Ciência Florestal

ISSN 1980-5098
Open access

Ci. Fl., Santa Maria, v. 32, n. 2, p. 979-995, Apr./June 2022 • https://doi.org/10.5902/1980509825216
Submitted: 14th/12/2016 • Approved: 23rd/09/2021 • Published: 24th/06/2022

Nota Técnica

Method of opening the fruits, biometry and anatomical description of embryonic development in pau-branco (*Cordia oncocalyx* Allemão) seeds

Método para abertura dos frutos, biometria e descrição anatômica do desenvolvimento embriogênico em sementes de pau-branco (*Cordia oncocalyx* Allemão)

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RESUMO

A exploração do pau-branco (*Cordia oncocalyx* Allemão), espécie de grande potencial madeireiro, é feita por extrativismo, sendo depredatória e sem garantia de oferta de matéria-prima suficiente e constante aos setores demandantes. A propagação da espécie em viveiros a partir de frutos e sementes poderia oferecer uma solução para esse problema. Os frutos obtidos de plantas nativas apresentam, no entanto, tamanhos diferentes e distintos graus de maturação, dificultando a germinação e o sucesso da propagação. Para estabelecer-se um protocolo de propagação para o pau-branco, partindo de um resgate com sucesso de sementes com embrião zigótico maduro, o conhecimento da anatomia da embriogênese zigótica final (na fase do fruto) é de grande importância. O presente trabalho objetivou determinar um método de abertura do fruto e estudar aspectos relacionados à microestrutura e anatomia do embrião da semente de pau-branco, inéditos até então, com o intuito de se determinar em qual fase de desenvolvimento torna-se apropriado o resgate de sementes para cultivo visando à obtenção de mudas. Frutos maduros e em diferentes estádios de desenvolvimento de pau-branco foram coletados para serem submetidos à investigação microscópica ou para a determinação da biometria. Para a descrição anatômica dos frutos e das sementes, seções transversais e longitudinais foram obtidas e processadas para microscopia óptica e eletrônica de varredura. Constatou-se que o fruto possui exocarpo e o mesocarpo, sendo este, dividido em mesocarpo sub-exocárpico e mesocarpo esclerenquimático, sendo esse último, o que confere dureza e rigidez ao fruto. O embrião torna-se visível em frutos com comprimento em torno de 1,5 cm, encontrando-se totalmente desenvolvido somente a partir de 2,0 cm, sendo este tamanho o recomendado para realizar sua excisão. A prensa hidráulica constitui-se em um método eficaz de abertura dos frutos. Conclui-se ainda que o embrião da semente de pau-branco apresenta reserva protéica.

Palavras-chave: Caatinga; Extrativismo; Pau-branco; Propagação; Anatomia

ABSTRACT

The tree species *Pau-branco*, *Cordia oncocalyx* Allemão has great economic potential, especially for logging, but its extraction is uncontrolled and can cause depredation. This type of harvest does not guarantee sufficient raw material to supply the demanding sectors such as furniture industries. The propagation of the species in plant nurseries starting from fruits and seeds could offer a solution to this problem. However, the fruits obtained from native plants comprise different sizes and different degrees of ripeness, making germination and propagation processes a difficult task. Knowledge of the final zygotic embryogenesis (fruit phase) anatomy is very important to establishing a propagation protocol for this species, especially to achieve a successful seed rescue of those containing mature zygotic embryos. The objectives of the present work was to establish a method to open the fruits and to study the microstructure and anatomy of the embryo in seeds of *pau-branco*, never shown before, with the goal of determining in which phase of development seed rescue is most appropriate for subsequent planting and seedling propagation. Mature fruits of *pau-branco* at different developmental stages were collected and subjected to microscopic investigation or biometrics determination. Transverse and longitudinal sections were obtained and processed for optical and scanning electron microscopy for the anatomical description of fruits and seeds. It was observed that the fruit has an exocarp and mesocarp. The mesocarp is divided into a sub exocarp-like mesocarp and a sclerenchymatous mesocarp, the latter of which gives hardness and rigidity to the fruit. The embryo, generally approximately 1.5 cm, is visible inside the fruits. When the embryo reaches 2.0 cm, it is considered fully developed, and it is recommend that excision can take place at this stage. the use of a hydraulic press was the most viable method for the extraction of seeds from the fruits. Furthermore, it was also concluded that the embryo of *pau-branco* seeds contain protein reserves.

Keywords: Caatinga; Wood extraction; *Pau-branco*; Propagation; Anatomy

1 INTRODUCTION

Data from the Brazilian Ministry of Environment (Ministério do Meio Ambiente - MMA) indicate that 69% (374.6 million hectares) of the Brazilian forest cover have potential productivity for the wood sector, including several potential species that have not been fully investigated or exploited (MMA, 2012). According to the Brazilian Association of Mechanically Processed Wood (Associação Brasileira da Indústria de Madeira Processada Mecanicamente – ABIMCI, 2014), round wood production has been increasing since 2009, reaching approximately 150 million m^3 in 2012. Exported forest products, only from the area known as the Legal Amazon, reached U$ 360 million in 2013. Native tree species present in the Caatinga biome, in addition to having favourable characteristics that allow their adaptation to specific environmental conditions, also have multi-use potential, such as logging, which is a viable activity of significant importance for the Brazilian economy.

