

Seed morphology of *Vriesea friburgensis* var. *paludosa* L.B. Sm. (Bromeliaceae)¹

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ABSTRACT - (Seed morphology of *Vriesea friburgensis* var. *paludosa* L.B. Sm. (Bromeliaceae)). The Brazilian Atlantic Forest is a biodiversity hotspot, and bromeliads are omnipresent in this biome. This study was aimed at characterizing both morphological and histochemical aspects of *Vriesea friburgensis* var. *paludosa* L.B. Sm. seeds. The seeds are filiform with a brown seed coat and yellowish-colored plumose appendages. Seeds are typically 4.4 mm long, 0.5 mm wide, and 1000 seeds weigh 683.8 mg. The seed coat presents phenolic compounds. Starch represents the main reserve compound in the endosperm; however, it is also present in smaller amounts in the embryo. The embryo occupies one-third of the seed and has a zone of constriction in the hypocotyl-radicle axis. The results of this work will contribute to the overall biology of *V. friburgensis* var. *paludosa*, as well as its taxonomic delimitation.

Keywords: anatomy, embryo, endosperm, histochemistry, seed coat

RESUMO - (Morfologia da semente de *Vriesea friburgensis* var. *paludosa* L.B. Sm. (Bromeliaceae)). A Mata Atlântica é um hotspot de biodiversidade, e as bromélias são um grupo taxonômico bastante comum presente neste bioma. O objetivo deste estudo foi caracterizar os aspectos morfológicos e histoquímicos das sementes *Vriesea friburgensis* var. *paludosa* L.B. Sm. As sementes são filiformes, com tegumento marrom e apêndices plumosos amarelados. Têm valores médios de 4,4 mm de comprimento, 0,5 mm de largura, e peso de 1.000 sementes de 683,8 mg. O tegumento apresenta compostos fenólicos. O amido é o principal componente de reserva no endosperma, porém ele também se apresenta em menores quantidades no embrião. O embrião ocupa um terço da semente e apresenta uma zona de constrição no eixo hipocotilo-radicula. Os resultados do presente trabalho contribuem na biologia e na delimitação taxonomia de *V. friburgensis* var. *paludosa*.

Palavras-chave: anatomia, embrião, endosperma, histoquímica, tegumento

Introduction

The Atlantic Forest biome contains high biodiversity and species endemism, and it is among the 25 hotspots on the planet (Myers *et al.* 2000). As far as plant diversity is concerned, the group of bromeliads plays an important role on this forest (Stehmann *et al.* 2009). The Bromeliaceae family includes 58 genera and 3172 species and subspecies (Luther 2008). It preferentially occurs in the tropics, ranging from North to South America, with a single species occurring in Africa, *Pitcairnia feliciana* (Benzing 2000). The Brazilian Atlantic Forest has a large number of representatives

from the Bromeliaceae family, with an estimated occurrence of 816 species, out of which 651 (80%) are endemic (Stehmann *et al.* 2009).

Recent phylogenetic analyses divided the Bromeliaceae family into eight subfamilies, Brocchinioideae, Lindmanioideae, Tillandsioideae, Hechtioideae, Navioideae, Pitcairnioideae, Puyoideae and Bromelioideae (Givnish *et al.* 2007). The subfamily Tillandsioideae is characterized by capsule-type fruits and seeds with plumose appendages adapted to wind dispersal (Smith & Downs 1977, Givnish *et al.* 2007). *Vriesea* is the second largest genus in the subfamily Tillandsioideae, which is

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composed of about 260 species (Luther 2008), and in the Atlantic Forest, this genus presents the greatest richness, with approximately 166 species (Martinelli *et al.* 2008, Stehmann *et al.* 2009). However, it remains difficult to accurately determine the specific taxonomic boundaries in the *Vriesea* genus (Martinelli *et al.* 2008). Therefore, detailed morphological studies are required for a complete taxonomic circumscription of these species.

Vriesea friburgensis is a medium-sized bromeliad, reaching more than 2 m of height during the reproductive stage with beautiful inflorescences which provide ornamental potential (Reitz 1983). This species grows as a terrestrial plant, forming dense clusters, but it may also occur on tops of rocks and on other plants (Reitz 1983). It is found in Brazil's southern, southeastern and northeastern regions, from Rio Grande do Sul to Pernambuco States (Martinelli *et al.* 2008), occurring in the Atlantic Forest (Stehmann *et al.* 2009) (figure 1a). However, wild populations of *V. friburgensis* have been reduced by recent destruction and fragmentation of its natural habitat due to human disturbance as well as illegal collections (Paggi *et al.* 2013). According to Martinelli *et al.* (2008), the conservation status of *Vriesea friburgensis* is endangered and vulnerable. Such species presents two varieties which differ mainly in the architecture and inflorescence color. The *tucumanensis* variety is characteristic of the plateau (planalto meridional), and the *paludosa* variety which is characteristic of Coastal Restinga (Reitz 1983).

