CELL WALL POLYSACCHARIDES OF COFFEE BEAN MUCILAGE. HISTOLOGICAL CHARACTERIZATION DURING FERMENTATION

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SUMMARY

The mucilage of pulped coffee beans before and after fermentation was examined by light microscopy. The mucilage of unfermented beans is constituted by two to three layers of elongated palisade-like cells with thin and folded walls attached at their base to the sclerenchymatous parchment. Ruthenium red staining specifically demonstrated the presence of pectic substances. After 20 hours of fermentation, the mucilage tissue is still present with apparently intact cell walls, but it is separated from the parchment. The walls are still stained by ruthenium red suggesting that pectic substances are still present. Therefore it is assumed that no pectinolysis occurs during fermentation or if so it must be to a very restricted and non detectable extent. In conjunction with our biochemical data, it is hypothesized that consumption of sugars by bacterial microflora induces an osmotic pressure gradient from outside to inside the mucilage layer, thus provoking a fracture of mucilage cell walls at their basal site of attachment to the sclerenchymatous parchment.

RESUMEN

El mucilago de las cerezas despulpadas de cafe antes y despues de fermentacion fue examinado con microscopia optica. El mucilago de las ceresas frescas se compone de dos o tres capas de largas celulas con delgados paredes. Estas celulas se pegan a sus bases con el esclerenquimatoso pergamino. La coloracion con el rojo de ruthenium permite demostrar la presencia de pectinas. Despues de 20 horas de fermentacion, el tejido mucilaginoso existe todavia con su paredes aparentemente intactos, pero esta separado del pergamino. Sus paredes estan todavia tenidos con el ruthenium rojo sugeriendo que las pectinas estan todavia presentes. Entonces, se supone que no hay pectinolisis durante la fermentation o que sino es a un nivel muy reducido y no detectable. De acuerdo con nuestros resultados bioquimicos, se supone que el consumo de los azucares por la microflora induce una presion osmotica de la parte externa a la parte interna del tejido mucilaginoso, provocando una rotura de los paredes de las celulas del mucilago a su base, sitio de adhesion al esclerenquimatoso pergamino.

COMMUNICATION

INTRODUCTION

Coffee cherry is a drupe consisting of smooth tough outer-skin or exocarp, soft yellowish pulp or outer mesocarp, mucilaginous layer or inner mesocarp, and greyish-green fibrous endocarp (parchment) surrounding seeds (Purseglove, 1974). In the wet processing of coffee, cherries are first pulped and then fermented to remove the mucilage; then wet parchment coffee is washed and dried. The mucilage,

formerly described as an hydrogel exhibiting no cellular organization (Carbonell and Vilanova, 1952 ; Coleman *et al.*, 1955) is a thin hydrated layer essentially made of pectic substances and soluble sugars (Oliveros and Gunasekaran, 1996 ; Avallone *et al.*, 1999a). Due to its viscous texture, pulped beans cannot be washed without preliminary removal of mucilage by natural fermentation or mechanical demucilagination. During fermentation, endogenous coffee and/or microbial enzymes are supposed to degrade pectic substances of the mucilage (Carbonell and Vilanova, 1952 ; Wilbaux, 1956 ; Rolz et al., 1982), changing its texture from an hydrogel to an hydrosol, but no definite proof was ever brought to sustain this assertion. Furthermore, *Erwinia dissolvens*, a major pectolytic microorganism of the microflora from fermenting beans, was shown to produce only an exo-pectate-lyase having a very restricted action on coffee high methoxyl pectins (Castelein and Pilnik, 1976). With regards to these conflictual assumptions, we decided to study by light microscopy the structure of the mucilage layer and its alterations during fermentation.

MATERIAL AND METHODS

Plant material

Coffee variety used in the present study was *Coffea arabica* L. var. *typica* Cramer. It was grown in Coatepec area (Xalapa, Veracruz, Mexico). Coffee cherries were harvested at the mature stage during the 1998 season.

