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# Pericarp ontogeny of *Tapirira guianensis* Aubl. (Anacardiaceae) reveals a secretory endocarp in young stage

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#### ABSTRACT

Most species of Anacardiaceae have drupes containing secretory structures. The substances produced by these structures may have importance to industry and folk medicine, and may even cause allergenic effects. This work describes the ontogeny of pericarp of *Tapirira guianensis* with an emphasis on the secretory structures present at different stages of development. Ovary and fruits in various stages of development were collected, fixed and processed for studies using light and scanning electron microscopy according to conventional techniques. Histochemical tests were employed to identify the major metabolites present in the tissues. The fruit is a drupe formed by exocarp, mesocarp containing secretory ducts and idioblasts, and endocarp with some lignified layers. Fruit growth occurs through the division and elongation of cells. The secretory ducts produce mainly phenols and lipids and are active during all stages of development. The secreted substances protect the fruit against pathogens and predators. In ripe fruits the cells of the mesocarp accumulate starch. This study is the first report of the presence of a secretory endocarp in young fruits of a species of Anacardiaceae. The substances produced by the endocarp in early developmental stages may play an important role in seed dispersal and germination.

Keywords: cashew family, drupe, fruit, mucilages, secretory ducts

## Introduction

Most species of Anacardiaceae have drupaceous fruits (Wannan & Quinn 1990; Gonzalez & Vesprini 2010). Wannan & Quinn (1990) studied fruits belonging to 29 genera of Anacardiaceae and recognized two basic types of endocarp: (1) the Spondias type - consisting of a mass of sclerenchyma with irregular orientation and (2) the Anacardium type - characterized by a lignified inner epidermis and a layered arrangement, including sclereids in palisade. The first type occurs in Spondioideae tribe and the second type in Anacardioideae tribe (Wannan & Quinn 1990; Pell *et al.* 2011).

Secretory structures are quite common in fruits of Anacardiaceae (Von-Teichman 1987; Wannan & Quinn 1990; Carmello-Guerreiro & Paoli 2000; 2002; 2005; Machado & Carmello-Guerreiro 2001; Lacchia & Carmello-Guerreiro 2009; González & Vesprini 2010). The most frequent structures are the ducts and cavities, both of which may

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produce resin, gum or a mixture of substances (Venning 1948; Metcalfe & Chalk 1950; Lacchia & Carmello-Guerreiro 2009). According to Barroso *et al.* (2007), the mesocarp of the representatives of this family can be fleshy (*Mangifera* and *Spondias*) or spongy with ducts or cavities (e.g., *Anacardium, Astronium* and *Myracrodruon*). In the latter, the secretory system is quite developed, and the ducts or cavities occupy almost the entire mesocarp (Carmello-Guerreiro & Paoli 2000). The substances produced may have importance in industry and folk medicine and can even cause allergenic effects (Dong & Bass 1993; Barroso *et al.* 2007; Pell *et al.* 2011).

Idioblasts (Carmello-Guerreiro & Paoli 2005), glandular trichomes (Li *et al.*1999) and pericarpial nectaries may also occur (Wunnachit *et al.* 1992; Rickson & Rickson 1998). These nectaries are already present in flowers and are maintained in the fruits, improving the viability and seed dispersal. None of these structures are exclusive to secreting fruits but basically occur in the whole plant (Lacchia & Carmello-Guerreiro 2009; Lacchia *et al.* 2016a; 2016b).

Tapirira belongs to tribe Spondioideae of Anacardiaceae (Pell et al. 2011). This genus includes about eight species of trees occurring mainly in tropical areas of America (Wendt & Mitchell 1995; Tropicos 2016). Tapirira guianensis is widely distributed throughout Brazil and other countries of South and Central America (Tropicos 2016), especially in areas of moist soil (Santana et al. 2009). It is a dioecious, important species for logging, medicinal use and may be employed in the recovery of degraded areas and riparian forests (Lorenzi 2002; Lenza & Oliveira 2005; Santana et al. 2009). The drupes of this species are greatly appreciated by birds (Corrêa 1978). Von-Teichman (1990) conducted an anatomical study of the ripe fruit of this species, which is classified as Spondias type. However, despite describing them, the author does not emphasize the secretory structures.