Studies related to seed morphology and anatomy may be helpful in clarifying the relationships amongst representatives of the Tillandsioideae family (Palací *et al.* 2004, Barfuss *et al.* 2005, Magalhães & Mariath 2012). Moreover, according to Buckeridge *et al.* (2004), enhanced knowledge about the chemical composition of seeds native to tropical forests is crucial. According to the mentioned authors, such information is important to assist in the production of high quality seedlings. From this perspective, additional knowledge about the characteristics of *V. friburgensis* var. *paludosa* seeds could improve germination studies and provide more insight into the taxonomic characteristics of this species. The present study provides a morphological and histochemical characterization of *V. friburgensis* var. *paludosa* seeds.

Material and methods

Plant material - *Vriesea friburgensis* var. *paludosa* seeds were obtained from mature fruits harvested from

plants grown in the bromeliad collection of the Centro de Ciências Agrárias, Universidade Federal de Santa Catarina, Brazil (figure 1b)

Morphological analysis - According to the Rules for Seed Analysis (Brasil 2009), a precision analytical balance (0.1 mg) was used to obtain the weight of 1000 seeds. As for the biometric description, the length and width of 50 seeds were randomly selected from five different specimens and then measured with a digital caliper individually, according to the method of Silva & Scatena (2011). These seeds were used for the viability test. The seeds were previously soaked in distilled water for 24 h and subsequently assessed by soaking longitudinal sections in an aqueous 1% (w v⁻¹) 2,3,5-triphenyl-tetrazolium chloride solution (TZ) (McGoverin *et al.* 2011), and incubated at 25 °C in the dark for 4 h. The living parts of a viable seed should be stained red (Patil & Dadlani 1993).

Scanning Electron Microscopy (SEM) - Samples with approximately 5 mm in length were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) plus 0.2 M sucrose overnight. The material was post-fixed with 1% osmium tetroxide for 4 h. The samples were dehydrated in ethanolic series, dried in the CO₂ Critical point dryer (EM-CPD-030, Leica, Heidelberg, Germany), and then sputter-coated with gold prior to examination. The samples were examined under SEMJSM 6390 LV (JEOL Ltd., Tokyo, Japan) at 20 kV.

Confocal laser scanning microscopy (CLSM) - The samples were analyzed in a laser scanning confocal microscope (Leica TCS SP-5, Wetzlar, Germany) with Leica HCX PLAPO lambda 63x/1.4-0.6 oil immersion objective. For nuclei detection, 0.5 mg mL⁻¹ DAPI (4',6-Diamidino-2-phenylindole dihydrochloride; Sigma-Aldrich, St. Louis, MO) was added to the samples during 30 minutes (Ouriques & Bouzon 2008) and observed by using a UV light-emitting diode (peak wavelength of 405 nm excitation and spectrum emission of 510-566 nm). The LAS-AF Lite program (Leica) was used for final processing of the confocal images.

Light Microscopy (LM) - The samples were fixed in 2.5% paraformaldehyde in 0.2 M (pH 7.2) phosphate buffer overnight. Subsequently, the samples were dehydrated in increasing series of ethanol aqueous solutions (Schmidt *et al.* 2009). After dehydration, the samples were infiltrated with Histoiresin (Leica

Historesin, Heidelberg, Germany). Sections (5 μm) were obtained by using a manual rotational microtome (Leica model RM 2135) with tungsten blades. The sections were stained with different histochemical techniques: Toluidine Blue O (TB-O) 0.5% aqueous solution, pH 3.0 (Merck Darmstadt, Germany) in order to identify phenols and acidic polysaccharides (O'Brien *et al.* 1964, Gordon & McCandless 1973); Periodic Acid-Schiff (PAS) to identify neutral polysaccharides (Gahan 1984); Lugol to identify starch grains (Johansen 1940); and Coomassie Brilliant Blue (CBB) 0.4% in Clarke's solution (Serva, Heidelberg, Germany) to identify proteins (Fisher 1968, Gahan 1984). Some sections were double-stained with PAS + CBB (Schmidt *et al.* 2012). As for the detection of total lipids, fresh material was used with longitudinal sections 10 μm thick frozen in Microtome Cryostat (Leica CM1850 UV). Sudan Black B (SB-B) staining was used to identify lipids (Johansen 1940). Sections were analyzed with an Olympus® DP 71 camera attached to an Olympus® BX-40 microscope.

