

## Ultrastructure of cocoa fruits (*Theobroma cacao*) of cultivars with contrasting susceptibility to *Moniliophthora roreri*

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**Abstract:** Moniliasis of cocoa is an important disease caused by the fungus *Moniliophthora roreri* (Cif & Park) (Evans *et al* 1978). A study was conducted on the external features of fruits of two cocoa cultivars with different degrees of susceptibility to *M. roreri*; also a comparative study of the exocarp tissues of healthy fruits was conducted. Scanning Electron Microscopy (SEM) and High Resolution Optical Microscopy (HROM) were used respectively. For SEM, sixty-day-old healthy fruits of cvs Pound-7 (susceptible) and UF-296 (moderately resistant) were collected from CATIE's germplasm collection in Turrialba and La Lola, Costa Rica. Apparently, no external anatomic differences between the fruits of the Pound 7 and the UF-296 (moderately resistant) cultivars exist, except for the star-like trichomes in the latter. Both show an alveolar epidermis on which there are noticeable anomocytic-type stomata and a large number of glandular trichomes. For HROM, healthy Pound-7 and UF-296 fruits from La Lola were used, as well as cv UF-273 resistant to the pathogen. The major histological feature of cv UF-273 and UF-296 was a cellular arrangement of the subepidermic parenchyma which was more compact, probably containing larger amounts of phenolic substances in the vacuoles, as compared to the Pound 7.

**Key words:** Moniliasis, *Moniliophthora roreri*, *Theobroma cacao* pod structure.

Cocoa (*Theobroma cacao*) is an economically important crop for small farmers of Costa Rica's low-land humid tropics. Diseases, however, seriously impair yields, for example, moniliasis, which is caused by *Moniliophthora roreri* (Thurston 1989). This disease was first detected in Costa Rica in 1978. If unchecked, it reduces yields by 80% (Costa Rica 1984). This disease has also been found in Ecuador, Colombia, Perú, Venezuela, Panamá and Nicaragua (Enriquez 1983).

The fungus infects almost exclusively the fruit. Infection is via the epidermis from which it spreads inter and intracellularly to the subepidermic tissues and the exocarp. Infection proceeds to the central tissues, including the seeds. The resulting necrosis spoils the harvest (Díaz 1956, Ampuero 1967, Jorgensen 1975, Evans *et al* 1978, Campuzano 1981). Sixty-day-old fruits are the most

susceptible to the fungus; older fruits are less susceptible (Bejarano 1961).

The fruiting body of cocoa has the seeds arranged in five to 10 longitudinal ridges with 16 to 64 seeds each. The gynoecium has five symmetrically distributed carpels. The development and growth of the berries are closely related to this symmetry (Roth & Lindorf 1971). In berries of 8,5 to 9,5 cm in length and 3 cm in diameter, a sclerenquimatous layer is formed. Cells are initially thin walled; then the walls become thick (Roth & Lindorf 1971).

Diseased fruits are eliminated to decrease inoculum potential, and cultural practices are adopted to make the microclimatological conditions unsuitable for the fungus (Barros 1977, Galindo 1990). The measures are effective provided resistant genotypes are used. There are differences in susceptibility to the fungus among CATIE's cultivars of cocoa (Phillips

1986). Since the 1960s efforts have been made to find some methodologies for the identification of resistant materials (Sotomayor 1965). The mechanisms for resistance are unknown. Putative structural and physiological mechanisms must be known in order to refine the screening process for selecting resistant cultivars and improve control measures. It would be very interesting to know, for instance, how the pathogen interfaces with the fruit surface and its microflora. The present work compares the external features and the exocarp of fruits of cultivars with contrasting degrees of susceptibility to the fungus *M. rozeri*. In the accompanying article information is presented on the prepenetration and infection processes of the fungus.

#### MATERIAL AND METHODS

Cocoa fruits were collected from Turrialba and La Lola, two sites from the low-humid tropics of Costa Rica in which CATIE (Spanish acronym for Tropical Agronomical Teaching and Research Center) maintains its germplasm collections. Only sixty-day-old fruits, obtained through artificial pollination, were used. Fruits from the two sites were compared to determine if there were site effects. Three cultivars were selected on the basis of their resistance to *M. rozeri*, cv Pound 7, highly susceptible, UF-296, moderately resistant, and UF-273, highly resistant (Brenes 1983, Phillips 1986).

