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Surface microsculpture and their contribution to distinguish seeds of *Eichhornia azurea* and *E. crassipes* (Pontederiaceae) of the Pantanal

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ABSTRACT – *Eichhornia azurea* and *E. crassipes* are morphologically easy to distinguish, however, their seeds are similar and differentiated only by size, which makes their identification difficult, especially when collected directly from the seedbank. We identified characters for distinguish seeds of these species through macrosculpture analyzes of the seed coat (size, shape, epidermal pattern, the structure of the primary and secondary microsculpture, funiculum, hilum, micropyle, raphe), using scanning electron microscopy. The species differ mainly by the secondary sculpture pattern, with perforated in *E. azurea* and wrinkled with micropapillae in *E. crassipes*; the external tissue of seed with smooth aspect in *E. azurea* and irregularly wrinkled in *E. crassipes*; and raphide crystals in *E. crassipes*. Our results contribute to taxonomy of the tribe *Eichhornieae* and future studies on the microsculpture analyzes of the macrophytes seed coat, in a comparative approach to understand ecological and evolutionary aspects.

Keywords: camalote, integument, micromorphology, *Pontederia*, water hyacinth

RESUMO – **Microescultura da superfície de sementes para distinguir *Eichhornia azurea* e *E. crassipes* (Pontederiaceae) do Pantanal.** *Eichhornia azurea* e *E. crassipes* são morfologicamente fáceis de distinguir, no entanto, suas sementes são semelhantes e diferenciadas apenas pelo tamanho, o que torna difícil a identificação destas, especialmente quando coletadas diretamente do banco de sementes. Identificamos caracteres para diferenciar as sementes dessas espécies através de análises de microescultura do revestimento das sementes (tamanho, forma, padrão epidérmico, estrutura da microescultura primária e secundária, funículo, hilo, micrópila, rafe), utilizando microscopia eletrônica de varredura. As espécies diferem principalmente pelo padrão secundário de escultura, sendo perfuradas em *E. azurea* e enrugadas com micropapilas em *E. crassipes*; tecido externo da semente com aspecto liso em *E. azurea* e enrugado irregularmente em *E. crassipes*; cristais de ráfide em *E. crassipes*. Nossos resultados contribuem para a taxonomia de *Eichhornieae* e para estudos futuros de análises de microesculturas do revestimento de sementes de macrófitas, em uma abordagem comparativa para entender aspectos ecológicos e evolutivos.

Palavras-chave: aguapé, camalote, micromorfologia, *Pontederia*, tegumento

INTRODUCTION

Pontederiaceae Kunth is a family that presents pantropical distribution, with many species occurring in the Neotropical region. This family includes about nine genera and 33 species, with the genus *Eichhornia* Kunth, popularly known as camalote, aguapé, or jacinto d'água, as part of the tribe Eichhornieae (Cook 1998).

Eichhornia azurea (Sw.) Kunth is a fixed floating aquatic plant with uninflated petiole, while *Eichhornia crassipes* (Mart.) Solms is a free-floating aquatic plant with inflated petiole (Nascimento *et al*. 2013). The former is distinguished by the presence of hairy tepals with fimbriated margin (Pott & Pott 2000). Both present leaf plasticity, adapting to natural changes in their habitats (Milne *et al*. 2006), and have already been confused with one another in botanical collections and in the systematic literature (Standley & Steyermark 1952). The fruits are dehiscent capsules with numerous ovoid seeds with obtuse extremities (Barroso *et al*. 1999, Pott & Pott 2000) that have limited buoyancy and usually sink in water after approximately 24 h (Barrett 1978). Even tough, they are considered hydrochoric and are dispersed by flooding (Cronk & Fenessy 2001, Pérez *et al*. 2011). These species are very distinct concerning their general morphology, however, their seeds are distinguished by their size – length: *E. azurea* with 2 mm and *E. crassipes* with 1 mm (Nascimento *et al*. 2013). When the seeds are collected far from the mother plant – such as in the seedbank or the water – and not directly from the fruit, they can be confusing and exceedingly difficult to identify, mainly due to size variation caused by the friction of seeds indumentum with the soil sediments and water.