Fluorescence microscopy (FM) - Representative samples of fresh material were longitudinally cut free-hand, mounted on slides, and analyzed using a UV light-emitting diode (wavelength of 405 nm) in the Epifluorescent Microscope (Olympus BX 41) equipped with the Image Capture Q Capture Pro 5.1 Software (Qimaging Corporation, Austin, TX, USA).

Results

Morphological analysis - *Vriesea friburgensis* var. *paludosa* has small seeds with yellowish-colored

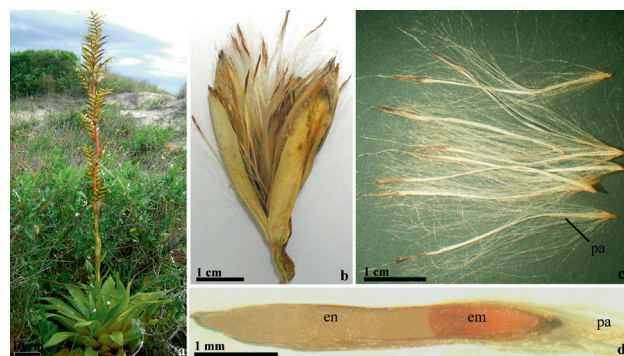


Figure 1. Morphological aspects of *Vriesea friburgensis* var. *paludosa* L.B. Sm. in the reproductive state. a. Plant in natural environment. b. Open capsules, exposing seeds. c. Seeds with plumose appendages. d. Longitudinal section of the seed submitted to tetrazolium test, showing internal structures: embryo (reddish coloring) and endosperm (colorless). pa: plumose appendage; em: embryo; en: endosperm.

plumose appendages longer at the micropylar end. The plumose appendages are located at the base of the fruit (figures 1b, c). The seeds are filiform with a brown seed coat. The weight of 1000 seeds is 683.8 mg. Regardless the appendages, the average seeds measure 4.4 mm long and 0.5 mm wide (table 1). All the seeds evaluated were viable under tetrazolium (TZ) solution. The embryo occupies about one-third of the seed with reddish coloring, while the endosperm is colorless (figure 1d).

Microscopic analysis: Seed Coat - SEM revealed the presence of undulated anticlinal walls of Seed Coat (figure 2a). This tissue surrounds the whole seed (figure 2b, c). The seeds have both testa (outer seed coat) and tegmen (inner seed coat). The testa consists of cells with lignified walls which form the plumose appendages. The tegmen is biseriate and composed of exo- and endotegmen. The exotegmen is formed by cells with thickened walls and without content in mature seeds (figures 2d-h). The cells of the endotegmen showed a greenish-blue color shift when stained with TB-O reagents, indicating the presence of phenolic compounds (figure 2d; table 2). For the endotegmen treated with SB-B, we have observed a slight reaction in the cytoplasm, with a pale darkish color, indicating hydrophobic radicals or lipids (figure 2e). Furthermore, the endotegmen cells were larger than other seed cells. The cytoplasm and the cell walls reacted positively to PAS, which indicates the presence of neutral polysaccharides (figure 2f). Other histochemical analyzes indicated the lack of starch reserves (figure 2g) and protein compounds (figure 2h) in this tissue. Moreover, autofluorescence was observed in the seed coat, probably caused by phenolic compounds found in the endotegmen (figure 2i).

Microscopic analysis: Endosperm - The seeds are classified as albuminous. The endosperm is located mainly in the seed chalazal region and is composed of two distinct tissues: the aleurone layer and the starchy parenchyma. The aleurone layer is composed

Table 1. Average size and weight of *Vriesea friburgensis* var. *paludosa* L.B. Sm. seeds. Means followed by standard deviation (\pm).

Variable	Means
Weight of 1,000 seeds	683.8 mg
Length	4.4 \pm 0.06 mm
Width	0.5 \pm 0.01 mm

of a cell layer found beneath the seminal tegument in the peripheral region of the endosperm. SEM image of aleurone layer showed cellular spaces, probably from the lipid materials extracted by ethanol during the dehydration process (figure 2b). At the micropylar end of the seed, in which the embryo can be seen,

the endosperm is reduced, and the aleurone layer is found on the periphery of the embryo (figure 2c). The aleurone layer cells have thick walls and vary from round to rectangular. These cells revealed the presence of acidic polysaccharides in the cytoplasm and on the cell wall by reaction to TB-O (figure 2d; table 2). In

