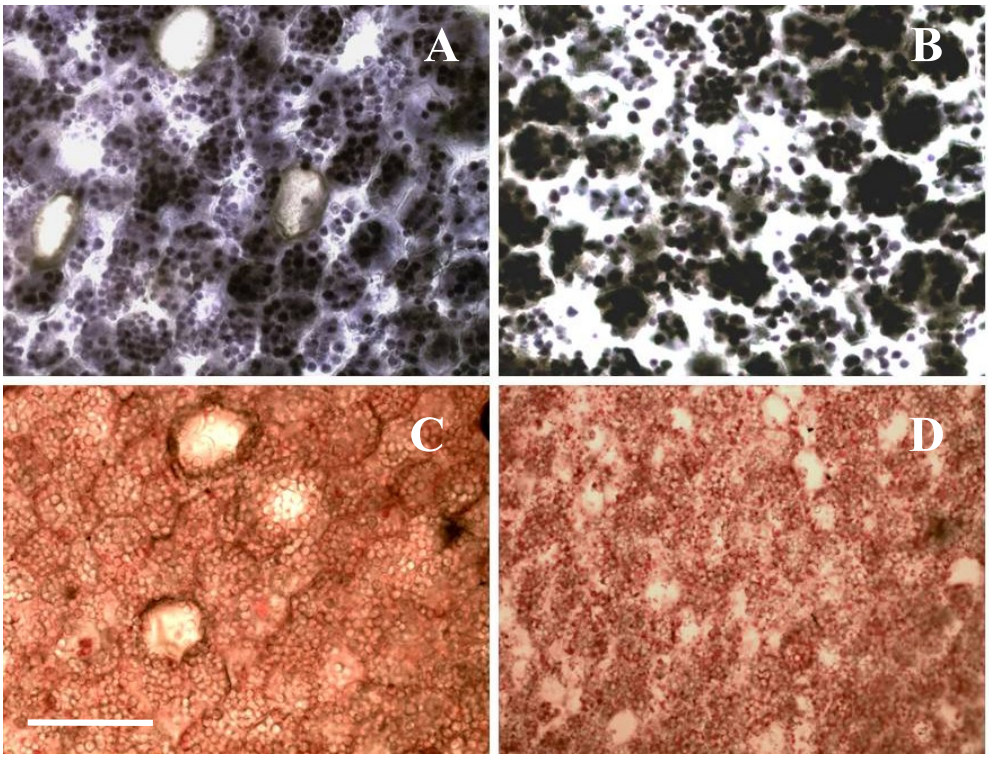

Figure 6. Histochemical tests of *Cryptocarya aschersoniana* seeds. A and B: Reaction with Lugol solution of seeds with a 49.4% and an 18.4% water content, respectively. C and D: Reaction with Sudan solution of seeds with a 49.4% and an 18.4% water content, respectively. Scale bar = 100 µm.

As the seeds dried, there was no significant change in the amount of lipids (Figure 5), but there were changes in the way lipids were found in the cells, as evidenced by the reaction with the Sudan solution (Figure 6C and 6D). This finding may indicate the melting of lipid bodies induced by drying (Silva & Ferraz, 2015) or some type of lipid oxidation with the release of peroxides that attack membranes (rich in unsaturated lipids), which leads to a loss of seed viability (Chandra & Keshavkant, 2018).

Under the experimental conditions, the drying time for the seeds collected in year 3 lasted up to 38 days (Figure 1), and the losses in seed quality during this time were a function of water loss and the time in which the seeds were exposed to the drying conditions. There was a decrease in seed viability over time at all water contents after drying, denoted by an increase in the percentage of dead seeds (Table 2). The highest percentages of dead seeds were observed after 38 days whether the seeds were dried to an 18.1% water content or maintained at the original water content. Comin et al. (2014) reported similar results in their study of *Eugenia uniflora* seeds.

Table 2. Mean percentage of germinated, dormant, and dead *Cryptocarya aschersoniana* seeds after 120 days under the germination test conditions.

Condition	Germinated seeds	Dormant seeds	Dead seeds
		%	
Freshly harvested	28 b	27 c	45 a
Moist (21 days)	26 b	16 bc	58 ab
Moist (38 days)	20 bc	12 bc	68 b
Dried for 21 days	59 a	3 ab	38 a
Dried for 38 days	4 c	0 a	96 c
CV (%)	27.7	15.6	55.9

A mean followed by the same letter in the same column does not differ significantly by Tukey's test at 5%.

Conclusion

C. aschersoniana seeds are sensitive to desiccation; the critical water content is approximately 30%, and the lethal water content is approximately 18%. These seeds are dispersed in a dormant state, and this dormancy can be partially overcome by partial drying. Some desiccation-protection systems, such as increased sucrose concentrations, are present in *C. aschersoniana* seeds, but they are not sufficient to maintain seed viability during drying. Ultrastructural changes were observed only when the seeds were completely unviable.

Data availability

Not applicable.

Acknowledgements

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