Journal of Applied Botany and Food Quality 93, 313 - 320 (2020), DOI:10.5073/JABFQ.2020.093.038

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The Influence of fermentation-like incubation on cacao seed testa and composition of testa associated mucilage

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(Submitted: June 13, 2020; Accepted: December 19, 2020)

Summary

Because the testa of *Theobroma cacao* L. is a physical barrier for mass transfer of water and nutrients during germination, as well as in the fermentation process, analysis of the testa is necessary to understand these processes. This research deals with the morphological structure of the testa and its influence during fermentation of cacao seeds on the production of the aroma precursors of chocolate. Investigations have been carried out using fermentation-like incubations. For the first 48 hours of fermentation-like incubations, the seed represents a closed system with no transport of matter between the incubation-medium outside and the cotyledons inside. At this stage, the testa influences the course of the incubations by its barrier function. Much of this activity involves testa-associated mucilage. The composition of testa-associated mucilage is described.

Keywords: *Theobroma cacao* L., cacao seeds, cocoa, fermentation, testa, raw cacao, scanning electron microscopy (SEM), anatomy, morphology, testa associated mucilage

Introduction

The fruit of cacao (*Theobroma cacao* L., Malvaceae, formerly Sterculiaceae), is a capsule containing 20-50 seeds; approximately 800 of these seeds are necessary to produce a kilogram of raw cacao. The seeds are surrounded by a mucilaginous pulp that is rich in sugars and pectin. Neither this pulp nor the seeds themselves have any "chocolate" flavor. The flavors and properties that are associated with chocolate are dependent on the method of processing the seeds after harvest. In contrast to normal germination, during post-harvest treatment for commercial production of cocoa, the fruits are harvested, opened, and the cacao seeds are put together in heaps or in containers in which the availability of water is restricted. Oxygen is initially present. Naturally occurring yeasts break down the fruit pulp, which is derived from the endocarp, and the medium becomes semi-anaerobic. Yeasts carry out alcoholic fermentation and produce ethanol, carbon dioxide and heat. Much of the fruit pulp is liquefied and liquid from the degrading fruit pulp is lost. In this way, holes are developed between the seeds and this leads to an aeration of the fermentation mass. The ethanol produced is a substrate for acetic acid bacteria that produce acetic acid. Together with the lactic acid produced by lactic acid bacteria, the pH is gradually lowered down to about 3.6. Heat is increasing as a byproduct of the activity of acetic acid bacteria and the temperature rises to about 50 °C. Acetic and lactic acids are taken up by the testa and the structure of the testa is modified. Under these conditions, the micropyle is damaged permitting transfer of additional heat and water containing acetic acid and lactic acid into the cotyledons resulting in the death of the embryo. In a later step, acidic water is taken up by horizontal diffusion through the testa into the cotyledons, where it is mainly transported to protein storage vacuoles (BIEHL et al., 1982). In this way chocolate flavor precursors are produced, many of them are amino acids or small peptides.

The flavors are developed by roasting the fermented and dried seeds and by a number of sequential steps that modify both physical and chemical properties of the so produced cocoa. The least understood part of preparing good quality chocolate depends on complex factors involved in the fermentation process. Much depends on the role of the testa or seed coat of cacao seeds.

Aim of this study

In this study, the testa functions in the early stages of the fermentation process are examined. In submicroscopic studies, the structural changes during early fermentation have been followed until the first ruptures in the testa are evident and the testa loses its regulatory function. The main focus is the biochemical analysis of the testa associated mucilage and identification of three proteins that are components of that mucilage.

Material and methods

Cacao fruits used for experiments were of the Forastero type from Ivory Coast. Each fruit contained up to 50 seeds of one to two grams each, which are surrounded by a sweet tasting pulp.

Incubations under fermentation-like conditions for investigation of influence on testa structure

Each batch of minimum eight cacao seeds in 200 mL of incubation medium (100 mM acetic or lactic acid, pH 4.5) was incubated for 4 days in a water bath initially set at 30 °C. After 24 hours, the water temperature was increased to 35 °C and after 48 hours to 55 °C. In the first 48 hours of incubation, oxygen was displaced by gassing with nitrogen. Samples of two seeds were collected after 24, 48, 72 and 96 hours. A sample of two seeds from each batch was taken before incubation was started.

Depulping the cacao seeds

The pulp was enzymatically removed from the seeds with Pectinex® BE XXL (Novozymes, Bagsvaerd, Denmark), consisting of polygalacturonase and pectin lyase. This was carried out overnight at 35 °C in distilled water at pH 4.5.

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Light microscopic analysis of cacao seeds

Embedding of seed tissue

Seed coats were removed with a razor blade and cut in approximately 4 mm^2 pieces, then fixed for at least 24 h in 4% formalin in distilled water. The testa sections were dehydrated in an ethanol series: two times for 30 min in 70% ethanol and 2 times for 30 min in 99.9% ethanol. Subsequently, the testa pieces were transferred into 100% LR White Medium Grade™ (water miscible) embedding medium (London resin company, Reading, England). This was replaced after an hour with fresh medium and the preparations incubated overnight in a refrigerator. The next day, while still impregnated with the liquid resin, testa pieces were transferred individually into 0.36 cm^3 gelatin capsules. These were then filled with LR White medium grade™ embedding medium and incubated for curing for 24 h at 60 °C in a drying cabinet. Before further processing, the capsules were hardened by storage for 48 hours at room temperature.