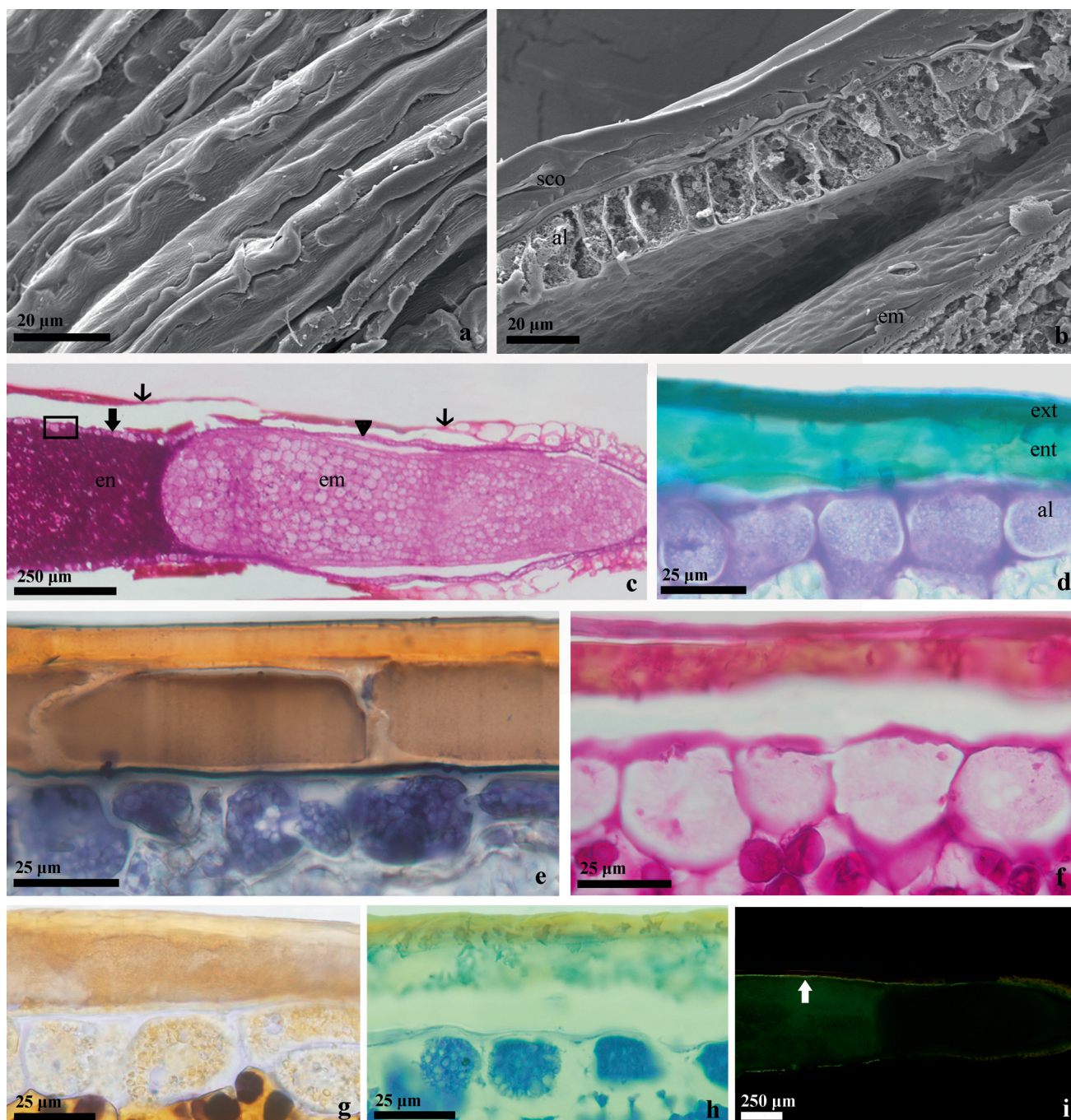


Figure 2. Seed coat and aleurone layer of *Vriesea friburgensis* var. *paludosa* L.B. Sm. seed. a-b. SEM images. c. Section submitted to PAS. Seed coat (open arrow), aleurone layer around the endosperm (black arrow) and around the embryo (arrowhead). d-h. Details of seed coat and aleurone layer (region marked with box in fig. c) under different histochemical tests: d. TB-O; e. SB-B; f. PAS; g. Lugol; h. CB-B; i. Fluorescence microscopy images. Autofluorescence caused by phenolic compounds (white arrow). al: aleurone layer; em: embryo; en: endosperm; ent: endotegmen; ext: exotegmen; sco: seed coat.

the cytoplasm, the presence of lipids was also observed (figure 2e). The neutral polysaccharides were found primarily as cell wall constituents, indicating the presence of cellulose and/or pectin (figure 2f). In these cells, no reserve compounds, such as starch (figure 2g) were identified, however CBB showed a great amount of protein bodies (figure 2h).

SEM images of endosperm showed starchy parenchyma composed of cells with high presence of starch grains of varying sizes (figure 3a). Confocal microscopy images showed the nuclei of these cells with fewer inflorescences than the cell nuclei in both aleurone layer and embryo (figure 3b). The reaction to TB-O showed the starchy parenchyma composed of several irregular cell layers with inconspicuous nuclei (figure 3c; table 2). Acidic polysaccharides were detected in the parenchyma cells, whereas the cytoplasm showed weak granulations (figure 3c).

