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Original Article

Ananas ananassoides (Baker) L.B.Sm. a bromeliad from the savanna: seed morpho-anatomy and histochemistry

Ananas ananassoides (Baker) L.B.Sm., uma bromélia da savana: morfoanatomia e histoquímica da semente

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Abstract

Ananas ananassoides (Baker) L.B.Sm. is a wild pineapple, commonly found in the savannas. This study aimed to describe the morpho-anatomy and histochemistry of its seed. The observations were made in the longitudinal and transverse sections, using an optical microscope. The cell arrangement in the seed coat, ripples in the integument, the ratio of embryo size and endosperm amount, and the number of strata in the aleurone layer are anatomical characteristics that may contribute to distinguishing this species. The starch in the endosperm, lipids and proteins in the embryo, constitute the seed's main nutritional reserves. The homogeneous embryo and phenolic compounds present in the seed coat and in the aleurone layer possibly contribute to the dormancy in this species. This study presents information relevant to the taxonomy and physiology of *A. ananassoides*, which represents contributions to the global knowledge of this species with a high potential as ornamental.

Keywords: embryo, endosperm, pineapple, seed coat.

Resumo

Ananas ananassoides (Baker) L.B.Sm. é um abacaxi silvestre, comumente encontrado nas savanas. Este estudo teve como objetivo descrever a morfoanatomia e histoquímica de sua semente. As observações foram feitas nos cortes longitudinais e transversais, em microscópio óptico. O arranjo celular no tegumento, as ondulações no tegumento, a relação entre o tamanho do embrião e a quantidade de endosperma, e o número de estratos na camada de aleurona são características anatômicas que podem contribuir para a distinção dessa espécie. O amido do endosperma e os lipídios e proteínas do embrião constituem as principais reservas nutricionais da semente. O embrião homogêneo e os compostos fenólicos presentes no tegumento e na camada de aleurona possivelmente contribuem para a dormência nessa espécie. Este estudo apresenta informações relevantes para a taxonomia e fisiologia de *A. ananassoides*, sendo contribuições valiosas para o conhecimento global dessa espécie com alto potencial ornamental.

Palavras-chave: abacaxi, embrião, endosperma, tegumento.

Introduction

Pineapple belongs to the large and diverse Bromeliaceae family, which contains about 3700 species (Gouda and Butcher, 2022). The bromeliads contribute to ecosystem stability due to their high levels of specialization (Benzing, 2000) and to important ecological role caused by their biotic interactions (Pederassi et al., 2012; Mendes et al., 2020). In fact, Paula Júnior et al. (2017) suggest including bromeliads in policies to protect biodiversity, considering their importance in maintaining biodiversity even in altered landscapes exposed to adverse conditions.

Ananas genus bear fused flowers that develop into the iconic sorose fruit that contributes to the aesthetic appeal of pineapple (d'Eeckenbrugge et al., 1997) and has several taxa with historical and modern economic importance (VanBuren, 2018). Potential uses have been explored for species of the genus *Ananas*, including the production of fibers to produce articles for the automotive industry (Zah et al., 2007), the production of polymeric composites (Souza et al., 2017a) manufacture of paper (Marques et al., 2007), proteolytic enzymes (Ramli et al., 2021) and secondary metabolites of great value for the

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cosmetic, pharmaceutical and food industry (Manetti et al., 2009; Banerjee et al., 2018), besides its great ornamental potential (d'Eeckenbrugge and Duval, 2009; Sanewski, 2009; Souza et al., 2012). Bromeliads are among the most valuable ornamental plants (Negrelle et al., 2012), and according to Souza et al. (2017b), the generation of new hybrids for ornamental use can reduce extractive activity and its negative impacts on natural populations. Therefore, it is important to develop studies focused on seeds since they are ideal structures to store the gene pool of a species, guaranteeing the conservation of genetic variability (Gosling, 2003).

Ananas ananassoides (Baker) L.B.Sm. is the most common and diverse form of wild pineapple, and it is the most likely ancestor of the cultivated pineapple (d'Eeckenbrugge et al., 2011). According to Souza et al. (2012), *A. ananassoides* can be used as parents in the genetic breeding program for the generation of pineapple ornamental hybrids. This species is a terrestrial and perennial bromeliad (Silveira et al., 2010; Stahl et al., 2012) found in Neotropical savannas or inland low-shade forests, growing on soils with limited water-holding capacity (sand, rocks). It forms populations of variable densities and regeneration can occur vegetatively and sexually (through seed production) (d'Eeckenbrugge and Leal, 2003; d'Eeckenbrugge et al., 2011). This species is considered a pioneer for presenting small and photoblastic seeds and higher germination rates in areas with high luminosity (Silveira et al., 2010).

