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Fate of proanthocyanidins and anthocyanins along fermentation of cocoa seeds (*Theobroma cacao* L.)

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Summary

Condensed tannins, also called proanthocyanidins, play a substantial role in the sensory characteristics of chocolate. Little is known about their fate along fermentation. In the present study, location and behavior of tannins in the cotyledons and *testae* of cocoa seeds are studied during fermentation by light and transmission electron microscopy. Tannins along with anthocyanosides were also measured.

Tannins are located in special tanniferous cells from both cotyledons and *testae* of native seeds. The most striking phenomena occurring during fermentation are an almost complete disappearance of the *testa* tannins and, in the cotyledons, a translocation without losses of the tannins from the tanniferous cells towards surrounding tissues. Quantitative analyses confirm these observations and show that little oxidation of the cotyledonary tannins occurs. The anthocyanosides from the cotyledons completely disappear.

It is assumed that this translocation could hinder proteolysis of the cotyledon proteins by formation of resistant tannin-protein complexes.

Introduction

The cocoa tree (*Theobroma cacao* L.), belonging to the *Malvaceae* (formerly *Sterculiaceae*) family, is grown for its fruits, actually berries. The fruit (also called a "pod", in French "cabosse") contains seeds (commonly called beans), which are fermented, and then dried to yield fermented dried cocoa beans, the starting material in chocolate making (ELWERS et al., 2010). Fermentation is a key step for the formation of aroma precursors, leading to fine and flavorful chocolate.

During this fermentation process, the activity of microorganisms on the cocoa pulp will produce alcohol, lactic acid, acetic acid, and generate heat that causes seed death. The chemicals and water from this cocoa pulp fermentation will penetrate slowly into the cocoa seeds causing them to swell and inducing (bio)chemical changes and development of the type and concentration of flavor precursors in the beans (HASHIM et al., 1998). With this respect, proteolysis occurring along fermentation will produce peptides and amino acids that contribute directly to the flavor of chocolate and to the Maillard reaction during roasting (DE BRITO et al., 2001). Thus, fermentation is actually a prerequisite to obtaining the characteristic cocoa-specific aroma upon roasting (ROHAN, 1964), since roasted unfermented cocoa beans do not generate cocoa aroma but produce an excessively bitter and astringent taste instead (LOPEZ et al., 1987).

Proanthocyanidins, also called condensed tannins, are present in most vascular plants and are thought to play diverse roles. They provide defense against herbivores and pathogens and protection against ultraviolet radiation. These secondary metabolites are polymers of catechins belonging to the vast family of flavonoids. The flavor quality of cocoa correlates with its content of oligomeric proanthocyanidins (COUNET et al., 2004). As tannins are known to form complexes with proteins (HAGERMAN and BUTLER, 1981), they could weaken the efficiency of endogenous proteolysis, thus lower the levels of aroma precursors. Moreover, these polymers causing an astringency mouthfeel which could be lessened by oxidation. It was of utmost importance to assess the fate of condensed tannins through fermentation in terms of quantity and localization.

Materials

Fresh cocoa fruits

A varietal mix of cocoa fruits was harvested in the Dominican Republic at physiological maturity and air-freighted within 24 h to our laboratory in a cooled container (4 °C). Seeds (pH 6.5) were removed from the fruit and processed immediately.

Fermentation

Cocoa fruits from the above harvest were opened with a knife, and seeds were recovered by hands with their covering mucilage, and then placed in wooden boxes. The cocoa beans were left to ferment at ambient temperature for 6 days with hand-mixing every two days. Fermented wet seeds (pH 4.5) were randomly withdrawn from boxes, and air-freighted as above.

Sampling

Specimens (1 mm^3) were obtained by piercing seeds (n = 10) transversally at the equator with a cork borer (diameter 5 mm). Cocoa seeds were also hulled with a scalpel, and sectioned transversally or longitudinally.

Methods

Light microscopy (LM)

Specimens were fixed in a glutaraldehyde/acrolein mixture with the presence of caffeine, dehydrated in a graded ethanol series, and embedded in resin (BRILLOUET and ESCOUTE, 2011). Thin sections (3 µm) were stained by Toluidine Blue O as described above. A fresh section of native seed was stained with dimethyaminocinnamaldehyde (DMCA) (BRILLOUET and ESCOUTE, 2011).