*Sample processing for scanning electron microscopy:* Exocarp samples were taken from the equator and the poles of fruits of cv UF296 and Pound 7, 1 cm<sup>2</sup> by 0.5 cm in depth. The segments were fixed in FAA, dehydrated in an increasing series of ethanol and dried in a critical point drier, three CO<sub>2</sub> changes were made. Samples were then gold coated (200-400 nm) in an ionic generator and observed in a scanning electron microscope.

*Sample processing for high resolution light microscopy:* Exocarp samples were taken from the equator and the poles of fruits of cvs UF296, Pound 7, and UF-273; then they were fixed in a Karnovsky fixer (Karnovsky 1965) and in a Sorensen's phosphate buffer (0.1 M, pH 7.4) for 20 hr at 4°C, which was followed

by three washes in the buffer. Samples were dried in an increasing ethanol series, then embedded, via propylene oxide, in Spurr's resin (Spurr 1969). Thin sections (1-2 µm) were obtained in an ultramicrotome and then stained according to Shoeder (Shoeder 1980) using Toluidine blue. Sections were observed and photographed in a light microscope.

#### RESULTS

*Scanning electron microscopy:* Sixty-day-old fruits of cvs Pound 7 and UF-296 showed an alveolar epidermis (Fig. 1) with numerous anomocytic stomata, with kidney-shaped occlusive cells, sometimes even over ridges in groups of two to 10 (Fig. 2). The ostiole, average 9 µm in length, was observed open (Fig. 3). The surface of the fruits showed numerous glandular trichomes, 9 µm in length (Fig. 4).

Longitudinal veins were observed on the fruit surface. Their epidermis showed numerous anomocytic stomata (Figs. 5, 6). The only striking difference between the fruits of the cultivars was the presence of star-like trichomes in cultivar UF-296 (Fig. 7).

Numerous bacteria were observed on the surface of the fruits of both cultivars, as microcolonies (Fig. 8), near trichomes or over them (Fig. 9) or on the surface (Fig. 10).

*Light microscopy:* Transversal sections of the exocarp of sixty-day-old fruits of cvs UF-273, UF-296 and Pound-7 showed a thin cuticle and an epidermis consisting of a monolayer of cells, rectangular with very regular borders (Figs. 11, 12, 13). Stomata were abundant, with small substomatic chambers (Fig. 14). Glandular trichomes were also observed, as well as numerous mucilaginous channels with a very well-defined epithelial layer; the ducts were observed either alone or in groups.

The vascular network was found well distributed in the tissues; vascular bundles were colateral. Numerous cell divisions were observed in different planes as the fruits were still growing.

In all the cultivars numerous epidermal and cortical cells loaded with dark substances in the vacuole, possibly phenolic in origin, were