Coffee wet processing

Coffee cherries were immediately pulped with a DV-255C PENAGOS® pulper. Pulped coffee beans were then conveyed in a water stream to the fermenting tank where they were left to ferment for 20 h. Samples of frozen (- 20 °C) coffee beans at times zero and 20 h of fermentation were rapidly air-freighted and delivered to our laboratory. Whole sound mature cherries were also kept for later light microscopy examination.

Light microscopy

Prior to fixation, the fleshy portion of whole mature cherries was obtained by hand-dissection while pulped beans (times zero and 20 h) were carefully husked to separate the mucilage and the parchment from the seeds. Samples were fixed in 2% glutaraldehyde (0.2 M phosphate buffer, pH 7.0 containing 1% acrolein and 1% caffeine) for 15 min under vacuum at room temperature, then for 24 h at 4 °C. Subsequently they were dehydrated in a graded ethanol series then impregnated for 24 h in a medium containing : Technovit 7100 resin (100 ml; Kulzer), Technovit 7100 accelerator (1 g; Kulzer), Technovit polyethylene glycol PEG 400 (1.5 ml; Kulzer) and triethylene glycol dimethacrylate (0.5 ml). They were finally embedded in the impregnation medium (5 ml) added with Technovit 7100 hardener (0.35 ml). Sections (3 µm) were obtained with a Historange LKB microtome and examined with a DMRB Leica microscope.

- ***Revelation of the cell wall polysaccharides (cellulose, hemicelluloses, pectic substances)*** : sections were oxidised for 5 min in 1% periodic acid, washed with distilled water, then stained for 10 min in the dark with Schiff reactant prepared as follows : *p*-rosaniline chloride (1 g) was dispersed in boiling water (200 ml) and the solution is cooled to 50 °C, then filtered ; sodium metabisulfite (2 g) and 1 M HCl (20 ml) were then added and, after 24 h in the dark, activated charcoal (0.5 g) was added and the medium filtered.
  Sections were then washed with water until washing liquor is colorless and stained with Naphtol blue black [NBB (1 g), acetic acid (7 ml), up to 100 ml water] for 5 min at 60 °C. Sections were washed with water, then treated with 7% acetic acid and finally dried for 15 min at 60 °C.
- ***Specific revelation of the cell wall pectic substances*** : sections were stained with 2% ruthenium red in water for 15 min at room temperature, washed with distilled water, then dried.

RESULTS AND DISCUSSION

Microscope examination before fermentation (time zero)

In a preliminary step we examined sections of different zones of whole mature cherries before they were pulped and fermented. The pericarp is constituted of :

- exocarp (= skin) consisting in a layer of small epidermic cells (~ 10 x 30 µm) (Photo1).
- outer mesocarp (= pulp)(thickness ~ 1 mm) built of roughly isodiametric cells of increasing diameter from outside (~ 40 µm), to middle portion (~ 100 µm), to inside (~ 110 µm) the cherry. Elongated cells are also present from (~ 30 x 80 µm) to (~ 50 x 100 µm). Cell walls are intensely stained fuschia red in the upper zone while in the bottom area the tissue seems a bit loosened with walls far less densely stained red. Tannin containing cells are visible in outer mesocarp (Photo 1).
- inner mesocarp (= mucilage)(thickness ~ 300 µm) constituted of two to three layers of elongated cells (length ~ 160 µm; width ~ 15-50 µm). Sectioning teared a bit the surface of the mucilage (Photo 2).
- endocarp (= parchment)(thickness ~ 50-110 µm) made of sclerenchyma with transverse sclereids (~ 25 µm)(Photo 2) and longitudinal sclereids (~ 150 x 25 µm) which are only visible in Photo 5.
- inner epidermis built of small flattened cells (length ~ 40 µm; width ~ 10 µm)(Photo 3).