Locally known as *pau-branco*, *Cordia oncocalyx* Allemão belongs to the Boraginaceae family and is characterized as a tree species measuring between 8 and 10 metres in height, with a leafy canopy, deciduous foliage, and anemochorous dispersal. This species is considered endemic of the Caatinga (BRITO; ARAÚJO, 2009). In addition to occurring very frequently in the state of Ceará, it is also found in some municipalities of the states of Rio Grande do Norte, Paraíba, Pernambuco, Pará, Minas Gerais and Bahia (ANDRADE-LIMA, 1989; CARVALHO, 2008).

Structural studies of the forest stratum of Caatinga have shown that this species has a high predominance and density, reaching forest composition values up to 50% (CAMPANHA *et al*., 2008). Several applications for the use of *pau-branco* wood can lead to extractive harvest, which, by itself, causes depredation and does not guarantee sufficient raw material supply to the demanding sectors. In 1862, Allemão and Allemão described this species as *Cordia oncocalyx*. In 1875, Miers, who was unaware of Allemão's description, described this same species as *Auxemma gardneriana*. Baillon (1890) recognized that these two descriptions referred to the same species and proposed to combine both names to *Auxemma oncocalyx* (Allemão) Baill. However, some authors prefer to adopt the first nomenclature and refer to this species as *Cordia oncocalyx* Allemão (Cordiaceae, Boraginales) (GOTTSCHLING; MILLER, 2006).

Cordia oncocalyx has a dry indehiscent fruit type that has a brownish colour, is 2 cm in length, and generally contains four seeds, from which only two are normal (MAIA, 2004). Although the seeds have a longevity greater than one year, the percent germination is low (18%) due to depredation by fungi that can lead to seedling death soon after germination. The wood is hard, heavy, and easy to handle, has no odour, and has a dark purplish-brown colour (MAIA, 2004).

According to Carvalho (2008), this species has high timber value with multiple regional uses, including wood work projects (fine furniture), planks, beams, stakes, fence posts, joists, chests, grain wood boxes, lath, as well as heavy building. This tree has also other important uses for farming, including use as forage crop, and is a medicinal, ornamental, and agroforestry species. Exploitation is conducted by extractivism, requiring support and presenting a high demand for the development of scientific studies, especially regarding in vitro propagation, to resolve issues related to its low germination rate, pericarp physical dormancy, high levels of predation in soil seed banks, and low seedling emergence. The scarce number of studies, along with improper management and intensive exploitation, might result in their reversible degradation of this plant community (RODAL; SAMPAIO, 2002).

In ecological terms, this species has a desirable characteristic in that it flowers from March to August followed by the production of a large number of fruits (CARVALHO, 2006). However, studies on the dynamics of the soil seed bank since 2002 have shown that this plant community has a low representation in the soil, suggesting a high predation level (BRITO; ARAÚJO, 2009). Guimarães *et al*. (2013) emphasized that *Cordia oncocalyx* requires an urgent conservation programme and is at risk of being classified as endangered in the near future.

In this context, seedling production could become an important strategy to secure the supply of *pau-branco* for the growing and promising furniture market in Ceará and help minimize extractive activities. However, germination tests have presented low success rates mainly due to the challenge in breaking the tegument and the pericarp. According to Lorenzi (1992), *Cordia oncocalyx* seeds in intact fruits can take from 70 to 120 days to germinate. Silveira (2002) reported the slow natural germination in *Cordia* spp., from the whole fruit, without removing the seeds, lasting from 100 days to one year. Silveira *et al*. (2005) attempted to stimulate the germination of *Cordia oncocalyx* seeds by breaking the pericarp with a hammer to remove the seeds followed by cultivation in vermiculite. The germination rate was only 18% due to seed deformation problems caused by the hammer, fungal deterioration of the emerging seedlings, and heterogeneous seed viability. Nursery workers also reported that the process of obtaining seedlings from whole seeds collected from the soil was very slow, taking several months or years to obtain a few seedlings. The attempt to rescue the seeds and proceed with germination would be an adequate solution to minimize or resolve the slow regeneration issue for this species.

The heterogeneous seed viability from collected fruits, related to the size (morphometry) and development of the fruit, is a problem that can be investigated using microscopic evaluation of the final zygotic embryogenesis of the seed. Determination of the fruit's developmental stages, with seeds showing well-developed and viable embryos, is fundamental for the selection of fruits for propagation programmes.

Knowledge of the final zygotic embryogenesis anatomy (in the fruit phase) is very important to establishing an adequate propagation protocol for *pau-branco* plants, which can begin with the successful rescue of seeds containing a mature zygotic embryo. Therefore, the main objectives of this present work were to establish a technique to open the fruits, minimizing the loss of seeds and to study the aspects related to the *pau-branco* microstructure and anatomy of the seed embryo, aiming to determine the most appropriate phase of development to perform seed rescue for planting while targeting seedling production.