Preparation of semi-thin sections

The cured, capsule-shaped synthetic resin products were trimmed with a razor blade under a binocular microscope and then clamped into an ultra-microtome. With freshly made glass knives, 2 to 2.5 µm thick sections were prepared and transferred onto glass slides. Each slide was placed for a few minutes on a hot plate at 50 °C in order to expand and relax the floating semi-thin sections and then permitted to dry which allowed them to become attached to the glass surface.

Staining of the slice preparations

Staining of the semi-thin sections through the cacao seed coat tissue was performed with Toluidine Blue O stain (Merck, Darmstadt, Germany) (modified following GUTMANN, 1995). Each slide was sprinkled with a solution of 0.05% (w/v) Toluidine Blue O in distilled water. After an exposure of only a few minutes, the slides were rinsed with distilled water and dried on a hot plate at 50 °C.

Scanning electron microscopy

For scanning electron microscopy, the seed pulp was removed with pectinase and the testa surface exposed. Then the testa was stripped from the seed so that the inner surface of the testa could be prepared. The samples were fixed in 4% formalin in distilled water and dehydrated in an ethanol series [30-100% (v/v) in water]. Then they were freeze dried, affixed to aluminum stubs and coated with gold in a sputter coater. Samples prepared in this manner were examined with a Philips XL 20 scanning electron microscope (Philips, Amsterdam, Netherlands).

Extracting the testa-associated mucilage (TA-mucilage)

After removal of the pulp, the seeds were again submerged in distilled water and allowed to swell for 24 hours at room temperature (RT). The mucilage was then manually removed with a scalpel and collected.

Quantitative determination of protein content of TA-mucilage

Measurement of the concentration and amount of protein in TA-mucilage was carried out using the Bradford protein assay (BRADFORD, 1976). 1 mL Bradford solution was added to 100 mL mucilage (Fig. 8), mixed well and incubated for 15 min at room temperature in the dark. The extinction coefficient was measured at a wavelength of 595 nm with a SmartSpec™ Plus Spectrometer (BIO RAD, Hercules, CA, USA). Distilled water was used as a control. Extinction coefficients were compared to a standard curve prepared with BSA between 10 and 150 µg mL^{-1} as the reference protein.

Analysis of free amino acids

TA-mucilage (1 mL) taken from the mucilage layer after 8 hours of swelling time of depulped seed was added to 4 mL of distilled water, which had been adjusted to pH 2.0 with 50% aqueous trifluoroacetic acid; the pH of the TA-mucilage-water mixture was then adjusted with 50% aqueous trifluoroacetic acid to pH 2.5. An additional 5 mL of distilled water pH 2.0 (as above) was added by rinsing the pH-electrode. This mixture was extracted on ice for 1 hour with a magnetic stirrer and the resulting suspension was centrifuged at 2,800 g (Heraeus Thermoscientific Megafuge 11R, Hanau, Germany) for 10 minutes. The clear supernatant was filtered through a 0.45 micron filter (Macherey-Nagel, Düren, Germany) and 30 µL of this filtrate was freeze-dried for one hour at -20 °C and 0.05 mbar in an HPLC-sample vial. For measurement, 800 µL borate buffer was added to the freeze-dried sample and 400 µL of *o*-phthaldialdehyde (OPA) reagent was added immediately before sample injection. After 2 minutes, 20 µL of this mixture was injected into the HPLC system (4-channel-degasser: Knauer, Berlin, Germany; HPLC pumps: Knauer, Berlin, Germany; Dynamic Mixing Chamber: Knauer, Berlin, Germany; Auto sampler: AS 2000 Merck-Hitachi; Darmstadt, Germany, column: LiChroCART® 250-4 Merck, Darmstadt, Germany; precolumn: LiChrospher®100 RP-18 (5µm) Merck, Darmstadt, Germany).

Reagent and buffer preparation

Borate buffer was prepared by dissolving 12.38 g of boric acid in 800 mL of water and adjusting to pH 9.5 by addition of concentrated KOH solution. This boric acid solution was boiled for 5 minutes and filled up to one liter with boiled distilled water. The buffer was stored in a refrigerator at 4 °C (200 mM, pH 9.5).

OPA reagent was prepared by dissolving 100 mg *o*-phthaldialdehyde in 2.5 mL of methanol. Before addition of the reagent, 22.4 mL of borate buffer pH 9.5 and 100 µL of 2-mercaptoethanol were added. The OPA reagent had to be prepared 24 hours prior to analysis.

HPLC analyses

The analyses were carried out with a gradient based on the following mobile phases (Tab. 1):

A: 1.6 L of 50 mM sodium acetate solution, pH 6.2, 50 mL methanol Lichrosolv (Merck, Darmstadt, Germany) gradient grade, 20 mL tetrahydrofuran Lichrosolv (Merck, Darmstadt, Germany)

B: 200 mL of a 50 mM sodium acetate solution at pH 6.2, 800 mL of methanol Lichrosolv (Merck, Darmstadt, Germany) gradient grade

Tab. 1: HPLC gradient for analysis of amino acids

time [min]	eluent flow [mL/min]	% A	% B
0	1.3	100	0
2	1.3	95	5
12	1.3	85	15
20	1.3	60	40
25	1.3	50	50
40	1.3	0	100
50	1.3	0	100
55	1.3	100	0
75	1.3	100	0