The seed surface has an outer covering called the ¨seed coat¨, also referred to as the testa, which is the main modulator between the internal seed structures and the external environment (Karcz *et al*. 2005). The functions of the seed coat structures are related to nutrition, protection, dehydration and imbibition, dispersal, and lastly, germination (Kigel & Galili 1995), and exhibit a complex and highly diverse morphology and anatomy (Barthlott 1981).

Seed microsculpture is very useful for the identification and taxonomic delimitation of various plant groups (Arabi *et al*. 2017). The ultrastructural and ornamentation patterns observed in the seed coat under scanning electron microscopy (SEM) are considered reliable and informative for the identification of species and the elaboration of phylogenetic and taxonomic hypotheses (Koul *et al*. 2000). Due to low plasticity and variation between individuals, these characters can reflect genetic and phylogenetic differences (Barthlott 1981).

Many studies show that seed microsculpture can provide important information about the taxonomic relations in some groups, such as in Alismataceae (Matias & Soares 2009); Convolvulaceae (Groth 2001, Abdel Khalik & Osman 2007); Cyperaceae (Da Silva *et al*. 2012); Fabaceae (Gurgel *et al*. 2014, Özkan *et al*. 2015); Plantaginaceae (Muñoz-Centeno *et al*. 2006); Solanaceae (Castellani *et al*. 2008); Caryophyllaceae (Ullah *et al*. 2019); subfamily Papilionoideae (Rashid *et al*. 2017); and *Eucalyptus* species (Myrtaceae) (Lugman *et al*. 2019). Despite this, the characteristics of the seed coat sculpture of these *Eichhornia* species have not yet been described, and are very useful for the taxonomy and identification of the seeds of these species. Similar studies have been carried out with aquatic species of Polygonaceae (Martin 1954), Onagraceae (Eyde 1978), and Alismataceae (Matias & Soares 2009).

The present work investigates the micromorphological characteristics of the seed coat in *E. azurea* and *E. crassipes* for the description of the taxonomic characters useful for distinguishing these taxa.

MATERIALS AND METHODS

Plant material

The seeds (*E. azurea* – CGMS 54547 – and *E. crassipes* – CGMS 37502) were obtained from 10 submerged mature fruits removed from three specimens of each species, collected in the Pantanal ponds (between coordinates 19°24’21, 89” S, 57°01’44.57” O and 19°14’45,14” S, 57°02’18,00” O), municipality of Corumbá, Mato Grosso do Sul, Brazil. After collection, the seeds were preserved in 70% ethanol, separately by species. The taxonomic classification followed the APG IV – Angiosperm Phylogeny Group system (2016). The scientific nomenclature was verified by the Brazilian flora species database (REFLORA) and specialized literature by Pott & Pott (2000).

Microscopic studies

For the study of morphological characteristics, the following characters were observed and measured from 24 seeds (12 from each species): the length (longitudinal), the width (of the median region), shape, seed surface characteristics (color, texture, consistence), structure of the primary and secondary microsculpture, shape of the funicle, hilum, micropyle and raphe (when visible). The terminology used for seed forms was based on Brasil (2009) and Barroso *et al*. (1999); for the seed surface descriptions the terminology followed Zeng *et al*. (2004), Barthlott (1981, 1984) and Behnke & Barthlott (1983). Moazzeni *et al*. (2007) was used to verify some terms related to primary sculpture. Seeds stored in 70% ethanol were separated and dried for SEM analysis. Seeds were dehydrated using an alcoholic series (80%, 90% and 100%). After cleansing and dehydration, the seeds were processed in a Critical Point Dryer with alcohol and carbon dioxide (Gordh & Haal 1979) and positioned in stubs using double-adhesive carbon tape with the dorsal, ventral and lateral surfaces facing upwards. Thus, the characteristics of all the surfaces could be observed and photographed. Afterwards, they were coated with gold and kept under vacuum until observation.

Images and statistical analysis

The samples were examined using Jeol JSM 6380 LV Scanning Electron Microscope at 15 K = kv and related electro-digital photomicrographs. To test the difference between the length/width between the seeds of the two species, we used Mann-Whitney Rank Sum Test ($p<0.05$) in the SigmaPlot® 12.0 program.