2 MATERIAL AND METHODS

2.1 Collection and morphological characterization of fruits

Pau-branco fruits in different stages of development were collected from a natural population located in the city of Fortaleza, Ceará, Brazil (03° 46'S, 38° 36'W) (Figure 1). Collection was carried out from fifteen adjacent individuals, located 3 to 5 m apart from each other and were conducted in March 2013, May 2014, and March 2015. The fruits were taken to the laboratory and then subjected to microscopic investigation or biometrics determination. Biometry was performed using a total of 420 mature brown fruits, which were characterized regarding the fruit length (with and without the calix that involves the fruit), transverse diameter, and number of normal and abnormal seeds per fruit according to the guidelines proposed by Carvalho and Nascimento (2008). Nucleus characterization was conducted using histology techniques for recent fruits belonging to different maturation stages. Groups of thirty fruits (without the calix) were subjected to opening methods trials. Different techniques were employed to open the fruits, including tools, liquid nitrogen, immersion in acids and use of a hydraulic press. The technique that provided the greater number of unbroken seeds was considered the most efficient one.

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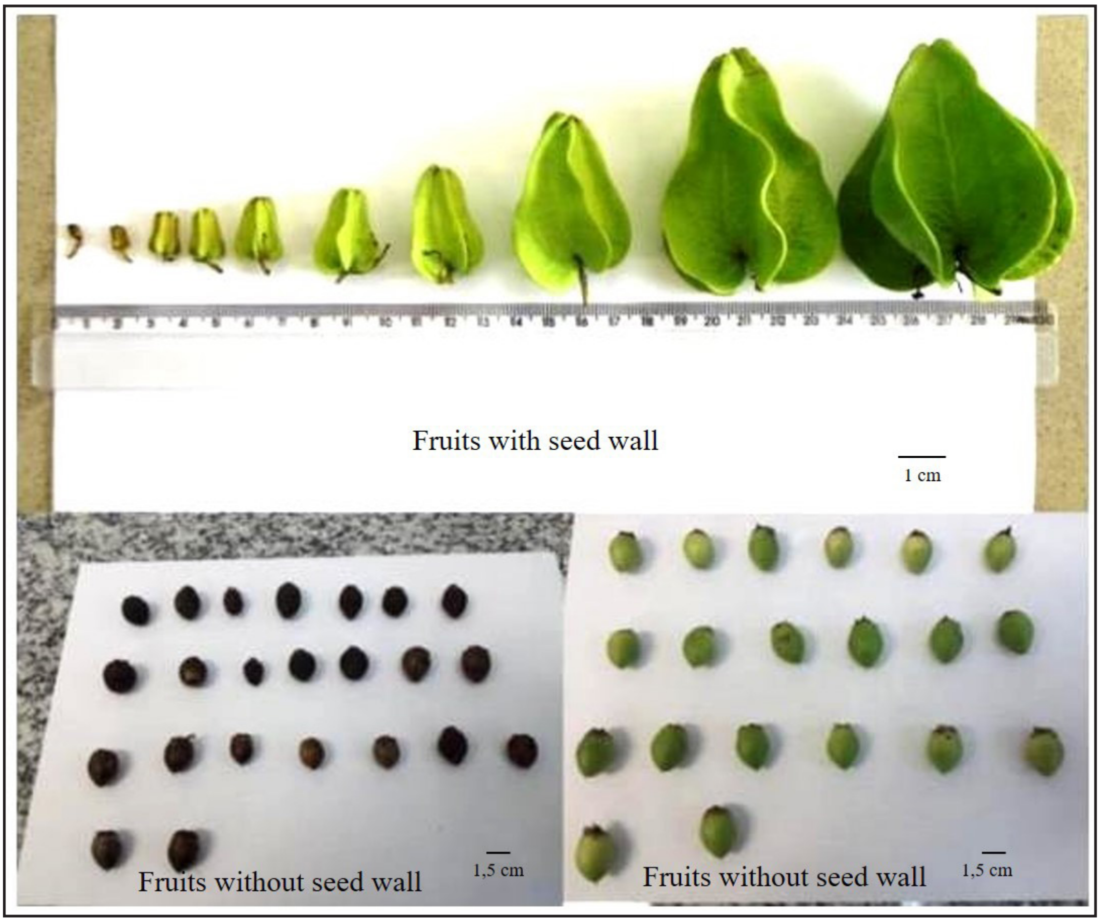
Figure 1 – Examples of fruits collected at different maturity stages, showing their different shapes



Source: Authors (2020)

2.2 Anatomical description of fruits and seeds using scanning electron microscopy (SEM)

For the anatomical description of fruits and seeds using scanning electron microscopy (SEM), transverse and longitudinal sections of the fruits were obtained and pre-fixed in a glutaraldehyde and paraformaldehyde solution. The samples were post-fixed in a 1% OsO_4 (osmium tetroxide) solution with 0.1M pH 7.2 cacodylate buffer. After fixation, the samples were dehydrated in a series of acetone solutions with increasing concentrations (30, 50, 70, 90, and 100%) and dried to the critical point using a drier machine (Emitech model K 850). Subsequently, the samples were placed on stubs and covered with a thin layer of platinum using a sputter-coating device (Emitech model K 550). The samples were then examined using a Zeiss DSM940A scanning electron microscope. Images were captured and digitalized using secondary electron emission under 15 kV acceleration voltage. The transverse sections of fruits were visualized under different magnifications and in different areas.