Mucilage left after industrial pulping is made of elongated cells attached to parchment sclereids (Photo 3). Residues of cytoplasmic material and nucleus stained greenish-blue by naphtol blue black are visible (Photo 3). Cell walls are thin and stained fuschia red confirming presence of polysaccharides. Accordingly, we have previously shown that mucilage alcohol insoluble residue contained cellulose in addition to pectic substances (Avallone *et al.*, 1999a). Thus the mucilage layer has actually a conventional tissue structure with cells surrounded by cell walls in contradiction with previous assertions describing the mucilage as an hydrogel having no cellular structure (Carbonell and Vilanova, 1952 ; Coleman et al., 1955). The loosened appearance of this tissue with thin walls might explain why these authors previously missed the presence of cell walls. However Beille (1947) followed by Rabéchault (1959) had already described in details the tissue structure of the mucilage layer : cells were drawn in a palisade-like arrangement like in our case with walls either dotted (Beille, 1947) or continuous (Rabéchault, 1959). It is worth to mention that Menezes and Maniero (1955) drawn the mucilage as one layer of cells with thick walls resembling palisade cells of seed coat of legumes (Esau, 1977) ; although having a palisade arrangement, cells of coffee mucilage have not secondarized walls.

Cells walls of the mucilage are stained pink by ruthenium red (Photo 4) confirming the presence of pectic substances (Coleman et al., 1955 ; Avallone *et al.*, 1999a) and not stained by the phloroglucinol/HCl reagent (Avallone *et al.*, 1999a) indicating abscence of lignin. These walls can be characterized as primary cell walls, conversely to the sclerenchymatous parchment not stained by ruthenium red. It is worth to mention that a noticeable amount of water-soluble pectic substances must have been lost during the fixation step when the samples were treated with aqueous glutaraldehyde (Avallone *et al.*, 1999b). Thus, pectic substances evidenced with ruthenium red (Photos 4, 6, 8) must be the water-insoluble fraction only. According to our observations, the tissue constitution of coffee cherry pericarp is summed up in Figure 1.

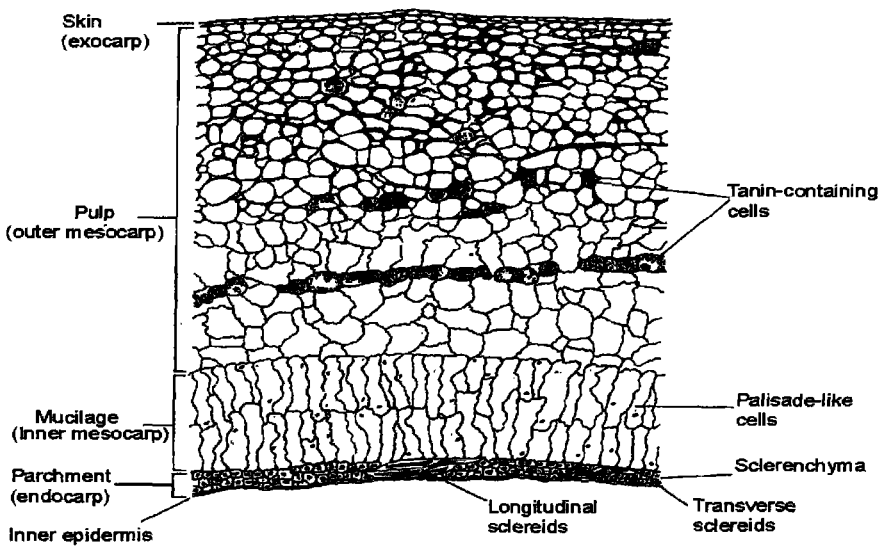


Figure 1. Tissue constitution of *Coffea arabica* L. var. *typica* Cramer pericarp. Microscope examination after fermentation (time 20 h)

First examinations of parchment from fermented beans revealed that cell walls of the mucilage layer were no longer visible except at their basal site of attachment to the sclerenchyma (Photos not shown). This tended to confirm previous assumptions on degradation (pectinolysis) of mucilage pectic substances by endogenous and/or microbial enzymes (pectinases)(Carbonell and Vilanova, 1952 ; Wilbaux, 1956 ; Rolz *et al.*, 1973). However, when sections were examined at a low magnification (Photos 5, 6), clusters of cells of the mucilage layer dissociated from the parchment with stained cell walls were visible. Higher magnification (Photos 7, 8) shows that cell walls of the mucilage layer at time 20 h are apparently intact. They are stained by ruthenium red indicating that pectic substances are still present. Fermenting bacteria are visible in Photo 7. Cells of the mucilage layer are seen in transverse section with ondulated walls while in Photos 2, 3 and 4 they are in longitudinal view.