Transmission electron microscopy (TEM)

Specimens were dipped in 50 mM sodium cacodylate buffer (pH 7.0) containing 6 % glutaraldehyde (w/v) (SIRONVAL et al., 1968) and 1 % caffeine (w/v) for 6 h, and then treated with 1 % osmium tetroxide (w/v) in water for 1 h. After dehydration, they were embedded in Epon EmBed 812. Sections were stained with 0.2 % Oolong tea (SATO et al., 2008). Ultrathin sections (60 nm) were visualised by an H-7100 Hitachi transmission electron microscope with 75 kV accelerating voltage.

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Measurement of condensed tannins

Condensed tannins were analyzed on freeze-dried defatted liquid nitrogen powders by the 2-mercaptoethanol technique according to ROUMEAS et al. (2013): briefly, aliquots in methanol containing 10% mercaptoethanol, and 0.1N HCl were heated at 40 °C for 2 h, and the thiol-adducts resulting from the depolymerization were analyzed by HPLC-DAD-MS. The tannins containing undepolymerizable resistant bonds resulting from oxidation appear as a hump underneath the individual thiol-adducts peaks: they were estimated by integration of the hump alone and expression as epicatechin equivalents.

Qualitative examination of anthocyanins

Anthocyanins were extracted on freeze-dried defatted liquid nitrogen powders by methanol containing 0.05M HCl and analyzed by HPLC-DAD-MS (λ = 530 nm).

Determination of pH

Unshelled seeds (10 g) were homogenized in distilled water (90 ml) with an Ultra-Turrax, and then pH was measured on the filtrate.

Results

General description of the seeds

Characteristics of the native ripe and fermented seeds are shown in Fig. 1. Native seed appears vaguely ellipsoidal and covered with a translucent mucilaginous tissue (1); after scraping off the mucilage (2), the outer face of the *testa* shows a longitudinal belt of vascular bundles. Its inner face (3) is slightly tan and dry. The cotyledons (4) appear as deep purplish-blue multi-lobed jointed structures, the interlobe space observed from their outer surface being filled with a discrete whitish material, the endosperm (LOPEZ et al., 1987). On a longitudinal section (5), the interlaced multi-lobed structure of the cotyledons is well visible; the color of the cotyledonary tissue is purplish-blue and the embryo is yellow; the outer layers of the lobes exhibit a brown color.

After fermentation, the mucilaginous tissue has almost disappeared (6) and the wet shining outer face of the *testa* is brown while its inner face is wet, shining, and deep reddish-brown (7). The inter-lobe endosperm has disappeared from the cotyledons (8), which suggests either it remained attached to the *testa* or the intervening along fermentation of endogenous pectinases. The most striking observation is that, after fermentation, the purplish-blue color of the cotyledon flesh has fainted dramatically, turning to a weak lilac nuance (9); moreover, the outer face of the epidermis visible on unsectioned lobes was chocolate brown (9).

Behavior of proanthocyanidins in the cotyledons

Samples were taken with a cork borer from the outer region of cotyledons from native and fermented seeds, fixed in glutaradehyde and acrolein, dehydrated in a graded ethanol series, and finally embedded in resin (BRILLOUET and ESCOUTE, 2011). Thin sections were stained with Toluidine Blue O or DMACA and observed under light microscope (BRILLOUET and ESCOUTE, 2011).

The tissue was made from the periphery of small epidermal cells. Tanniferous cells are rather evenly distributed within the cotyledonary storage parenchyma with a slight predominance in the subepidermal tissue (Fig. 2). Their size range from 20 to 50 µm and they are ellipsoidal in shape. They contain Toluidine Blue O strongly positive material which is made of giant chlorotannic accretions (BRILLOUET et al., 2014) filling entirely the vacuole, and punctuated with *lacunae*. These last authors described the (chloro)tannic accretions as gatherings of thousands of shuttles (visible in Fig. 2, insert), themselves made of packings of thousands of tannosomes, the organelle responsible for polymerization of condensed tannins (BRILLOUET et al., 2013). Other parenchymatous cells show rather indistinct contents with small lipid vacuoles and starch.

