Journal of Applied Botany and Food Quality 93, 248 - 256 (2020), DOI:10.5073/JABFQ.2020.093.031

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Histological and cytological comparison of seed structures of *Theobroma cacao*, *Theobroma grandiflorum* and *Theobroma bicolor* relevant to post-harvest processing and product quality

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(Submitted: July 9, 2020; Accepted: September 27, 2020)

Summary

For the present study, anatomical details of seeds of *Theobroma cacao* L., *Theobroma grandiflorum* (Willd. ex Spreng.) Schum. and *Theobroma bicolor* Humb. & Bonpl. were investigated using light and transmission electron microscopy (LM and TEM). The focus was on features with possible significance for the course of fermentation and drying to generate cocoa-like aroma. It was found that the seed coat of *T. cacao* has the smallest diameter and contains only a thin sclereid layer, whereas *T. grandiflorum* and especially *T. bicolor* exhibit much more massive palisade layers. The thicker seed coats of *T. grandiflorum* and *T. bicolor* could delay the passage of liquids during fermentation and seed deshelling may require more effort than with *T. cacao*. In addition, existing differences in the amount of plant mucilages and phenolic substances in the testa may affect the composition and activity of the microflora during fermentation. Differences were also evident in the cotyledon tissue, which is processed to obtain cocoa-like products. Besides a higher degree of maceration of the parenchyma of *T. grandiflorum* and *T. bicolor*, the main distinction was the different number and distribution of polyphenol cells. There were also differences in the species-specific fat and protein content.

Introduction

All species of the genus *Theobroma* L. (*Malvaceae*) produce fruits with medium to large, recalcitrant seeds which are rich in fat, proteins and other nutrients. They are surrounded by a fibrous seed coat, which in turn is embedded in a mucilagenous pulp (Lopez et al., 1987). As the essential raw material for chocolate, the cocoa tree *T. cacao* (Fig. 1) is cultivated almost in the entire humid tropics of the world. With a total annual production of around 4 million tonnes of raw cocoa, the fermented and dried cocoa seeds, it is one of the most important world economic plants.

The other species of the genus *Theobroma* L. are almost unknown outside their natural distribution areas in South America, although the seeds of at least 12 other *Theobroma* species are used locally for the manufacture of chocolate-like products (Cuatrecasas, 1964). Regarding the cupuaçu tree *T. grandiflorum* (Fig. 1b), there exists a widespread cultivation in the Amazon Basin (Venturieri, 1993). Actually, mainly the pulp of the cupuaçu fruits is used as a popular ingredient of juices and sweets, but especially in recent years, there have been various initiatives to generate chocolate-like products from the seeds of *T. grandiflorum* that are seeking their market niche in the high-end chocolate sector. As documented in the Cocoa Atlas (Rohsius et al., 2010), occasional trade batches of dried cupuaçu seeds arrive in Europe as supposed "white cocoa". The further establishment and professionalization of a „cupulate" (chocolate from cupuaçu seeds) industry would increase the market value of cupuaçu fruits significantly.

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Fig. 1: Opened fruits of *T. bicolor* (left), *T. grandiflorum* (middle) and *T. cacao* (right). Pictures kindly provided by C. Rohsius

Same as *T. grandiflorum*, the macambo tree *T. bicolor* (Fig. 1a) is commonly found in cultivation. In the case of this species, the production area comprises the entire humid tropical America, from southern Mexico to Bolivia and Brazil (Cuatrecasas, 1964). Similar to the use of *T. grandiflorum*, the sweet-aromatic fruit pulp is frequently eaten while the seeds are used in many places for home-made chocolate-like products like those of *T. grandiflorum*. Also in the case of *T. bicolor*, there are attempts to create brands that attract international gourmets.

Cocoa-specific aroma precursors are generated during fermentation of cocoa seeds by an acid-induced proteolysis of the cotyledonar storage proteins by endogenous proteases (Voigt et al., 1995). The seed acidification during the fermentation process is caused by microbial production of lactic and acetic acid as a consequence of the pulp components (Schwan and Fleet, 2015).

For both *T. bicolor* and *T. grandiflorum*, Reisdorff et al. (2004) demonstrated the potential for the generation of a chocolate-like aroma based on the storage proteins and enzymes present in the cotyledons. The majority of investigations aimed at elucidating the nature of chocolate flavour have been directed at the biochemistry of post-harvest processing. However, the fermentation process is also influenced significantly by the histological and cytological structure of the seeds. The permeability of the seed coat to the microbial metabolites produced in the pulp, which are necessary for the curing of the seed, determine the rate of fermentation and therefore the quality of the product (Lopez et al., 1987). The seed coat also protects the cotyledons from the microflora during fermentation (Lopez et al., 1987). Chemical reactions in cocoa seeds during fermentation and roasting depend on the composition and post-mortem structural changes of the mesophyll cells (Biehl et al., 1981).

To date, few studies exist that focus on the histological and cytological characteristics of *T. bicolor* and *T. grandiflorum*, although

these features have a significant influence on the course of the post-harvest processing, which is essential for the generation of cocoa-like flavour. Already in 1998, a comparison of the seed structures of *T. grandiflorum, T. bicolor* and *T. cacao* was carried out as part of a diploma thesis under the supervision of Prof. Dr. Reinhard Lieberei (MÜLLER, 1998). The intention was to to provide a comparative description of the microstructure of the seeds suitable for the production of chocolate-like products as an important information for developing adjusted post-harvest processes for the different *Theobroma* species. Although the work was completed a considerable time ago, the results are still up to date. The present publication is intended to make them available to a wider public for the first time. The data were completely revised and placed in relation to relevant scientific findings that have been published since then. The universal approach that characterised all studies published under the supervision of R. Lieberei was retained.

Material and Methods

Material

Five ripe fruits of *T. grandiflorum* and four ripe fruits of *T. bicolor* were obtained in 1998 from the experimental field and the surroundings of the German-Brazilian scientific cooperation project SHIFT (Studies On Human Impact on Forests and Floodplains in the Tropics) near Manaus, Brazil. Five ripe fruits of *T. cacao* were bought at a fruit market in Manaus, two further cocoa fruits were kindly provided by the Technical University of Braunschweig at a later point of time. All cacao fruits visually corresponded to the 'Cacao Comum' type that is frequently cultivated in Brazil, with yellow fruit husk, as shown in Fig. 1. The export of all *Theobroma* fruits and corresponding preparations was carried out with the approval of the Brazilian project partners and in accordance with the then valid Brazilian guidelines.

Preparation for light microscopy

Whole *Theobroma* seeds were cut into 1 mm thick sections and fixed for at least 24 h in 4% formaldehyde in water, according to the instructions of GERLACH (1984). The fixation reduced oxidation-related browning of the polyphenol-containing tissue and ensured long-term preservation of the histological and cytological structures. For further processing into semi-thin sections, storage cotyledons were cut with a razor blade into small blocks of approx. 1 mm^3. Seed coat tissue was removed from the storage tissue and also cut into small fragments. The tissue samples were transferred into snap-cap jars and stored therein at room temperature until further use.