RESULTS

The length ($U = 0$, $p<0.001$) and width ($U = 17.50$, $p < 0.001$) (Fig. 1 and Tab. 1) differ between the seeds of the two species. Both (Figs. 2, 3, and 4) have seeds that are terete, nearly oblong, with color varying from yellowish-brown to black (Figs. 2A, 3A, 3B and Tab. 1).

Table 1. Comparative characteristics of *Eichhornia azurea* and *E. crassipes* seed coat, removed from the fruit.

Characteristic	*Eichhornia azurea*	*Eichhornia crassipes*
Seed length (mm)	1.5-2.3	0.9-1.3
Seed width (mm)	0.7-1.4	0.6-0.7
Seed shape	Terete/oblong	Terete/oblong
Color	Yellowish-brown/ black color	Yellowish-brown/ black color
Testa	With ribs/glabrous	With ribs/glabrous
Primary sculpture	Reticulated pattern	Reticulated pattern
Secondary sculpture	Perforated	Rugate
Raphides crystals	Absent	Present

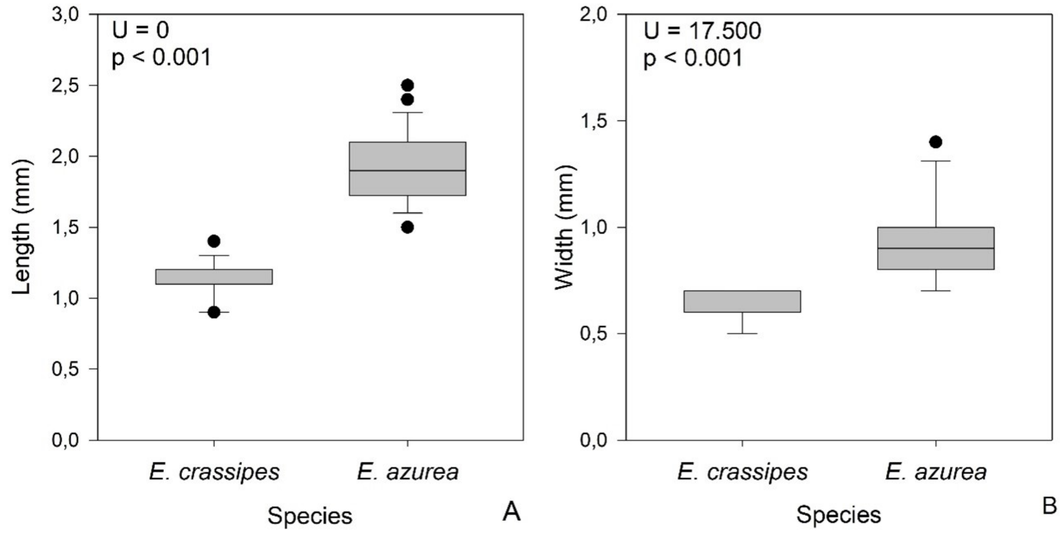

Figure 1. A. Average length and **B.** width of *Eichhornia crassipes* and *Eichhornia azurea* seeds (Mann-Whitney Test, $p < 0.001$).