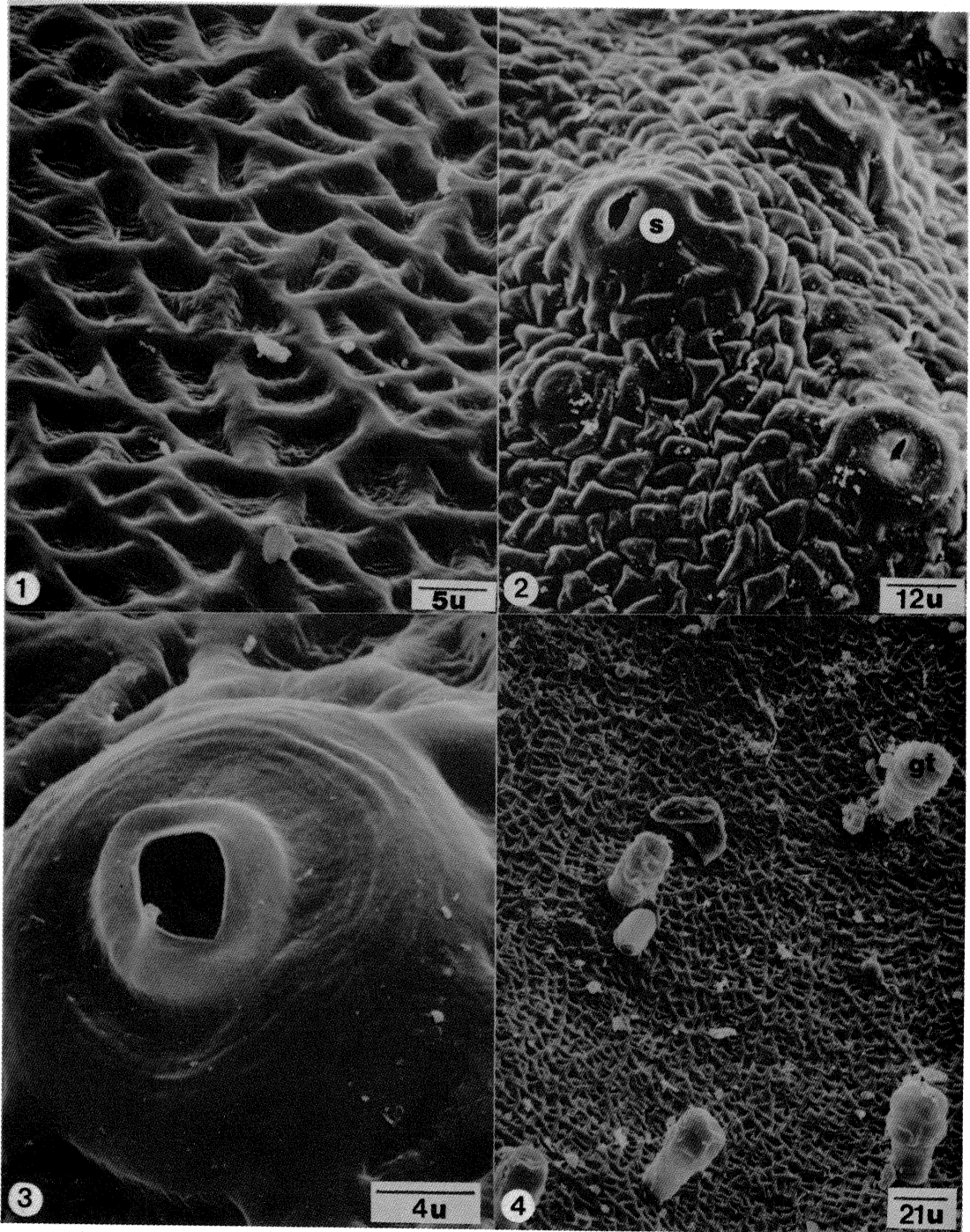


Fig. 1. Cocoa pod's surface showing alviolae.

Fig. 2. Cocoa pod's surface showing clustering of stomata.

s:stomata

Fig. 3. Detail of stoma in cocoa pod.