For embedding, the plant tissue was dehydrated in an ethanol dilution series. The preparations were incubated twice for half an hour in 70% ethanol and twice for half an hour in 100% ethanol. The tissue blocks were then transferred into 100% LR-White Medium Grade (London Resin Company), which was replaced by fresh LR-White after 1 hour, in which the material remained in the refrigerator overnight. The following day, after removing the excess LR-White, the tissue preparations were transferred into gelatine capsules (Pohl-Boskamp, volume 0.36 cm^3). These were completely filled with LR-White.

The embedding medium was polymerized in a drying oven for 20-24 hours at 60 °C. Prior to further processing, the LR-White cylinders were stored at room temperature for several days for complete curing. The LR-White cylinders were trimmed with a razor blade to a pyramid shape, whereby a trapezium-shaped cut surface was worked out. The area of the pyramid tip was limited to a maximum of 1 mm^2. To make the semi-thin sections, the specimen holder with the trimmed LR-White cylinder was clamped into an ultramicrotome (Leica, Ultracut S) with the broad side of the trapezoid facing downwards. The trapezoidal surface was aligned parallel to the blade of a glass knife. The feed rate of the specimen was adjusted to 2 to 2.5 µm.

The collecting basin attached to the glass knife was filled with filtered aqua bidest. in which the semi-thin sections obtained were floating. From here, they were transferred to a drop of water on the slide placed on a heating plate and fixed at a temperature of about 50 °C.

For semi-thin sections (2 µm) of the seed coats and the cotyledon tissue a staining with Toluidine blue O according to GUTMANN (1995), slightly modified was applied. The semi-thin sections fixed on a microscope slide were incubated for 30 s in a solution of sodium hypochlorite (ca. 13% Cl, Fluka, Buchs, Switzerland). After rinsing with aqua dest. and drying the slides, the samples were incubated for 5 min in a solution of 0.05% (w/v) Toluidine Blue O in aqua dest. Then the slide was rinsed with aqua dest. again and subsequently dried. The treatment causes a greenish-black or (due to oxidation) brownish colour of phenolic compounds, whilst proteins and cell walls are stained blue and lipids remain colourless.

Some semi-thin sections of the seed coat of *T. cacao* (thickness approx. 2.5 µm) were stained with Fast Green FCF and Safranin using a modified procedure according to GERLACH (1984). For this the samples were incubated overnight in Fast Green FCF (Serva, 0.1% solution in ethanol, 92%) and the following day they were rinsed thoroughly with tap water and dried. Afterwards, the semi thin sections were stained for several minutes in a solution of 1% Safranin (Sigma) in 50% undenatured ethanol. After rinsing again with tap water, the preparations were dried, enclosed in Styrax (Sigma) and covered with a cleaned cover glass. The Safranin stains all cell walls bright red, while cytoplasm and proteins are stained green by the Fast Green. All semi-thin sections were analyzed with an Olympus BH-2 light microscope. Pictures were taken with an Olympus OM-2 camera.

Preparation for transmission electron microscopy

The electron microscopic preparations carried out for this work were largely based on a method developed by SPURR (1969), modified by H. Quader (Institute of Plant Sciences and Microbiology, former Institute of Botany, Hamburg). They were made from cotyledon tissue of all three *Theobroma* species.

Storage cotyledonary tissue of all three *Theobroma* species was cut into small blocks (about 1 mm^3) in phosphate buffer (50 mM, pH 7.2). This buffer solution was used for the entire preparation. The tissue blocks were fixed in 2% glutaraldehyde (Merck) in phosphate buffer for a total of two hours. After one hour fixation time, they were degassed with a water jet pump. After completion of the fixation, the tissue blocks were washed out with phosphate buffer and then fixed for 60 min in a 2% aqueous solution of osmium tetroxide (Plano). After renewed washing in phosphate buffer, the material was dewatered in an acetone dilution series with 30%, 50%, 75%, 90%, and 95% acetone in aqua bidest. for 10 min at a time. The remaining water was removed by adding 100% acetone, whereby the medium was exchanged once after 10 min and twice after 15 min.

After complete drainage of the tissue, incubation in Spurr embedding medium (Plano) was performed. The material was first added to a 3/1 (vol/vol) acetone-Spurr mixture for 60 min, then to a 1/1 (vol/vol) acetone-Spurr mixture for another 60 min. Overnight, the specimens were incubated in a 3/1 (vol/vol) acetone-Spurr mixture, leaving the snap-on lid vials slightly open to allow the acetone to evaporate. The next day, the medium was exchanged for 100% Spurr. After another 240 min, the tissue blocks were transferred into gelatine capsules (see above), which were filled with 100% Spurr and polymerised for 24 hours at 70 °C in a drying oven.

Trimming of the Spurr cylinders was carried out according to the procedure described for the LM preparation in 2.2, with trapezoid

areas of be less than 1 mm^2. Copper grids (Agar Scientific, 100 mesh, Pioloform) were used as slides. Ultra-thin sections with a thickness of 65 nm were prepared at the ultramicrotome (see 2.2).
The preparations were post-treated with uranyl acetate (Serva) and lead citrate (Sigma). The copper grids were first placed on a drop of a 5% uranyl acetate solution for 12 min; they were then rinsed with aqua bidest. and transferred to the drop of solution of 0.2 mol lead citrate in aqua bidest.. After a further 10 min, the contrasting process was stopped by rinsing the nets in aqua bidest. The ultrathin sections were studied and photographed with a Zeiss 109 electron microscope at magnifications from 2500-20000.

Measurement of the preparations
The measuring grid of a Thoma counting chamber with a small-square edge length of 50 µm was used as a comparison object for size determination in light microscopic investigations. In the TEM investigation a measuring grid (edge length 0.82 µm * 1.64 µm) photographed at magnifications from 2500-16000 was used.

Determining the proportions of reserve substances in the storage cells
LM and TEM images of the storage cells of all analyzed *Theobroma* species were photocopied. The area portions of storage protein vacuoles, lipid vacuoles and starch grains were cut out and weighed separately to calculate the share of the corresponding storage material in the reserve material content. The proportions of the storage substances were expressed as volume percentages. In the described manner, a total of 93 storage cells of *T. cacao*, 126 storage cells of *T. grandiflorum* and 83 storage cells of *T. bicolor* were measured with their ingredients. Cell sections of all segments and cutting angles were assessed to determine the percentage share of the different storage compounds also on a three-dimensional level.

Results and discussion

The common structure of the seed coat
The shells of the seeds of all investigated *Theobroma* species emerge from two integuments, the outer (testa) and the inner integument (tegmen). In all cases, the testa is clearly thicker and more differentiated (Fig. 2). The parenchyma of the tegmen is composed of only a few cell layers, which are strongly compressed. The contents of some of these cells can be stained greenish with Toluidine Blue Rangel et al. (2012) and Cerri et al. (2019) interpret the contents as phenolic substances. The outer epidermis of the tegmen is composed of a single layer of lignified, thick-walled cells that will be described below in more detail. The basic tissue of the testa consists of numerous layers of collapsed and deformed cells, which are somewhat larger than the parenchyma cells of the tegmen. Again, the contents of some of these cells can be stained with Toluidine Blue O, especially in the case of *T. bicolor* (Fig. 2).
The following structures are embedded in the collapsed parenchyma of the testa:
- vascular bundles of mostly elliptical shape, which consist mainly of spiral tracheas
- large, elliptical mucosal caverns, often chambered by radial septums, which (mainly in the case of *T. cacao* and *T. bicolor*) swell strongly in aqueous media.