After fermentation, the cotyledonary tissue showed mesophyll cells entirely filled with spherical empty bodies resulting from the fusion of lipids. Intercalated between lipid bodies strong Toluidine Blue O-positive stuff were seen (Fig. 3, pink arrowheads). The most striking phenomenon was the almost total disappearance of the (chloro)tannic accretions from the tanniferous cells; indeed, only remnants of them were observed.

TEM examination of the cotyledonary tissue from fermented seeds revealed, as LM, fusion of lipid into spherical bodies (Fig. 4). Patches of osmium-positive material were observed between lipid bodies (red arrows); they correspond to the intercalated stuff described above. This material is unambiguously made of condensed tannins: indeed, one can see that for a given micrograph (e.g. Fig. 4B), these patches appear wavy, undulations of one patch being parallel, and undulations from different patches being also parallel in between them. This is a typical TEM artifact: actually, tannins form along

Fig. 1: Views of (1) native ripe seed with covering mucilage, (2) native ripe seed without mucilage, (3) native *testa* (reverse), (4) native cotyledons, (5) longitudinal section of native cotyledons with yellow embryo, (5a) transverse section of native cotyledons, (6) fermented seed, (7) fermented *testa* (reverse), (8) fermented cotyledons, (9) longitudinal section of fermented cotyledons, (9a) transverse section of fermented cotyledons. Arrow: endosperm (4); the brown interlobe surface is marked with an asterisk (5,9).

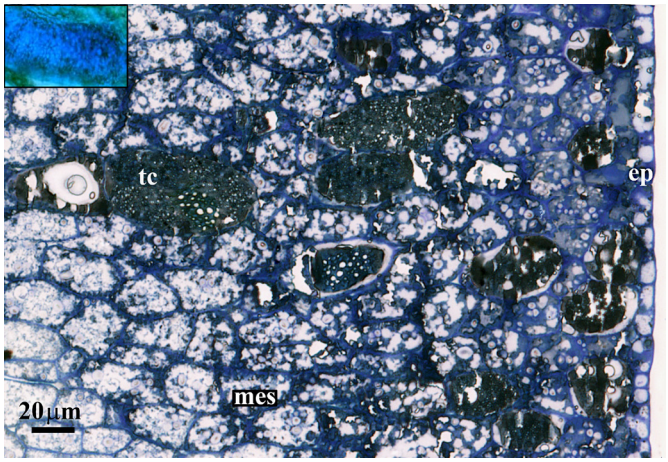

Fig. 2: Section of cotyledon from native seed (Toluidine Blue O staining). tc, tanniferous cell; ep, epidermis; mes, mesophyll. Insert: a tanniferous cell (fresh seed) stained with DMACA and showing numerous shuttles.

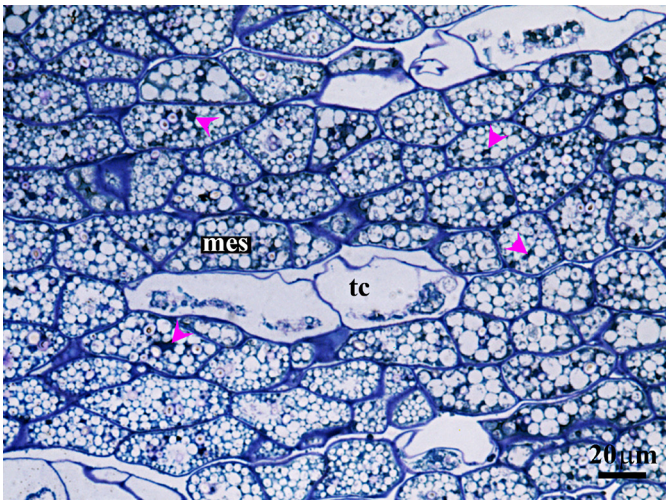

Fig. 3: Section of cotyledon from fermented seed (toluidine blue staining). Translocated tannins are shown as pink arrowheads.

