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Histological features of phenolic compounds in fine and bulk cocoa seed (*Theobroma cacao* L.)

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Summary

Phenolic compounds contribute substantially to the quality of cocoa. In the present study the storage of polyphenols in ripe cocoa seed cotyledons from different genetic origins is compared via light microscopy. Besides, polyphenol accumulation is observed in maturing cocoa cotyledon tissues.
About 10 % of the cocoa cotyledon parenchyma is formed by polyphenol cells, which occur in clusters and lines of up to ten cells. Higher amounts of polyphenol cells are located around the vascular bundles. Cotyledons of Criollo genotypes are composed of larger storage cells than the ones of Forastero and Nacional cocoa. In general during ripening, the polyphenol accumulation is completed earlier than that of fat and storage proteins.
It is assumed that the larger polyphenol and storage cells of Criollo seeds may contribute to the unique quality of this fine flavour cocoa.

Introduction

Cocoa and chocolate are extraordinarily popular consumer goods. They are based on raw cocoa, the fermented and subsequently dried seed of the cocoa tree *Theobroma cacao* L. Originating in the Amazon area of South America, cocoa today is cultivated throughout the entire tropical belt of the earth (BARTLEY, 2005).

Traditionally the cocoa tree is classified into five different types: Upper Amazon Forastero (UAF), Lower Amazon Forastero (LAF), Nacional, Trinitario and Criollo. While seeds from the first two cocoa types generally are processed to mass or bulk cocoa, the other three cocoa types are primarily used for fine or flavour cocoa. Among these, the rare Criollo type is supposed to produce one of the best cocoa qualities (CUATRECASAS, 1964). According to recent molecular studies the populations of *Theobroma cacao* must be considered to be composed of ten closely related genetic clusters (MOTAMAJOR et al., 2008).

Phenolic compounds amount up to 18 % of fat-free dry weight in unprocessed cocoa seed (KIM et al., 1984). According to some authors the reaction products of phenolic substances belong to the most important components of cocoa flavour (ROHAN et al., 1964). CLAPPERTON et al. (1994) as well as COUNET et al. (2004) correlate details of the individual flavour quality of cocoa samples with their content of mono- and oligomeric procyanidins. Complex condensation products of phenolic cocoa compounds generated during fermentation and drying are responsible for the brown colour of raw cocoa and chocolate. Due to their anti-oxidative potential, cocoa polyphenols are supposed to have a protective effect against arteriovascular diseases, cancer and inflammatory processes in the human body (ENGLER et al., 2004; YAMAGISHI et al., 2000).

Most of the cocoa phenolic seed compounds are located within large vacuoles of so-called polyphenol cells in the cotyledon tissue. These idioblasts are also characteristic for other *Theobroma* species (MARTINI et al., 2008). They amount about 10 % of the cocoa storage parenchyma. Besides phenolic compounds, many of the polyphenol cells also contain starch granula and lipid vacuoles (JAENICKE, 1973). These two components are also present in the ordinary cotyledon storage tissue cells, which additionally contain storage proteins.

To produce a proper cocoa flavour, a fermentation and subsequent drying of fresh and totally ripe cocoa seeds is necessary. During the early stages of this process, a disintegration of cotyledon cells takes place (BIEHL et al., 1977; DE BRITO et al., 2001). Subsequently, substantial amounts of phenolic compounds leak from the seeds while others form complex oxidation products with each other and with the surrounding proteins. Towards the end of fermentation only 10-35 % of the original amounts of phenolic compounds are left in the seed cotyledons (KIM et al., 1984; KEALEY et al., 1998; ROHSIUS, 2007). LAMBERT et al. (2003) describe a genotype-specific loss of phenolic seed compounds during fermentation and drying. This may be caused by differences in the histological seed structure which influences the progression of flavour-relevant chemical reactions during fermentation and drying.

For the present study semi-thin sections of cotyledons from Nacional, LAF, UAF and Criollo cocoa seeds were compared via light microscopy. In the same manner, ripening stages of the seed of two different cocoa clones were analysed. In both cases the main focus was on histological and cytological aspects of the polyphenol cells. The objective was to analyse potential differences between the respective cocoa types which may have relevance to quality and may have new aspects about the accumulation of cocoa seed compounds during ripening.