Fragments of the mucilagenous pulp which belongs to the mesocarp (Roth, 1977) are closely attached to the outer epidermis of the testa. The parenchyme of the pulp comprises large tubular cells which serve as substrate for the microorganisms during fermentation (Lopez, 1987). They are seperated from the seed coat by a single layer of smaller epidermal cells, the endocarp. When comparing cross sections of seed coats of *T. cacao*, *T. grandiflorum* and *T. bicolor*, their different diameters are immediately apparent (Fig. 2). The seed coat of *T. grandiflorum* with an average of 520 µm is clearly thicker than that of *T. cacao* (385 µm). The biggest difference, however, is to *T. bicolor*, whose seed coat far exceeds the diameter of the other two species, with an average measured 1670 µm. The thickness of

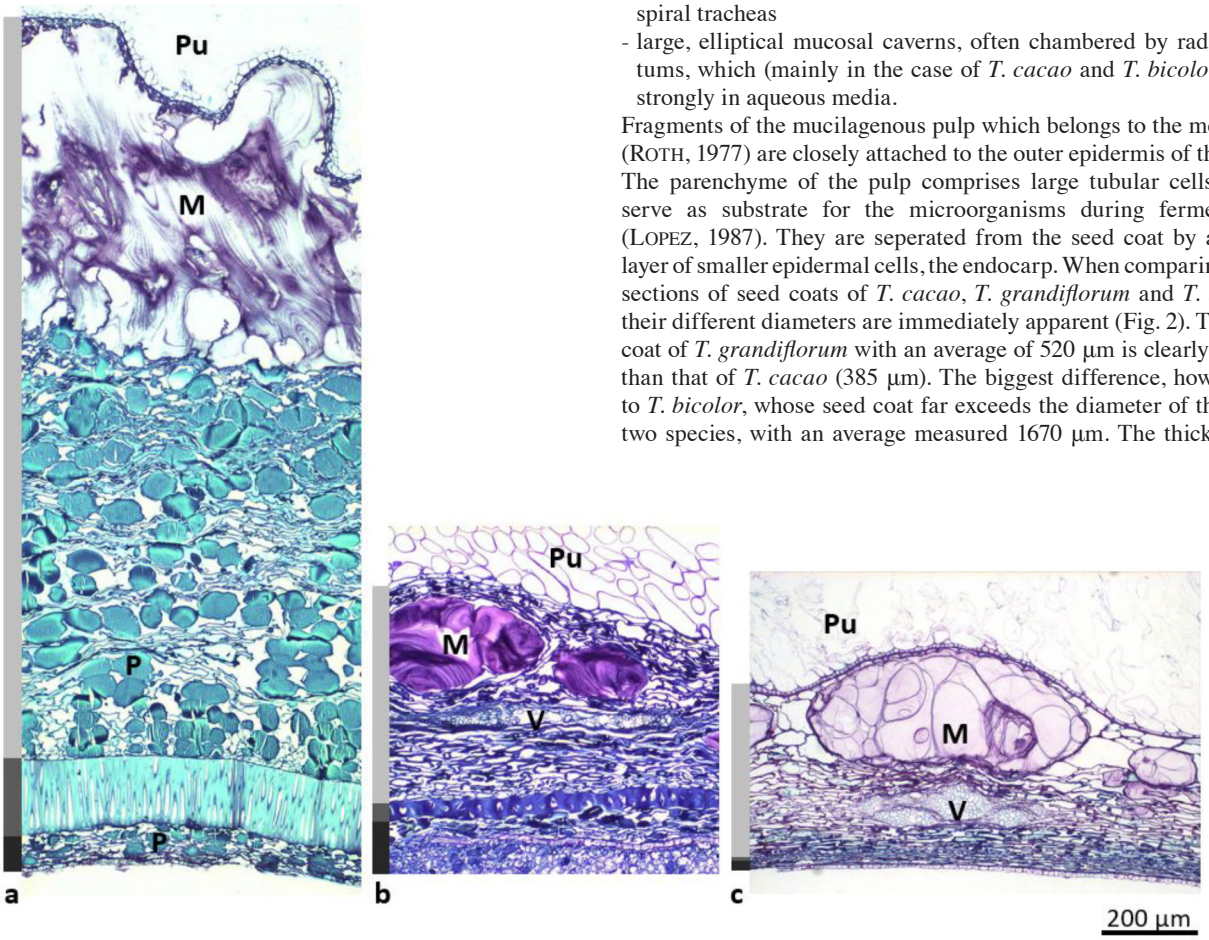

Fig. 2: Longitudial sections through the seed coats of *T. bicolor* (a), *T. grandiflorum* (b) and *T. cacao* (c)
M: mucus caverns, V: vascular bundles
Marking (left margin): testa (light grey), exotegmal sclereid or palisade layer (dark grey), parenchyma of the tegmen (black)
Semi-thin sections, stained with Toluidine Blue O

the seed coats of *T. cacao* and *T. bicolor* crucially depends on the swelling grade of the mucus caverns which multiply their volume after the uptake of water. The mucus chambers of *T. grandiflorum* do not show comparable swelling as they are often smaller and generally are not located directly below the extensible outer epidermis of the seed coat (Fig. 2). For the highly compressed parenchymal tissue of the tegmen a thickness of about 80 µm was determined for *T. grandiflorum*. It is thus more pronounced than in *T. cacao* (33 µm) and *T. bicolor* (70 µm) (Fig. 2).

One of the most striking differences between the seed coats of the three compared *Theobroma* species is the massive disparity in the expression of the outer epidermis of the tegmen. In the case of *T. cacao*, it is a simple sclereid layer whose individual cells are on average 15 µm long and 9 µm thick (Fig. 3). *T. grandiflorum* and *T. bicolor* have a much more massive palisade cell layer (Fig. 3b, c). In both species it consists of cells whose walls are strongly thickened laterally and towards the tegmen. The cells have only a small lumen. The palisade cell layer of *T. grandiflorum* consists of cells with an average length of 17 µm and a thickness of 29 µm. *T. bicolor* has a palisade cell layer which, with an average thickness of 127 µm, has by far the largest diameter of all three species. The length of the individual cells averages 20 µm.

The seed coat of *T. cacao* does not contain any substances that are involved in the formation of the chocolate flavour (Gassner et al., 1989). Nevertheless, it has a decisive influence on the quality of the fermentation product: While it prevents the colonisation of the embryonic tissue by microorganisms, it also represents an obstacle which must be overcome by the decomposition products of the pulp penetrating into the embryonic tissue. Especially the infiltration of the cotyledon tissue with acetic acid and other microbial degradation products, which is of great importance for the generation of the typical cocoa aroma during fermentation (Biehl et al., 1981; Voigt and Biehl, 1995).