Fig. 4. Glandular trichomes in cocoa pod. g.t. glandular trichome

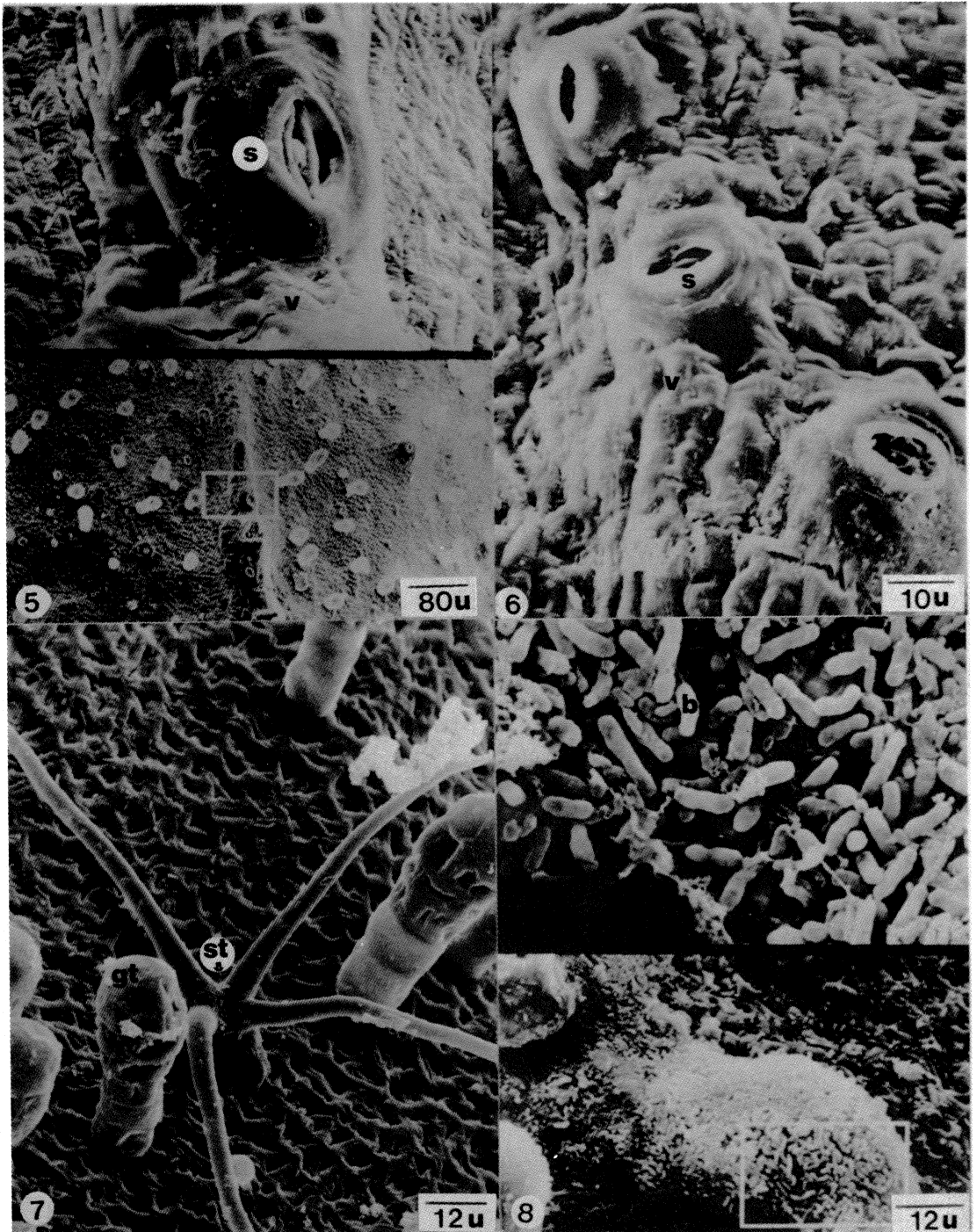


Fig. 5. Detail of vein in cocoa pod. v: vein, s: stoma

Fig. 6. Estomata located on vein in surface of cocoa pod. v: vein, s:stoma.

Fig. 7. Trichomes in pod of cocoa cultivar "UF-296". g.t. glandular trichome, s.t. star trichome.

Fig. 8. Close up of microcolonies of epiphytic bacteria on cocoa pod. b: bacteria.