Experimental

Cocoa Samples

Ripe and unfermented seed samples of the following clones and varieties were analysed: EET 95 (Nacional type), Catongo (LAF), NA 33 (UAF) as well as Pentagona, Porcelana, Guasare and Criollo Mérida (all representing Criollo type cocoa). The Nacional, LAF and UAF samples were obtained from the gene banks of the Malaysia and Ghana Cocoa Board (Tawau and New Tafo) as well as from the Cocoa research Unit of Trinidad and Tobago and from the greenhouse collections of Reading, UK. The Criollo samples were obtained from different experimental fields of the INIA (Instituto de Investigaciones Agrícolas) Mérida, Venezuela.

All mature samples except for Criollo seeds were obtained as fresh, ripe fruits via air express service. Seeds from the centre of the fruits were extracted and incubated in 4 % aquaeous formol solution for at least 48 h.

As for the Criollo type cocoa, seed samples were transferred into 4 % aquaeous formol solution before being sent to Hamburg for analysis.

In general, three seeds per fruit were selected for microscopical preparation. At least three series of semi-thin sections were produced from each seed. Thus about 9 microscopical preparations were obtained from each fruit (see Tab. 1).

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Tab. 1: Survey of the microscopical preparations of mature cocoa seeds.

cocoa type	n sampled fruits	n sampled seeds	n preparations
Criollo	10	29	87
LAF	5	15	47
Nacional	5	15	45
UAF	6	16	48

The Cocoa seed samples of defined ripening stages originated from a farm formerly belonging to Nestlé near Quevedo in Los Rios, Ecuador. They were extracted from cocoa pods which had developed after hand-pollination of two clones, a Trinitario (ICS 95) and a Nacional (EET 95). The date of pollination served as reference for the age of the sampled seeds. They were directly transferred into vessels containing 4 % aquaeous formol solution. Within this solution, the samples were sent to Hamburg for microscopical analysis. Seeds of the following ripening stages were harvested:
EET 95: 100, 120, 140 and 154 days after pollination.
ICS 95: 100, 120 and 170 days after pollination.

Chemicals

Unless otherwise specified, all chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany). The Water was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Methods

Cocoa seeds were fixed in 4 % aquaeous formol solution for at least 48 h. Cotyledon tissue from the seed centre was cut into cuboids of ca. 1 mm^3. The sections were put into 2 ml *Eppendorff tubes* and dehydrated in a graded ethanol series (two times 70 %; two times 100 %; 30 min each). Subsequently the preparations were transferred into 100 % LR White Medium Grade™ (London Resin Company, Berkshire, UK). After 60 min the medium was exchanged for fresh LR White Medium Grade ™ in which the preparations were incubated for at least 12 h at 4 °C. Subsequently each tissue sample was put in a 0.36 cm^3 gelatine capsule (Pohl-Boskamp, Hohenlockstedt, Germany). The capsules were filled with LR White Medium Grade ™ and were then cured in a cabinet dryer for 24 h at 60 °C.

The cooled-down, fully crystallised preparations were trimmed with a razor blade. Sections of 2 µm to 2.5 µm were cut with an ultra-microtome (Ultracut S, Leica, Wetzlar, Germany) using freshly prepared glass cutting blades. The sections were fixed on a microscope slide and subsequently stained with Toluidin blue O according to GUTMANN (1995) slightly modified. The semi-thin sections were incubated for 30 s in a solution of sodium hypochlorite (ca. 13 % Cl, Fluka, Buchs, Switzerland). After rinsing the sections with aqua dest. and drying the slides, the samples were incubated for 5 min in a solution of 0.05 % (w/v) Toluidin blue O in aqua dest. Then the slide was rinsed with aqua dest again and subsequently dried. The treatment causes a greenish-black staining of phenolic compounds, whilst proteins and non-lignified cell walls are stained blue and lipids remain colourless.

The stained tissue sections were embedded in Roti®-Histokitt (Carl Roth, Karlsruhe, Germany) and covered with a coverslip. After curing, the preparations were analysed with an Olympus (Hamburg, Germany) BH-2 light microscope in bright field at magnifications of 100x and 200x. Digital pictures were taken with a ColorView (Soft Imaging System, Muenster, Germany) camera.

From each preparation of ripe cocoa seeds five polyphenol cells and five storage cells were coincidentally selected on the screen. Subsequently, the cell faces were calculated via analySIS (Soft Imaging System).
By this means the faces of 15 storage cells and 15 polyphenol cells per seed were measured.

Statistical analysis

Statistical analysis was performed with STATISTICA (version 6, StatSoft, Inc., Tulsa, OK, USA). Data were subjected to correlation analysis and to analysis of variance (one-way ANOVA) with subsequent Scheffé post-hoc- test on a level of significance of $p < 0.05$.