2.3 Anatomical description of the final zygotic embryogenesis

For the histological studies, fruits with different development sizes were fixed in 1% glutaraldehyde and 4% formaldehyde with a 0.2 M pH 7.2 phosphate buffer for a period of 24 hours. After fixation, the samples were progressively dehydrated in an ethanol series ranging from 10% to 95% and then infiltrated and embedded in Leica Historesin. Longitudinal and transverse sections (5 mm thick) were obtained using a rotary microtome. Section coloration was conducted using 0.05% toluidine blue for 1 minute. The images were obtained using an Axioscope coupled microscope with a photographic camera.

3 RESULTS AND DISCUSSION

Fruit characterization and biometrics provided information to determine the variability among species of a certain population. A correlation between biometrics data and the presence of seeds can provide supporting details and can also aid in the selection of seeds for genetic improvement programmes or propagation protocols aimed at seedling production. The fruits showed an elliptical shape and either a green colour when immature or a brown colour when mature (Figure 1).

Table 1 – Descriptive statistical analysis of data obtained for length with and without the seed wall (mm) as well as the transverse diameter of the *pau-branco* fruits

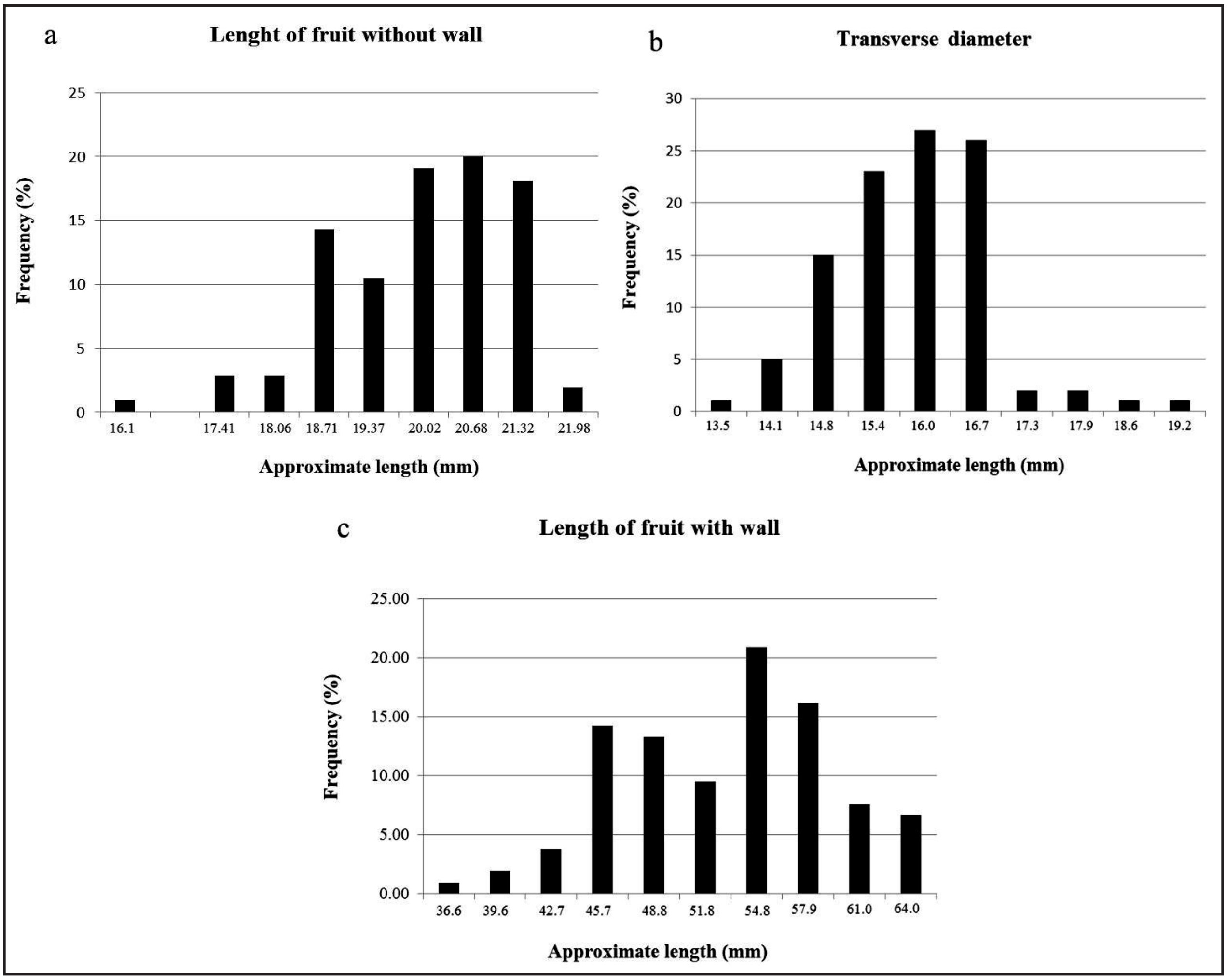
	Length of fruit with seed wall(mm)	**Length of fruit without seed wall(mm)**	**Transverse diameter (mm)**
Mean	52.25	19.93	15.64
Standard error	0.65	0.13	0.10
Median	52.75	20	15.7
Standard deviation	6.66	1.31	1.06
Sample variance	44.40	1.72	1.11
Minimum	36.55	16.1	13.5
Maximum	67.05	22.63	19.85

Source: Authors (2020)

A high degree of variation between the minimum and maximum values of the measurements of each variable was observed (Table 1). It was also evident that the wall is much bigger than the fruit itself and that the transverse diameter is slightly smaller than the fruit length, conferring an elliptical shape instead of spherical aspect to the fruit. The transverse diameter also presented a lower value of sample variation indicating that this is a more constant feature within the evaluated fruits.