Therefore, this study aimed to examine the ontogeny of the pericarp of *Tapirira guianensis* with an emphasis on secretory structures present in different stages of development. Through histochemical tests the secretion produced by different structures, in different stages of development, was characterized. We also describe the first case of a secretory endocarp in Anacardiaceae.

# **Materials and methods**

#### Plant material

Anthetic female flowers and fruits at various developmental stages of *Tapirira guinanensis* Aubl. were collected in three areas in the state of São Paulo, Brazil: the Itirapina experimental station (22°13'S; 47°51'W), the Mogi Guaçu experimental station (22°10'S; 47°07'W) and an additional area of cerrado (Brazilian savannah) in the District of Sousas, Campinas (22°51'S; 46°57'W). The Itirapina

experimental station includes vegetation of Cerrado and Campo Cerrado, while the Mogi Guaçu experimental station comprises *sensu lato* Cerrado vegetation, according to the classification of Ribeiro & Walter (1998). Collections were made from March to December 2011 and from January to February 2012. Vouchers are deposited in the UEC herbarium (UEC 182229).

### Light microscopy (LM)

For anatomical studies the samples were fixed in FAA (formaldehyde, acetic acid, 50% ethanol) for 24 h (Johansen 1940). The material was then dehydrated in an ethanol series and embedded in hydroxyethyl methacrylate resin (Historesin<sup>®</sup> Leica), according to Gerrits & Smid (1983). Transverse and longitudinal sections 8  $\mu$ m thick were obtained using a Microm HM340E rotary microtome and stained with 0.05% Toluidine Blue in sodium acetate buffer with a pH of 4.7 (O'Brien *et al.* 1964). All slides were mounted with water and the images captured with an Olympus DP71 digital camera coupled to an Olympus BX51 microscope.

#### Histochemistry

For the histochemical tests, the material was fixed in FAA (for hydrophilic substances) for 24 h (Johansen 1940) and in BNF (buffered neutral formalin, for lipophilic and phenolic substances) for 48 h (Lillie 1965). The material was then also dehydrated in an ethanol series and embedded in hydroxyethyl methacrylate resin (Gerrits & Smid 1983). Transverse and longitudinal sections 8  $\mu$ m thick were obtained using a Microm HM340E rotary microtome. The treatments performed can be found in Table 1. The results were recorded using an Olympus DP71 digital camera coupled to an Olympus BX51 microscope.

### Scanning electron microscopy (SEM)

For micromorphological analysis, samples fixed in FAA were dehydrated in an ethyl series, critical point dried, and sputter coated with gold. Observations were carried out using a Jeol JSM 5800 LV scanning electron microscope at 10 kV equipped with a digital camera.

### Stages of development

Based on the anatomical changes that occur during fruit development, the results were grouped into four stages: (i) ovary of the anthetic flower, (ii) very young fruit (3-5 mm in length), (iii) immature fruit with verified elongation or cell growth (5.1-8 mm in length) and (iv) ripe fruit (8.1-10 mm in length) (Fig. 1). The pericarp is divided into three clearly differentiated parts in all phases of development: exocarp, mesocarp and endocarp.

## Results

#### Stage I

The outer epidermis of the ovary is uniseriate (Fig. 2A-B), composed of juxtaposed cells coated with a thick cuticle and containing stomata (Fig. 2B). The ovary is covered with glandular and tector trichomes (Fig. 2C-F). The glandular trichomes contain a bicellular, uniseriate stalk while the secretory head is multicellular and multiseriate (3-4 rows) (Fig. 2E). The non-glandular trichomes are elongated, multicellular and uniseriate with tapered apex and thick wall (Fig. 2F). The ordinary epidermal cells and secretory trichomes accumulate phenolic substances (Fig. 2G, Tab.2). Furthermore, the trichomes showed a positive reaction to lipids and polysaccharides (Tab.2).

The ovarian mesophyll can be divided into three regions based on the size and arrangement of the cells. The outermost region underlying the external ovarian epidermis consists of parenchyma cells in an intense process of cell division (Fig.2A). This region consists of eight to ten layers of cells with evident nuclei and thin walls (Fig. 2A-B). Druses are distributed throughout this region (Fig.2A). The middle portion contains vascular bundles and secretory ducts (Fig.2A). The secretory ducts have a one layered epithelium (Fig. 2H) which releases a secretion into the lumen formed by droplets and a more fluid portion composed of lipids, phenolic compounds and mucilage (Tab.2). The internal region of the ovarian mesophyll consists of 12-15 cell layers of parenchyma in an intensive process of division (Fig. 2A) with evident nuclei and thin cell walls.