Results

Cotyledon tissues of EET 95 and ICS 95 seed to the time of 100 days after pollination are presented in Figs. 1 a and b. Both images are representative for the clone-specific appearance of the cotyledon tissue at this stage of development. Additionally, parts of the cotyledon epidermis are displayed (1 a: left side, 1 b: above). The epidermal cells contain large, colourless vacuoles. Due to the different angle of cutting, the sizes of the epidermal cells in Figs. 1 a and 1 b are significantly different.

The storage tissue of the cotyledons commences directly below the epidermis. 100 days after pollination most of the tissue cells remain more or less unstained by the method applied. Similar to the epidermal cells, these future storage cells contain a large central vacuole as well as isolated starch bodies. The ripening polyphenol cells are characterized by ring-shaped inclusions. Due to their colour, these inclusions were identified as phenolic compounds which start to accumulate close to the tonoplasts.

Details of EET 95 and ICS 95 cotyledon tissue to the time of 120 days after pollination are presented in Figs. 1 c and d. Macroscopically, these seeds resemble fully ripe cocoa seeds. However, the accumulation of lipids and proteins is not concluded at this stage of development. Especially the ordinary storage cells of EET 95 (Fig. 1 c) still contain large, achromatic vacuoles. Besides starch granula, which are already present at the time of 100 days after pollination (Figs. 1 a and b), some of the storage cells of EET 95 (Fig. 1 c) contain small quantities of lipid vacuoles. Compared to EET 95 the development of the ICS 95 storage cells is further advanced at 120 days after pollination (Fig. 1 d). These cells contain more lipid vacuoles, and blue regions indicate the accumulation of storage proteins which are not present in EET 95 tissue at the same stage.
Both, in EET 95 and ICS 95 tissue, the accumulation of phenolic compounds is almost finished at 120 days after pollination (Figs. 1 c and d).

At the stage of 140 days after pollination cotyledon tissue cells of EET 95 exhibit all cytological details of full ripeness (Fig. 2 a). 165-day-old tissue cells of the same clone differ from the earlier stage by slightly larger protein vacuoles (Fig. 2 b). This probably indicates a water uptake caused by a beginning germination. Cotyledon tissue of ICS 95 at the time of 175 days after pollination (Fig. 2 c) does not exhibit any significant cytological differences to the ripe cotyledon tissue of EET 95 (Fig. 2 a).
The typical storage cells of ripe cocoa cotyledons contain fat, storage proteins and starch in separate compartments. Achromatic lipid vacuoles dominate in most of the storage cells. They determine the shape of the protein vacuole which is stained blue. The vacuoles of the ripe polyphenol cells are stained dark green.
In all analysed samples of mature cocoa seeds the polyphenol cells are distributed quite evenly within the cotyledon tissue, but slightly