Neutral polysaccharides were also found to be cell wall constituents, indicating the presence of cellulose and/or pectin. In particular, they were found as starch grains in amyloplasts in the cytoplasm, which turned red when double-stained with PAS + CBB (figure 3d). Furthermore, we observed a conspicuous presence of protein materials in the shape of disorganized amorphous grains in the cytoplasm of cells, as identified by Coomassie blue staining (figure 3d). Conversely, just a few lipids were present (figure 3e). A complex of stacked cell walls was also observed in the endosperm, on the opposite side of the cotyledon apex along with a large amount of starch grains, whereas proteins were not detected (figures 3f, g).

Microscopic analysis: Embryo - It was possible to distinguish the uniseriate protoderm, a tenuous procambium, the cotyledon, as well as shoot and root apical meristems (figure 4a). The embryo presented

Table 2. Compounds evaluated by histochemical tests in *Vriesea friburgensis* var. *paludosa* L.B. Sm. seeds. + Positive reaction; - negative reaction; ± weak reaction.

Structure	Figure number	Histochemical test	Reaction	Compounds
Seed coat	2	TB-O	+	Phenolic compounds
		SB-B	±	Lipids and tannins
		PAS	+	Neutral polysaccharides
		LUGOL	-	
		CBB	-	
Aleurone layer	2	TB-O	+	Acidic polysaccharides in the cytoplasm
		SB-B	+	Lipids in the cytoplasm
		PAS	+	Neutral polysaccharides in the cell wall
		LUGOL	-	
		CBB	+	Total protein in the cytoplasm
Starchy parenchyma	3	TB-O	+	Acidic polysaccharides in the cell wall, weak reaction in the cytoplasm
		SB-B	±	Lipids in the cytoplasm
		PAS	+	Neutral polysaccharides in the cell wall and in the cytoplasm.
		LUGOL	+	Starch in the amyloplast
		CBB	+	Total protein in the cytoplasm
Embryo	4	TB-O	+	Acidic polysaccharides
		SB-B	+	Total lipids
		PAS	+	Neutral polysaccharides
		LUGOL	+	Starch
		CBB	+	Proteins

a constriction zone between the shoot and the root portion (figure 4b). The presence of polysaccharides, proteins and lipids was identified (figures 4c-e; table 2). The starch grains could be visualized after positive reaction to PAS or Lugol (figure 4f, g). The apical part of the cotyledon has a smooth surface and it is immersed in the seed endosperm which fills the chalazal portion of the seed. The cotyledon cells are

bigger than the other embryo cells and it is composed of a uniseriate epidermis and parenchyma cells. In parenchymal cells, the onset of procambium, which extends throughout the embryo, could be distinguished (figure 4h). Three-dimensional reconstruction in CLSM allowed visualization of the expanding cotyledon on the shoot apical meristem, covering it like a sheath.