Studies on the characterization of seeds from native species are important for the maintenance of biodiversity as they can support programs for the recovery of degraded areas (Oliveira et al., 2006). The chemical composition of seed reserves can influence germination, vigor, and storage potential (Bewley et al., 2013). However, anatomical and histochemical studies of tropical seeds are still considered scarce (Barbosa et al., 2021; Ferreira et al., 2020; Ribeiro et al., 2021).

Given the ecological and economic importance of the representatives of the Bromeliaceae, studies with these approaches have been carried out on the seeds of some species of this family (Cecchi et al., 1996; Cecchifiordi et al., 2001; Morra et al., 2002; Palací et al., 2004; Magalhães and Mariath, 2012; Prado et al., 2014; Chilpa-Galván et al., 2018; Mendes et al., 2018, Silva et al., 2020a; Martelo-Solorzano et al., 2022; Mendes et al., 2021; Silva et al., 2021). However, for the species *A. ananassoides,* few studies have focused on these reproductive structures. Studies carried out with this species have evaluated aspects of floral biology (Stahl et al., 2012), the spatial distribution pattern (Elias et al., 2017), and the generation of new hybrids with ornamental purposes (Souza et al., 2009). There are some studies on the seeds of this species, but these were focused on germination, investigating the effects of light, temperature and storage (Silveira et al., 2010), and evaluating pre-germination treatments (Anastácio and Santana, 2010). Other studies only used seeds to obtain material that would later be evaluated under different *in vitro* conditions (Silva et al., 2017; Silva et al., 2020b). Given the scarcity of detailed information about *A. ananassoides* seeds, the present work proposes to describe the morpho-anatomy and histochemistry of its seed. Results from this study will contribute to further investigations in the field of taxonomy, ecology, breeding systems, and propagation, among others. We also anticipate that our study will provide global knowledge for this species which has high potential for agricultural development in the tropic due to its ornamental features.

2. Material and Methods

2.1. Material collection and morphological characterization

Ripe fruits (n = 30) were collected from three natural populations in the sub-region Savannas in the municipality of Galeras (9°1'44"N and 74°58'19"W; 9°1'43"N and 74°58'22"W; 9°1'40"N and 74°58'20"W) Sucre, Colombia, with the altitudinal range varying from 29 to 35 m above sea level. The mean monthly annual temperature ranged from 28ºC, with an average annual precipitation of 1233 mm. The localities are characterized by presenting a continuous matrix of herbaceous vegetation and scattered small trees. Soils are poorly drained and remain flooded for several weeks. The localities are surrounded by bushy vegetation, and low pastures for cattle. Also, human intervention takes place in the area. Voucher specimens were deposited in the Herbarium HEUS, University of Sucre.

Length and width of the fruits were measured with a Vernier caliper. The pericarp was removed manually with a domestic knife to obtain the seeds. Subsequently, the number of seeds per fruit was counted and then these were described in terms of their shape, color and biometrics (seed width and length). The seeds were soaked in distilled water for 24 h and then cut with a steel razor blade. The seed portion occupied by the embryo is the mean value obtained by dividing the embryo length by the seed length (Magalhães and Mariath, 2012) analyzed with Image J software version 1.8.0 (National Institutes of Health, Bethesda, Maryland, USA).

2.2. Seed anatomy and histochemical characterization

Samples were fixed in FAA (formalin, acetic acid, 50% ethanol) (Johansen, 1940) and dehydrated in a graded ethanolic series. The material was embedded in histological paraffin (Johansen, 1940) and transversal and longitudinal sections (10 µm thick) were made with a rotary microtome (MRP 2015 Lupetec). For the anatomical evaluation the material was stained with 0.5% Toluidine Blue O, at pH 4.7 (O'Brien et al., 1964). To verify the nature of the substances accumulated in the seeds, some histochemical tests were performed, such as Toluidine Blue O, Ferric Chloride solution, Ruthenium Red, Periodic Acid-Schiff, Coomassie Brilliant Blue and Lugol's reagent (Table 1). The permanents preparations were mounted in Canada balsam. Fresh material were also stained with Sudan III (Table 1). Photographs of the anatomical aspects of the seeds were taken with a digital camera coupled with a light microscope (Labomed Lx400). The terminology used for seed coat description follows Corner's (Corner, 1976) classification. Thus, the outer seed coat is called the testa, and the inner seed coat is the tegmen.