Mass spectrometric characterization of the TA-mucilage proteins

Aliquots of fresh TA-mucilage were mixed with Laemmli buffer (concentrated 5-fold: 5 mL 10% sodium dodecyl sulfate (SDS) solution, 1.25 mL of 2 mol/L Tris pH 6.8, 0.7707 g dithiothreitol (DTT), 3 mL glycerol, and 2 mg bromophenol blue) and then heated for 5 min

at 95 °C. The proteins were separated by SDS-gel electrophoresis on a separating gel consisting of 12% acrylamide (120 V) and a stacking gel of 4% acrylamide (64 V) (molecular weight marker: PageRuler Protein Ladder, Thermo Fisher scientific, Waltham, Massachusetts, USA). These gels were then stained with Coomassie G stain. The most intense bands were excised, proteins reduced with DTT (10 mM, 56 °C, 30 min), the cysteines modified with iodoacetamide (55 mM, RT, 20 minutes in the dark) and modified proteins in the gel digested with trypsin (5 ng/µL trypsin in 50 mM NH_4HCO_3 solution, 37 °C, overnight). After digestion, the gel pieces were extracted repeatedly with a mixture of 50% acetonitrile and 5% formic acid. The combined extracts were dried in a lyophilizer and then dissolved in a mixture of 5% methanol and 5% formic acid. The dissolved peptides were desalted on a C18mZipTip syringe (Merck Millipore, Darmstadt, Germany) and eluted with 1 µL of a mixture of 60% methanol and 5% formic acid. The sample was analyzed by nano-electrospray mass spectrometry on a QTOF II tandem mass spectrometer (Micromass, Manchester, UK). MS/MS spectra, obtained by collision-induced fragmentation of the peptides, were evaluated by means of manual and Mascot MS/MS ion search algorithm.

Results

Seed anatomy

As is true for most plants, the fruit of cacao possesses three distinct layers: the exocarp, mesocarp and endocarp. Seeds of the plant occur within the endocarp in tissues derived from the endocarp and the fertilized ovule. During development of the seeds, water and nutrients are transferred into the ovule via the placenta from the parent plant. This occurs via the funiculus and the raphe, a large vascular bundle originating from the placenta that terminates at the chalaza end of the developing ovule at the hypostasis (Fig. 1). When the seed is removed from the fruit, the raphe ceases to be connected leaving behind a structure called the hilum.

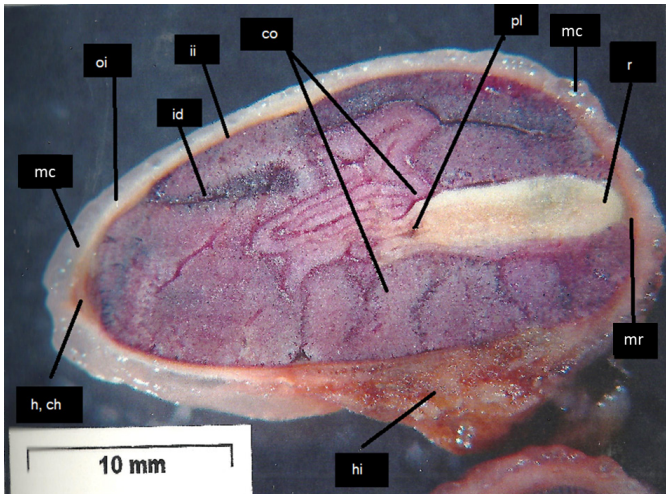

Fig. 1: Longitudinal section of a cacao seed: after pectinase treatment fruit pulp has been removed and testa associated mucilage has taken up water and covers the testa as a continuous layer. The hypostasis (h), chalaza (ch), hypodermal mucilage caverns (mc) outer integument (oi), inner integument (ii), cotyledons (co), plumule (pl), idioblasts filled with flavonoid phenolic compounds (id), radicula (r), micropylar region (mr), and hilum (hi).

The cross-section of a fresh cacao seed with adherent fruit pulp consists of a number of clearly identifiable structures (Fig. 1, Fig. 2). The outermost feature of the seed is a slightly slimy, sweet-sour-tasting pulp formed from tubular cells of the endocarp (Elwers, 2008). Inside the fruit pulp is a complex seed coat or testa that arises from the integuments of the ovule and is divided in the mature state into the exo- and the endotesta (Fig. 3).

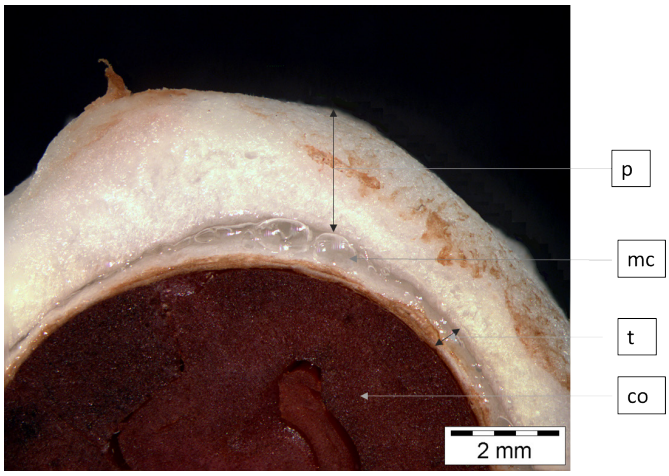

Fig. 2: Cross section of a cacao seed with pulp, mucilage caverns, testa and cotyledons, p = pulp, mc = mucilage, stored in mucilage cavern, co = cotyledon tissue, composed of two folded cotyledons. The fruit pulp (p) is derived from the endocarp. The mucilage (mc) is found in the outer layer of the testa (t). The cotyledon tissue (co) is surrounded by the testa (see Fig. 3 for greater detail). The boundary between the endotesta and the exotesta as seen in Fig. 3 is not seen in Fig. 2 because the magnification is not sufficiently high.