The histograms presented in Figure 2 show that 21% of fruits with the seed wall had a length of approximately 54.85 mm, 20% of the fruits without the seed wall had a length of approximately 21 mm, and 27% of the fruits had a transverse diameter of approximately 16 mm. Silveira *et al*. (2005) found similar values, with an average length of the fruit without the seed wall of approximately 22 mm and diameter of approximately 17 mm. These results suggest that there is little difference among fruit measurements of different individuals during distinct collection times. The differences observed in the length could have occurred due to genetic variability present among the studied trees.

Figure 2 – Frequency histograms of the biometric measurements of *pau-branco* fruit. (a) Fruit length with the seed wall. (b) Fruit length without the seed wall. (c) Transverse diameter of fruit without the seed wall



Source: Authors (2020)

The frequency of seeds developed inside the collected fruits was also evaluated, showing that 35% of the fruits did not have any developed seeds, 40% had only one developed seed, 13% had two developed seeds, and only 1.5% had 3 developed seeds inside the fruit. Therefore, it was necessary to open the fruit in a transverse manner. Figure 3 shows a transverse section of a fruit containing 3 developed and 1 undeveloped seeds. Silveira *et al*. (2005) detected partial seed loss in 26% of the studied fruits, mainly due to predation caused by insects.

Figure 3 – Transverse section of a *pau-branco* fruit showing 3 developed and 1 undeveloped seeds

Source: Authors (2020)

The opening of fruits for the collection and determination of viable seeds was very challenging due to the extreme rigidity of the fruit's mesocarp, given the sclerification processes. Attempts to open the fruit were conducted using tools such as a hacksaw and pliers and were proven unsuccessful due to the hardness of the fruit and the potential for accidents. Freezing in liquid nitrogen was also performed aimed at cracking the fruit; however, the fruit remained intact and immune to this process. The fruits were also immersed in sulfuric and hydrochloric acid, which allowed the removal of only the exocarp and collenchymatous mesocarp, with the remainder of the fruit remaining intact and closed.

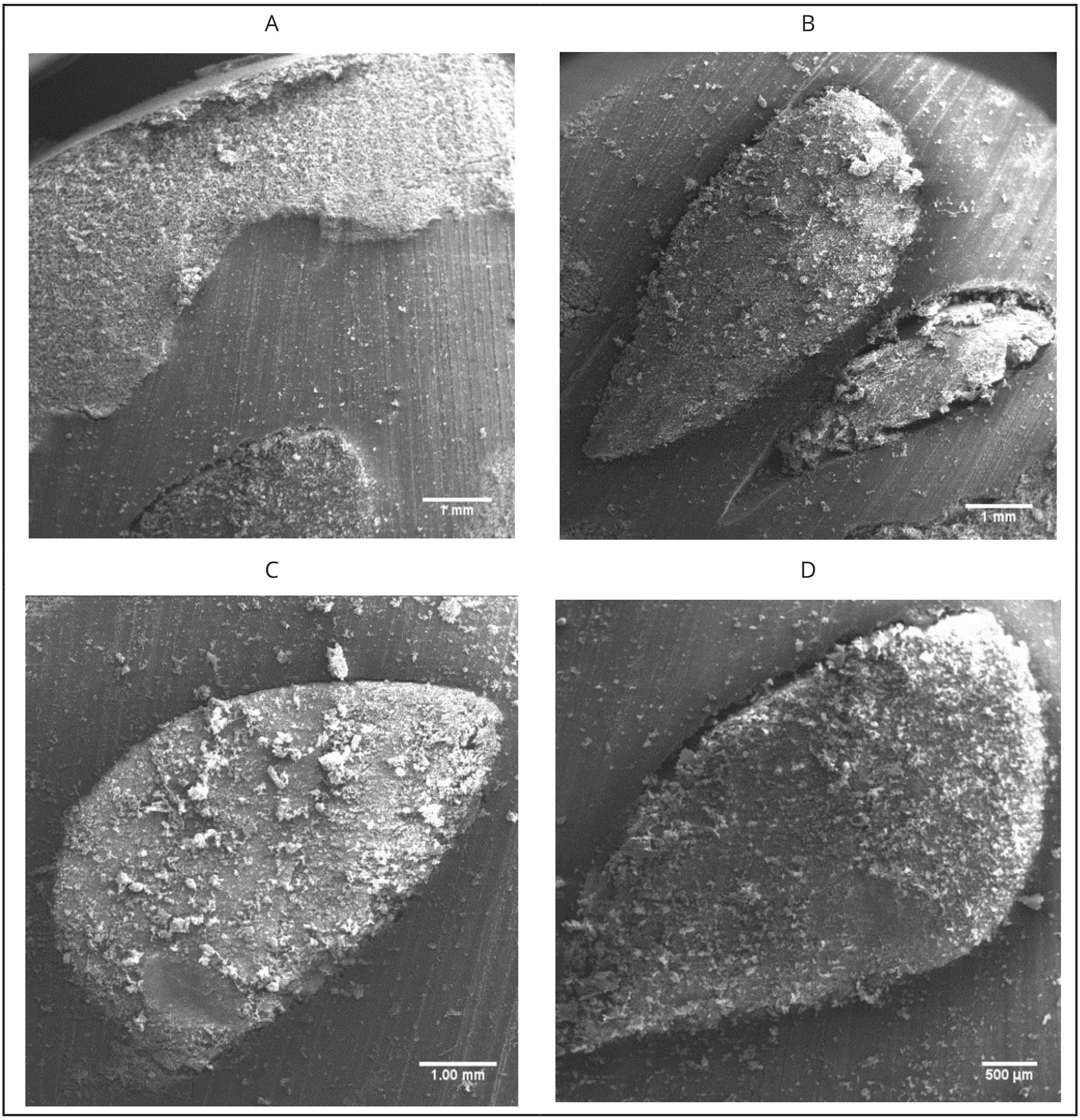
Next, the fruits were opened using a hydraulic press, in which the fruit was subjected to mechanical stress until breakage, enabling the opening of the fruit's mesocarp and collection of the intact seeds (Figure 4).