Table 1. Histochemical tests in *Ananas ananassoides* seed structures.

Staining procedure	Target compounds	References	Seed structures		
			Seed coat	Endosperm	Embryo
Toluidine Blue O	acidic polysaccharides Pectin/ mucilage phenolic compounds	O'Brien et al., 1964	+	*	-
Ferric Chloride	phenolic compounds	Johansen, 1940	+	*	-
Ruthenium red	Pectin/mucilage	Johansen, 1940	+	+	-
Periodic Acid-Schiff	Neutral polysaccharides	O'Brien and McCylly, 1981	+	+	-
Coomassie Brilliant Blue	Proteins	Fisher, 1968	+	*	+
Sudan III	Total lipids	Pearse, 1972	+	*	+
Lugol	Starch grains	Johansen, 1940	-	+	-

+Positive result. *Positive result for aleurone layer. -Negative result.

3. Results

3.1. Morphological characterization

Ananas ananassoides presented an ovoid-shaped fruit, yellow at maturity and with a leafy crown on top (Figure 1a). It was a multiple fruit formed by the fusion of small berry-like individual fruits. It had a hard rind formed by floral bracts, under which are perianth remains. Each of the flowers was subtended by a bract and the ovaries were inferior and trilocular (Figure 1a). When seeds were formed, they developed on the middle axis of the fruit.

The multiple fruit presented an average of 4.34 small seeds (Table 2). The seed was rounded at one end, tapering towards the other. It was dark brown, with a mucilaginous envelope. The seed coat was tough and leathery with numerous ridges running along the length of the seed (Figure 1b).

3.2. Seed anatomy and histochemical characterization

In the evaluated material the seed coat, endosperm and embryo were differentiated (Figure 2a). Several cell layers made up the seed coat. These cells had thickened walls that contained phenolic compounds (lignin) and pectin inside, which gave the seed coat a brownish color (Figure 2b-2d, Table 1). They also positively reacted to Periodic Acid-Schiff, which detects polysaccharides (Figure 2e).

The testa was made up of colorless cells with thickened walls (Figure 2d). A biseriate tegmen was identified, consisting of cells with reduced lumen, with thickened walls. The exotegmen was constituted by a palisade of lignified Malpighi cells while the endotegmen was collapsed. The cells of the two integuments (testa and tegmen) show pronounced ridges (Figure 2b, 2f and 2h).

The endosperm represented more than 90% of the seed (Figure 2a, Table 2). Surrounding the endosperm cells was a layer of smaller cells filled with protein granules (Figure 2f). This aleurone layer was made up of one to four cell strata. Generally, several strata were presented

Table 2. Dimensions of fruits and seeds of *Ananas ananassoides*.

Parameters	Mean ± standard error
Fruit length (mm)	91.92 ± 0.76
Fruit width (mm)	68.68 ± 0.97
Number of seeds per fruit	4.34 ± 0.05
Seed length (mm)	4.56 ± 0.09
Seed width (mm)	2.25 ± 0.08
Embryo/endosperm (%)*	8/92

*Seed portion occupied by the embryo and endosperm.

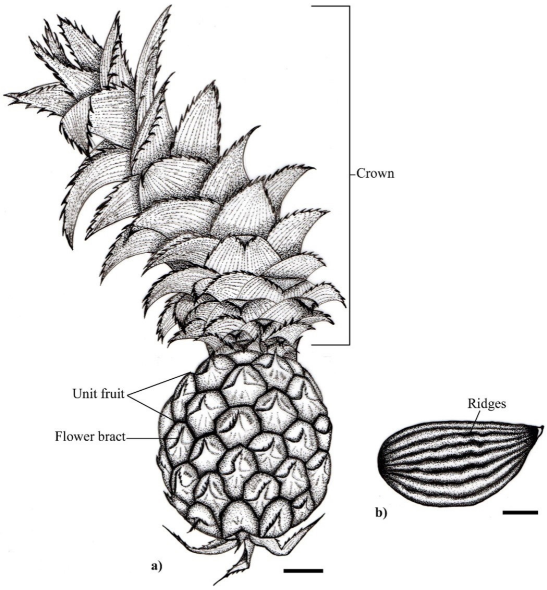

Figure 1. Morphology of *Ananas ananassoides*: (a) Fruit and (b) seed. Scale bars: (a) = 1 cm; (b) =1 mm.