The speed at which the decomposition products of the pulp penetrate through the seed coat during cocoa fermentation depends on the permeability of the seed coat. Assuming the same substance constant, the largest permeability of the seed coat of *T. cacao* and the lowest of *T. bicolor* can be deduced from the widely differing diameters of the seed coats of the three species studied.

According to Andersson et al. (2006), the sclereid layer of *T. cacao* is encrusted with lignin, which can be assumed analogously for the palisade layers of *T. grandiflorum* and *T. bicolor*. Andersson et al. (2006) demonstrated that liquids penetrate the testa of *T. cacao* relatively quickly via the apoplastic pathway and then spread vertically within the sclereid layer. From here, the fluids are transported into the tegmen through the symplasts, starting from plasmodesmata found in the sclereid layer.

As the palisade layers of *T. grandiflorum* and in particular *T. bicolor* have a multiple of the diameter of the simple sclereid layer of *T. cacao*, their thickened cell walls can be assumed to be an even greater obstacle for the penetration of aqueous solutions. While in most cells of the palisade cell layer of *T. grandiflorum* the typical cell wall thickenings are perforated in many places, probably for plasmodesmata (Fig. 3b), such perforations are hardly found in the palisade layer of *T. bicolor* (Fig. 3c). In this respect, a considerably increased barrier effect can be expected, especially from the palisade cells of *T. bicolor*.

Since the palisade cell layer is interrupted in the area of the micropyle (Müller, 1998), it does not exercise its barrier function in the whole seed coat. At the gap, which is about 1 mm^2 in size at this point, fluids penetrating from the outside may only have to overcome parenchymal tissue. Though Andersson et al. (2006) describes a greater lignification in this area in the seed coat of *T. cacao*, Rohsius (2008) shows evidence that the acidification of the seed interior during fermentation usually starts in the area adjacent to the micropyle. One explanation is the swelling and stretching of the germ root in the initial phase of fermentation, which causes the formation of fine fissures in the area of the micropyle, making it permeable to acetic acid and other liquids.

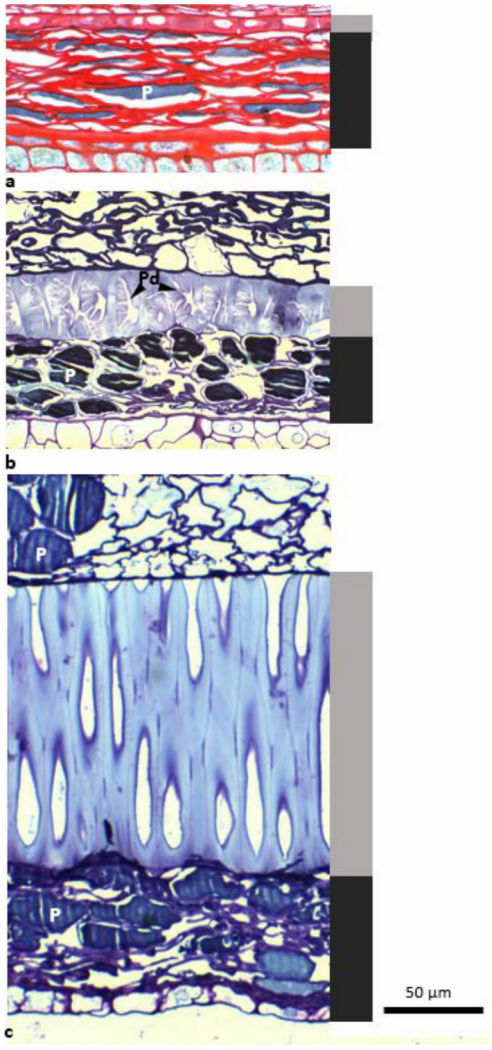

Fig. 3: Longitudial sections through the tegmen of *T. cacao* (a), *T. grandiflorum* (b) and *T. bicolor* (c)
P: polyphenol cells; Pd: cell wall perforation for plasmodesmata
Marking (right margin): exotegmal sclereid or palisade layer (light grey), parenchyma of the tegmen (black)
Semi-thin sections, stained with Safranin/Fast green (a) and Toluidine Blue O (b, c)

A more detailed examination of this tissue section in all three *Theobroma* species is therefore necessary in order to assess its permeability and dynamics during fermentation. To complete the present results, further structures of the *Theobroma* seed coats, as described by Rangel et al. (2012) for *T. cacao*, such as hilum, raphe and chalaza, should be investigated as well. This is even more important as according to Andersson et al. (2006), different physical properties are to be expected here.

Within the present work, no experiments on the permeability of the individual seed coat structures were carried out. Therefore, it cannot be stated how differently the seed coats of *T. cacao*, *T. grandiflorum* and *T. bicolor* are penetrated by acetic acid, which is necessary for the formation of the aroma precursors. However, it seems to be out of the question that the seed coats of the three *Theobroma* species studied, which differ greatly in diameter and structural characteristics, have

different permeabilities. This information may help to design adapted fermentation systems for *T. grandiflorum* and *T. bicolor.*

In addition to the permeability to liquids penetrating during fermentation, the seed coat may also have an impact on the microbiome present during fermentation.

According to LOPEZ et al. (1987), the subepidermal caverns filled with plant mucilages are preserved during fermentation. The influence of these structures on the fermentation process must therefore be considered. According to BAHMANN (2014), the mucus inside the seed coat of *T. cacao* consists of proteins and carbohydrates and not of pectins like a multitude of other plant mucilages. The proteins involved included osmotin, chitinase and glucanase, all of which are related to the defence against plant-pathogenic fungi. In fact, in vitro the mucus from *T. cacao* displayed growth inhibition against various yeasts and fungi while not affecting the growth of acetic and lactic acid bacteria (FAHRURROZI et al., 2013). As both yeasts and the above-mentioned groups of bacteria are important for fermentation, it can be assumed that the mucus plays an important role in the course of this process. Against this background, further comparative studies of the mucus of the different *Theobroma* species are essential for evaluating the individual impact on post-harvest processing, also in view of the very differently pronounced mucus caverns of the three *Theobroma* species studied.

In addition, the presence of phenolic constituents in the seed coat is likely to have an effect on the colonization with microorganisms during fermentation due to their antibacterial properties. Especially in *T. bicolor*, the green coloration indicates a large accumulation of these substances, mainly in the testa (Fig. 3c).

Finally, the question of the technical removal of seed coat in the further processing of *T. grandiflorum* and *T. bicolor* needs to be considered. Even the comparatively thin seed coat of *T. cacao* requires a considerable effort for removal, as is required in the production of cocoa liquor. Undoubtedly, the removal of the much more massive seed coat of *T. grandiflorum* and *T. bicolor* is even more difficult and would need adapted technical solutions in a mechanized process.