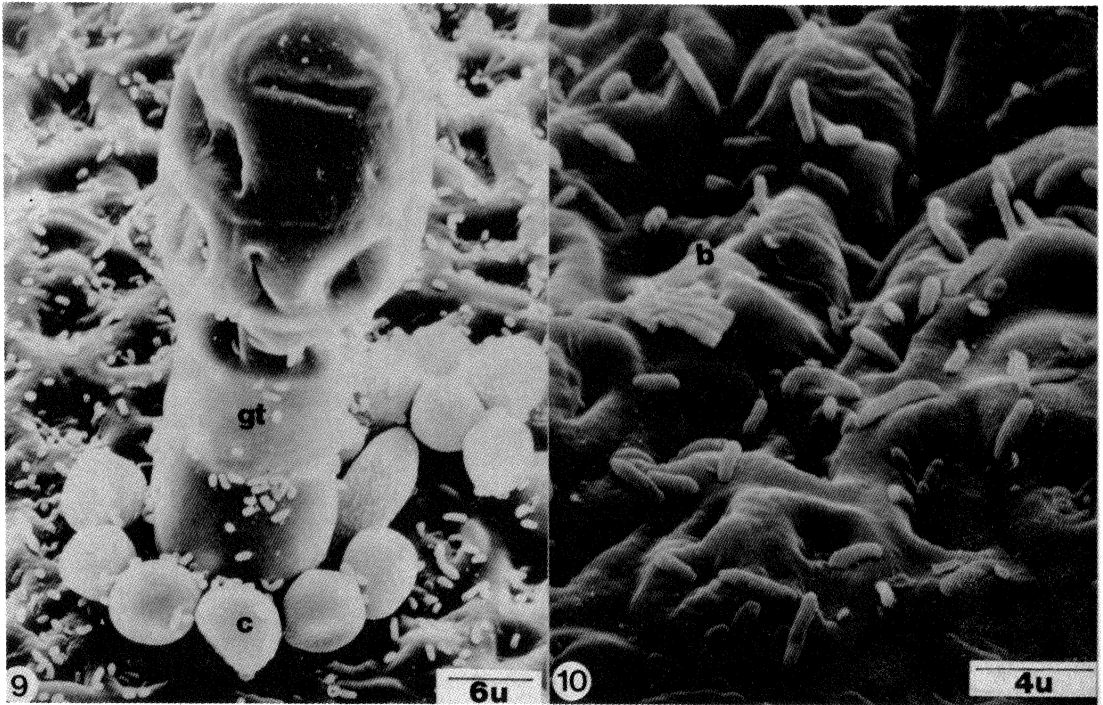


Fig. 9. Close up of glandular trichome on cocoa pod. g.t. glandular trichome, c. conidia.

Fig. 10. Cocoa pod's surface showing bacteria. b: bacteria

observed. The number of layers, though, in the subepidermal parenchymatous cells varied in each of the cultivars. In cv UF-273, seven continuous layers were observed; cells showed a larger accumulation of phenolic substances in comparison with cv UF-296, which showed five cell layers, or Pound-7, which showed eight layers but with very little accumulation of phenolic substances (Figs. 12, 13).

The cellular organization in the subepidermic parenchyma differed in the 3 cultivars; cv UF-273 showed a more compact cellular arrangement, a greater number of crystals and thicker basal walls in the epidermal cells. In cv UF-296 the subepidermic parenchyma presented a cellular arrangement not so compact, and in cv Pound-7 the cellular arrangement was not compact but with great intercellular spaces.

#### DISCUSSION

This study was conducted to explore if there were anatomical differences either on the

surface or the exocarpic tissues of cocoa berries that could be associated with a differential susceptibility of cultivars of cocoa.

The observations made on the fruit surface of cvs Pond-7 and UF-296 (susceptible and moderately resistant) did not reveal major differences between them. The cellular differences, though, showed a more compact cell arrangement, and higher content of phenolic compounds may be related to a higher resistance to the pathogen in cv UF-296. However, according to Rocha and Jiménez (1966), the concentration of polyphenols varied inversely with fruit age. Young fruits have the highest concentration (Manotas 1953), especially in the exocarp. These experts have suggested that the fungistatic effect of the phenolic substances in the exocarp of cocoa fruits vary with age.

It remains to be determined if phytoalexins, phenolics with antimicrobial properties (Friend 1979), are formed as a result of infection by *M. royeri*. If phytoalexins differ between cultivars, these could well be a resistant