The inner epidermis of the ovary is uniseriate, formed by juxtaposed cells with evident nuclei in central position (Fig. 2I). These cells undergo periclinal divisions forming a biseriate inner epidermis (Fig. 2I).

#### Stage II

At this stage the increase of pericarp layers primarily occurs. The exocarp, derived from the ovarian outer epidermis, is quite similar to the previous stage (Fig. 3A), except that a significant loss of trichomes occurs.

The mesocarp develops from the fundamental ovarian tissue and is divided into three zones: outer, median and

Table 1. Histochemical tests used in the characterization of the substances.

Test	Substance detected
Sudan black B (Pearse 1980)	lipids
Nile blue (Cain 1947)	acidic and neutral lipids
Lugol's reagent (Johansen 1940)	starch
Ferric chloride (Johansen 1940)	phenolic compounds
Wagner's reagent (Furr & Mahlberg 1981)	alkaloids
Schiff's reagent (PAS) (McManus 1948)	carbohydrates
Ruthenium red (Gregory & Baas 1989)	acidic mucilages
Tannic acid and ferric chloride (Pizzolato & Lillie 1973)	mucilages
Copper acetate and rubeanic acid (Ganter & Jollés 1969; 1970)	fatty acids
Aniline blue black (Fisher 1968)	proteins



**Figure 1.** Stages of development of *Tapirira guianensis* fruit under stereomicroscope. Stage I: ovary of the anthetic flower, Stage II: very young fruit (3-5 mm in length), Stage III: immature fruit with verified elongation or cell growth (5.1-8 mm in length) and Stage IV: ripe fruit (8.1-10 mm in length).

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Test	outer epidermis/ epicarp	glandular trichomes	outer mesocarp	median mesocarp	inner mesocarp (idioblasts)	endocarp	secretory ducts
Sudan black B	-	+ (I, II)	-	-	-	+ (II)	+ (I, II, III, IV)
Nile blue	-	+ (I, II)	-	-	-	+ (II)	+ (I, II, III, IV)
Lugol's reagent	-	-	+ (IV)	+ (IV)	-	-	-
Ferric chloride	+ (I, II, III, IV)	+ (I, II)	-	-	+ (II, III)	-	+ (I, II, III, IV)
Wagner's reagent	-	-	-	-	-	-	-
Schiff's reagent (PAS)	-	+ (I, II)	-	-	-	+ (II)	+ (I, II, III, IV)
Ruthenium red	-	-	-	-	-	-	+ (I, II, III, IV)
Tannic acid and ferric chloride	-	-	-	-	-	-	+ (I, II, III, IV)
Copper acetate and rubeanic acid	-	-	-	-	-	-	-
Aniline blue black	-	-	-	-	-	-	-

Table 2. Histochemical tests in Tapirira guianensis fruits at different stages of development.

Notes: I (stage I), II (stage II), III (stage III), IV (stage IV), + (positive reaction), - (negative reaction).

inner mesocarp (Fig. 3A). In the outer mesocarp, there is an increased number of layers, which comprise about 20 layers of parenchyma cells, which are still in the process of cell division in several planes. In median mesocarp the secretory ducts are distributed. In this phase the secretory ducts are delimited by a one layered epithelium surrounded by a sheath (2-3 layers) (Fig. 3B). The secretion responded positively to lipids, total polysaccharides, phenolic compounds and mucilage (Fig. 3C-F) (Tab.2). The epithelial cells degenerate adding to part of the secretion (Fig. 3G), while the sheath cells undergo periclinal divisions renewing the epithelium. In the inner mesocarp, idioblasts with phenolic content appear (Fig. 3H) (Tab.2). This region presents cell divisions in several levels.

The endocarp, derived from inner ovarian epidermis, consists of two layers of secretory cells (4A-D). Under SEM, several drops of secretion were observed in the endocarp (Fig. 4E), which were also observed on the developing seed coat (Fig. 4F). The endocarp secretion responded positively to lipids, mucilages and polysaccharides tests (Fig. 4A-D) (Tab.2).