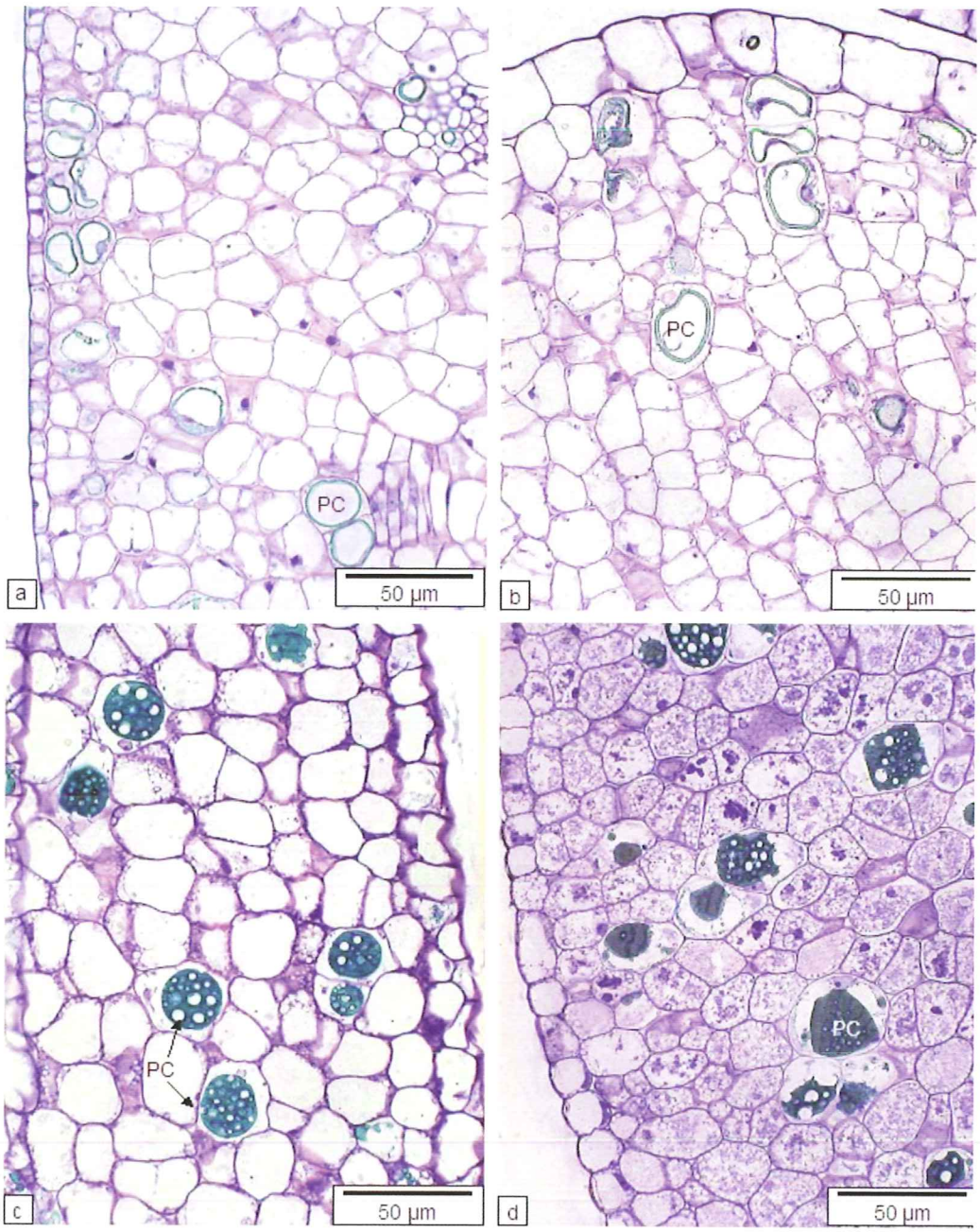

Fig. 1: Ripening stages of cotyledon tissue of the clones EET 95 and ICS 95, 100-120 days after pollination.
a) EET 95, 100 days after pollination; **b)** ICS 95, 100 days after pollination; **c)** EET 95, 120 days after pollination; **d)** ICS 95, 120 days after pollination.

higher amounts are located within the outer parts of the parenchyma. Most of the idioblasts are arranged in clusters of up to ten cells. Besides, lines of up to ten polyphenol cells are located beneath the adaxial epidermis of the cotyledons (Figs. 3 a and b). Larger amounts of polyphenol cells are found around the vascular bundles.

Trichomes which sporadically occur on the cotyledon epidermis also contain high amounts of vacuoles whose contents are stained green by Toluidin Blue O (Figs. 3 c and d). These vacuoles differ from the ones found in the storage tissue in their smaller size and because they frequently occur in groups of 2-4 vacuoles in the same cell (Fig. 3 d). In all analysed cocoa preparations the average polyphenol cells are significantly larger than the surrounding storage cells. Due to their more prosenchymatic shape, the variability of the measured polyphenol cell faces is higher than that of the rather isodiametric storage cells (Fig. 4).

In Fig. 5 the type-specific mean values of the cell faces of the analysed storage cells (a) and polyphenol cells (b) are compared. It becomes evident that Criollo type storage and polyphenol cell faces of the cotyledon tissue are significantly larger than the ones of the Nacional, LAF and UAF samples. This result is confirmed by ANOVA with a subsequent Sheffé post-hoc test for both the faces of the polyphenol cells ($F = 25.03$, $p < 0.005$) and the normal storage cells ($F = 34.88$, $p < 0.005$).

