Stereo and scanning electron microscopy of cocoa beans (*Theobroma cacao* L.): fungi spoilage susceptibility

H. H. Kreibich¹, E. M. Oliveira² E. H. S. Moecke³ and V.M. Scussel¹

¹Laboratory of Mycotoxicology and Food Contaminants, Department of Food Science and Technology, Federal University of Santa Catarina, Av. Admar Gonzaga, Itacorubi, 1346, Florianópolis, Santa Catarina, Brazil.
²Central Laboratory of Electron Microscopy, Federal University of Santa Catarina, Florianópolis, SC, Brazil.
³Microscopy Laboratory, Department of Food Science and Technology, Federal University of Santa Catarina, Av. Admar Gonzaga, Itacorubi, 1346, Florianópolis, Santa Catarina, Brazil.

The dry cocoa beans (*Theobroma cacao* L.) post-fermentation morpho-histological susceptible characteristics to living organism proliferation were investigated through stereo (SM) and scanning electron microscopy (SEM). Selected fungi spoiled bean were utilized. The following characteristics related to shell and edible parts were identified. **Shell:** the pulp residues (highly hydrophilic and nutritious fine tissue remaining on shell); and the wrinkled and uneven surface (rough tissue - humidity absorption and conidia deposition, respectively). **Edible part:** the uneven and sectioned parenchyma (trapping tissue for fungi conidia and humidity entrance). Different fungi spoiled cocoa bean shell and edible part are also shown with SEM micrographies. Due to its pulp residues and its above characteristics, cocoa bean shell is quite prone to living organism proliferation after fermentation. Therefore moisture control should be applied to prevent and/or reduce proliferation. Knowledge on cocoa beans morpho-histological susceptible characteristics is of interest to help chocolate industries to improve final products quality.

**Keywords:** *Theobroma cacao*; adulteration; scanning electron microscopy; spoilage; fungi

1. Introduction

After cocoa fruits are harvested, their seeds have the surrounding pulp extracted and sent to natural fermentation. The desirable types are yeasts and lactic / acetic acid bacteria, which through enzymes catalysis produce alcohol and lactic / acetic acids [1, 2]. That process causes beans swelling and brownish color development. The moisture content (mc) is high - ca. 60% [3, 4]. From that stage, the seeds are called beans (no germination capacity) and are sent to sun drying on cement platforms (mc: around 7.5%) [5, 6, 7]. Despite those procedures for quality, difficulties maybe be faced which need to be controlled, such as, fungi proliferation due to climatic factors (rain/heat: high moisture/temperatures) and pH changes (organic acids produced during fermentation) which can lead to production quality losses up to 40% [8, 9, 10, 11].

The presence of biological contaminants (fungi / insects) in the cocoa paste (chocolate preliminary step) is indication of lack of environment control during the phases involving its initial production, i.e., from seed to bean transformation (pulp extraction / fermentation / drying / storage) [7, 12].

Scussel et al.[13] studying the Brazil nuts morpho-histological characteristics, reported that some features/ tissues can allow living organisms entrance and, as long as they find favorable environment conditions (humidity and temperature), their proliferation takes place. The cocoa morphological characteristics are shown through stereo (SM) and scanning electron microscopies (SEM) [14, 15], but studies have not shown microscopy in cocoa beans deteriorated and the presence of fungi.

Considering that, in-shell dry cocoa beans post-fermentation can be spoiled by fungi/insects thus reducing the sensory quality parameters (prior cocoa paste production) of fine chocolate products, this work reports an investigation on cocoa beans living organisms spoilage susceptible structures through SM and SEM. Some fungi infection in selected spoiled beans parts are also shown.

2. Material and Methods

2.1. Material

(a) **Sample:** selected cocoa beans (in-shell) (a.1) healthy i.e., showing no visible cracks or fungi/stains on shell surface (5 kg) and (a.2) fungi spoiled/damaged, obtained after the fermentation and drying processes (mean moisture content: 7 %) of Forasteiro variety.