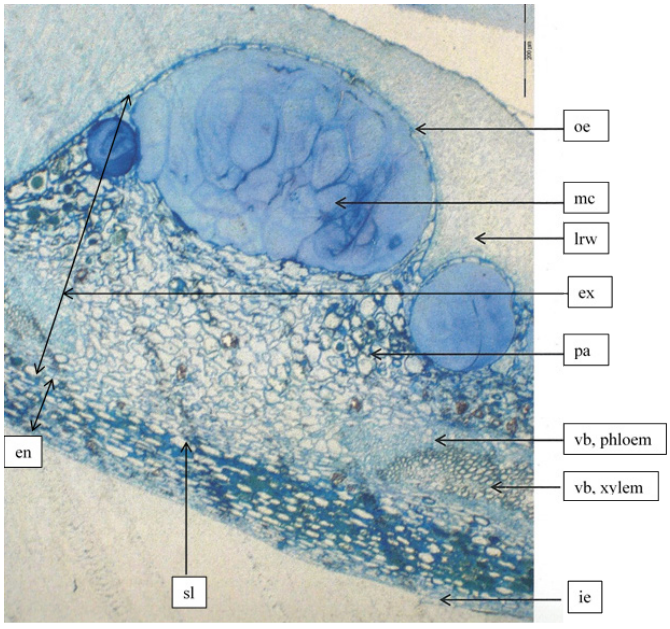

Fig. 3: Light microscope picture of the testa from depulped seed with mucilage caverns, vascular bundles and blue colored polyphenol storage cells (this slice has been stained with Toluidine Blue O), oe = outer epidermis, mc = mucilage, pa = parenchyma (in this case, loosely stained parenchyma), vb, phloem = vascular bundles, phloem tissue, vb, xylem = vascular bundles, xylem tissue, ie = inner epidermis, lrw = embedding medium (LR-White Medium Grade). The limits of the exo (ex) and endotesta (en) are seen by a sclereid layer (sl). Neither the cotyledons nor the fruit pulp are to be seen in this testa preparation. The non-cellular silvery layer is adjacent to the inner epidermis but is not seen due to the preparation method of the testa. The magnification is indicated in the right upper corner and indicates 200 µm.

In the exotesta, there is a clearly discernible accumulation of "caverns" separated from the epidermis, in which a colorless mucilage is stored (CHEESMAN, 1927). In the process of germination, this mucilage layer binds water. The mucilage caverns do not possess a cell wall, but are limited by the surrounding cells. Without strong supporting data, CHEESMAN (1927) postulated that the mucilage caverns initially possessed cell walls that disappear on seed maturation by lysogenic or rhexigenic action (Fig. 1, Fig. 2). A cross-section through the testa of a depulped cacao seed shows the large mucilage caverns directly beneath the outer epidermis in the exotesta (Fig. 3). Beneath the mucilage caverns is first a loose parenchymal tissue in which the vascular bundles are located. When a cacao seed is freed from fruit pulp, the contours of the vascular bundles of the exotesta are clearly visible on the outside of the seed (Fig. 4).

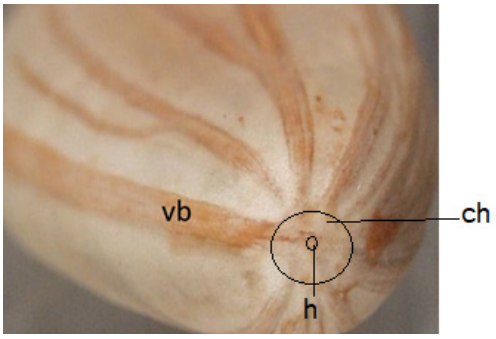

Fig. 4: Vascular bundles (vb) in the testa of a depulped seed. The vascular bundles approach, but are not continuous at the chalaza (ch). The hypostasis (h) at the center of the chalaza is not clearly to be seen. The micropyle is at the opposite end of the seed.

Scanning electron microscopy

In addition to data from light microscopic studies, additional data concerning the anatomical structure of the testa have been obtained from scanning electron microscopy of sections of the testa. In scanning electron micrographs of cross sections of the testa of depulped cacao seeds, stacking of the individual cell layers is apparent. Mucilage caverns directly beneath the outer epidermis are clearly visible in recordings with the scanning electron microscope. The vascular bundles are clearly visible and are located on the border of loosely packed to densely packed parenchyma (Fig. 5).

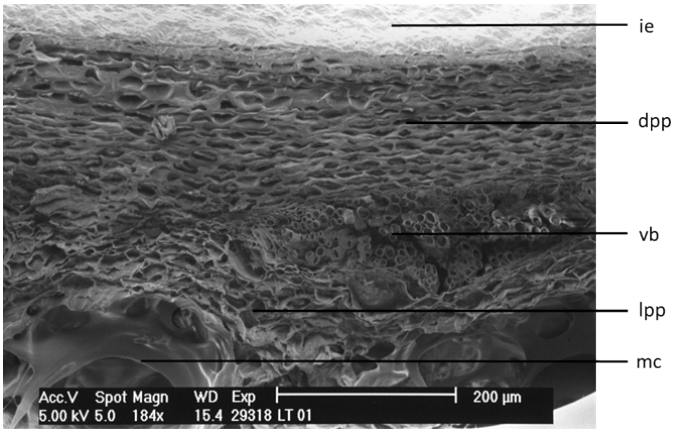

Fig. 5: SEM image of a testa cross-section from a fresh but depulped cacao seed. The mucilage caverns are oriented to the bottom; the inner epidermis is directed to the top of the figure. vb = vascular bundle, mc = mucilage cavern, ie = inner epidermis, dpp = densely packed parenchyma, lpp = loosely packed parenchyma. The location of the silvery layer would be adjacent to the inner epidermis of the endotesta.