Figure 4 – Fruits opened by the hydraulic press containing three viable seeds and one undeveloped seed

Source: Authors (2020)

Images obtained using SEM are shown in Figures 5 and 6. Transverse sections of the fruit were visualized under specific levels of magnification at different areas. The fruit has an exocarp and a mesocarp, which is divided in two regions: the sub-exocarpic mesocarp and sclerenchymatous mesocarp (Figure 5A). The latter imparts stiffness and rigidity to the fruit. The mature seed has an ellipsoidal shape (Figure 5B, C and D), is of an exotestal type with an outer epidermis, has an ivory-white colour, and presents a distal length between 5 and 6 mm. Souza (2008) carried out the anatomical analysis of the developmental pericarp and seed of the *Cordia trichotoma* (Vell.) Arrab.ex I. M. Johnst diaspora. For this species, the seed was also exotestal with outer epidermis of the coat-seed with reticulate thickened cell-walls.

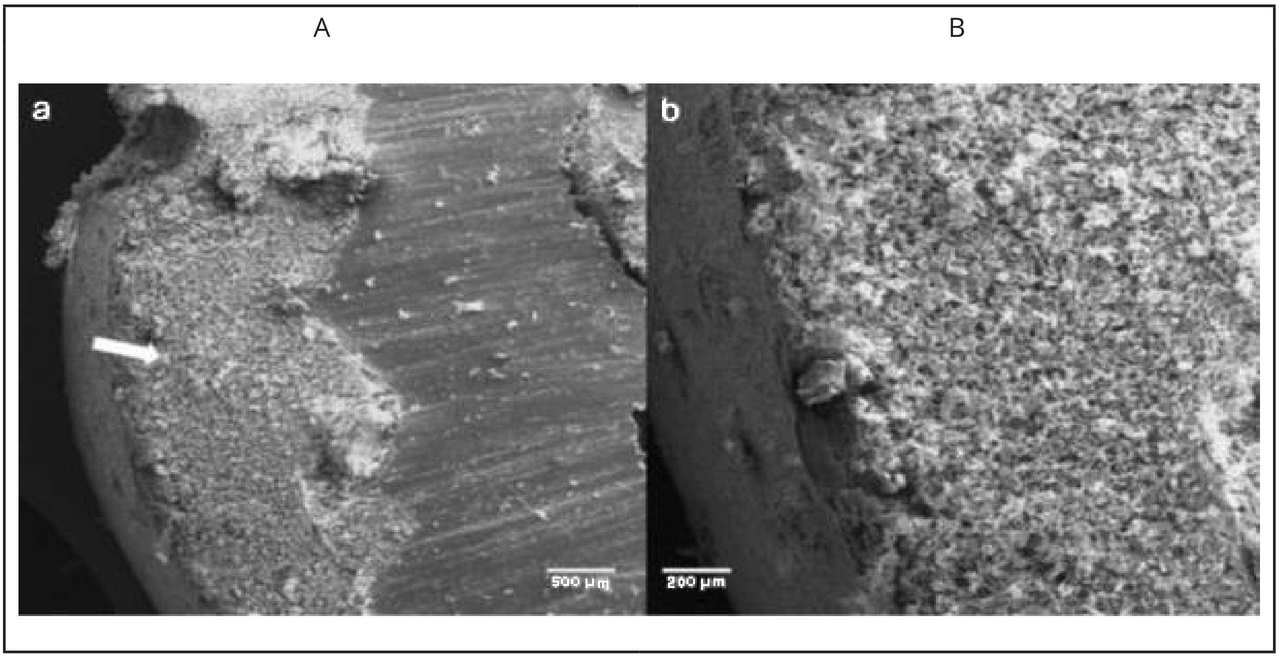
Figure 5 – Transversal section of the fruits visualized using SEM. (a) Details of the exocarp and mesocarp; the white arrow indicates the exocarp and the yellow arrow indicates the sclerenchymatous mesocarp; 13x magnification. (b, c, d) Seed transverse sections inside the fruits



Source: Authors (2020)

Details of the sub-exocarpic mesocarp are shown in Figures 6A and 6B. In Figure 6B, it is possible to observe thick parenchyma cells with a spherical shape according to the images obtained by histology shown below.