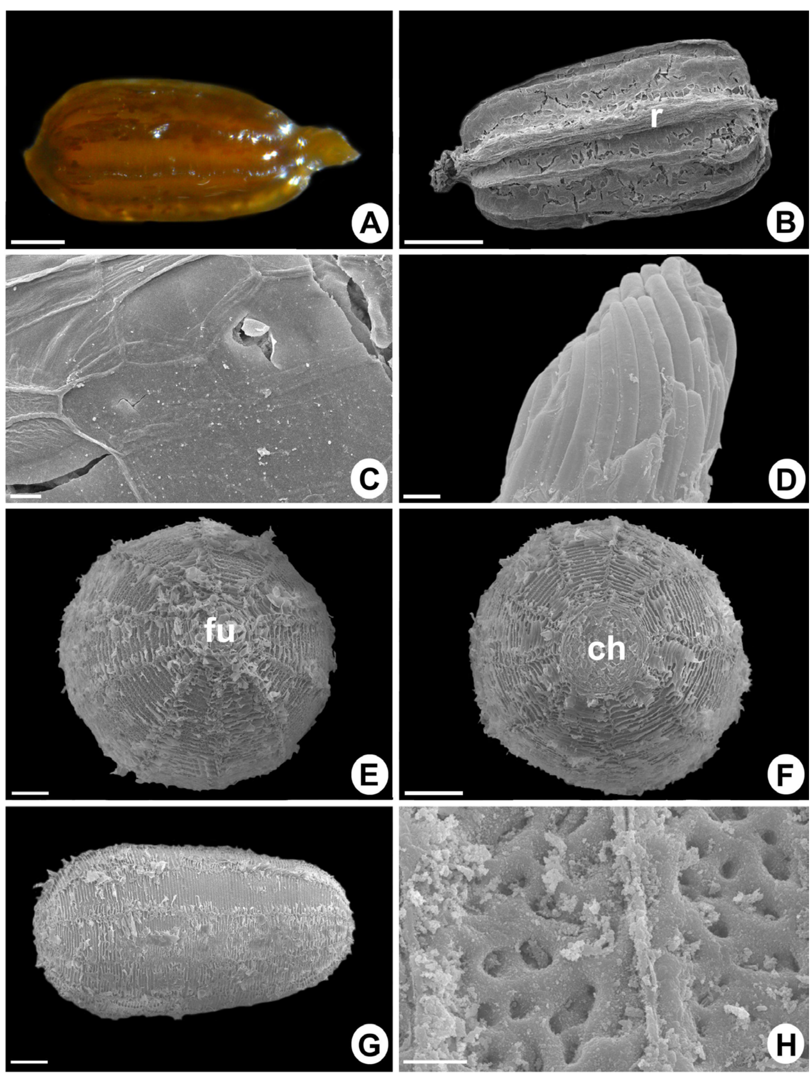

Figure 2. *Eichhornia azurea* seeds. **A.** general aspect; **B.** seed involved in an external tissue hiding the secondary sculpture; **C.** surface detail of the external tissue; **D.** detail of funicle with a striated microsculpturing; **E.** seed in apical view with central funicle; **F.** chalazal region; **G.** seed without the external tissue; **H.** the secondary sculpture with a perforated pattern. (r = raphe; fu = funicle; ch = chalaza). Bars: **A-B** = 500 µm, **C** = 10 µm, **D-E** = 100 µm, **F-G** = 200 µm, **H** = 5 µm.

The seeds of *E. azurea* were frequently found covered with a thin external tissue (Fig. 2B) with smooth aspect (Fig. 2C) that covered the entire surface, including structures like the chalaza and the ribbed testa. The seeds of *E. crassipes* may also present this external tissue (Fig. 3B), however, the surface sculpture is irregularly wrinkled (Fig. 3C). In *E. azurea*, when this external tissue is present, the apical funicle forms a central elevated projection with, around 0.35 mm length, and a striated aspect (Fig. 2D), and when absent, the funicle was indistinct (Fig. 2E). Likewise, the funicle of *E. crassipes* is a central elevated projection, but smaller (0.15 mm of length) and with a perforated aspect (Fig. 3D).

The seed base is truncated, and the chalaza position is discernible by a circular spot covered by residual tissues, that gives a scales appearance in the *E. azurea* (Fig. 2F). In *E. crassipes*, terminal chalaza has a darker circular spot (Fig. 3E), smooth.

In both species, the testa has a glabrous surface, with straight periclinal and anticlinal cell walls, forming rectangular cells, evenly arranged in long distinct rows. The primary sculpture has a reticulated pattern, and the seed surface presents cells with expansions of the anticlinal wall. The cell-wall appears to be thicker in the longitudinal axis than in the transverse one (Fig. 3F). The joining of more prominent expansions with inconspicuous expansions, confer the ribbed appearance of the seeds (Figs. 2G and 3G). The external periclinal face has a secondary sculpture with a perforated pattern (Fig. 2H) in the *E. azurea* and a rugate secondary sculpture, with inconspicuous micro papillae (Fig. 3H) in the *E. crassipes*.

In the *E. crassipes*, the seeds present idioblasts (Fig. 4A) with calcium oxalate crystals forming raphides (Figs. 4B and 4C), abundantly distributed almost exclusively in the raphe region (Fig. 4D).