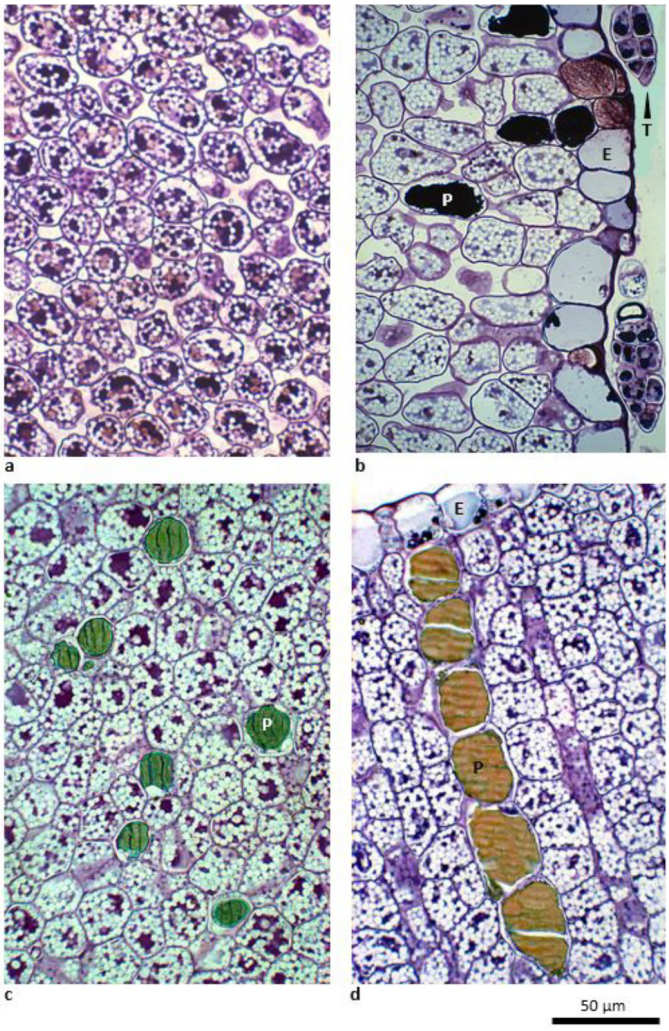

Fig. 4: Sections through the storage parenchyma of cotyledons of *T. bicolor* (a), *T. grandiflorum* (b) and *T. cacao* (c, d)
P: polyphenol cells, E: epidermis, T: epidermal trichome
Semi-thin sections, stained with Toluidine Blue O

The structure of the cotyledon tissue

The largest part of the cotyledon tissue consists of thin-walled storage cells, which have a polyhedral, slightly elongated shape. Their average dimensions for all three species were in a comparable range of about 25*20 µm (Fig. 4, 5). In ripe seeds, the cotyledon tissue of *T. grandiflorum* and *T. bicolor* seems to be macerated stronger than in seeds of *T. cacao* (Fig. 4a, b). A higher degree of maceration could accelerate the penetration and run-off of liquids such as acetic acid during fermentation once the seed coat is passed. However, since such a difference is not described by MARTINI et al. (2008A), it would have to be verified by further investigations.

The storage cells of all three *Theobroma* species studied are almost completely filled with the stored reserve substances fat, proteins (aleuron) and starch (Fig. 5). These are enclosed in a hydrophilic, quantitatively subordinated basic plasma, whose continuous, closed network penetrates even the narrowest gaps (Fig. 6).

Of the storage substances, starch makes up the smallest proportion. It is stored in the starch granules (amyloplasts), of which there are several in each storage cell. In TEM preparations, the amyloplasts are contrasted with stripes (Fig. 6). The starch granules measure about 2.5*3.5 µm on average and tended to be a bit larger in tissues of *T. bicolor.* The starch grains of the three examined species have a similar ultrastructure. Differences in starch compaction give rise to the striped electron microscopical contrastation of the starch grains (Fig. 6). The storage proteins are located in protein vacuoles surrounded by a membrane, also known as aleuron. In TEM preparations their electron dense content can be clearly distinguished from the basic plasma (Fig. 6a).

The protein vacuoles of most storage cells are pressed into a forced shape by the densely packed lipid vacuoles (Fig. 4-6), which is in agreement with the findings of JAENICKE (1973).

The cotyledon fat is embedded in the storage cells in the form of lipid vacuoles. These are round inclusions in the cytoplasm, some of which are intertwined but always isolated from each other. Even in the densest packing, plasma boundaries between the lipid vacuoles are always visible in intact storage cells of the cotyledon mesophyll of the three species studied (Fig. 5). The lipid vacuoles of *T. grandiflorum* are 1-7 µm (average 4 µm) in size, while the size of lipid inclusions of *T. cacao* is 1-4 µm (average 3 µm) and of *T. bicolor* 1-5 µm (average 3 µm).

With regard to the percentage of starch, fat and proteins stored in the cells, *T. bicolor* differed from the two other species investigated by a higher amount of storage proteins and a lower fat content (Tab. 1). It must be pointed out that the applied method for proportion determination refers only to the volume occupied in the storage cells and not to the percentage of dry weight. However, the finding of a lower fat and higher protein content in *T. bicolor* is consistent with the results of MARTINI et al. (2008B). According to CARPENTER et al. (1994) only 38% fat is found in the seeds of *T. bicolor* while seeds of *T. cacao* usually contain a fat content of about 50%. According

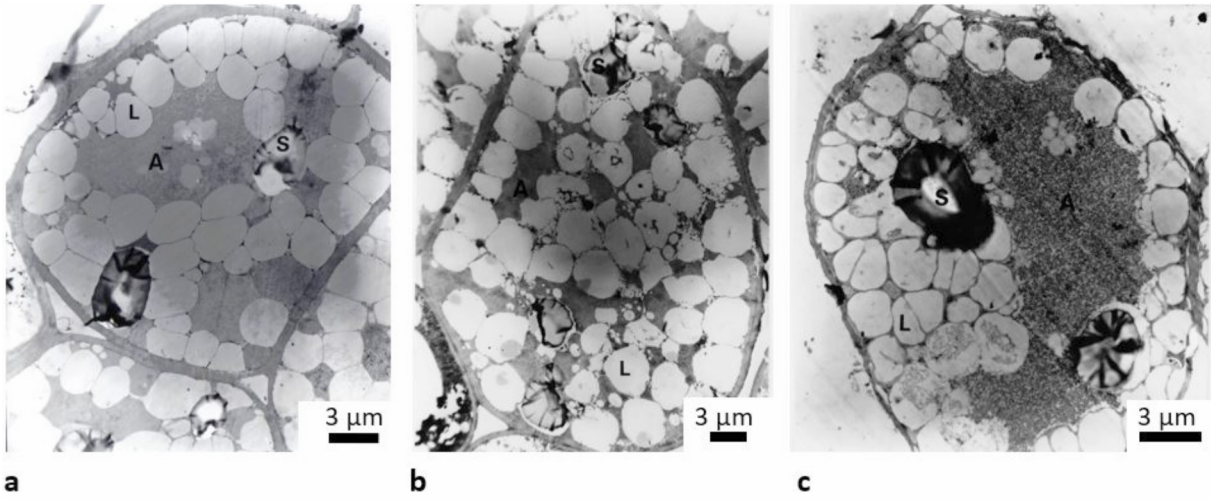

Fig. 5: Storage cells of *T. cacao* (a), *T. grandiflorum* (b) and *T. bicolor* (c)
A: aleuron, S: starch grain, L: lipid vacuole
Ultra-thin sections