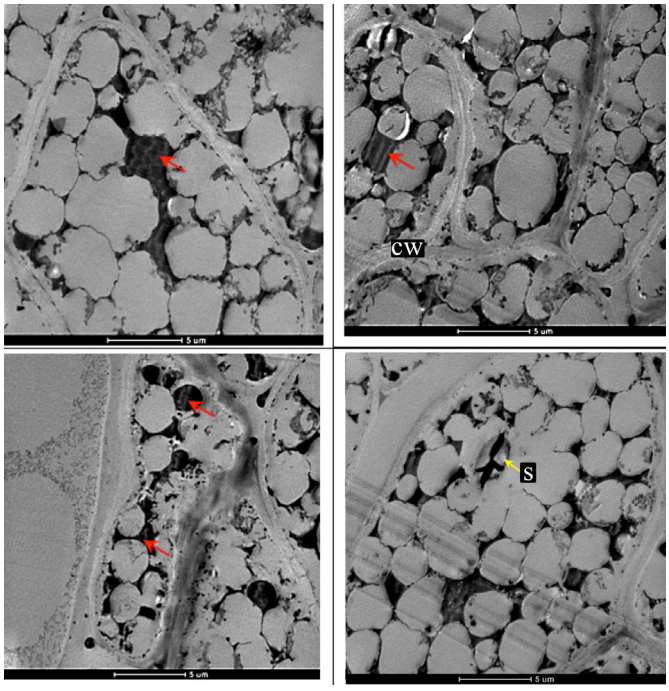

Fig. 4: TEM micrographs of the cotyledonary mesophyll of fermented seed. Note patches of condensed tannins (red arrows) intercalated between lipid bodies. cw, cell wall; s, starch (yellow arrow).

the (fixation/post-fixation) steps a very hard material which, after resin embedding, provokes vibrations of the diamond knife during sectioning (see BRILLOUET et al., 2014: Fig. 1f). Thus, it appears that proanthocyanidins, formerly entrapped in tanniferous cells (Fig. 2), have diffused into the entire storage parenchyma, and got intercalated between lipid bodies.

Behavior of proanthocyanidins in the *testa*

Sections of *testae* from native and fermented seeds were obtained as above and stained with Toluidine Blue O. The structures of both *testae* were basically the same (Fig. 5): a thin layer of epidermal cells covers 6-7 layers of elongated cells; then size of cells increases forming 10-12 layers of giant cells. However, a great difference was noted in the meso*testa*: in the native *testa*, some cells contained spheroidal tannic accretions (Fig. 5A) stained deep blue-black, while they were absent from the fermented beans where cells appeared with no cell content (Fig. 5B). One also must note a general expansion of meso*testa* cells.

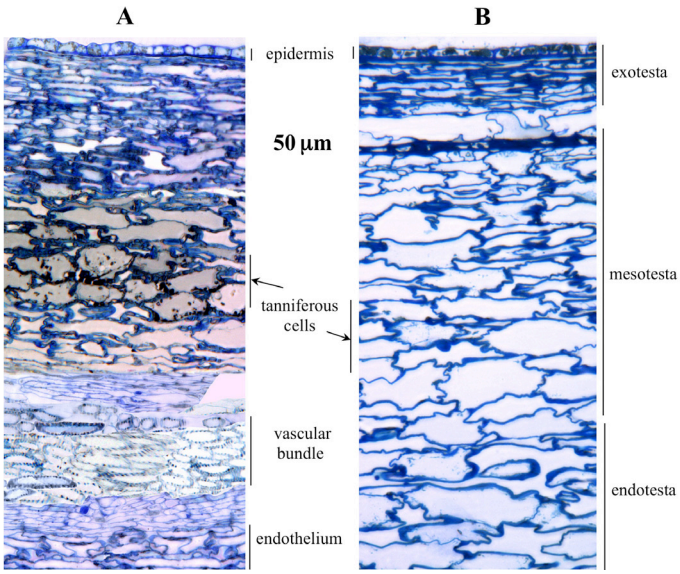

Fig. 5: Sections of native (A) and fermented (B) *testae*. Toluidine Blue O staining. Location of tanniferous cells in the fermented *testa* was obtained by counting cell layers from the epidermis of native *testa*. Spheroidal (chloro)tannic accretions: red arrow.