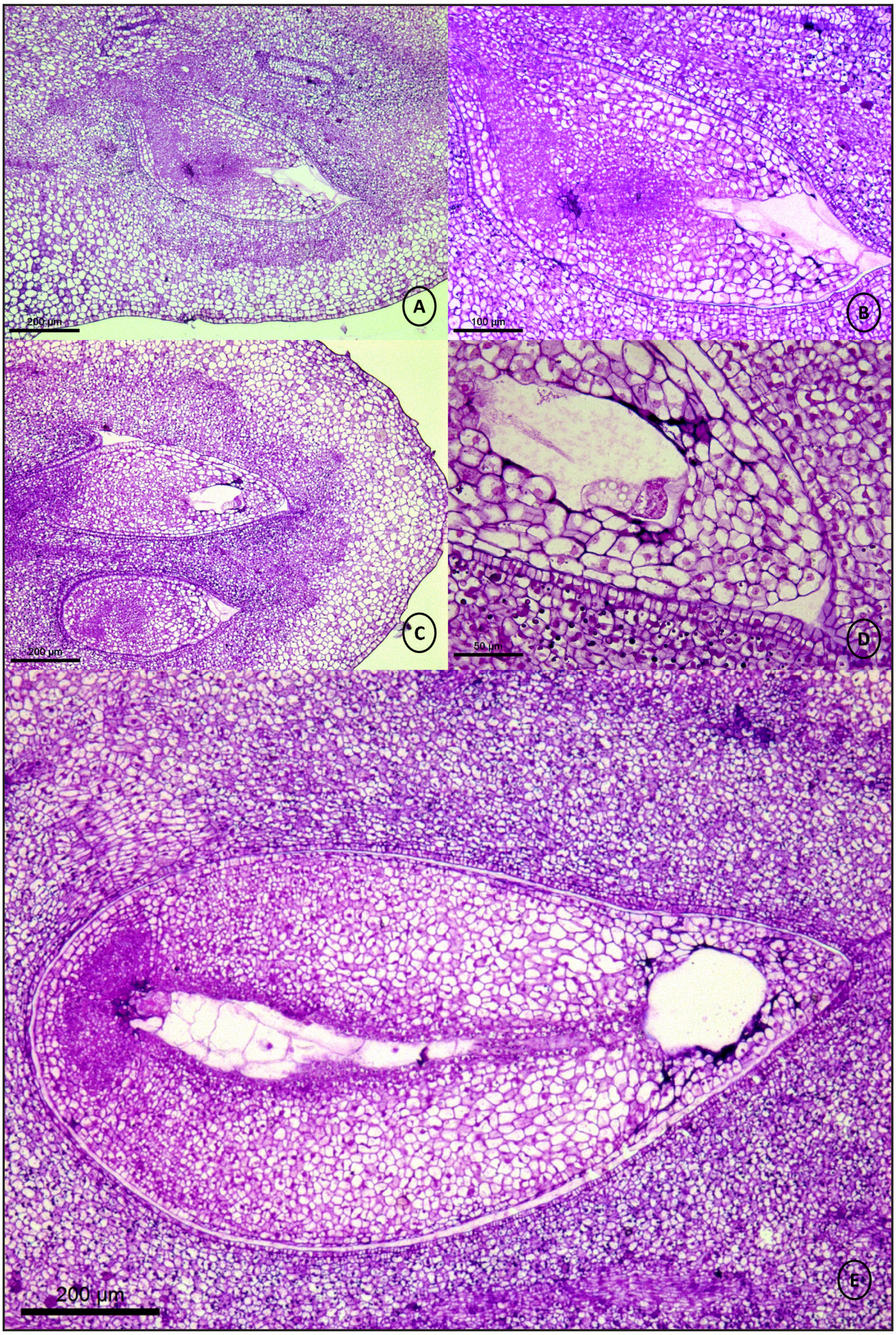
Figure 6 – Details of the sub-exocarpic mesocarp. (a) Transverse section of the fruit showing two mesocarpic layers; white arrow: sub-exocarpic mesocarp, 20x magnification. (b) Sub-exocarpic mesocarp, 50x magnification



Source: Authors (2020)