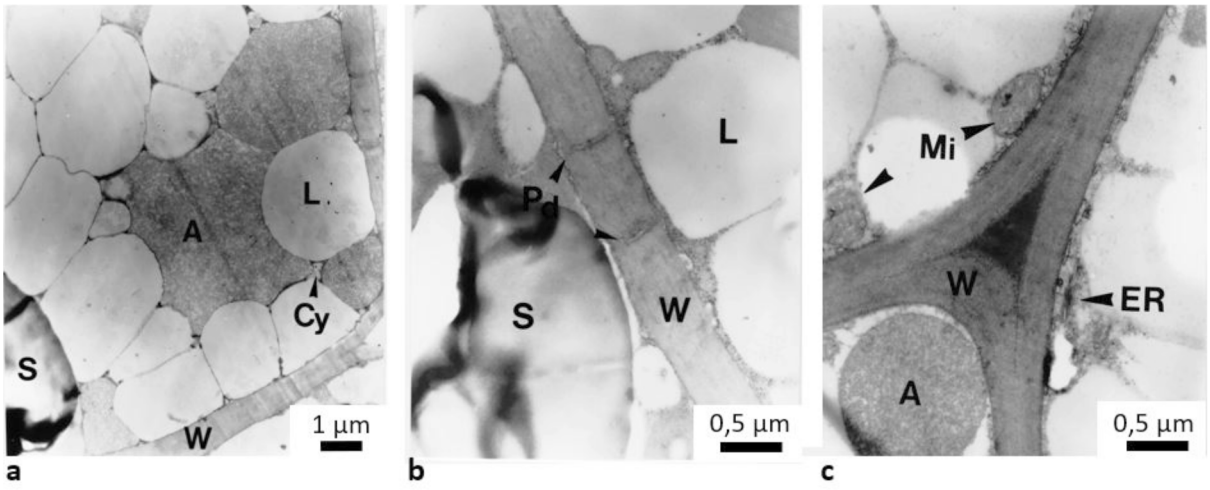

Fig. 6: Details of storage cells of *T. cacao*
A: aleuron, S: starch grain, L: lipid vacuole, W: cell wall, ER: endoplasmatic reticulum, Mi: mitochondrion, Pd: plasmodesm, Cy: cytoplasma
Ultra-thin sections

to the authors, the fat composition of *T. grandiflorum* and *T. bicolor* also differs significantly from that in *T. cacao*. As the proportion and composition of the fat also have a major influence on product quality, for example on its melting point and flow behavior, deviations from *T. cacao* can be expected for chocolates made from *T. bicolor* or *T. grandiflorum*, which may be adjusted by adding cocoa butter.

In addition to the storage substances, the cytoplasm of the electron microscopic preparations of *T. cacao* contained isolated mitochondria and parts of the endoplasmic reticulum (Fig. 6c). The storage cells are interconnected via plasmodesmata (Fig. 6b). Scattered among the storage cells are larger idioblasts whose interiors are filled almost entirely by one single vacuole. These cells are recognized as polyphenol cells and contain virtually all of the seed's polyphenolic material and alkaloids (Fig. 4, 7). With an average of around 25*35 µm, this type of cells is significantly larger than the storage cells described above.

The content of the polyphenol vacuoles either appears as rather uniform or has non-contrast inclusions (Fig. 7b). in inmature stage, they only show spherical accumulations of polyphenols alongside the tonoplast, as described by ELWERS et al. (2010). In the cytoplasm outside the large vacuoles, the polyphenol cells occasionally contain starch granules and lipid vacuoles (Fig. 7). The dark red or purple colour of the storage cotyledons of many varieties of *T. cacao* is caused by anthocyanins which are embedded in the polyphenol vacuoles. The content of the polyphenol cells of *T. grandiflorum* is basically colourless in the unoxidized, unstained state. In the cotyledon tissues of *T. cacao* and *T. grandiflorum*, the polyphenol cells were found in a portion of 8-11%. While the distibution of the polyphenol cells of *T. grandiflorum* is rather equal in the whole tissue, more idioblasts of

Tab. 1: Percentage by volume of lipid vacuoles, proteins and starch in the storage cells of cotyledonous tissue of *T. cacao*, *T. grandiflorum* and *T. bicolor*

Species	[%]		
	Fat	Protein	Starch
T. cacao	70	26	4
T. grandiflorum	65	29	5
T. bicolor	54	40	6

T. cacao appear close to the upper epidermis of the cotyledones where they often form small groups and rows (Fig. 4d). A larger amount of polyphenol cells attached to the cotyledon vascular bundles was observed in both species (Fig. 8).

The cotyledon tissue of *T. bicolor* contains a much smaller amount of polyphenol cells, exclusively in direct surroundings of the vascular systems which are distributed sporadically in the cotyledon tissue. This result is consistent with MARTINI et al. (2008A, B), who also find much lower amounts of polyphenols in the storage cotyledon tissues of *T. bicolor* exclusively in the area of vascular bundle systems. The significantly lower proportion of phenolic substances is already obvious when cutting fresh seeds of *T. bicolor*, as the cut surfaces remain yellowish white and do not – as in *T. cacao* and *T. grandiflorum* – turn brown within minutes by oxidation of the polyphenols. As a consequence, an oxidative browning of the storage tissue of *T. bicolor* will also be omitted during post-harvest processing.

As the polyphenol vacuoles in the cotyledonar tissue are destroyed during fermentation, their contents tan significant portions of the surrounding proteins (BIEHL et al., 1983). The function of the products resulting from the tanning process as flavour components is disputed (ZIEGLEDER and BIEHL, 1988). However, as the tanning prevents large amounts of storage proteins from proteolytic cleavage during fermentation, there is no doubt that this reaction effects the flavour