#### Stage III

At this stage, all trichomes of the exocarp are lost. The cells of the outer mesocarp present pectic-cellulosic thickening becoming collenchymatous (Fig. 5A-B), the thickest being close to the exocarp (Fig. 5B). The median mesocarp increases the parenchyma cell layers between the secretory ducts and the vascular bundles, now well-developed (Fig. 5A). In the inner mesocarp, the most striking differences arise. Intercellular spaces become quite conspicuous among the parenchyma cells (Fig. 5C). In the last 3-4 layers, the vast majority of internal mesocarp cells differentiates, forming elongated sclereids in longitudinal, transverse and oblique directions (Fig. 5C). The cells that do not lignify remain parenchymatic with many containing

crystals (Fig. 5C). In the endocarp, the layer adjacent to the inner mesocarp differs in elongated sclereids with the last layer, in contact with the locule, remaining non-lignified (Fig. 5C). In this phase, the endocarp is not secretory; however, secretions produced in the previous stage remain covering the entire endocarp and seed coat.

#### Stage IV

The most evident change in the ripe fruit exocarp is the appearance of lenticels (Fig. 6A). The external and median mesocarp cells accumulate starch (Fig. 6B-C) (Tab.2). Parenchyma cells in the median mesocarp layers divide and stretch in several directions (Fig. 6D). In the inner mesocarp, some of the idioblasts that accumulated phenolic compounds are now differentiated in sclereids (Fig. 6E). In the endocarp, the layer in contact with the locule (which was secretory) now also lignifies, forming sclereids (Fig. 6F).

## Discussion

The fruit of *T. guianensis* is classified as a drupe since the exocarp and mesocarp are fleshy and, the endocarp is formed by several layers of sclerified cells (Von-Teichman 1990). In drupes, the exocarp acts as a protective outer layer, the mesocarp is usually parenchymal and endocarp is hard, with layers that protect the seed (Roth 1977; Spjut 1994). According to Roth (1977), the exocarp and the endocarp may be formed by a single layer derived from the outer and the inner ovarian epidermis, respectively. In this case, they are called *sensu stricto* exocarp or endocarp. When they also include derived mesocarp layers, they are called sensu lato exocarp or endocarp. The exocarp of T. guianensis is formed by the outer layer, derived from the outer epidermis of the ovary, and by several layers of collenchymatous cells formed from the outer mesocarp. Therefore, in this species the exocarp is known as *sensu lato*, according to Roth (1977).



**Figure 2.** Structural and histochemical aspects of *Tapirira guianensis* ovary. (A) General aspect of the ovary in longitudinal-section. Note the cells in intense process of division (arrows). (B) Outer epidermis of the ovary in cross-section. (C-D) Electron micrographs of trichomes in the outer epidermis. (E) Glandular trichome in longitudinal section. Note the bicellular and uniseriate stalk and the multicellular and multiseriate secretory head (F) Elongated, multicellular and uniseriate non-glandular trichome in longitudinal section. (G) Outer epidermis showing positive reaction to ferric chloride. (H) Fundamental tissue in cross-section showing the secretory ducts and vascular bundles still in development. The secretory ducts have an epithelium which releases a secretion into the lumen. (I) Inner epidermis in cross-section showing the periclinal divisions (arrows). Abbreviations: ct, cuticle; dr, druse; ep, epithelium; ft, fundamental tissue; ie, inner epidermis; lu, lumen; oe, outer epidermis; sd, secretory duct; st, stomata; vb, vascular bundle.

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**Figure 3.** Structural and histochemical aspects of *Tapirira guianensis* pericarp in Stage II. (A) General aspect of the pericarp in crosssection. (B) Detail of the secretory ducts with uniseriate epithelium and multiseriate sheath. (C) Secretory duct showing positive reaction to Schiff reagent. (D) Secretory duct showing positive reaction to Sudan black B. (E) Secretory duct showing positive reaction to ferric chloride. (F) Secretory duct showing positive reaction to Nile blue sulphate. (G) Detail of the epithelium. Note the degeneration of cells, eliminated together with the secretion, while the sheath cells undergo periclinal divisions renewing this epithelium. (H) Idioblasts showing a positive reaction to ferric chloride Abbreviations: dr, druse; ec, exocarp; en, endocarp; ep, epithelium; id, idioblast; im, inner mesocarp; mm, median mesocarp; om, outer mesocarp; vb, vascular bundle.