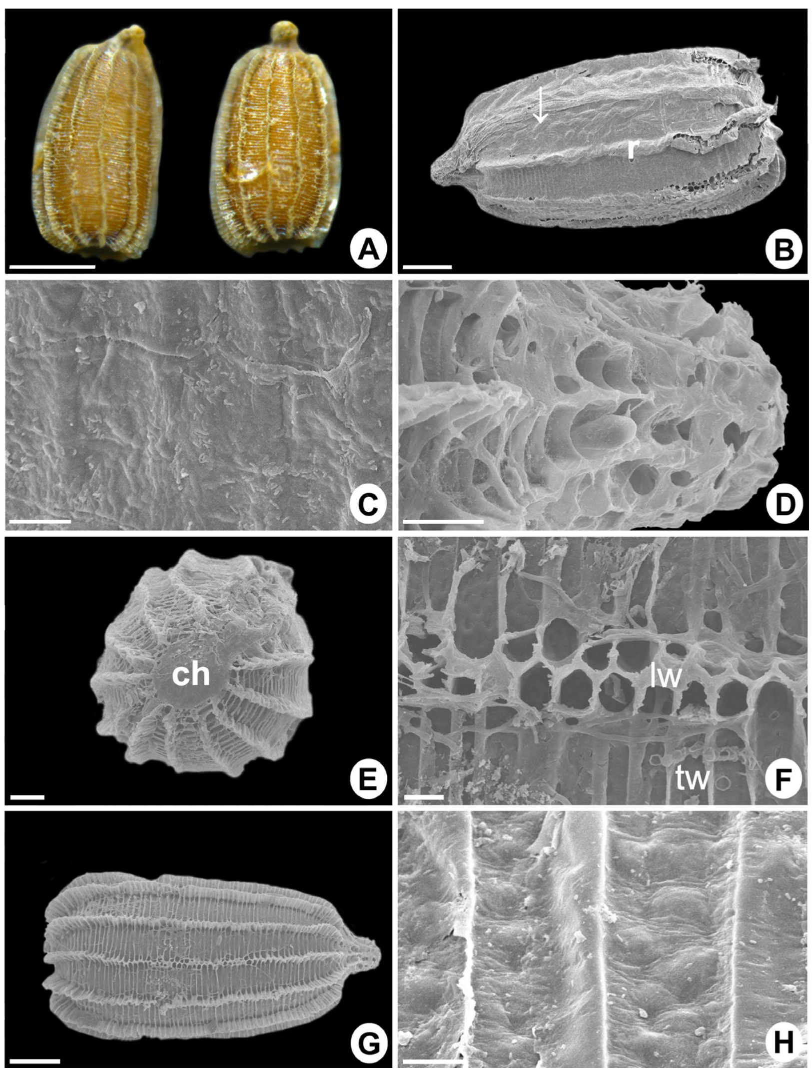

Figure 3. *Eichhornia crassipes* seeds. **A.** seeds photographed with a stereomicroscope; **B.** seed involved in an external tissue hiding the secondary sculpture, arrow showing the idioblasts; **C.** detail of the surface of the external tissue; **D.** detail of funicle with a perforated pattern; **E.** chalazal region; **F**. difference between the expansions in the anticlinal walls; **G.** primary sculpture with reticulate pattern – rib appearance; **H.** secondary sculpture with a rugate pattern with micropapillae. (r = raphe; ch = chalaza; lw = longitudinal anticlinal wall; tw = transversal anticlinal wall). Bars: **A** = 500 µm, **B-G** = 200 µm, **C-H** = 10 µm, **D** = 50 µm, **E** = 100 µm, **F** = 20 µm.