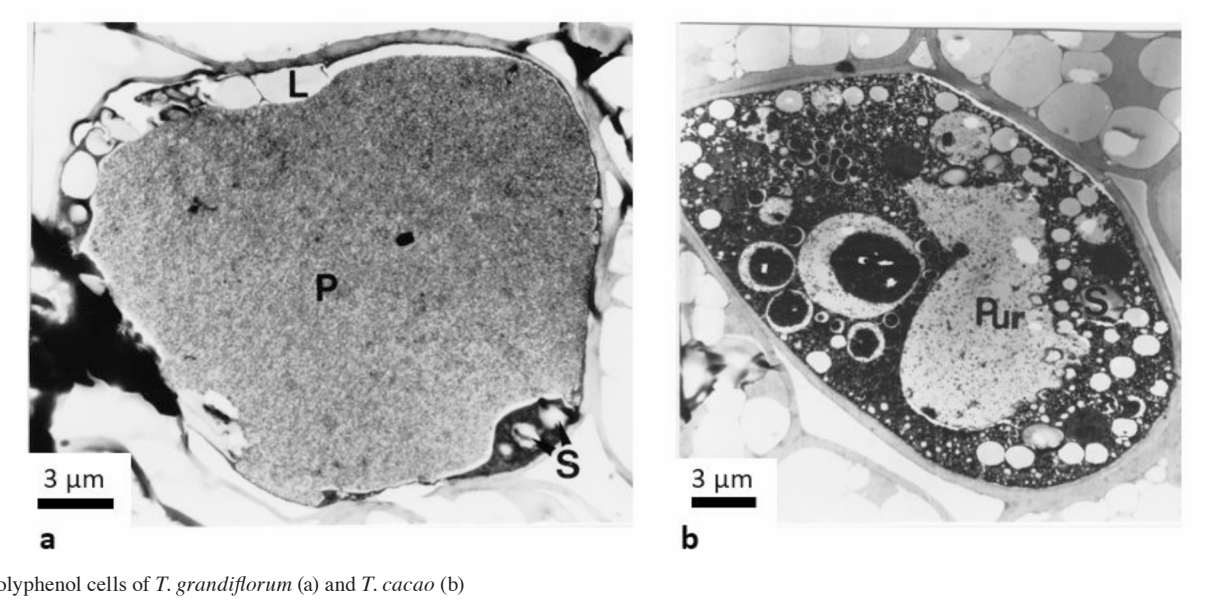

Fig. 7: Polyphenol cells of *T. grandiflorum* (a) and *T. cacao* (b)
P: polyphenol vacuole, S: starch grain, Pur: purine inclusions
Ultra-thin sections

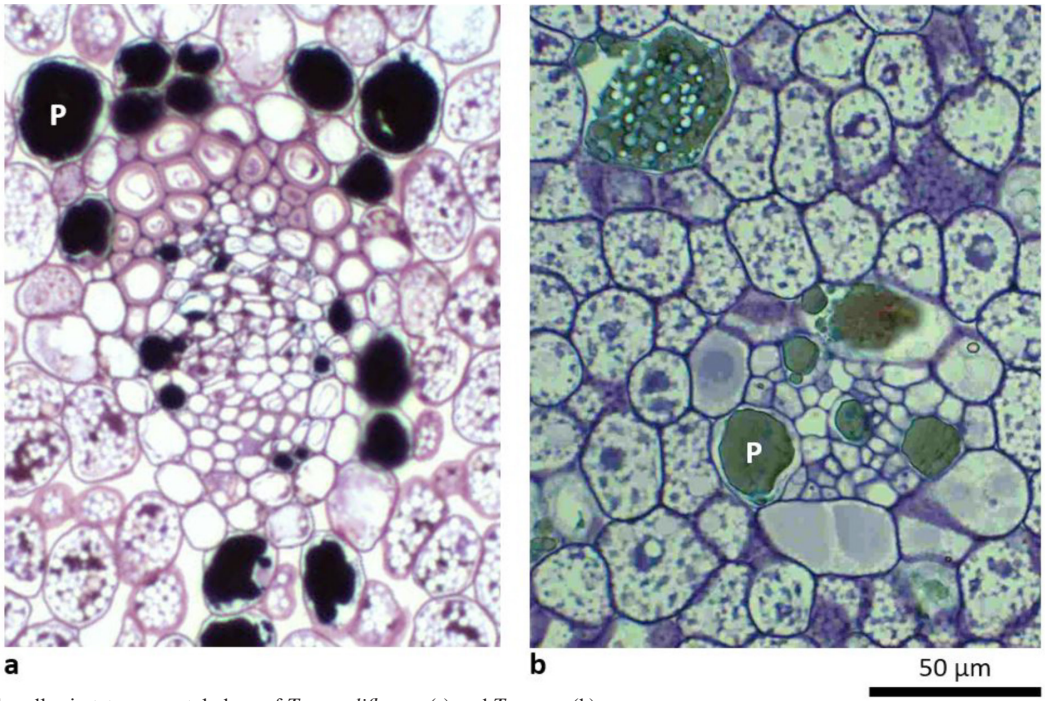

Fig. 8: Vascular bundles in tstorage cotelydons of *T. grandiflorum* (a) and *T. cacao* (b)
P: polyphenol cell
Semi-thin sections, stained with Toluidine Blue O

development. In a cocoa-like product made from the seeds of *T. bicolor*, the astringency and bitterness of many phenolic constituents are missing, as well as the purine alkaloids which are also stored in the polyphenol vacuoles. In summary, it can be concluded that the differences between the three examined *Theobroma* species in presence and distribution of polyphenol cells in the cotyledonar tissue result in a deviation in the flavour development during post-harvest processing.

Conclusions

The microscopic examinations carried out in the present study revealed the following differences between the three investigated *Theobroma* species, which can be expected to be relevant when processed into a cocoa-like product:

1. General thickness of the seed coat and sclereide respectively palisade layer: *T. grandiflorum* and especially *T. bicolor* have a significantly thicker seed coat. The exchange of liquids during cocoa fermentation (and partly also during drying), such as the infiltration of lactic and acetic acid and the washing out of phenolic substances, is most likely delayed by this aspect. This assumption is reinforced by the much more massive outer epidermis of the tegmen of *T. bicolor* and *T. grandiflorum*, which should further reduce the permeability of the seed coats.
   With increasing thickness of the seed coat as well as the sclereid or palisade layer, it becomes more difficult to detach the shells for further processing into a cocoa-like product.
2. Protein mucilages and phenolic ingredients in the seed coats.
   Although all three investigated species show both mucus caverns and phenolic substances, their expression is again clearly different. *T. grandiflorum* is the species with the least mucilage development, *T. bicolor* has both the largest mucilage caverns and the most phenolic substances. As both substance groups are assumed to inhibit the growth of microorganisms, a corresponding influence on the microbiome colonizing the seed coat during fermentation can be assumed.
3. Quantity distribution and properties of the storage substances
   Fat, protein and starch are stored in the normal storage parenchyma cells of all investigated *Theobroma* species. While the proportions of these storage substances do not differ significantly between *T. cacao* and *T. grandiflorum*, the fat content of *T. bicolor* is considerably lower. Together with known differences in fat composition, these differences will affect the texture of cocoa-like products compared to real chocolate. The three investigated species also show known differences in the properties of the stored storage proteins and proteases. However, all three species have the potential to generate the unique cocoa aroma.
4. Number and distribution of polyphenol cells in storage cotyledon tissue
   T. cacao and *T. grandiflorum* are relatively similar in size and number of polyphenol cells in the storage parenchyma and show only slight differences in their distribution in the tissue. In contrast, *T. bicolor* is characterized by the almost complete absence of these idioblasts in the storage tissue, which can only be detected in the area of the vascular systems. As a consequence, an oxidative browning of the storage tissue of *T. bicolor* almost does not take place after cutting or during post-harvest processing. Furthermore, the astringency and bitterness typical of cocoa is not to be expected in a product made from *T. bicolor* seed, as the responsible purine alkaloids and polyphenols are missing.