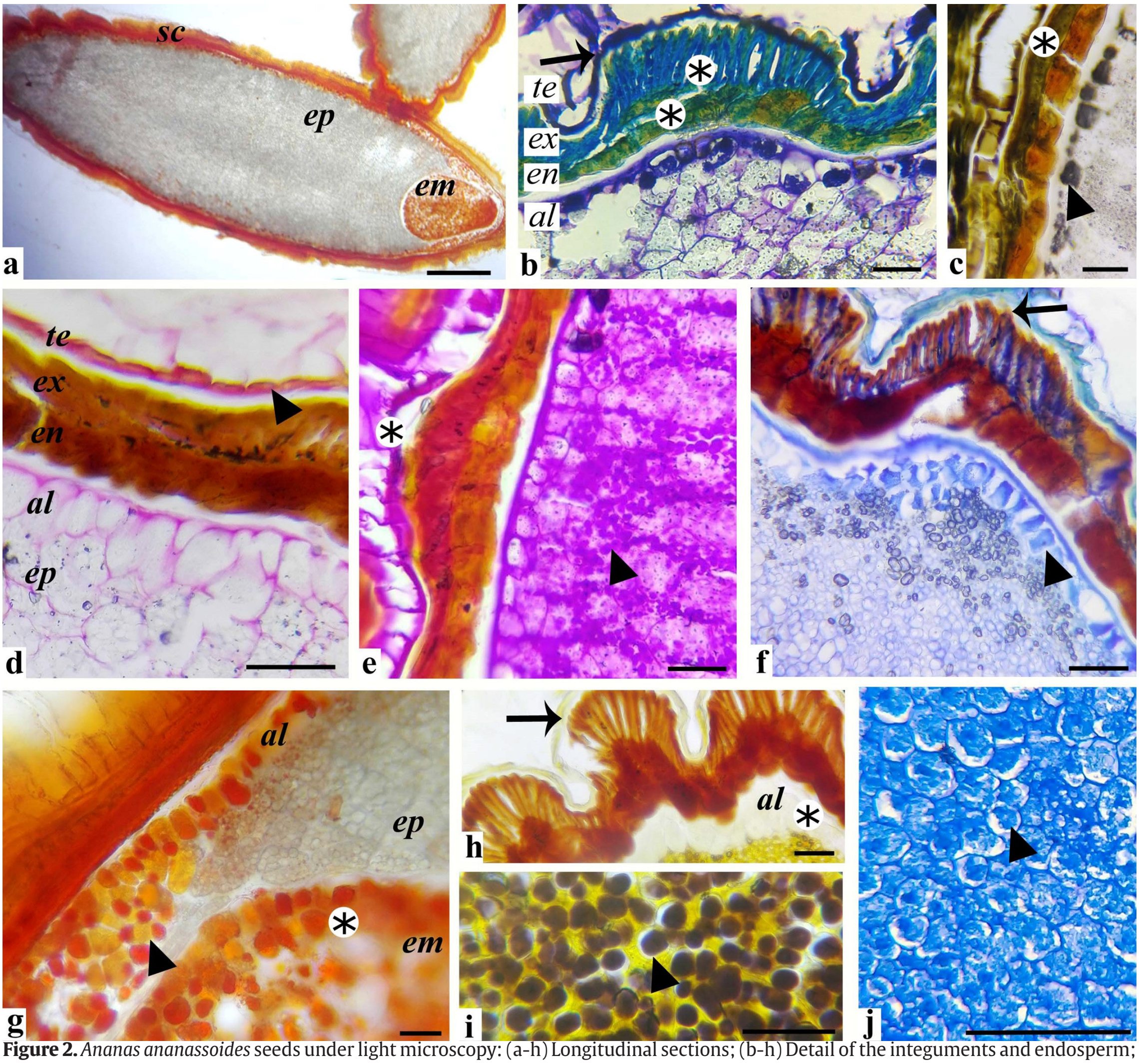

Figure 2. *Ananas ananassoides* seeds under light microscopy: (a-h) Longitudinal sections; (b-h) Detail of the integuments and endosperm; (i) Cross section of the endosperm; (j) Cross section of the embryo; (a) Positive reaction to Sudan III in integuments and embryo. The three basic components of seeds are identified: seed coat, endosperm and embryo. The embryo occupies less than 10% of the volume of the seed. (b). Positive reaction to Toluidine Blue O indicating presence of phenolic compounds and pectins in the integument (asterisk). Pronounced ridges in the integuments (arrow); (c) Positive reaction to ferric chloride indicating presence of phenolic compounds in the integument (asterisk) and aleurone layer (arrowhead); (d) Positive reaction to Ruthenium Red indicating the presence of pectins in cell walls. Testa with thickened walls (arrowhead); (e) Positive reaction to Periodic Acid-Schiff Neutral polysaccharides in the testa (asterisk) and starch grains in the endosperm (arrowhead); (f) Positive reaction to Coomassie Brilliant Blue. Aleurone layer with protein granules (arrowhead). Pronounced ridges in the integuments (arrow); (g) Positive reaction to Sudan III. Aleurone layer made up of several strata in the region around the embryo (arrowhead). Oil in the embryo (asterisk); (h) Negative reaction to Lugol's reagent in aleurone layer (asterisk). Pronounced ridges in the integuments (arrow); (i) Positive reaction to Lugol's reagent in the endosperm. Starch grains (arrowhead); (j) Positive reaction to Coomassie Brilliant Blue in the embryo. Protein (arrowhead). al: aleurone layer; em: embryo; en: endotegmen; ep: endosperm; ex: exotegmen; sc: seed coat; te: testa. Scale bars: a = 500 µm; b-j = 100 µm.

in the region around the embryo (Figure 2g). This layer was formed by cubic or irregular shape cells, with thick cell walls rich in polysaccharides, mainly pectins (Figure 2b-2d). Phenolic compounds, evidenced by the positive reaction (black color) to ferric chloride and lipid content evidenced by the positive reaction to Sudan III, were observed in the cellular cytoplasm (Figure 2c and 2g). Starch was not detected (Figure 2h). The endosperm was formed by larger irregular cells with thin walls and inconspicuous nuclei. The photomicrographs of the stained cross-sections showed numerous starch grains in the endosperm (Figure 2e and 2i).