**Figure 4.** Histochemical and SEM of the endocarp in Stage II. (A) Endocarp and idioblasts in Toluidine blue coloration. Note the secretion droplets (\*). (B) Endocarp and idioblasts showing positive reaction to Schiff reagent. (C) Endocarp showing positive reaction to tannic acid and ferric chloride. (D) Endocarp and idioblasts showing positive reaction to Nile blue sulphate. Note the secretion droplets (\*). (E) Electron micrograph of secretion droplets (arrow) in endocarp. (F) Electron micrograph of secretion droplets (arrow) in funicle. Abbreviations: en, endocarp; id, idioblast

Von-Teichman (1990), despite having studied just the ripe fruit, also considers the exocarp of this species as *sensu lato*. In this case the external mesocarp is considered part of the exocarp due to their functional aspect (Roth 1977). In general, the exocarp of the Spondioideae representatives, a tribe belonging to *T. guianensis*, consists of small, thin walled, tightly packed parenchyma cells, which may develop thick cellulosic walls (Wannan & Quinn 1990). In Anacardioideae, the exocarp may be sclerified, e.g., *Lithraea molleoides* (Carmello-Guerreiro & Paoli 2005) and *Schinus terebinthifolius* (Carmello-Guerreiro & Paoli 2002). The ovarian epidermis and developing fruit are covered by tector and glandular trichomes. This characteristic was not observed by Von-Teichman (1990) since he studied only the ripe fruits. The trichomes play an important role in mechanical protection of the fruit in development, and also act in protection against ultraviolet radiation (Roth 1977). This protection is enhanced by phenolic compounds produced by the epidermis and the glandular trichomes (Castro & Demarco 2008), these substances assist in protection against herbivory (Fahn 1979; Calvo *et al.* 2010) and against the microorganism proliferation (Calvo *et al.* 2010).

Secretory ducts are widely distributed in the median region of the mesocarp. They play an important role during all phases since they remain active during



**Figure 5.** Structural aspects of *Tapirira guianensis* pericarp in Stage III. (A) General aspect of the pericarp in cross-section. (B) Exocarp and outer mesocarp in cross-section. Note the pectic-cellulosic thickening and druses. (C) Detail of inner mesocarp and endocarp. Note intercellular spaces (\*) and the formation of sclereids. The layer in contact with the locule, remaining non-lignified. Abbreviations: cr, crystal; ct, cuticle; dr, druse; ec, exocarp; en, endocarp; id, idioblast; im, inner mesocarp; mm, median mesocarp; om, outer mesocarp; pc, pectic-cellulosic thickening; sc, sclereid; st, stomata; vb, vascular bundle.

the whole fruit development. They produce the same substances independent of the phase in which the fruit is. In Anacardiaceae several studies have mentioned the presence of resiniferous ducts in fruits, always associated with vascular bundles (Von-Teichman 1987; 1990; Wannan & Quinn 1990; Von-Teichman & Van-Wyk 1993; 1994; 1996; Carmello-Guerreiro & Paoli 2000; 2005; Machado & Carmello-Guerreiro 2001; González & Vesprini 2010). This is a constant feature for the family, regardless of the tribe to which the species belong.

Lacchia & Carmello-Guerreiro (2009) closely studied the formation of these ducts in *T. guianensis* fruit, as well as the secretory mechanism. The authors concluded that the formation of the ducts is schizogenous and the secretory mechanism is eccrine. These ducts have a mixed secretion, containing lipids, polysaccharides and phenolic substances. It is possible to verify the disruption of the epithelium cells and consequently extravasation of the secretion into the lumen with cell debris with continuous replacement of the epithelium by the meristematic activity of the sheath, which characterizes the mode of secretion as holocrine. The occurrence of a parenchymatous sheath surrounding secretory ducts producing new epithelium cells has been reported in several studies (Monteiro *et al.* 1995; 1999; Machado & Carmello-Guerreiro 2001; Bennici & Tani 2004; Rodrigues *et al.* 2011a; 2011b).