From inside the dissection of the chalaza region, it can be seen that several vascular bundles arise at the hypostasis. These vascular bundles of the endotesta have no direct contact with the vascular bundles entering via the raphe (Fig. 6).

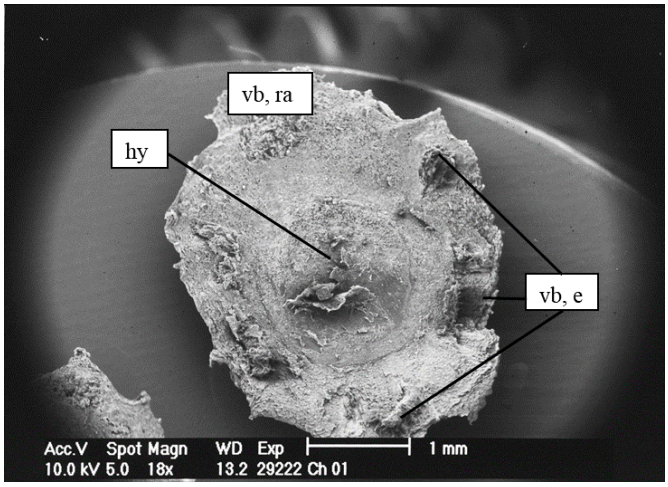

Fig. 6: SEM image of the chalaza from inside. hy = hypostasis, vb ra = vascular bundle from the raphe, vb e = vascular bundle entering the endotesta, magnification: see scale.

The extent of mucilage caverns in relation to the testa is evident in the scanning electron micrographs as it was in the optical micrographs (Fig. 2, Fig. 3). The mucilage caverns are not surrounded by a cell wall, but are located in the intercellular spaces between the other much smaller cells directly beneath the outer epidermis and may achieve a longitudinal extent of about 100 microns to 200 microns. As the caverns mature, accumulation of large amounts of mucilage occurs. Caused by lyophilization for SEM preparation the mucilage dried out and the caverns themselves appear hollow in scanning electron recordings (Fig. 5, Fig. 7).

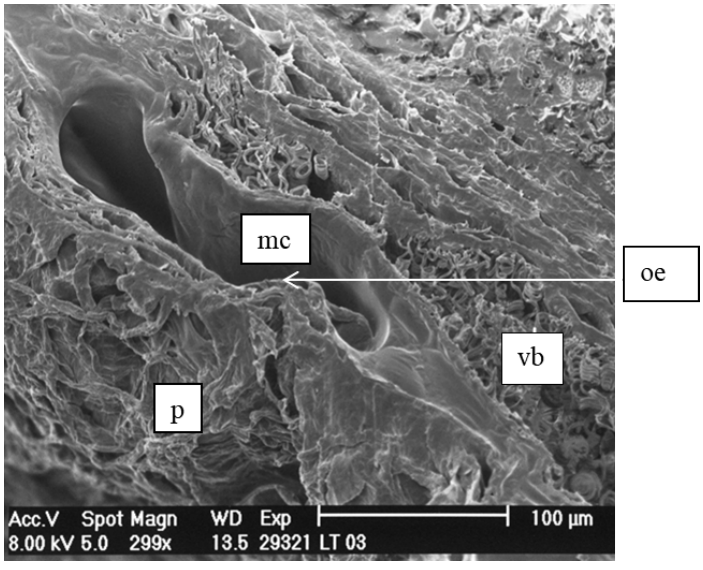

Fig. 7: SEM image of a mucilage cavern, p = fruit pulp located outside the testa directly attached to the outer epidermis (oe) of the exotesta, pulp cells are desiccated and dried by preparation during lyophilization, mc = mucilage cavern, vb = vascular bundle. Parenchyma tissues are adjacent to the outer epidermis. The endotesta (en) is not seen in this sample.

The testa associated mucilage

When incubated in water, the mucilage from the mucilage caverns of the testa of cacao seeds (testa associated mucilage / TA-mucilage) binds water and expands (Fig. 2, Fig. 3). The TA-mucilage only appears after the fruit pulp is removed and the aqueous phase can reach the mucilage caverns. At that point, a large volume of mucilage develops and covers the seed, surrounding it with a continuous layer (Fig. 3 and Fig. 8). This TA-mucilage is stored in the caverns directly under the epidermal layer of the testa.

Chemistry and properties of the mucilage

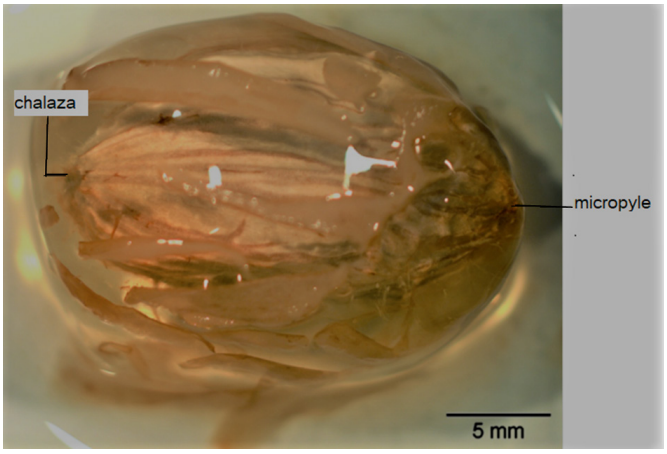

Fig. 8: Swollen TA-mucilage layer of a depulped cacao seed after 8 hours in water. The micropyle is visible covered by the swollen mucilage layer. Vascular bundles are evident on the surface of the testa. They are not connected to the micropyle. The chalaza is at the opposite end of the seed from the micropyle. Layers from the outer part of the exotesta are also visible.