The embryo is typically of a monocotyledonous. It was small, basal, undivided, homogeneous, and without apparent delimitations of its main structures. Likewise, it was slightly curved and more pointed at one end than the other. It occupied less than 10% of the volume of the seed and was located at its narrow end (Figure 2a). It is made up of isodiametric cells filled with oil and protein (Figure 2g and 2j).

4. Discussion

The morphology and anatomy of the fruits and seeds are relevant for the taxonomy of Bromeliaceae (Smith and Downs, 1974; Fagundes and Mariath, 2014; Magalhães and Mariath, 2012; Silva et al., 2020a). Our morphological description is the same observed in several species of the Bromelioideae subfamily in which the seeds have a mucilaginous coat and the fruits are fleshy (Benzing, 2000; Pereira et al., 2008; Silva and Scatena, 2011; Martelo-Solorzano et al., 2022; Silva et al., 2020a). According to Silva et al. (2020a), the fleshiness of fruits is considered a synapomorphy of Bromelioideae.

Regarding the color of the seed coat, Bewley et al. (2013) indicates that it is a distinguishing feature of many seeds, but sometimes cannot be used taxonomically because they may change due to environmental and genetic influences during development. The reduced number of seeds per fruit found in *A. ananassoides* is related to the self-incompatibility present in most *Ananas* wild types (including *A. ananassoides*), and to sterility factors probably accumulated due to dominant vegetative reproduction (d'Eeckenbrugge et al., 1993; Souza et al., 2017b).

We classified the seed coat strata of *A. ananassoides*, using as reference the ontogenetic analysis of the seeds of *A. comosus* (Rao and Wee, 1979) and other species (*Aechmea bromeliifolia, Billbergia distachia* and *Neoregelia bahiana*) of the Bromelioideae subfamily (Silva et al., 2020a). Silva et al. (2020a), indicated that there are different ontogenetic processes during seed development among bromeliad subfamilies. According to these authors, in Bromelioideae species, all cell layers of both outer and inner integuments are persistent, forming the seed coat, which is thus composed of testa and tegmen. We clearly identify these layers in *A. ananassoides*.