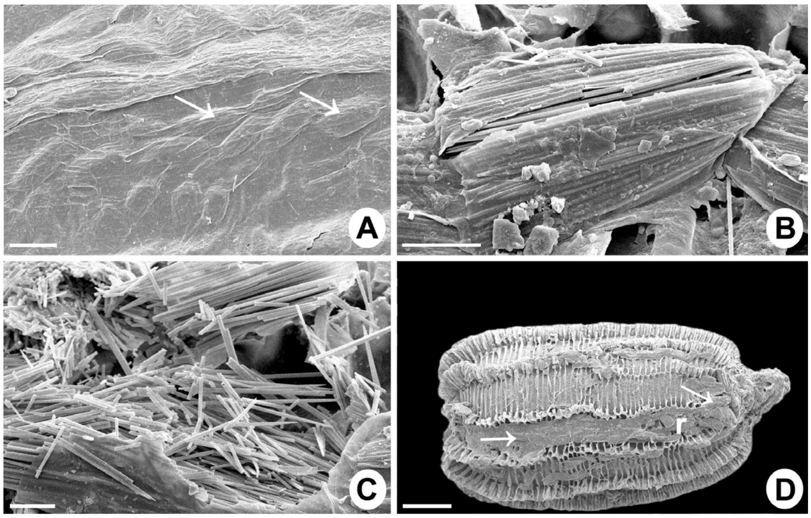

Figure 4. *Eichhornia crassipes* idioblasts. **A.** raphes region with groups of idioblasts (arrows); **B.** idioblast with raphide crystals; **C.** detail of a ruptured idioblast showing raphide crystals; **D.** highlight of the raphe region (arrow) with idioblasts. (r = raphe). Bars: **A =** 50 µm, **B-C =** 10 µm, **D** = 200 µm.

DISCUSSION

The seeds of *E. crassipes* and *E. azurea* resemble each other because they present terete shapes, primary sculpture with a reticulated pattern, as described by Horn (1987), and longitudinal wings that insure a ribbed appearance to the seeds. According to Agostini (1974), anatropous ovules are characterized by apical hillum with funicle commonly persistent and chalaza opposite to funicle. However, we found a secure way of differentiating the species using the secondary pattern of microsculpture; which is perforate in *E. azurea* and rugate in *E. crassipes*.

According to Karcz *et al*. (2005), the pattern of secondary sculpture refers to the thin cuticle ornamentation of the cell walls. Cuticles are frequently present in the seed coat, which might be found on the surface of the seed or internally, between the testa and tegmen, or between the seed coat and the nucellus (Kigel & Galili 1995). Thus, although we did not test for the presence of a cuticle, we assume that the secondary sculpture in both species is evidence of this.

As described by Nascimento *et al*. (2013), *E. azurea* seeds are characterized by their around 2 mm length, while *E. crassipes* around 1 mm. However, considering the results obtained in this study, there is a size variation that exceeds the values described by the authors. Instead, distinction using micromorphology seed coat characters is highly reliable.

Crystals are widely distributed in angiosperms and may be present in the seed coat, whether in parenchyma or sclerenchyma cells, as stated by Kigel & Galili (1995), commonly presenting idioblasts with raphides, as observed in the raphe-chalazal region of *E. crassipes*. Pereira *et al*. (2010) observed calcium oxalate crystals in the blade of *E. crassipes*, suggesting an association with the promotion of greater rigidity and support capacity for the organ, thus maintaining the structure of parenchyma cells. This characteristic was confirmed by Pereira (2010), mainly for the palisade and spongy parenchyma. Such structural function can be present in the seed to maintain the raphe configuration, and, consequently, the communication between the funicle and chalaza, preventing damage to vascular bundles.

Another factor that can be related to the presence of raphide crystals is stress. Mahmood *et al*. (2005) suggested that crystals can be associated with high levels of calcium in plants, as individuals in stressful situations showed crystals in parenchyma cells of vegetative organs. Mazen & El Maghraby (1997) found that *E. crassipes* is able to store heavy metals like cadmium, lead, and strontium into crystals, thus preventing them from causing greater damage to the plant. The presence of these crystals guarantees *E. crassipes* potential use in phytoremediation techniques, thus to its ability to absorb and crystallize such metals.

The concentration of these raphide crystals in the raphe region can be related to the strategy to protect the embryo, preventing contaminants from being transferred from the mother plant to the seed. However, more studies are necessary to verify if this is truly occurring.

We highlight the fact that *E. azurea* and *E. crassipes* seeds can be recognize based on the characteristics of their secondary sculpture. The external surface of the seed coat was the most important characteristic for delimiting seeds found in the seedbank and will contribute to the taxonomy of the tribe *Eichhornieae*. Our results also open avenues for future studies on the microsculpture analyzes of the seed coat in other species of aquatic macrophytes in a comparative approach to better understand their ecological and evolutionary aspects.

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