In contrast, when the TA-mucilage of cacao is hydrolyzed, the content of individual amino acids increases considerably (Fig. 9), whereas untreated TA-mucilage contains only small amounts of free amino acids. The most abundant amino acids are glutamic acid, lysine and aspartic acid. Glutamine, histidine, and tryptophan were present in detectable amounts only after hydrolysis (Fig. 9). These data indicate that TA-mucilage contains proteins or peptides, as demonstrated by the elevated levels of free amino acids found after hydrolysis. The basic material of the TA-mucilage in which the enzymatic active proteins are integrated in is at this time unknown.

The amount of protein in seed coat mucilage was determined by Bradford Protein Assay to be 11.4 µg mL^{-1} extracted seed coat mucilage and 0.46 mg per complete seed as estimated from the volume of the mucilage per seed after three days of swelling time.

Separation of the TA-mucilage proteins was carried out by SDS gel-electrophoresis. TA-mucilage contains several proteins of different molecular weight (Fig. 10). They are contaminated with polygalacturonase and pectinlyase (molecular weights of approximately 40 and 50 kDa) from Pectinex® BE XXL that was used to digest and remove the fruit pulp.

After tryptic digestion of excised bands and use of nano-spray mass spectrometry, the sequences of the resulting peptides were identified by comparison to EST databases of *Theobroma cacao* (ARGOUT et al., 2008). From these ESTs, the sequences of the respective proteins could be assembled and identified by comparison to sequences of other species. Three of the most important proteins were chitinase, glucanase and osmotin. The composite sequences of the proteins obtained by comparison with EST databases data are shown in Tab. 2.

The effects of acid on the testa, the sclereid layer and the micropyle

Water and acid break down the structure of the testa within the first two days of fermentation, including the permeability of the sclereid layer and permit acid to enter the seed and cotyledons. The micropyle is damaged permitting entry of water and acid directly into the seed (Fig. 11 a and b).

Discussion

The testa or seed shell of cacao seeds serves to protect the embryo from mechanical damage but also represents a physical barrier for microorganisms and plays an important role in protection of the nutrient-rich cotyledons in the early germination phase. Because of this, the testa also influences the course of fermentation in the production of raw cacao.

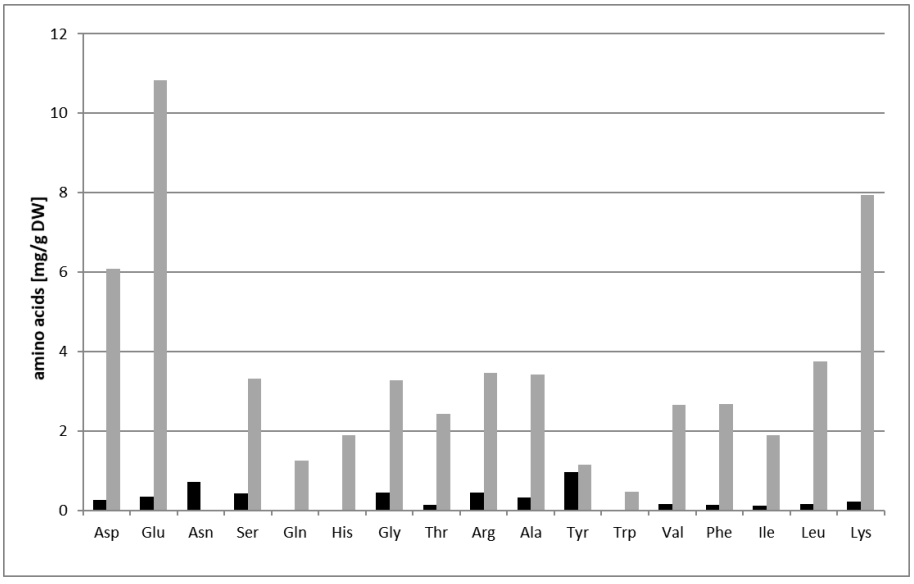

Fig. 9: Content of the individual free amino acids before (dark symbols) and after (gray symbols) acid hydrolysis of the TA-mucilage. The average value of two measurements is given. n = 2.

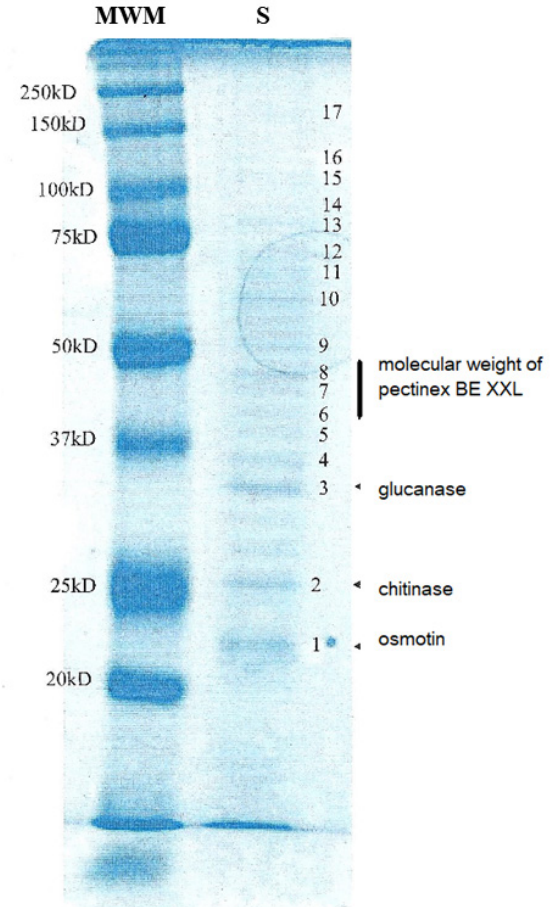

Fig. 10: SDS gel electrophoresis of the proteins and peptides from TA-mucilage. MWM = molecular weight marker, S = TA-mucilage, the numbers mark the visible proteins later identified by HPLC-MS 1 = osmotin, 2 = chitinase, 3 = glucanase, 4-17 = unidentified proteins.