Though there exists a slightly positive correlation between the size of the cell faces and the average seed dry weight of the respective of

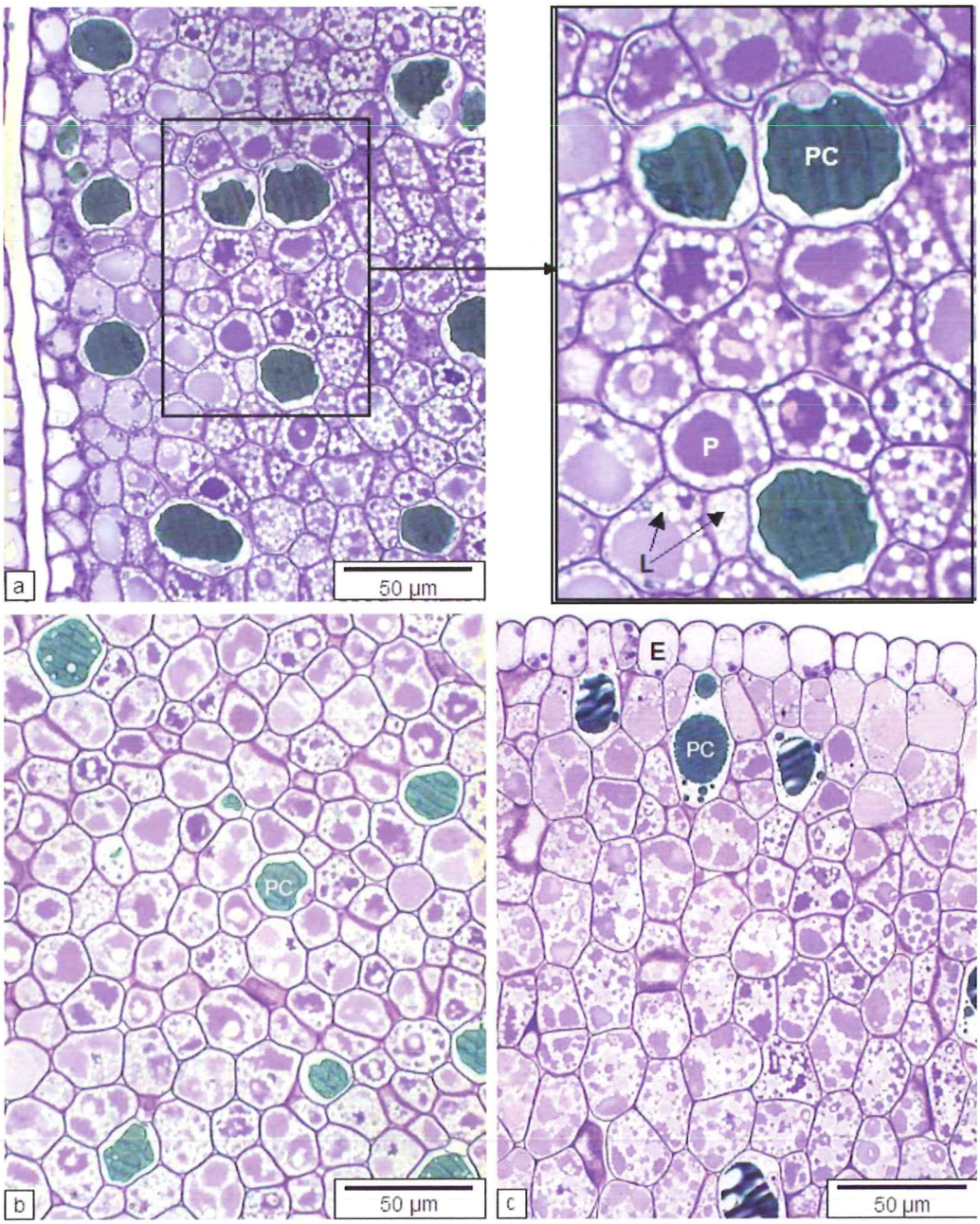

Fig. 2: Ripening stages of cotyledon tissue of the clones EET 95 and ICS 95, 140-175 days after pollination.
a) EET 95, 140 days after pollination; b) EET 95, 165 days after pollination; c) ICS 95, 175 days after pollination

clone, the seed weight does not determine the type-specific size of cell faces found in the present study. According to Figs. 6 and 7 most of the Criollo seeds do not only exhibit large cell faces but also comparatively high dry weights. However, the only Criollo sample with extraordinary light seeds (Figs. 6 and 7, circuited) also contains cell faces which are above the average. Additionally, Nacional type samples which show the highest seed weights do not have the largest cell faces.

Discussion

The classification of cytological details in the cocoa cotyledon tissue was to a large extent adopted from JAENICKE (1973).

The trichomes that grow sporadically on the cotyledon epidermis correspond to the typical ton-shaped glandular trichomes of the *Malvales* described in UPHOF and HUMMEL (1962). Glandular trichomes often exude phenolic compounds to the epidermal surface. Here, the film of phenolic compounds may serve as protection against excessive UV irradiation and herbivores (JUNIPER and JEFFREE, 1983). Due to their low density, such a protective function is irrelevant in case of the trichomes on cocoa cotyledons. However, similar trichomes are present in much higher densities on photosynthetic cocoa leaves (unpublished data).