According to our biochemical (Avallone *et al.*, 1999b). and histological (this study) data and contrary to previous assumptions (Carbonell and Vilanova, 1952 ; Wilbaux, 1956 ; Rolz *et al.*, 1982), mucilage pectic substances are either not splitted during fermentation by endogenous and/or microbial enzymes or, if they are splitted, it must be to a very restricted and non detectable extent Our findings are in agreement with the work of Castelein and Pilnik (1976) who found that *Erwinia dissolvens*, a major coffee fermenting bacteria, produces only an exo-pectate-lyase which is unable to depolymerize coffee mucilage high methoxyl pectins.

At time zero just after pulping, the mucilage layer forms a continuous, although loosened, tissue covering the parchment (Figure 2-A). Mucilage being exposed to air, natural microbial flora starts developping on the surface of the mucilage layer; sugars are progressively metabolised inducing an osmotic pressure gradient from outside to inside. Thus a water flux will compensate leading to turgor of the cells. Although it had not been possible to model this phenomenum, it is possible that such an osmotic pressure gradient would exert a pressure sufficient enough to rupture cell walls at the basal site of attachment of the cells (Figure 2-B). Indeed, this zone of junction between walls of two different tissues, a parenchyma with thin primary walls and a lignified secondarized sclerenchyma, was likely more cohesive than in the rest of the mucilage.

Simultaneously organic acids are produced inducing a pH gradient. It is likely that the pH lowering induces physico-chemical alterations in the cell walls (*e.g.* activation of cell wall enzymes, alteration of the pectic gel)(Carpita and Gibeaut, 1993). There is definitively an unknown alteration of the cell walls since after fermentation they have stiffened and were less hydratable than before fermentation ;

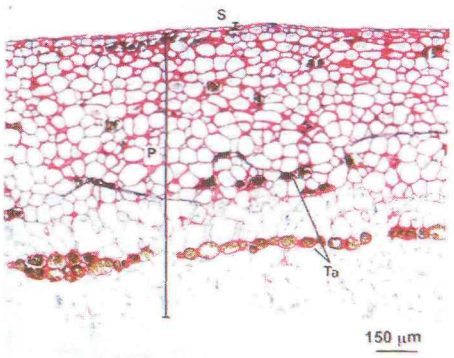


Photo 1. Outer portion of coffee cherry pericarp.
S : skin (exocarp). P : pulp (outer mesocarp). Ta : tannin containing cells.
Periodic acid-Schiff-Naphtol blue black.

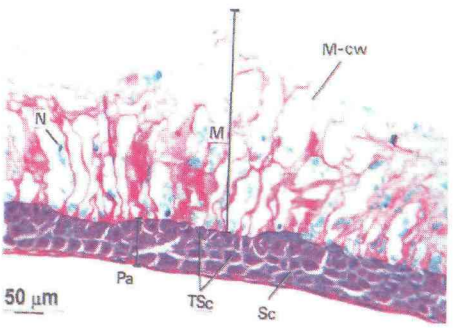


Photo 2. Inner portion of coffee cherry pericarp.
M : mucilage (inner mesocarp). M-cw : mucilage cell walls. Pa : parchment (endocarp). Sc : sclenrenchyma. TSc : transverse sclereids. N : Residual nuclei.
Periodic acid-Schiff-Naphtol blue black.

Photo 3. Mucilage layer and parchment of coffee bean <u>before</u> fermentation. (right)
M-cw, Pa, Sc as in Photo 2. IE : Inner epidermis.
Periodic acid-Schiff-Naphtol blue black.