Quantitative analysis of the proanthocyanidins

Measurement of condensed tannins in native and fermented cotyledons (Tab. 1) showed that they were made of procyanidins, exclusively constituted of epicatechin. Their content in starting cotyledons was 4.2% FW (6.1% DW), and in fermented cotyledons 3.3% FW (5.5% DW). Expressed per cotyledon, one sees that intercalated tannins in fermented cotyledons represented the bulk of condensed tannins present in native ones. The tannins present in native cotyledons exhibited some degree of oxidation: indeed, 42% of these tannins were not depolymerizable by the current technique (ROUMEAS et al., 2013). This proportion increased to 47% after fermentation while the total amount of tannins (unoxidized + oxidized) remained the same.

The *testae* from native and fermented seeds contained 3.7% FW (12.2% DW) and 1.2% FW (4.0% DW) of condensed tannins, respectively. Expressed per *testa*, one sees that 20.7% of the tannins were oxidized in the native seed, while this proportion increased to 63.3% in the fermented seed. Most importantly, 73.0% of the *testa* tannins disappeared after fermentation. This loss is essentially due to the unoxidized tannins (87.5%). It is worth to note that the mucilaginous tissue entrapping the native cotyledons is void of tannins (data not shown).

Fate of anthocyanins in the cotyledons

Anthocyanins were extracted from native and fermented seeds and separated by HPLC-DAD-MS (Fig. 6). In agreement with FORSYTH and QUESNEL (1957), the native seeds contained two major anthocyanins, cyanidin 3-*O*-galactoside and cyanidin 3-*O*-arabinoside (Fig. 6A). Surprisingly, these free anthocyanins disappeared completely after fermentation (Fig. 6B) when the cotyledon flesh was

Tab. 1: Analysis of the condensed tannins.

		native testa	native cotyledon	native seed	fermented testa	fermented cotyledon	fermented seed
mass	(g)	0.6 ± 0.1	1.6 ± 0.1	2.2 ± 0.2	0.5 ± 0.1	2.1 ± 0.1	2.6 ± 0.2
water	(%)	69.7	31.7		70.2	40.4	
testa/seed	(%)	25.4			20.3		
cotyledon/seed	(%)		74.6			79.7	
condensed tannins		mg/testa	mg/cotyledon	mg/seed	mg/testa	mg/cotyledon	mg/seed
(sensitive bonds)		17.6 ± 1.1	38.7 ± 3.1	56.3 ± 4.2	2.2 ± 0.1	36.7 ± 2.1	38.9 ± 2.2
(resistant bonds)		4.6 ± 0.5	28.6 ± 1.8	33.2 ± 2.0	3.8 ± 0.4	32.1 ± 1.9	35.9 ± 2.0
total		22.2	67.3	89.5	6.0	68.8	74.8

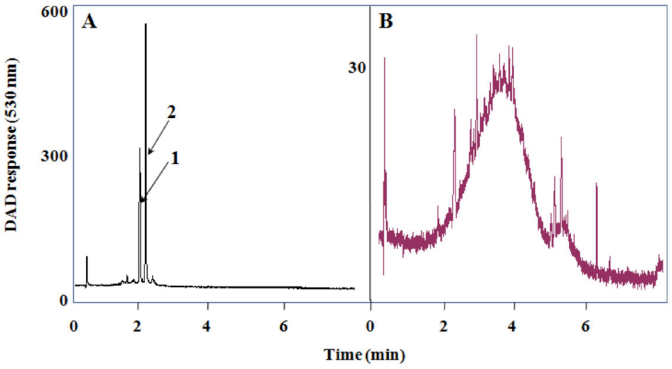

Fig. 6: HPLC chromatograms of methanolic extracts from native (A) and fermented (B) cotyledons (λ = 530 nm). (1) cyanidin 3-*O*-galactoside; (2) cyanidin 3-*O*-arabinoside.

still faint lilac (Fig. 1; no. 9). Instead of the two anthocyanin peaks, a hump was observed absorbing very faintly at 530 nm. When left on the bench for days, the methanolic extract from native seeds turned from deep pink to brownish, while the orange red extract from fermented beans remained the same.