(b) **Equipment:** microscopes - (b.1) stereo microscope (10x and 0,71-11,5x for ocular and objective lenses, respectively), model MZ16 with stereo-light source, Leica Microsystems (Heerbruch, Switzerland) coupled to a color image-capture camera, model OPT14 MP, Opticam Microscopy Technology (Doral, Fl., USA); (b.2) light microscope (100 – 400x), model BX40, Olympus (Tokyo, Japan); (b.3) scanning electron microscope (5000x), model JSM-6390LV, Jeol (Peabody, Mass., USA) and gold coating machine, model EM-Scd500, Leica (Leider, Ill., USA).
2.2 Methods

(a) Sample preparation: the healthy cocoa beans (in-shell) were sectioned (whole/shell/edible part) for SM and SEM analysis of the main structures, as reported by Scussel et al. [15]. (a.1) whole cocoa bean half sections - SM: in-shell were sectioned using a fine saw and sliced (shell and edible part) with a scalpel. (a.2) cocoa bean parts - SEM: shell and edible parts (parenchyma portions - nibs) were cut into small cubes, with the fat previously extracted (edible part) [16, 17]. (a.3) stub mounting and gold coating: the samples were fixed on stubs containing double-sided carbon tape and coated with gold for SEM analysis [15].

![Figure 1. Stereo micrographs of in-shell dry cocoa bean (*Theobroma cacao* L.) post-fermentation - SHELL: (a) sound with cracks and closer view of pulp residue; (b) fungi deteriorated with visible pulp residues [7.1 to 40x].](image-url)
b) SM and SEM observation: (a) SM - each cocoa bean half-section was observed through SM to determine the location of each structure. Micrographies were saved using the SM image capture software (magnification from 7.1 to 115x). Note: the details of the bean shell surfaces (outer and inner) were also observed by SM for further elucidation by SEM; (b) SEM - each area of interest of the sample was scanned and its characteristics observed [16, 17] at different magnifications (ranging from 70 to 2,000x).

3. Results and Discussion

From the surface and slices of sound whole cocoa beans observation by SM and SEM, it was possible to register some shell and edible part spoilage susceptible characteristics. They were for shell, its surface remaining pulp residues (left overs of the pulping process prior fermentation) and cracked / uneven morphology. For the edible part, they were mainly the surface and parenchyma groove tissue morphology (formed during fermentation by the multiple reactions, gas release and pressure). Figures 1, 2b, 4 show the morpho-histological spoilage susceptible characteristics and Figures 2a, 3, 5 to 7 the fungi infection of the visibly spoiled (mycelia / hyphae / reproductive structures) cocoa bean by both microscopies.

Figure 2. Stereo micrographies of in-shell dry cocoa bean (*Theobroma cacao* L.) post - fermentation – shell surface PULP FUNGI SPOILED: (a.1/2) pulp tissue with fungi proliferation [7.1x] ; (b.1/2) mycelia growth [10.5x] and (c.1/2) by optical microscopy - conidia and hyphae [200x].
Figure 3. Stereo micrographies of dry cocoa bean (*Theobroma cacao* L.) post-fermentation: (a/b) shell outer /inner surface and (c) edible part sulky parenchyma(c.1) surface and (c.2) cross section (20 to 40x).

3.1. Sound cocoa bean spoilage susceptible structures – SM and SEM

The characteristics related to shell structures, which allow living organisms proliferation and/or conidia and insects (flies - *Drosophila melanogaster*; ants – *Tapinoma sessile*) entrance, as well as humidity absorption to the inner bean (parenchyma) were identified as follows. Shell: the pulp residue, a highly hydrophilic and nutritious fine tissue remaining on shell that is used as a rich substrate for fungi conidia proliferation and insect infestation (Figures 2a, 3, 4b, by SM and SEM). In addition, the shell, which is rather thin (100 to 250 µm) when compared to Brazil nut’s [18], has a cracked / crinkled and uneven surface with a rough tissue that allows humidity absorption and so fungi conidia deposition. That also, adds onto the deterioration process conditions as reported by Scussel at al. [18, 13] for Brazil nuts. Figures 1, 2b.1, 2b.2, 4 and 5 show those characteristics by SM and SEM, respectively. Edible part: the uneven with groove parenchyma surface and inside its inner soft tissue. The edible part, acts as a trap region for fungi conidia and humidity entrance (Figure 1a). Due to shell pulp (nutritious / hygroscopic) residues and its (cracked & crinkled) surface, the whole cocoa bean (especially when in-shell) is quite prone to living organism proliferation.
3.2. Spoiled cocoa beans fungi infection - SM and SEM

The main sign of fungi presence (apart from some edible tissues visibly deteriorated), is their mycelia colonies morphology visualization by microscopy (SM / light / SEM) and/or through a series of mycology tests [18, 20]. Depending on the infection intensity and the extent of affected area, the fungi reproductive structures, apart from mycelia, can be observed, allowing genera and species identification.