Tab. 2: Proteins identified by their derived EST sequences.
The sequences in bold, correspond exactly to those found in the EST sequences of the corresponding protein, whereas the sequences in gray only partially coincide.

protein	derived EST-sequence
osmotin	MSSFKTLPTLSFLFVTLFSLAHAATFDIR**NNCPYTVWAAAVPG GGR**RMNR**GETWQISAAPGTTQAR**IWARTNCQFDASGRGK **CQTGDCGGVLECK**GYGSPPNTLAEYAIGQFANQDFIDISNI DGFNVPMEFSSNSPGCTRVIKCTADIVGQCPNELKVPGGCN GPCPVFKTEEHCCNSGNCGPTNFSKFFKDRCPDAYSYPKDD **PTSLFTCPTGTNYK**VIFCPStop
chitinase	MRFLTLLVACILASLSRALADITPLISR**DLFNEMLK**HRNDA NCPGNGFYTYDGFIAAAN**SFGAFGTTGDVDTR**KREIAAFL AQTSHETTGGWATAPDGPYAWGYCFVQEQGNPGDYCVAS QEWPCVPGKKYFGR**GPIQISYNYNYGPAGKAINVDLLNN PDAVAR**DPTISFKTALWFWMTPQSPKPSCHDVITGQKPSAQ DTAAGRVPGYGVITNIINGGIECGKGSNPQVEDRIGFYKRY CDIL**QVSYGDNLD****CYNQRPFA**Stop
glucanase	MGKSYLAGNFGFMASTMLLFGLLVASLRTTSAQVGVCYG MLGNNLPPRPEVIALFNQRNIRRMRLYGPDKPALEALRGSN IELMLGVPNDK**LEGLAANQANANAWVQDNVR**NYGNVKFR **YIAVGNEVKPSDPAARFVVPAMQNIRNAIVGAGLGNQIK VSTAIDTVTLGESFPPSK**GSFRPDYRPLLDPIIRFLVNNK**APL LVNLYPYFSYDDNSDIR**LDYALFK**AQNPVVSDPPLQYR**NL FDAILDAVYAALEK**AGGGSLEIVVSESGWPSAGGR**KRGA TNIDNARTYNQNLIQVKPPGKPIEAYIFAMFDENNKQGEEIE RHWGLFLPNKQPKYPINFNStop

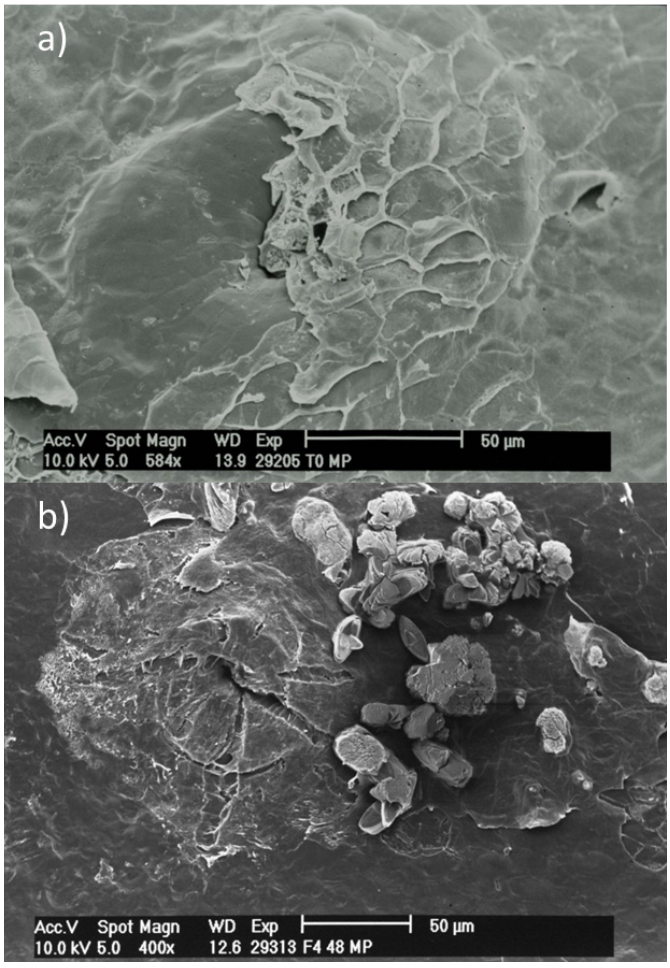

Fig. 11: a) SEM image of cacao micropyle from the inside of the testa of the seed before incubation. b) SEM image of cacao micropyle from the inside of the testa of the seed after 48 h of incubation. Note the cracks surrounding the micropylar area.