The large amount of phenolic substances found in *T. guianensis* fruit, stored in ducts, exocarp and idioblasts, is also found in other species of the family, referred to as tanniferous substances (Von-Teichman 1987; Von-Teichman & Van-Wyk 1993; 1994; 1996; Piennar & Von-Teichman 1998; González & Vesprini 2010). Von-Teichman (1990) also report these substances in ducts and idioblasts of *T. guianesis* fruits. However, it does not perform tests to confirm the chemical nature of these substances. There are several functions of the phenolic substances, among

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**Figure 6.** Structural and histochemical aspects of *Tapirira guianensis* pericarp in Stage IV. (A) Lenticel formation in cross-section. (B) Outer mesocarp showing positive reaction to Lugol. (C) Median mesocarp showing positive reaction to Lugol. (D) General aspect of the fruit in cross-section. (E) Detail of inner mesocarp and endocarp. Note intercellular spaces (\*) and the fact that some of the cells that accumulated phenolic compounds now are differentiated sclereids (arrows). (F) Endocarp. Note that the layer in contact with the locule now also lignifies. Abbreviations: ec, exocarp; el, elongated cell; en, endocarp; im, inner mesocarp; mm, median mesocarp; om, outer mesocarp; sc, sclereid; vb, vascular bundle.

them chemical defense against pathogens, herbivory and ultraviolet radiation (due to its antioxidant power) and an aid in the dispersal by birds (inducing regurgitation) (Roshchina & Roshchina 1993; Von-Teichman & Van-Wyk 1993; 1994; Aguilar-Ortigoza & Sosa 2004; Castro & Demarco 2008).

Another important feature in mesocarp is the elongation of cells located between the secretory ducts and the vascular bundles. These elongated cells were also observed by Von-Teichman (1990). The cell divisions that occur in this region are responsible for the separation that occur between the vascular bundles and the ducts. In young stages we observe the secretory ducts and the vascular bundles very close. Furthermore, these cells are the main site of starch accumulation in the last stage of development, an energetic substance demanded by dispersers (Roth 1977).

According to the organization of the endocarp, Wannan & Quinn (1990) proposed a classification of two kinds of pericarp for Anacardiaceae: (1) the Spondias type with endocarp comprising a mass of sclerenchyma with irregular orientation and (2) the Anacardium type with endocarp in layers, comprising a lignified outer epidermis and parenchyma arranged in layers, including sclereids in palisade. Thus, the characteristics of the *T. guianensis* endocarp fall under the Spondias type. Von-Teichman (1990) studied the structure of the ripe fruit of *T. guianensis* and found that the endocarp is not massive, but relatively thin in comparison to another species of the same tribe, i.e., Lannea discolor Engl. (Von-Teichman 1987). We not report the presence of operculum, which agrees with the observations of Von-Teichman (1990). Moreover, the T. guianensis endocarp is considered sensu lato since the fully developed fruit includes the sclerified layers derived from the inner mesocarp. A novel aspect observed in *T. guianensis* is the presence of a secretory endocarp in unripe fruits. The production of hydrophilic mucilages by the endocarp may facilitate seed hydration (Western 2012). In cases in which the mucilage covers the seed, such as in *Euphorbia* species, the mucilage may mediate germination under waterlogged conditions, prevent seed predation by adherence to soil and promote seed dispersal by attachment to animals (Demarco & Carmello-Guerreiro 2011; Western 2012). These mucilages are acids or neutral complex polysaccharides of high molecular weight (Fahn 1979) that undergo substantive swelling upon hydration (Western 2012). The production of lipids may be an important chemical defensive against fungi and other microorganisms (Fahn 1979). As described for Heeria angentea (Von-Teichman & Wan-Wyk 1996), in *T. guianensis* the parenchymatous cells of the endocarp is replaced by a sclerenchymatous endocarp at the last stage of fruit development. According to Von-Teichman (1990), the endocarp hardening can be related to the seed protection for seeds lacking a mechanical protective layer. The secretory endocarp is naturally replaced by a sclerenchymatous endocarp, once the seed has not a mechanical protective layer (Von-Teichman 1990).

#### Conclusions

The results described herein suggest that fruits of *T. guianensis* have several characteristics related to fruit protection against pathogens and predators due to the presence of ducts secreting gum-resin, idioblasts containing phenolic substances and druses widely distributed in the mesocarp. The substances produced by the endocarp in young stages may play an important role in seed dispersal and germination. The presence of a secretory endocarp is first reported in the family.

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