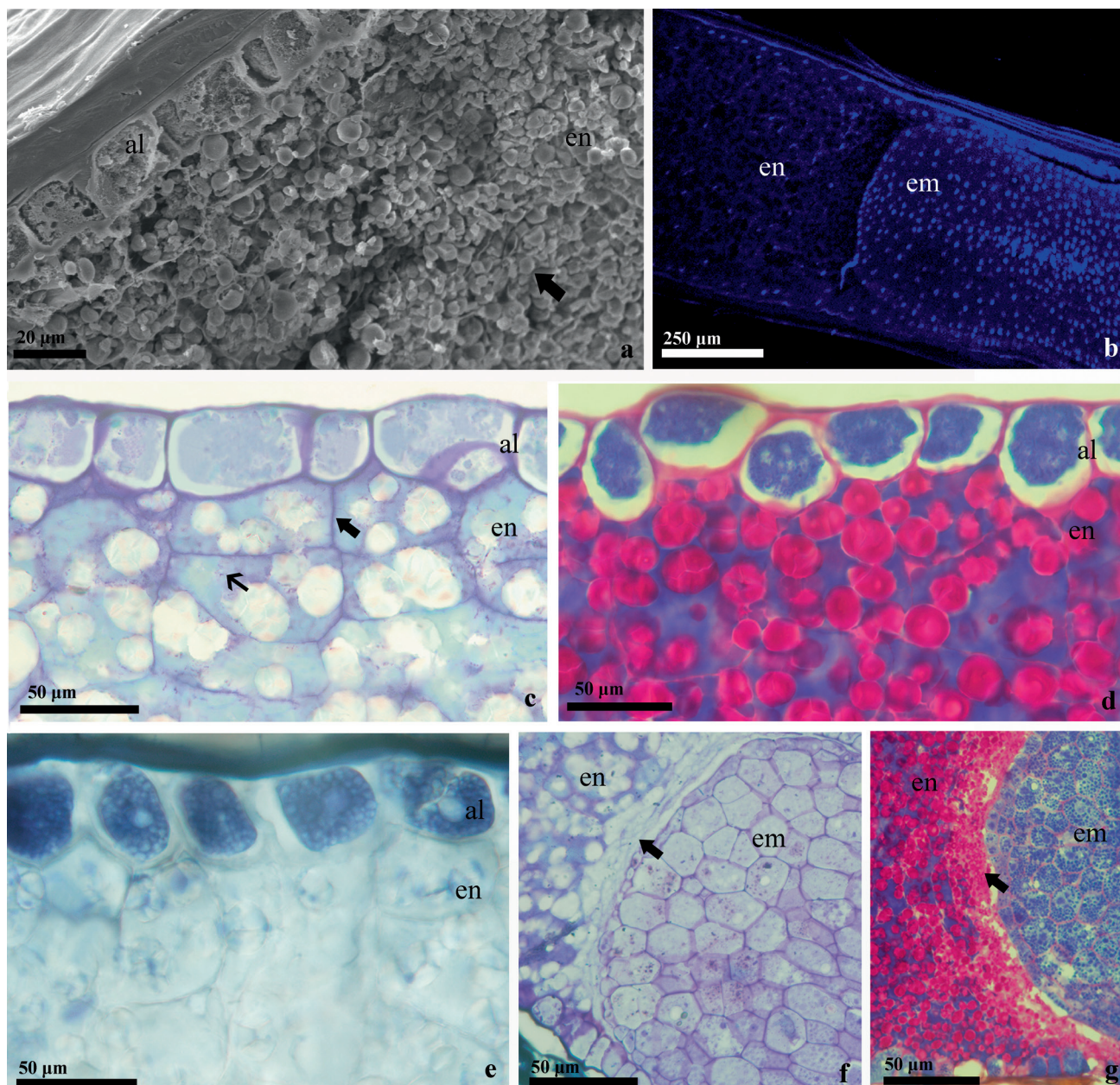


Figure 3. Longitudinal sections of the endosperm of *Vriesea friburgensis* var. *paludosa* L.B. Sm. seed. a. SEM image showing starch grains (arrow) b. Confocal microscopy image. Details of endosperm under different histochemical tests: c. TB-O showing acidic polysaccharides in the cell walls (arrow) and cytoplasm with weak acidic granulations (open arrow); d. double-stained with PAS + CB-B, indicating the red-colored starch and blue-colored proteins; e. Sudan Black B (SB-B); f. endosperm under TB-O test with cell wall complex stacked (arrow) near the cotyledon; g. same region under double-stained with PAS + CB-B, showing the absence of protein compounds, but a rather large amount of starch grains (arrow). al: aleurone layer; em: embryo; en: endosperm.

Discussion

The data obtained from this study are similar to the morphological characteristics of the seeds found on other species of this genus (Pereira *et al.* 2008, Magalhães & Mariath 2012). According to Benzing (2000) and Scatena *et al.* (2006), the presence of winged structures, such as plumose appendages, facilitates long-distance dispersal of seeds, enabling them to reach, as well as thrive in, inhospitable microenvironments. This type of appendage has been found on seeds of other bromeliads which represent the subfamily Tillandsioideae (Scatena *et al.* 2006, Pereira *et al.* 2008, Pereira *et al.* 2009, Silva & Scatena 2011, Magalhães & Mariath 2012). The plumose appendages of *Vriesea friburgensis* var. *paludosa* show the type of structural arrangement suggested by Magalhães & Mariath (2012) for the genus *Vriesea*: the exo- and mesotesta are split in the chalazal region, while remaining attached to each other in the micropylar region to form a parachute-like structure. According to Palaci (2004), the genus *Vriesea* has seed appendages of cell rows derived from longitudinal splitting of the outer integument, which strongly elongates at the base throughout seed maturation.

The tetrazolium (TZ) biochemical test is based upon the activity of the respiration enzymes in seeds. The dehydrogenase enzymes reduce the colorless tetrazolium salt solution to a chemical compound that stains living cells (respiring) with a red color, whereas dead cells (not respiring) remain colorless (Elias & Garay 2004). The areas of vital importance in interpreting the staining pattern in monocots are the shoot and root apical meristems and the portion in which the embryo is attached to the cotyledon (Patil & Dadlani 1993). By using TZ, it was found that *V. friburgensis* var. *paludosa* seeds were viable, presenting embryonic coloring. However, the endosperm remained unstained due to the absence of respiratory activity in this tissue. Mature seeds of several monocot genera showed no staining in the endosperm when they were subjected to TZ (Hands *et al.* 2012). This coloration showed that the embryo occupied one-third of the seed. In accordance with Magalhães & Mariath (2012), the genus *Vriesea* has a small embryo which occupies 27 to 33% of the seed.