According to Crang et al. (2018) during desiccation of seeds at the final stage of their ripening, the seed surface relief, acquires a characteristic pattern that is stable and taxon-specific. For example, the mucilaginous nature of the *A. ananassoides* seed is the result of the enlargement of the cells of the outer integument due to the accumulation of compounds, observed in later stages of seeds development in Bromelioideae species (Benzing, 2000; Silva et al., 2020a). This mucilage possibly prevents desiccation, helps their fixation on appropriate places for germination, and is related to dispersal by animals (Pereira et al., 2008; Silva and Scatena, 2011; Silva et al., 2020a).

A. ananassoides seeds proved to be similar in size and shape to other species of the Bromelioideae genera: *Ananas, Aechmea, Billbergia, Neoregelia* and *Bromelia* (Pereira et al., 2008; Silva and Scatena, 2011; Silva et al., 2020a; Martelo-Solorzano et al., 2022). However, anatomical analyzes indicate that in the seed of *A. ananassoides*, there is a palisade of lignified Malpighi cells, which constitute the exotegmen. This has previously been reported for the genus *Ananas* and *Bromelia* (Rao and Wee, 1979; Martelo-Solorzano et al., 2022). In addition, *A. ananassoides*, as well as in *A. comosus*, present ridges in the two integuments (testa and tegmen) that have not been anatomically evidenced in other genera of bromeliads (Rao and Wee, 1979; Prado et al., 2014, Magalhães and Mariath, 2012; Chilpa-Galván et al., 2018; Silva et al., 2020a; Martelo-Solorzano et al., 2022). We observed that the ridges in the endotegmen of *A. ananassoides* are more pronounced than in *A. comosus*. On the other hand, several studies have reported the presence of polysaccharides and phenolic compounds in the seed coat of bromeliad (Prado et al., 2014; Magalhães and Mariath, 2012; Chilpa-Galván et al., 2018; Silva et al., 2020a; Martelo-Solorzano et al., 2022).

According to the presence of endosperm, *A. ananassoides* seed is classified as albuminous. However, the space occupied by the endosperm in bromeliads varies according to the genus. In *Ananas* and *Bromelia* genera, it has been reported that the endosperm occupies more than 90% of the seed (Rao and Wee, 1979; Martelo-Solorzano et al., 2022), in *Vriesea* occupies about 70% (Magalhães and Mariath, 2012; Prado et al., 2014), and in *Tillandsia* it may occupy up to 60% of the seed interior or be completely consumed (Magalhães and Mariath, 2012; Chilpa-Galván et al., 2018; Martelo-Solorzano et al., 2022).

In the cereals, the majority of cells in the endosperm accumulate reserves inside and are nonliving at maturity. But on the outside of the endosperm remains a living tissue, the aleurone layer, which is responsible for the production and release of enzymes for the mobilization of these reserves (Bewley et al., 2013). The distinction of an aleurone layer was recorded in *Ananas* comosus by Rao and Wee (1979). These authors showed that this aleurone layer is one cell thick except for the region around the embryo, like what we observe in *A. ananassoides*. In *Bromelia* genus, an aleurone layer made up of three cell strata has also been observed, but these can appear surrounding the embryo or the rest of the endosperm (Martelo-Solorzano et al., 2022). Studies with others genera of bromeliads also indicated the presence of this layer, which can appear made up only of a cell stratum (Cecchifiordi et al., 2001; Palací et al., 2004; Prado et al., 2014; Martelo-Solorzano et al., 2022). Magalhães and Mariath (2012), suggested that the presence/absence of the aleurone layer in the region in contact with the embryo provides an anatomical character that can help distinguish *Vriesea* and *Tillandsia* genera.