Further research is needed to clarify whether the cotyledonar trichomes also contain terpenoids. These substances contribute to specific flavour components especially of fine or flavour cocoas (ZIEGLEDER, 1990).

All analysed cocoa types exhibit a uniform distribution pattern of polyphenol cell clusters in their storage parenchyma. Thus, the cells

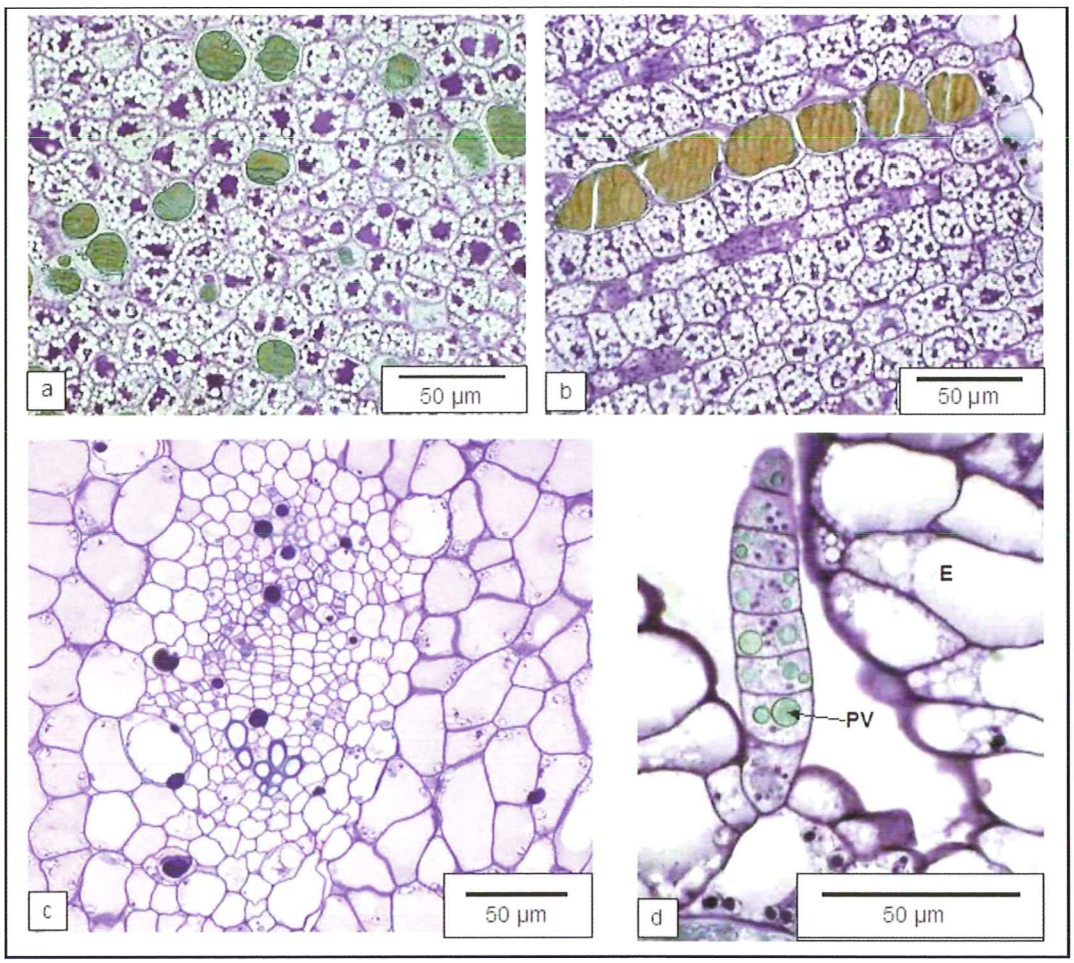

Fig. 3: Ripe cocoa cotyledons. a, b: cotyledon parenchyma with polyphenol cells in clusters (a) and in lines (b). c: vascular bundle with polyphenol cells (coloured). d: epidermal trichome with polyphenol vacuoles (VS), E = epidermal cell.