Discussion

General

Compared with native cotyledons, fermented ones have taken up 27 % of water (Tab. 1), which is in agreement with Biehl et al. (1982). This water uptake is reflected by the wet surface of the *testa* (Fig. 1; no. 7). After fermentation, the *testae* kept their water load unchanged although a tremendous swelling of the cells was observed (Fig. 5). Due to this observation we assume that water transiently moved from the mucilaginous tissue into the cotyledons.

Accumulation of proanthocyanidins in tanniferous cells of cocoa seed cotyledons follows the general trend observed in the Tracheophyta (Brillouet et al., 2013, 2014): accordingly, most tanniferous cells were seen entirely filled with stained material, the proanthocyanidins (Fig. 2), with some *lacunae* (Ewers et al., 2010: Fig. 1c, d): in fact, this apparently homogenous vacuolar content is made of tannic accretions of various sizes (Martini et al., 2008: Fig. 12), themselves made of thousands of tannic shuttles [Fig. 2, insert; also (Brillouet et al., 2014)]. The frequent fragmentation of the tanniferous cell contents in *Theobroma microcarpum* (Martini et al., 2008: Fig. 21) does not correspond to a biological event but is actually the normal *in vivo* spheroidal status of the accretions (Brillouet et al., 2014).

Histological translocation of tannins

Similarly to our data and based on scanning electron micrographs of fermented seeds showing empty tanniferous cells, Lopez et al. (1987) hypothesized a diffusion of polyphenols (actually proanthocyanidins) from the tanniferous cells to the surrounding tissue; however, no histochemical proof of that phenomenon was provided. An almost complete disappearance of (chloro)tannic accretions from tanniferous cells was also observed in roasted seeds by light microscopy after Toluidine Blue O staining (De Brito et al., 2001). The authors stated that native seeds showed a great number of "phenolic bodies" (De Brito et al., 2001: Fig. 1a) [i.e. the (chloro) tannic accretions contained in tanniferous cells (Brillouet et al. (2014)], and that they were still present after 24 h of fermentation; as the fermentation progressed, polyphenols "diffused" throughout the whole cotyledon up to 48 h. After 72 h, no phenolic material could be detected by microscopy. However, at 72 h, the stained section displayed in their article (Fig. 1b) was taken from roasted seeds not fresh fermented ones. Nevertheless, after a careful examination of Fig. 1b, one sees that the cell walls of the storage parenchyma cells at 72 h were strongly stained by Toluidine Blue O contrary to walls from native cotyledons (Fig. 1a): it is therefore possible that tannins had diffused throughout the parenchyma and impregnated the cell walls. However, after examination by TEM (Fig. 4), we did not notice in the parenchyma an increase of the cell wall osmiophily, which would have indicated the presence of cell wall-bound tannins. It is possible that the heat treatment (sun drying and roasting) applied to the sample shown in their Fig. 1b might have altered the reactivity to Toluidine Blue O. In the end, although their statement remains valid and confirms our observations, their histochemical data are not conclusive. At that point, a contradiction must be underlined: indeed, the authors stated that polyphenols diffused out of the cotyledons, which is in agreement with Forsyth (1952), while total phenolics measured after aqueous acetone extraction by the Prussian blue technique did not significantly differed before and after fermentation, which is in agreement with our data. This contradiction can be easily overcome in our case: proanthocyanidins actually diffused from the tanniferous cells towards the storage parenchyma cells where they remained until the end of fermentation without any losses. It is finally possible that De Brito et al. (2001) did not notice the cytoplasmic patches of tannins intercalated between lipid globules.

Now, one must discuss the determinism of the diffusion of the proanthocyanidins into the storage parenchyma. Firstly, since tannins are not soluble molecules evolving in cytosol of the cells, but are packed in multimembrane structures [tannosome, shuttle, and (chloro)tannic accretion] (Brillouet et al., 2013, 2014; Brillouet, 2014, 2015), these microbodies must be disrupted or, at least, permeability of their membranes must be altered. Secondly, the tannins must undergo a solubilization prior to diffusion. Ethanol is transient-