The presence of the endosperm indicates albuminous seeds. The ability of the endosperm to store starch so proficiently is a characteristic associated with monocot orders, including the

Poales (Hands *et al.* 2012). However, the reserves accumulated in the endosperm of bromeliads vary according to the species, and the space occupied by the endosperm in the seeds can also vary according to the genus (Magalhães & Mariath 2012). Nevertheless, the genus *Vriesea* is characterized by the accumulation of starch as a reserve compound (Magalhães & Mariath 2012).

Seed coats develop from the integuments which surround the ovule and, after fertilization, can include extensive differentiation and accumulate substances that can also contribute to overall seed morphology (Moïse *et al.* 2005). Thus, the features observed in the seed coat of *V. friburgensis* var. *paludosa* determine its role in protecting the embryo and regulating germination. In the present study, the phenolic compounds were identified mainly by autofluorescence and reaction with TB-O in the endotegmen. However, according to Geier (1980), PAS also reacts with phenolic compounds when highly concentrated. In bromeliads, the endotegmen of *Vriesea* and *Tillandsia* genera is formed by cells with phenolic compounds (Magalhães & Mariath 2012). The presence of these compounds in the endotegmen of mature seeds may be a distinctive feature of Poales (Nakamura *et al.* 2009). Phenolics are secondary metabolites produced by plants to defend themselves against pathogens (Freeman & Beattie 2008). The content of phenolic compounds found in seeds may be related to the permeability of seed coats to water (Marbach & Mayer 1974) and may restrict oxygen uptake (Bewley & Black 1994). These compounds also cause the seed coat structure to be harder and act as germination inhibitors (Werker 1997).

According to Magalhães & Mariath (2012) the endotesta cells of *Vriesea*, have irregular secondary thickening (undulations) on their anticlinal walls, and the connections between cells may be straight or slightly curved. Only curved connections between cells of *V. friburgensis* var. *paludosa* were observed.

In the mature seed, both the aleurone and the embryo remain alive, but they maintain a basal level of metabolic activity (Leprince *et al.* 1993). On the other hand, most endosperm cells die during seed maturation (Berger 1999). Some monocot species undergo programmed cell death in the endosperm without degeneration (Sreenivasulu & Wobus 2013). Moreover, the cytoplasmic contents of the cells are replaced by the stored food reserves (Bewley & Black 1994).

CLSM allows images with different depths and more contrast, as well as three-dimensional

information of the sample (Martinez-Nistal 2002). DAPI identified nuclei of embryonic cells and aleurone layer in of *V. friburgensis* var. *paludosa*. However, the nuclei of endosperm cells were not stained probably degraded DNA in such cells. These nuclei were conspicuous under light microscopy.

Apart from its role as a reserve tissue, the endosperm of *V. friburgensis* var. *paludosa* could also regulate embryonic development through the provision of several cell layers opposite the apex of the cotyledon. This has been previously suggested by Cecchi-Fiordi *et al.* (2001) in seeds of the genus *Tillandsia*. Several species of the genus *Vriesea* also showed these layers of compressed cells (Magalhães & Mariath 2012). According to the mentioned authors, their cell contents were consumed by the growing embryo. However, in this region, *V. friburgensis* var. *paludosa* presents reserve compounds such as starch. Therefore, the absence of reserve compounds

in this region cannot be a characteristic of the genus *Vriesea*. Furthermore, the embryo of *V. friburgensis* var. *paludosa* showed the presence of starch. However, Magalhães & Mariath (2012) found no starch in the embryos of several species of the genus *Vriesea*.

Area constriction in the radicle-hypocotyl axis of the embryo has been observed on species of genus *Tillandsia* (Cecchi-Fiordi *et al.* 1996, Morra *et al.* 2002, Magalhães & Mariath 2012). According to Magalhães & Mariath (2012), the most notable difference between the genera *Vriesea* and *Tillandsia* results from the presence/absence of that constriction zone. However, that region is present in the *V. friburgensis* var. *paludosa* embryo. Therefore, this feature cannot be used for the delimitation *Vriesea* and *Tillandsia*.