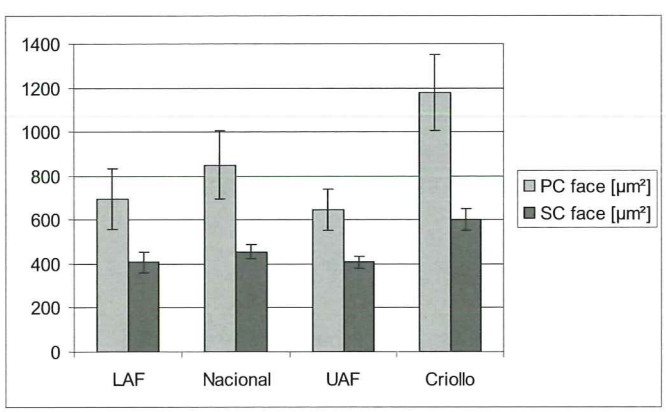

Fig. 4: Mean values of the polyphenol cell (pc) and storage cell (sc) faces of the LAF, Nacional, UAF and Criollo cotyledon tissues.

distribution of polyphenol cells in the storage tissue is not responsible for the different flavour potentials of mass and fine flavour cocoas. Higher amounts of polyphenol cells in a lengthwise association with vascular bundles of cocoa seed were also found by MARTINI et al. (2008). According to these authors, such structures are also present in other *Theobroma* species. Polyphenol cells in the vicinity of vascular elements may protect against fungal infestation. For instance, DAAYF et al. (1997) find a higher accumulation of phenolics in root vascular bundles of fungus-resistant cotton plants (*Gossypium spec.*). NAGY et al. (1998) detect elevated amounts of phenolic compounds in vascular sytems of *Picea abies* L. after infection with *Ceratocystis polonica*. (Siem.) C. Moreau.

The accumulation processes of storage compounds observed in ripening seeds of *T. cacao* L. relates to the findings of JONES and ROST (1989) for *Oryza sativa* L. Similar to cocoa, in rice embryos starch was detected first, followed by fat and subsequently by storage proteins. The stock-piling of phenolic compounds is finished earlier than the one of lipids and storage proteins. For this reason, cocoa embryos are already violet-coloured and thus resemble ripe embryos before they really reach maturity. By macroscopical means, such embryos can hardly be distinguished from ripe cocoa embryos. Thus, it is difficult to find the correct point of time at which cocoa seeds are fully ripe, especially as ripe cocoa fruits remain on the trees and also there exist significant differences in maturing times between different cocoa varieties (EFOMBAGN et al., 2004). This may lead to the use of immature cocoa seed for processing, provoking heterogenity of harvested material and, consequently, give rise to immense deficits in cocoa flavour (HOSKIN and DIMICK, 1994).

Though both longitudinal and transversal sections of ripe cotyledon tissue were analysed the average size of Criollo cell faces is significantly larger than the one of UAF, LAF and Nacional type cocoa. This demonstrates that Criollo cocoa owns cotyledon tissue