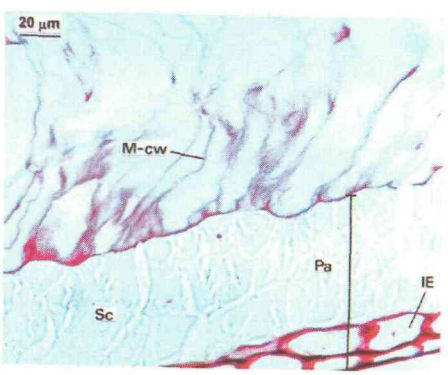
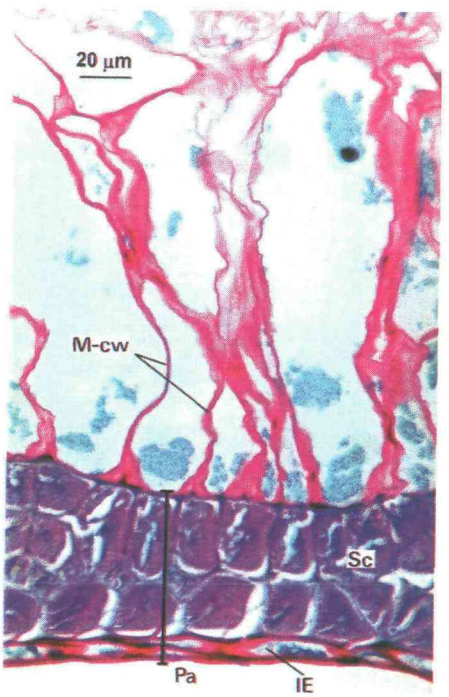


Photo 4. Mucilage layer and parchment of coffee bean <u>before</u> fermentation.
M-cw, Pa, Sc as in Photo 2. IE : Inner epidermis.
Ruthenium red.



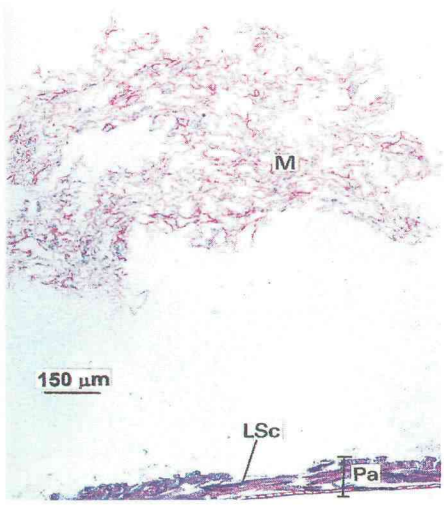


Photo 5. Mucilage layer and parchment of coffee bean <u>after</u> fermentation.
M, Pa as in Photo 2. LSc : Longitudinal sclereids.
Periodic acid-Schiff-Naphtol blue black.

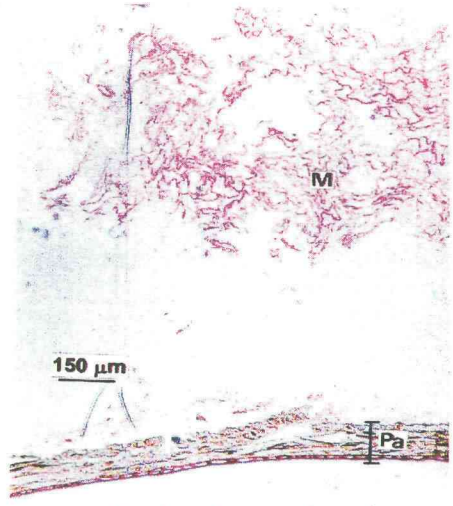


Photo 6. Mucilage layer and parchment of coffee bean <u>after</u> fermentation.
M, Pa as in Photo 2.
Ruthenium red.