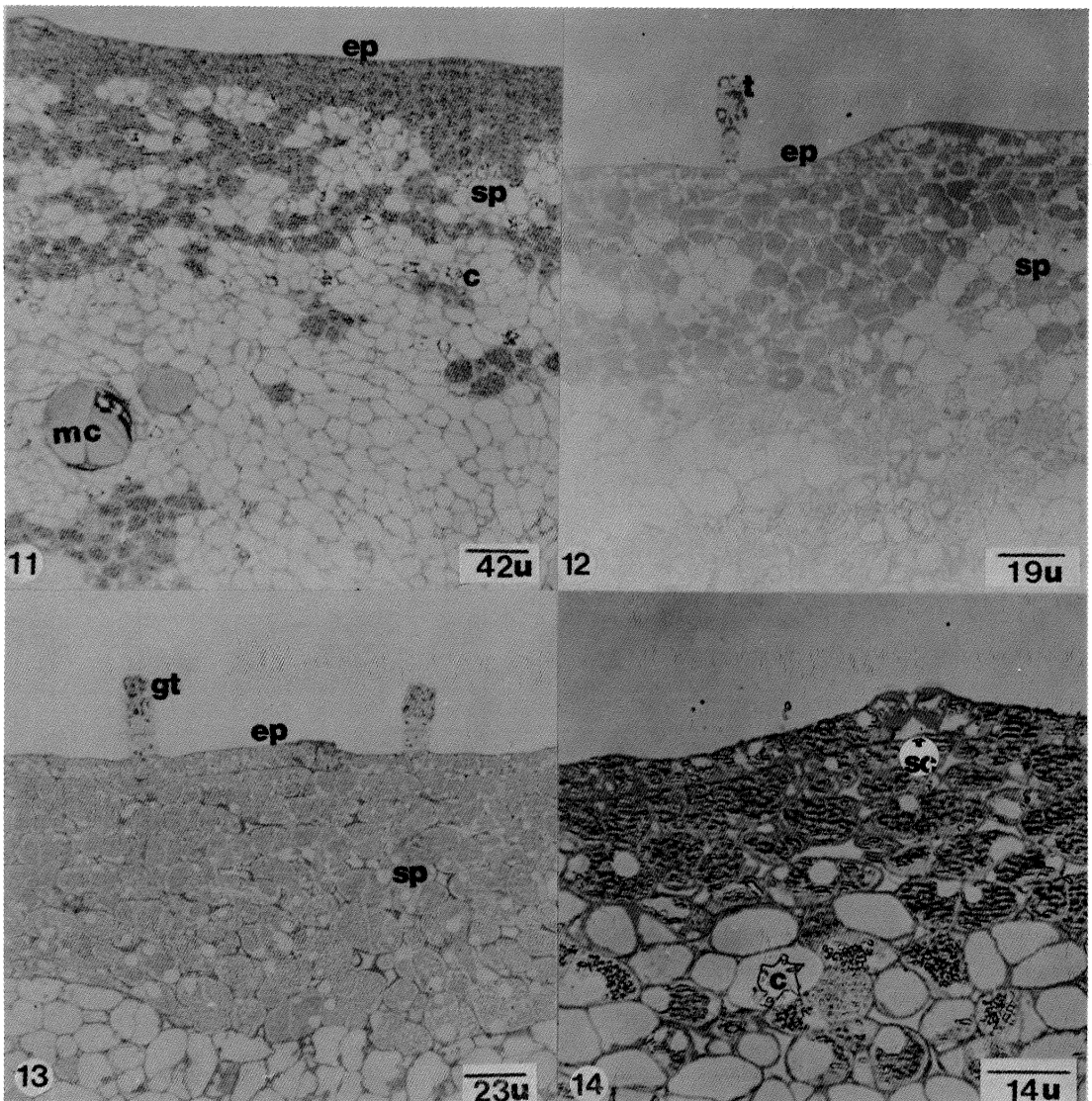


Fig. 11. Transversal section of healthy tissue of pod of cocoa cultivar "UF-273". Observe accumulation of possible phenolic substances in layers of subepidermic parenchyma. e.p. epidermis, s.p. subepidermic, c. crystals, m.c. mucilaginous channel.

Fig. 12. Transversal section of healthy tissue of pod of cocoa cultivar "UF-296". Observe distribution of possible phenolic substances in layers of subepidermic parenchyma. e.p. epidermis, t: trichoma, s.p. subepidermic parenchyma.

Fig. 13. Transversal section of healthy tissue of pod of cocoa cultivar "Pound-7". Observe distribution of possible phenolic substances in layers of subepidermic parenchyma. e.p. epidermis, g.t. glandular trichoma, s.p. subepidermic parenchyma.

Fig. 14. Stoma of cocoa pod in transversal section. s.c. substomatic cavity, c: crystals.

mechanism worth exploring. It would also be necessary to study the phenolic metabolism of the pathogen, as virulent isolates of other fungi seem to detoxify phytoalexins (Friend 1979). Ripened fruits are in general naturally resistant (Diaz 1956). Thus in older fruits other factors

may be involved. Aside from the concentration and types of phenolic substances, there may also be sclerenchymatous cells with lignified walls (Roth & Lindorf 1971). Lignin is an effective barrier because it prevents microbial penetration and mechanical damage (Vance 1980).

Another factor to take into account is the concentration of organic substrates on the fruit. Environmental factors, as well as genetic ones and age, may influence these substrates. Thus the concentration of sugars decreases with age (Manotas 1956, López 1980), and the spores may be deprived of an external source of substrates (Diaz 1956). However, it remains to be shown whether these substrates are necessary for infection to occur.

*Superficial features of the cocoa fruits:* The number of both stomata and trichoma on the surface of the fruits is defined during the initial stages of fruit development (O'Brien 1969). Thus the density of these structures varies with age. If trichomes are infection courts, as observations suggest (Flores, Galindo & Ramírez, this issue), the infection may as well change with age. Sixty-day-old fruits were chosen because of their higher susceptibility (Bejarano 1961).