Similar to *A. ananassoides*, other species of bromeliads also have proteins and lipids in the aleurone layer and a high concentration of starch grains in the other cells of the endosperm (Cecchifiordi et al., 2001; Palací et al., 2004; Scatena et al., 2006; Magalhães and Mariath, 2012; Prado et al., 2014; Chilpa-Galván et al., 2018; Mendes et al., 2021; Martelo-Solorzano et al., 2022). However, the type of reserves accumulated in the endosperm of bromeliads may vary according to the species (Magalhães and Mariath, 2012; Chilpa-Galván et al., 2018; Martelo-Solorzano et al., 2022). Cecchifiordi et al. (2001) also reported that, in addition to starch, the endosperm in *Tillandsia* seeds also contains calcium oxalate. However, such oxalate crystals were not seen in *A. ananassoides*. On the other hand, in *A. comosus,* Rao and Wee (1979) stated that running through the center of the endosperm is a longitudinal dark groove resulting from the partly collapsed endosperm cells. In *A. ananassoides* this groove was not identified in the endosperm. According to Mendes et al. (2021) the Bromeliaceae family presents the helobial type of endosperm, which is a synapomorphy for the group.

The homogeneous and undivided embryo found in *A. anannassoides*, contrasts with embryos with advanced stage of development found in other bromeliad species (Magalhães and Mariath, 2012; Martelo-Solorzano et al., 2022). The *A. ananasoides* embryo shows a high concentration of protein and lipid, similar to that described for *A. comosus* seeds, but unlike this species, the procambium was not evidenced in *A. ananasoides* (Rao and Wee, 1979). Marcos-Filho (2005), indicates that seeds with a large amount of starch have high storage potential. However, the abundant presence of lipids and proteins in the embryo, as recorded in *A. ananassoides* can reduce the storage time potential, due to the lower chemical stability of the lipids in relation to starch and the high affinity of the proteins with water. According to d'Eeckenbrugge et al. (2011) *Ananas* seeds can maintain viability for 2 years or more in dry and cool conditions, opening the possibility of a pineapple seedbank, provided that procedures can be optimized. These authors indicate that seed conservation techniques would be particularly interesting for wild germplasm. It should be noted that the chemical composition of seeds is ultimately determined by genetic factors and hence varies widely among species and their varieties and cultivars. Some modifications of composition may be imposed by environmental conditions prevalent during seed development and maturation, but such changes are usually minor (Bewley et al., 2013).

Previous studies on *A. ananassoides* revealed high percentages (>90%) of germination and viability of seeds. However, germination was slow, taking up to more than three weeks and spreading over time (Anastácio and Santana, 2010; Silveira et al., 2010). Anastácio and Santana (2010), found differences in the percentage of germination between newly collected and stored seeds, suggesting the presence of immature embryos in this species. According to Amaral et al. (2020), the presence of embryos with these characteristics is related to the low performance of the seeds in germination. Our observations confirm the poor development of the *A. ananasoides* embryo in ripe fruits, indicating a morphological dormancy. In addition, although the integument can provide physical dormancy (Molina-Guerra et al., 2020), Anastácio and Santana (2010), indicate that the integument of *A. ananassoides* is permeable and therefore the seeds do not have tegumentary dormancy. Nevertheless, the seed coat may contain chemical inhibitors, such as phenolic compounds that prevent embryonic growth (chemical dormancy) (Bewley et al., 2013). We identify these compounds in the seed coat and the aleurone layer of *A. ananassoides*. This layer presents several strata in the region around the embryo, which potentially contributes to the growth inhibitory effect. In species of the genus *Bromelia*, Martelo-Solorzano et al. (2022) related the high accumulation of phenols in the tegument with the longer time necessary for the seeds to germinate. Besides, it has been reported that some bromeliads of *Tillandsia* genus that do not accumulate reserves in the endosperm, germinate faster and require a shorter time to the seedling establishment than species with a copious endosperm (Montes-Recinas et al., 2012).

To our knowledge, this is the first study that documents the morpho-anatomy and seed histochemistry of *A. ananassoides*. The richness of information that seeds can provide is highlighted, since the characters described in this work present information relevant to the taxonomy and physiology of the studied species. Our results provide anatomical characteristics that may contribute to distinguishing this species. Among them are: cell arrangement in the seed coat; ripples in the integument; the ratio of embryo size and endosperm amount and the number of strata in the aleurone layer. On the other hand, carbohydrates in the form of starch in the endosperm, and lipids and proteins in the embryo, constitute the seed's main nutritional reserves. The homogeneous embryo and phenolic compounds present in the seed coat and in the aleurone layer possibly contribute to the dormancy in this species. Lastly, we recommend conducting ontogenetic studies of *A. ananassoides* to better understand the origin of each seed coat layer.

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