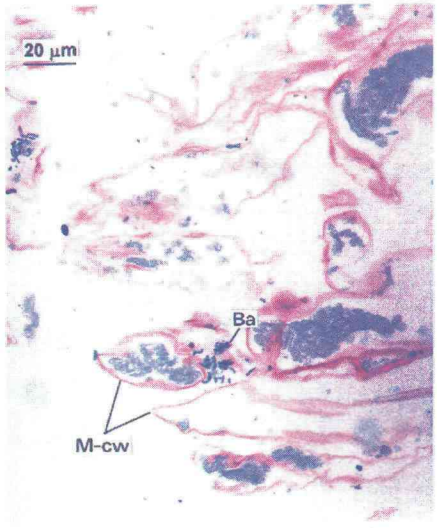


Photo 7. Mucilage layer of coffee bean <u>after</u> fermentation.
Cells are seen in tranverse section. M-cw as in Photo 2. Ba : bacteria.
Periodic acid-Schiff-Naphtol blue black.

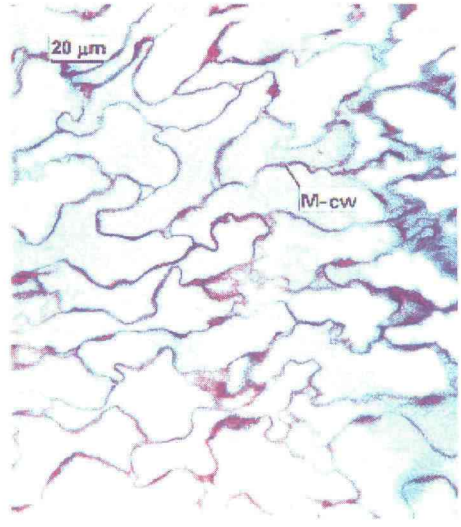
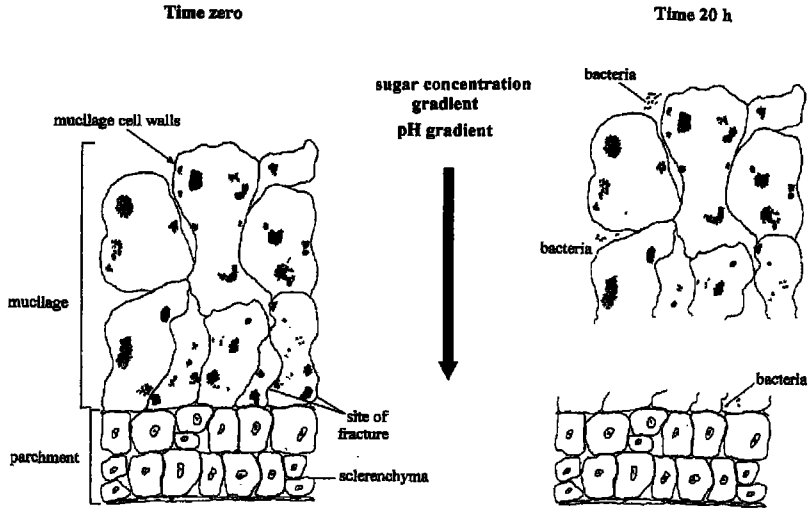


Photo 8. Mucilage layer of coffee bean <u>after</u> fermentation.
Cells are seen in tranverse section. M-cw as in Photo 2.
Ruthenium red.

however with regards to their polysaccharide composition, both types of cell walls exhibit no measurable differences (Avallone *et al.*, 1999b).



2-A 2-B

Figure 2. Schematic view of events occuring during fermentation of coffee bean mucilage. (2-A) before fermentation ; (2-B) after fermentation.

CONCLUSION

After fermentation, the mucilage tissu is still organized with larg cells and its cell walls still contains polysaccharides and pectins. During this step, there is not a total pectic degradation of the tissu and its components as it is said in the literature, but just a separation from the parchment. In pratical terms, this mean that if we want to control the fermentation step with an inoculum addition, it would be better to use a good fermentative strain than a pectinolytic micro-organisms.

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