The rugosity of the fruit surface, especially in the proximity of the trichomes and stomata, would allow the formation of water films; thus germination of spores of *M. rozeri* is more likely to occur here than in other flatter surfaces of the fruit because free water is necessary for this process to take place (Bastidas 1953, Merchan 1982, Campuzano 1981). This is relevant to infection as will be shown in the accompanying article.

Alternatively natural resistance could well be related to antagonistic microflora that flourishes in response to the availability of fruit substrates and organic detritus. Bacteria were ubiquitous on the surface of both cultivars. Antagonistic bacteria have been isolated from this niche. When they were sprayed on the surface of cocoa fruits disease incidence sharply decreased (Jiménez *et al* 1986). As bacteria could vary from cultivar to cultivar, it would be interesting to further study the relationships of the bacterial microflora with spore germination and infection (Blaha and Paris 1985).

*Host-pathogen interactions:* The internal tissues of the exocarp of cocoa fruits of cvs UF-273 and UF-296, the resistant cultivars, did show a more compact cellular arrangement with phenolic substances in comparison with Pound-7.

The distribution of conidia on the fruit surface may be a factor to consider. Spores always accumulated in preference at the base of glandular trichomes, both in inoculated and naturally infected fruits (Flores, Galindo & Ramírez, this issue). This could be due to the accumulation of water that favored the deposition of spores here. If this pattern of deposition is constant, it would be pertinent to establish the role of secretions on spore adherence and germination, as well as their concentration and composition.

Although structural barriers may account for resistance to plant pathogens, resistance in many cases depends on cell reaction to infection that leads to the production of substances that halt further infection by the pathogen (Kosuge 1969).

It has been mentioned by Koller (1980) that the cuticle thickness of the fruit may be a factor in resistance, although this factor may vary with environmental conditions (Martin and Juniper 1970). The results here presented do not show any difference in this characteristic between cultivars. No difference was found in the thickness of the pericarp between cvs Pound-7 and UF-296, either (Engels 1981).

The first barrier that a plant pathogen encounters is indeed the cuticle, a structure consisting of cutine, a biopolyester composed of hydroxy and epoxy fatty acids, 16-18 carbon atoms in length (Kolattukudy 1980), often impregnated with waxes. Thus plant pathogenic fungi produce cutinases and esterases, which degrade cutin and facilitate entry to the plant. Alternatively, infection sometimes involve mechanical piercing of the cuticle. Both mechanisms may also be involved (Baker & Bateman 1978, Yoder & Gillian 1985). Cuticle thickness does not appear to be a barrier that prevents infection (Jaroz *et al* 1982). Cuticle chemical composition may change with environmental conditions (Martin & Juniper 1970). During infection of tomato fruits by *Bothrytis cinerea*, based on ultrastructural evidence, enzymatic dissolution of the cuticle occurs (Rijkenberg *et al* 1980). Preliminary evidence do not support this mechanism of penetration in cocoa fruits by *M. rozeri*.

## RESUMEN

La moniliasis del cacao es una enfermedad importante causada por el hongo *Moniliophthora roreri* (Cif. & Park) Evans *et al.* Se llevó a cabo un estudio sobre las características externas de frutos de dos cultivares de cacao con diferentes grados de susceptibilidad a *M. roreri*; también se realizó un estudio comparativo de los tejidos del exocarpo de frutos sanos. Para el análisis de los tejidos se usó Microscopía Electrónica de Rastreo (MER) y microscopía de luz de alta resolución (MOAR). Para MER, frutos sanos de 60 días de edad de los cultivares Pound-7 (susceptible) y UF-296 (moderadamente resistente) fueron colectados del banco de germoplasma que el CATIE posee en Turrialba y La Lola, Costa Rica. Aparentemente no existen diferencias anatómicas entre los frutos del cultivar Pound-7 y el cultivar UF-296, excepto el tipo de tricoma estrellado presente únicamente en el cultivar UF-296. Ambos muestran una epidermis alveolar en la cual hay estomas del tipo anomocítico y una gran cantidad de tricomas glandulares. Para MOAR, se usaron frutos sanos de Pound-7 y UF-296 provenientes de la Lola, así como frutos del cultivar UF-273 resistente al patógeno. La principal característica histológica de los cultivares UF-273 y UF-296 fue el arreglo celular del parénquima subepidérmico, el cual fue más compacto y probablemente contenía mayores cantidades de sustancias fenólicas en las vacuolas, comparados con el Pound-7.

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