The facts presented here can help to optimally exploit the so far little used potential of *T. grandiflorum* and *T. bicolor* seed for transformation into cocoa-like products. The knowledge about the specific anatomical and histological characteristics, the similarities and differences compared to *T. cacao*, make an important contribution to the development of adapted post-harvest processing strategies.

Acknowledgement

This publication was prepared in memory of Prof. Dr. Reinhard Lieberei, who inspired and advanced the preceding thesis with his universal approach and great spirit of research.

Conflict of Interest

No potential conflict of interest was reported by the authors.

References

Andersson, M., Koch, G., Lieberei, R., 2006: Structure and function of the seed coat of *Theobroma cacao* L. and its possible impact on flavour precursor development during fermentation. J. Appl. Bot. Food. Qual. 80, 48-62.

Bahmann, C., 2014: Biotechnologie von *Theobroma cacao* L.. PhD thesis, University of Hamburg, 203 pp..

Biehl, B., Passern, D., Sagemann, W., 1981: Effect of acetic acid on subcellular Structures of cocoa bean cotyledons. J. Sci Food Agric. 33, 1101-1109. DOI: 10.1002/jsfa.2740331107

Biehl, B., Adomako, D., 1983: Die Kakaofermentation (Steuerung, Acidation, Proteolyse). Lebensm. Chem. Gerichtl. Chem. 37, 57-63.

Carpenter, R.R., Hammerstone, J.F., Romanczyk, L.J.Jr., Aitken, W.M, 1994: Lipid composition of Herrania and *Theobroma* seeds. J. Am. Oil. Chem. Soc. 71, 845-851. DOI: 10.1007/BF02540460

Cerri, M., Reale, L., Zadra, C., 2019: Metabolite Storage in *Theobroma cacao* L. Seed: Cyto-histological and phytochemical analyses. Front. Plant Sci. 10(1599). DOI: 10.3389/fpls.2019.01599

Cuatrecasas, J., 1964: Cacao and its allies. A taxonomic revision of the genus *Theobroma*. Contrib. US. Nat. Herb. 35(6), 379-605.

Elwers, S., Zambrano, A., Rohsius, C., Lieberei, R., 2010: Histological features of phenolic compounds in fine and bulk cocoa seed (*Theobroma cacao* L.). J. Appl. Bot. Food. Qual. 83(2), 182-188.

Fahrurrozi, F., Bahmann, C., Lieberei, R., Bisping, B., 2013: Antifungal activity in seed coat extracts of Theobroma cacao L.. Poster, Annual Conference VAAM, Bremen.

Gassner, G., Hohmann, B., Deutschmann, F., 1989: Mikroskopische Untersuchung Pflanzlicher Lebensmittel. 5th ed., Fischer, Stuttgart, 234-238.

Gerlach, D., 1964: Botanische Mikrotechnik – Eine Einführung. 3rd ed., Thieme, Stuttgart, 311 pp.

Gutmann, M., 1995: Improved staining procedures for photographic documentation of phenolic deposits in semithin sections of plant tissue. J. Microsc. 179, 277-281. DOI: 10.1111/j.1365-2818.1995.tb03642.x

Jaenicke, J., 1973: Elektronenmikroskopische Untersuchungen an Embryonen von *Theobroma cacao* L. (Kakao) während der Embryogenese und Samenkeimung. PhD thesis, Tierärztliche Hochschule Hannover, Hannover.

Lopez, A.S., Dimick, P.S., Walsh, R.M., 1987: Scanning Electron Microscopy studies of the cellular changes in raw, fermented and dried cocoa beans. Food Microstruct. 6, 9-16.

Martini, M.H., Figueira, A., Gonçalves Lenci, C., de Queiroz Tavares, D., 2008A: Polyphenolic cells and their interrelation with cotyledon cells in seven species of *Theobroma* (Sterculiaceae). Revista Brasil. Bot. 31, 425-431. DOI: 10.1590/S0100-84042008000300006

Martini, M.H., Lenci, C.G., Figueira, A., Tavares, D.D.Q., 2008B: Localization of the cotyledon reserves of *Theobroma grandiflorum* (Willd. ex Spreng.) K. Schum., *T. subincanum* Mart., *T. bicolor* Bonpl. and their analogies with *T. cacao* L.. Revista Brasil. Bot. 31 (3), 147-154. DOI: 10.1590/S0100-84042008000100013

Müller, S., 1998: Histologische und cytologische Studien an Samen von

Theobroma-Arten. Diploma thesis, University of Hamburg.

Rangel-Fajardo, M.A., Zavaleta-Mancera, H.A., Córdova-Téllez, L., López-Andrade, A.P., Delgado-Alvarado, A., Vidales-Fernández, I., Villegas-Monter, A., 2012: Anatomy and histochemistry of the mexican cacao (*Theobroma cacao* L.) seed [Anatomía e histoquímica de la semilla del cacao (*Theobroma cacao* L.) criollo mexicano]. Rev. Fitotec. Mex. 35 (3), 189-197.

Reisdorff, C., Rohsius, C., Claret de Souza, A., Gasparotto, L., Lieberei, R., 2004: Comparative study on the proteolytic activities and storage globulins in seeds of *Theobroma grandiflorum* (Willd ex Spreng) Schum and *Theobroma bicolor* Humb Bonpl, in relation to their potential to generate chocolate-like aroma. J. Sci. Food. Agric. 84, 693-700. DOI: 10.1002/jsfa.1717

Rohsius, C., 2008: Die Heterogenität der biologischen Ressource Rohkakao (*Theobroma cacao* L.). PhD thesis, University of Hamburg, 233 pp.

Rohsius, C., Elwers, S. Lieberei, R., 2010: Cocoa-Atlas. DVD, Foundation of the German Cocoa and Chocolate Industry, Bonn, Hamburg.

Roth, I., 1977: Fruits of Angiosperms. In: Zimmermann, W.,Carlsquist, S., Wullf, H.D. (ed.), Encyclopedia of Plant Anatomy, Volume 10 (2), 483-494. Gebrüder Bornträger, Berlin, Stuttgart.

Schwan, F.R., Fleet, H.G., 2015: Cocoa and coffee fermentations. Boca Raton, CRC.

Spurr, A.R., 1969: A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26, 31-43. DOI: 10.1016/S0022-5320(69)90033-1

Venturieri, G.A., 1993: Cupuaçu: a espécie, sua cultura, usos e processamento. 1st ed., Clube do cupu, Belém, 3-39.

Voigt, J., Biehl, B., 1995: Precursors of the cocoa-specific aroma components are derived from the vicillin-class (7S) globulin of the cocoa seeds by proteolytic processing. Bot. Acta 108, 1-6. DOI: 10.1111/j.1438-8677.1995.tb00496.x

Ziegleder, G., Biehl, B., 1988: Analysis of cocoa flavour components and flavour precursors. Mod. Meth. Plant Analys. 8, 322-341. DOI: 10.1007/978-3-642-83343-4_14

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