ly produced in the mucilaginous tissue at the beginning of fermentation by yeasts; however the low level of production (2 to 60 mg/g of wet mucilage), its early generation when "phenolic bodies" are still present (i.e. after 24 h fermentation; De Brito et al., 2001), and the necessity for ethanol to diffuse into the cotyledons does not suggest a participation of ethanol to tannin solubilization. Indeed, ethanol is rapidly consumed by the microflora outside the seeds. Then, acetic acid is produced by bacteria fermenting ethanol from the mucilaginous tissue. Slow diffusion of acid into the cotyledons lowers pH from 6.5 to 4.5 at the end of fermentation and cause the death of embryo. At pH 4.5, half of acetic acid is under non-protonated form: it is thus possible that the weakly hydrophobic acidic water phase provokes the solubilization of tannins and their further migration towards parenchyma cells where they are stopped by complexing with cytoplasmic proteins (Hagerman and Buttler, 1981).

The situation is different in the *testa*: the proanthocyanidins disappeared from this compartment (loss estimated at ~31 % of total seed tannins) without a concomitant increase in the cotyledons. Since they are not prone to rapid biological cleavage, a possibility is that they diffused countercurrently to acetic acid into the mucilage tissue. However, their final fate remains mysterious.

Technological consequences of the translocation

Since we have demonstrated that there were no losses in cotyledonary tannins along fermentation but a translocation from tanniferous cells into the reserve parenchyma accompanied by a slight oxidation, it is now worth questioning the consequences such a phenomenon has on the quality of the final product, chocolate. It is known that flavor precursors are formed in the cotyledonary storage parenchyma by proteolysis of reserve proteins, albumin and vicillin, by endogenous proteases generating aminoacids and peptides (Lima et al., 2011). It is also known that proteolysis kinetic is highly variable depending on several parameters (origin of the seeds, fermentation conditions, s.o.) (Hue et al., 2016). Tannins are known to form complexes with proteins (Hagerman and Butler, 1981), thereby being strong enzymatic inhibitors. It is possible that this translocation can interfere in the proteolysis: indeed, unknown proportions of these reserve proteins, percents being possibly different between albumin and vicillin since the strength of complexation is not the same according to the protein structure, will be complexed with the translocated tannins, thus becoming resistant to proteolysis. Some of the proteases themselves could also be complexed thus rendered partly inactive. It is likely that a complex mixture of these events is at work, and more detailed studies needed to enlighten this phenomenon.

Oxidation of the tannins

It has been stated that unfermented and roasted cocoa seeds are excessively astringent (Biehl and Voigt, 1996 cited in Misnawi, 2002), and that disappearance of astringency in the final product would be due to oxidation of the tannins along processing (fermentation, drying, and roasting). If their fate along fermentation is now well characterized, i.e. no losses and a weak oxidation (De Brito et al., 2001 and this article), this is not the case for drying, and roasting. Indeed, tannins were measured only as reducing compounds by the Prussian blue technique (De Brito et al., 2001), and were not finely characterized as in this article. It is thus possible that these authors measured other phenolics on top of tannins; nevertheless, they reported high tannin losses during drying (26% compared to fermented seeds, and further roasting (additional 12%). However, these losses do not necessarily mean a disappearance of tannins as in the case of *testa* tannins (this article): a complexation with proteins could have hindered their solubilization in aqueous acetone (De Brito et al., 2001) prior to their measurement. Finally, a strong oxidation must occur along sun drying since the dried seeds exhibit strong chocolate brown cotyledons (data not shown), but a fine characterization is needed (as in this article) to ascertain their fate and level of oxidation.

Fate of the color

The disappearance of anthocyanins from the cotyledonary tissue after fermentation has already been described (Forsyth and Quesnel, 1957b). These authors stated that bleaching of the cotyledons occurring along fermentation, i.e. from purple to "white", would be due to the activity of endogenous glycosidases freeing the anthocyanidin, cyanidin. The aglycone would exist as a pseudobase under the reaction conditions, and would be stable under acidic conditions. However, our acidic methanolic extract from fermented seeds was pinkish orange, and its color was stable for weeks on the bench, contrary to the extract from native seeds. It is thus possible that quinonoid anhydrobase chromophores derived from liberated cyanidin would form in the cotyledons and be stabilized by substitution with reactive tannins, as described in wine (Somers, 1971).

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