In the current study, by utilizing the selected spoiled cocoa beans, the shell pulp residue and surface cracks, as well as the edible part parenchymal tissue had fungi (conidia and hyphae structures) of different genera identified. Despite that, none of them were from fungi toxigenic genera. Figures 5 to 7 show the diversity of mycelia and conidia among the cocoa bean shell and edible part tissues. Figure 7 shows a closer view of the reproductive structures, where one can observe the conidia differences in shape, size and surface, distributed through the spoiled cocoa bean tissues. Whether

![Figure 4. Scanning electron microscopies of SOUND dry cocoa beans (Theobroma cacao L.): (a / b) SHELL (a) uneven surface with cracks, (b) pulp residue; (c) EDIBLE PART surface [22 to 1,700x].](image1)

![Figure 5. Scanning electron microscographies of selected FUNGI SPOILED dry cocoa beans (Theobroma cacao L.) surfaces: (a/b) SHELL and (c) EDIBLE PART: showing fungi proliferation, either on the cracks tissues (conidia deposition / entrance) [300 to 2,700x].](image2)
the cocoa beans environment surrounding conditions are adequate and nutrients are available for those conidia growth, their proliferation can intensify.

**Figure 6.** Scanning electron micrographs of selected FUNGI SPOILED dry cocoa beans (*Theobroma cacao* L.) – EDIBLE PART: (a/b/c) parenchymal cells showing fungi proliferation (with different mycelia / hyphae / conidia formats) [180 to 1,700x].

**Figure 7.** Scanning electron microscographies of REPRODUCTIVE STRUCTURES of selected fungi spoiled dry cocoa beans (*Theobroma cacao* L.): (a/b) *Rhizopus / Mucor* whole (vesicles & stipes) and conidia; (c/d) *Aspergillus / Penicillium* diverse conidia morphology [1,700 to 10,000x].
3.3. Living organisms - Control methods

Although, studies have reported toxigenic fungi in dried nuts and seeds from different growing area none were observed in the cocoa bean samples studied. Only spoilage fungi, such as those from *Rhizopus* and *Mucor* genera were visualized by SEM (Figures 6 and 7). Despite that, any fungi infection, means cocoa bean tissues spoilage and also bean compounds (lipid / protein / carbohydrates) degradation both by air (oxygen) and endogenous enzymes catalysis, thus affecting the further expected chocolate flavor quality. To prevent that, application of controlled atmospheres could contribute to improve safety despite the susceptibility of beans structures to fungi entrance. In addition, thermometry (temperature control) in cocoa bean storage facilities (whether bulky or in bags) is also recommended, as applied for cereals and other grains and nuts (high quality storage) [23, 24].

These results may serve as subsidies for chocolate industries (high demand on cocoa beans quality selection) to ensure that safety continues from that raw material (fermented and dry) to its final product (chocolate). Knowledge on cocoa beans morpho-histological susceptible characteristics are of interest to help chocolate industries to improve final products quality either by (a) applying efficient cocoa pulp extraction procedure prior & after fermentation, (b) keeping post-fermentation beans as dry as possible apart from the (c) controlled atmosphere (ozone/ carbon dioxide / vacuum / hermeticity) application.

4. Conclusion

This is the first study able to identify through SM and SME, the fungi spoilage morphological susceptible structures of cocoa beans, including the cocoa infection spoilage mycelia/conidia characteristics. The pulp residues, the cracked cocoa beans surface and groove out and inner parenchyma (post-fermentation) are the main sites responsible for either, living organism entrance or humidity absorption leading to deterioration. It is important to emphasize that mycotoxin contamination can take place on those affected cocoa beans susceptible sites, as long as toxigenic fungi strains reach / infect them and find optimal/adequate environmental conditions for them to grow.

**Acknowledgements** The authors are grateful to the CEPLAC, located in Ilheus city, at Bahia state, Brazil, for providing the cocoa samples, CAPES for the grant provided to H.K. and the Central Laboratory of Electron Microscopy (LCME) of the Federal University of Santa Catarina for technical support.

**References**