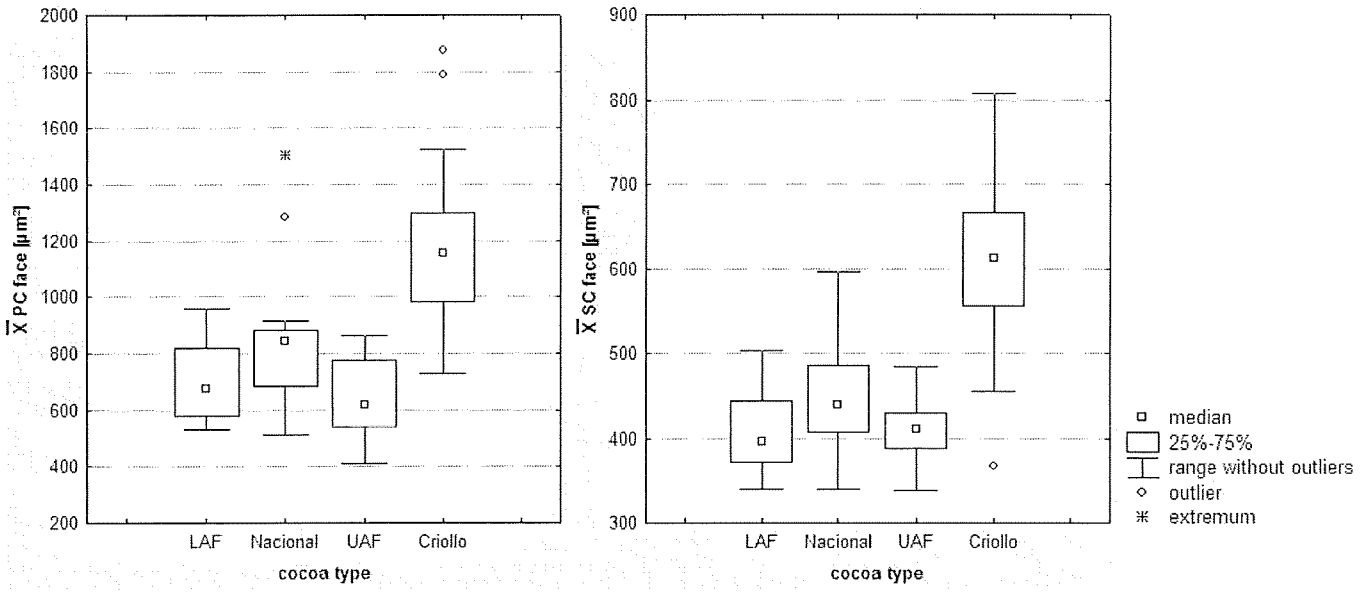

Fig. 5: Comparison of mean values of the type-specific polyphenol cell (pc) and storage cell (sc) faces of the cotyledon tissue of *T. cacao* L..

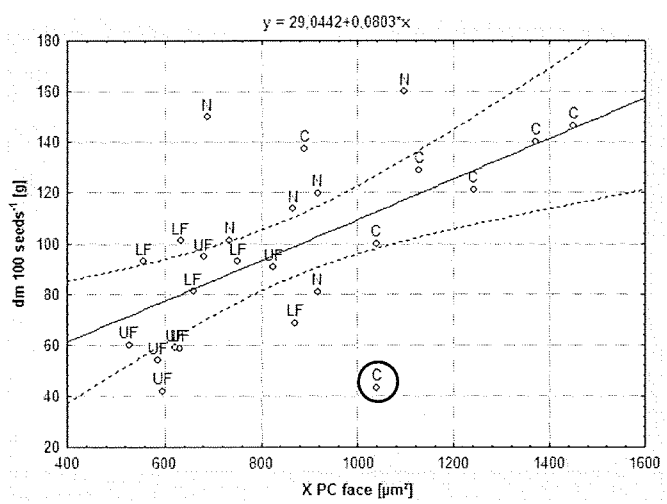

Fig. 6: Scatterplot of the sample-specific polyphenol cell faces and the respective 100-bean-weights with classification of the respective cocoa type Criollo (C), UAF (UF), LAF (LF) and Nacional (N).

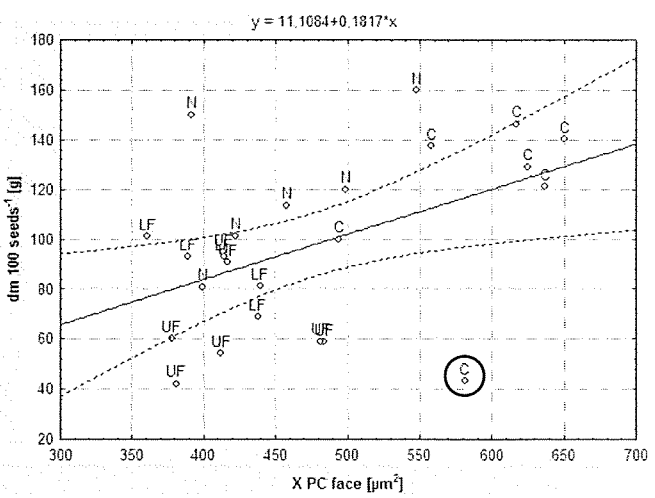

Fig. 7: Scatterplot of the sample-specific storage cell faces and the respective 100-bean-weights with classification of the respective cocoa type Criollo (C), UAF (UF), LAF (LF) and Nacional (N).

cells of significantly larger volume. No significant differences could be found between the cell sizes of the other three analysed cocoa types.

It is not known if there is any influence of the size of cotyledon tissue cells on the biochemical processes which produce the precursors of cocoa aroma during fermentation and drying. Differences in cell sizes may modify the invasion of liquids like acetic acid into the seed. Tissue cells with larger volume may provide larger spaces for the chemical reactions subsequent to cell decompartmentation as described by BIEHL (1973) and BIEHL et al. (1977).

In fact, Criollo cocoa seeds are fully fermented after three days whilst most other cocoa seeds are fermented for at least five days (BUTLER, 2006). Further studies are needed to find out if the size of parenchyma cells matters in this context.

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