Conclusions

The morphological and histochemical features of seeds provide important information for an infrageneric

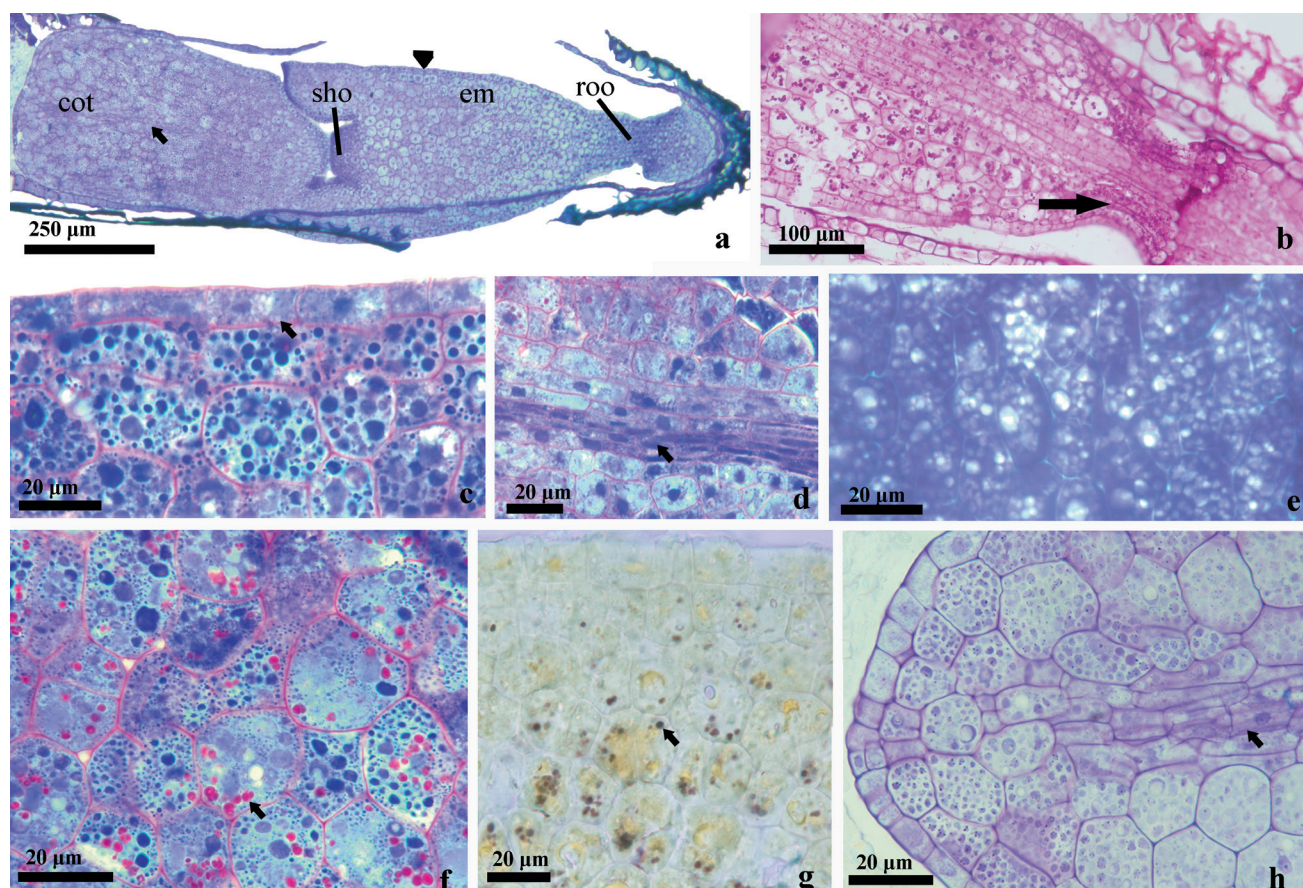


Figure 4. Longitudinal sections of the embryo of *Vriesea friburgensis* var. *paludosa* L.B. Sm. a, h. TB-O test; b. PAS; c, d, f. double-stained with PAS + CBB; e. SB-B and g. Lugol reagent. a. Structures of the embryo: protoderm (arrowhead), procambium (arrow), cotyledon, shoot and root apical meristems. b. Detail of constriction zone (arrow). c. Detail of protoderm (arrow). d. Detail of procambium elongated cells (arrow). f, g. Embryo cells with small starch grain (arrow). h. Details of cotyledon. Uniseriate epidermis and parenchyma cells with the onset of procambium (arrow). cot: cotyledon; em: embryo; sho: shoot meristem; roo: root meristem.

delimitation of bromeliads. The knowledge gained from the present study of *V. friburgensis* var. *paludosa* seeds is suggestive of strategies used by this species, including the presence of plumose appendices and accumulation of phenolic compounds in the seed coat, to establish itself and survive in inhospitable microenvironments. This species has characteristics that differ from other members of the genus *Vriesea*. Among them are: smaller amounts of starch in the embryo and the presence of a zone constriction in the hypocotyl-radicle axis. The results of this work contribute to the overall biology of *V. friburgensis* var. *paludosa*, as well as its taxonomic delimitation.

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