At the beginning of the fermentation process, the testa serves as a natural barrier between the fermentation mass and the embryo. The testa is impermeable to movement of water and of microorganisms. The sclereid cell layer initially is shown to be impermeable to acetic acid by exclusion of a fluorescent tracer (Lucifer Yellow CH) with the same dissociation constant as acetic acid (ANDERSSON et al., 2006). The assumption of initial impermeability of the testa is also supported by the observation that the purple discoloration and later browning of the seed tissue begins during fermentation at the micropyle, progresses along the seed axis and later reaches the outer regions of the seed (ROHSIUS et al., 2006). If transport of acids through the testa did occur initially, the purple discoloration and later browning would begin in the outer regions of the seed adjacent to the exotesta, but this does not occur.

At the beginning of the fermentation process, the radicula absorbs water initially provided by the raphe, elongates and pierces the testa at the micropyle. After penetration of the micropyle by the radicula, mass transport between the inside of the seeds and the outside fermentation medium occurs (VOIGT and LIEBEREI, 2014). No direct connection of the vascular bundle of the raphe to the vascular bundles of the seed exists. Although it might seem possible for mass transfer through the raphe to the inside of the seed to occur, this is limited because there is no direct and continuous linkage between the vascular bundles in the testa and the bundles of the raphe at the chalaza. Further, transverse transport through the testa, regardless of

the presence of vascular bundles, stops at the sclereid layer that separates the outer from the inner integument (Andersson et al., 2006). All this suggests that the seed initially represents a closed system and is best understood as an isolated reaction chamber.

The function of the TA-mucilage during germination

The testa-associated mucilage (TA-mucilage) present in the first sub epidermal layer of the exotesta and the sclereid layer appears to limit transport processes within the testa, exerts control of transport of substances into the seed and forms a barrier for the embryo against external substances. In addition to the potential importance of the TA-mucilage for fermentation processes, the ecological role of this protective, preformed layer in germination under natural conditions needs to be further examined. The TA-mucilage probably exerts a controlling influence on microbial processes during normal seed germination as well as in the different phases of fermentation.

The mucilage stored in the mucilage caverns of cacao seed is composed mainly of proteins instead of carbohydrates as in many other plant mucilages (Tomoda et al., 1987; Cui et al., 1994). Our biochemical investigation revealed that the TA-mucilage protein does not consist of a single protein, but at least out of 17 different proteins, three of which, osmotin, chitinase and glucanase, have been identified. Each is known to have functions in plant defense against pathogenic fungi (Abad et al., 1996; Collinge et al., 1993; Gomes et al., 1996). The activity of osmotin, chitinase and glucanase against microorganisms involved in cacao fermentations has to be investigated.

Osmotin acts in the plant as an antifungal regulator. This enzyme prevents germination of fungal spores and induces lysis of germ tubes, both leading to decreased survival of the fungus (Anil Kumar et al., 2015). The inhibitory effect of osmotin is based on the abolition of the pH gradient across the membranes of fungal cells (Abin et al., 2011). Heterologically expressed peptides of a cacao osmotin-like protein (TcOsm1) have an expressed antifungal property (Falcao et al., 2016).

The inhibitory activity of chitinase against pathogenic fungi is based on the ability of this enzyme to break down newly formed chitin walls of higher fungi, resulting in inhibition of growth of fungal hyphae (Kasprzewska, 2003). Chitinases degrade the chitin of fungi to oligosaccharides, which act as elicitors or signaling molecules that can induce defense reactions in plants (Kasprzewska, 2003). Glucanases, as well as chitinases, also attack the cell walls of the fungi (Arlorio et al., 1992). Glucans form the main component of fungal cell walls of oomycetes and related fungi and can be degraded by the glucanases. Experiments of Arlorio et al. (1992) have shown that a combination of chitinase and glucanase is particularly effective against pathogenic fungi.

Each of the three proteins present in TA-mucilage is known to exert fungicidal action in plants.

The TA-mucilage only begins to swell after the fruit pulp layer is removed. In the early phase of fermentation, yeasts produce pectinases that lead to the breakdown of the pulp layer. After drainage of the degraded fruit pulp, the TA-mucilage is exposed to water and forms a mucilage layer that may be inhibitory to fungal growth (Abad et al., 1996; Collinge et al., 1993; Gomes et al., 1996). At this phase of fermentation, populations of acetic acid bacteria increase, they oxidize ethanol formerly produced by the yeasts, and form acetic acid resulting in acidification of the seed tissues (Camu et al., 2007). These processes are accompanied by a considerable increase in temperature. Besides the production of acetic acid and the temperature increase up to 50 °C the possibly antifungal action of proteins of TA-mucilage may be an explanation for the decrease of yeast population during cacao fermentation after 2 days (Schwan and Wheals 2004; Camu et al., 2007, De Vuyst and Weckx, 2016).

Abbreviations used in figures in the text:

ch	chalaza
co	cotyledon
cs	cacao seed
dpp	densly packed parenchyma
ec	exocarp
en	endotesta
ex	exotesta
f	funiculus
h	hypostasis
hi	hilum
id	idioblast cells
ie	inner epidermis
ii	inner integument
lpp	loosely packed parenchyma
mc	mucilage or mucilage caverns
mr	micropyle or micropylar region
oe	outer epidermis
oi	outer integument
p	fruit pulp
pa	loose parenchyma
pl	plumule
r	radicula
ra	raphe
sl	sclereid layer
t	testa
vb	vascular bundle

In this article, we have used the term „cacao“ to indicate fruit material, unprocessed seeds and fermented seeds from *Theobroma cacao*. The term „cocoa“ is used for fermented seeds after roasting. Chocolate is used to refer to cocoa that has been additionally processed, usually including the addition of sugar, milk products, and other substances.

Cit.: https://writingexplained.org/cacao vs. Cocoa.: Cacao vs. Cocoa – what is the Difference?

Conflict of interest

The authors declare no conflict of interest.

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