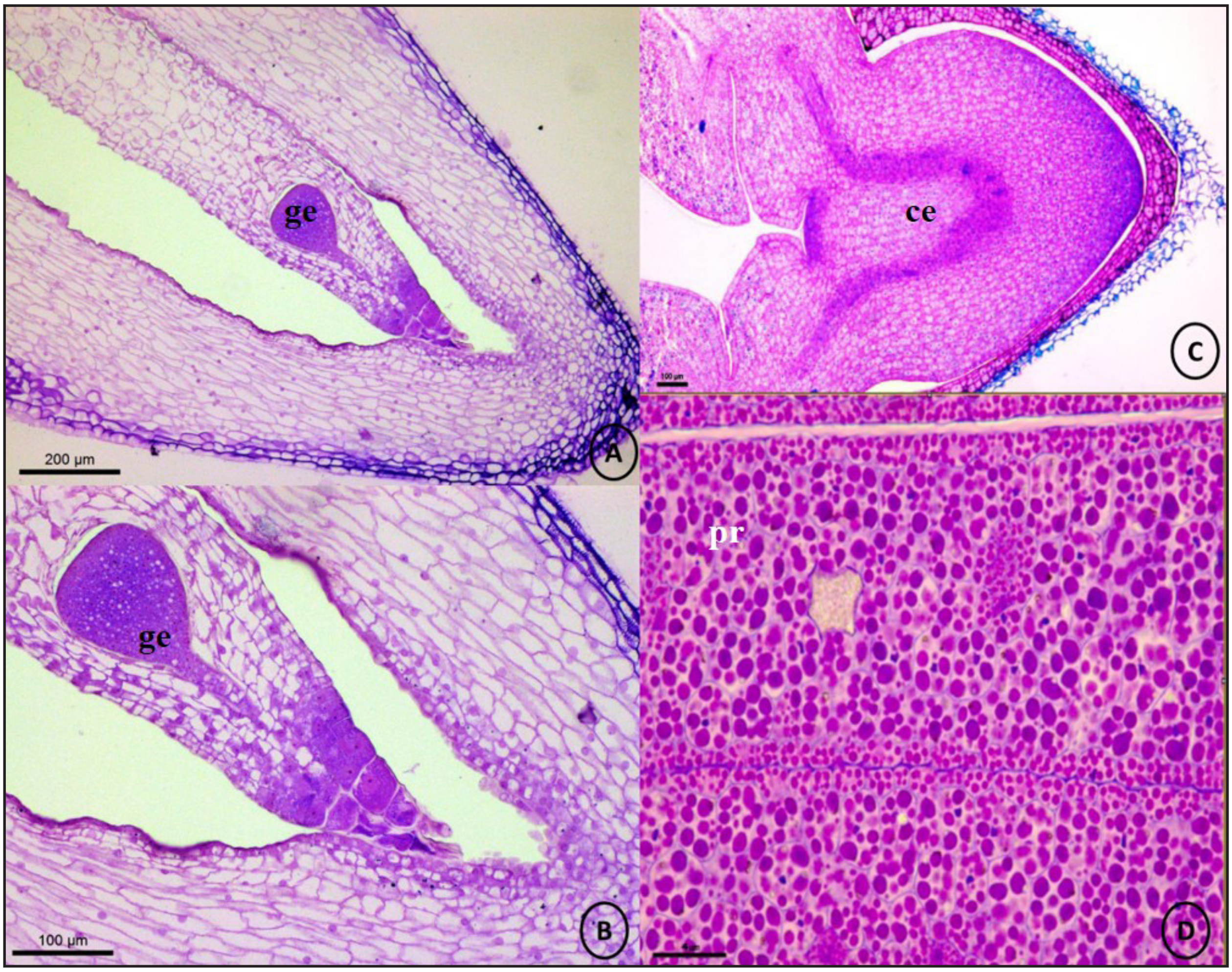
The exocarp is of a uniseriate type formed by squared and rectangular-shaped cells under longitudinal section. The external mesocarp has thick parenchyma cells. The seeds have a single, reduced tegument. Nuclear endosperm is visible in Figures 7A and 7B. According to Kigel and Galili (1995), a woody and indehiscent fruit type is a typical characteristic of *pau-branco*. Seeds that are retained within the fruit can be a result of the protective role being transferred from the tegument to other fruit tissues. This transfer causes a consequent structural reduction of the seminal wraps. This typical feature is clearly noted in the *pau-branco* fruit hardiness. Initial development of the zygot is seen in Figure 7E.

Figure 7 – Longitudinal section of young *pau-branco* fruits (0.7 and 1.9 cm in length, without a seed wall). (A,B) Nuclear endosperm - ne. (C,D) Zygot - zy. (E) Cellular endosperm



Source: Authors (2020)

Figure 8 – Embryo structure of *pau-branco* in longitudinal sections. (A, B) Globular embryo - ge (fruits with 6,0 cm of length). (C) Cotyledonar embryo - ce. (D) Proteins reserve - pr



Source: Authors (2020)

As observed by Souza (2008), who analyzed the morphology and anatomy of the *Cordia trichotoma* (Vell.) Arrab.ex I. M. Johnst diaspore, cotyledons are plicate and also present asymmetrical and heterogeneous mesophyll, indicating some similarities among mature seed characters for these Boraginaceae genera. However, he detected an oily content in the endosperm instead of protein reserves (Figure 8), emphasizing the difference between these species.

4 CONCLUSIONS

The intense hardening of the *pau-branco* fruit mesocarp may contribute to the physical dormancy previously reported and the use of a hydraulic press was the most viable method for the extraction of seeds from the fruits. Embryos become visible in fruits with a length of approximately 1.5 cm and it is fully developed inside fruits with a length greater than 2.0 cm, thus this is the best size to collect fruits for propagation purposes. Seed embryos of *pau-branco* have protein reserves.

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How to quote this article

Muniz, C. R.; Correia, D.; Soares, A. A. Method of opening the fruits, biometry and anatomical description of embryonic development in pau-branco (Cordia oncocalyx Allemão) seeds. Ciência Florestal, Santa Maria, v. 32, n. 2, p. 979-995, 2022. DOI 10.5902/1980509825216. Available from: https://doi.org/10.5902/1980509825216.