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# Preinfection process in cocoa fruits by *Moniliophthora roreri*

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**Abstract:** *Moniliophthora roreri* (Cif & Park) (Evans *et al* 1978) infects cocoa (*Theobroma cacao* L) fruits causing great losses. A study was conducted of the pathogen-host relationships on the fruit considering fungal spore distribution, germination, germ tube formation and infection using Scanning Electron Microscopy. Sixty-day-old fruits, inoculated and not inoculated, of cv 'Pound-7' (susceptible) and 'UF-296' (moderately resistant) were collected from two sites of Costa Rica's low humid tropic, La Lola and Turrialba. Conidia were found mostly at the base of trichomes. Spores germinated similarly on both cultivars. Germination occurred through a polar germination pore or alternatively through the lateral spore wall 7-8 hr after inoculation of the fruits. The number of germinated spores was low. Germtube infection of the fruit via stomata was not common. Preliminary evidence suggests infection via direct penetration of epidermal cells and trichomes. Hyphae that emerged from the trichomes spread and infected other sites in the epidermis, including trichomes, stomata, injuries and other epidermal cells. Apressorium-like structures or terminal swellings on germ tubes were observed occassionaly.

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

Cocoa (*Theobroma cacao* L.) is an economically important crop for small farmers of Costa Rica's low-land humid tropics. Diseases, such as moniliasis, caused by *Moniliopthora roreri* (Thurston 1989) seriously impair yields. Moniliasis was first detected in Costa Rica in 1978 (Enriquez and Suarez 1978). This disease has been found also in Ecuador, Colombia, Peru, Venezuela, Panamá, and Nicaragua. If unchecked, yields may be reduced by 80%.

*M. roreri* infects primarily the fruit. Infection occurs through the epidermis, from which it spreads inter- and intra-cellularly to the subepidermal tissues and the exocarp. Infection proceeds to the central tissues, including seeds, and necrosis develops back to the epidermis. Externally, the infection appears initially as very small and circular oily spots which then coalesce to a yellowish to brown lesion with irregular borders (Merchan 1981, Galindo 1987). In 3-4 days, white mycelium develops over these lesions, and later conidia appear that confer a cream to brown color to the fruits (Galindo 1987). Fruits mummify and continue to produce conidia up to a year or more (Campuzano 1981, Merchan 1981).

The necrosis resulting from infection damages the seeds (Evans *et al* 1978, Campuzano 1981). Fruits younger than 60 days old are the most susceptible to *M. roreri*, older fruits being less susceptible.

Infected fruits are eliminated periodically to decrease inoculum. Cocoa trees are pruned to reduce shade and change the microclimatological conditions to diminish infection by *M. roreri* (Barros 1977, Galindo 1987). These measures are effective provided resistant genotypes are used. Considerable variation in susceptibility to the pathogen exists among cultivars of cocoa (Phillips and Galindo 1986). Since the 1960s efforts have been made to develop methods to identify resistant

materials, but the mechanisms for resistance are not known. Putative structural or physiological mechanisms must be known in order to refine the screening process for selecting resistant cultivars and improve control measures. Information is presented on the preinfection activities of propagules of *M. roreri* on fruits of cocoa.

## MATERIALS AND METHODS

Cocoa fruits were collected from Turrialba (640 meters above sea level, average temperature of 22ºC, 2700 mm annual rainfall) and La Lola (40 masl, 26,5ºC average T, 3670 mm annual rainfall), which are 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 hand pollination, were used. Fruits from the two sites were compared to determine if there were site effects. The cultivars were selected on the basis of their resistance to *M. roreri*, cv 'Pound 7', highly susceptible, and 'UF-296', moderately resistant (Phillips and Galindo 1986).

Fruits were inoculated with conidia of *M. roreri* according to Philipps and Galindo (1986). The conidial suspension ( 2 x $10^5$ $mL^{-1}$) was prepared in distilled water with 0,01 % Tween-80 from a 16 day old culture of the fungus grown on V-8 medium (V-8 20%, $CaCO_3$ 0,3% and agar 1,8%) at 22-25ºC. The spore suspension was applied to the surface of the fruit with a DeVilbiss atomizer No. 15. Fruits were covered for 2 days with a plastic bag with a humid towel to provide conditions which favor infection as occurs in nature. Samples were taken every hr for 8 hr to observe spore germination and every 6 hr for 66 hr to study germ tube development.

**Sample processing for scanning electron microscopy.** Exocarp samples were taken from the equator and the poles of fruits of cv 'UF-296' and 'Pound 7', 1 $cm^2$ 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 $CO_2$ changes were made. Samples were then gold coated (200-400 nm) in an ionic generator and observed in a scanning electron microscope.

## RESULTS

Conidia of *M. roreri* appeared mostly at the base of glandular trichomes or on the trichomes themselves (Figs. 1, 2).

Mature conidia are unicellular, with an ornamented wall (Fig. 3) and variable shape –spherical (6.4 µm D), elipsoidal or elongated (10.3 x 4.8 µm) (Fig.4). Conidial sizes were based on an average of 10 measurements. Sometimes chains of spores were observed on inoculated fruits (Figs. 5, 6). A germination pore was located at terminus of the spore (Fig. 3).

Only a few of the conidia germinated on both resistant and susceptible, 66 hr after inoculation. Those that germinated did it with a unique germtube, 8 hr after inoculation (Fig. 7), but in very few instances two germtubes were seen (Fig. 8). The germtube generally emerged directly through the terminal germination pore (Fig. 7), at a subterminal location (Fig. 9) or in few occasions laterally (Fig. 10). Often the spores collapsed upon germination (Figs 11, 13). Sometimes the germtube bifurcated after 18 hr (Fig. 13). Germtubes often showed a swelling at the growing tip (Figs 12, 14, 15), a constriction was also observed in the distal extreme, at the spore side (Fig. 11).

The germtubes occasionaly showed an appressorium-like structure (Fig. 14) and the emission of what appears to be a thin infection hyphae from appressorium (Fig. 14). In spite of the relatively high stomatal density, their direct penetration by germtubes was not common (although suggested in Fig. 8), even when the germination occurring near the stomatal ostiole (Fig. 16) was observed. However, penetration via stomata was observed later, 42 hr after inoculation, by hyphae growing from adjacent glandular trichomes (Fig. 17) or stomata (Fig. 18). This occurred after primary infection (about 20 hr after inoculation). Alternatively, some of the germtubes that grew over the alveolar fruit epidermis directly penetrated (20 hr after inoculation) the cuticle (Figs 19, 20) without apparent involvement of an appressorial structure.

Although germtubes of spores close to the trichomes did not necessarily grow toward the trichome, infection by this route was suggested but not shown (Fig 21), via short germtubes. In later stages, more than 45 hr after inoculation,

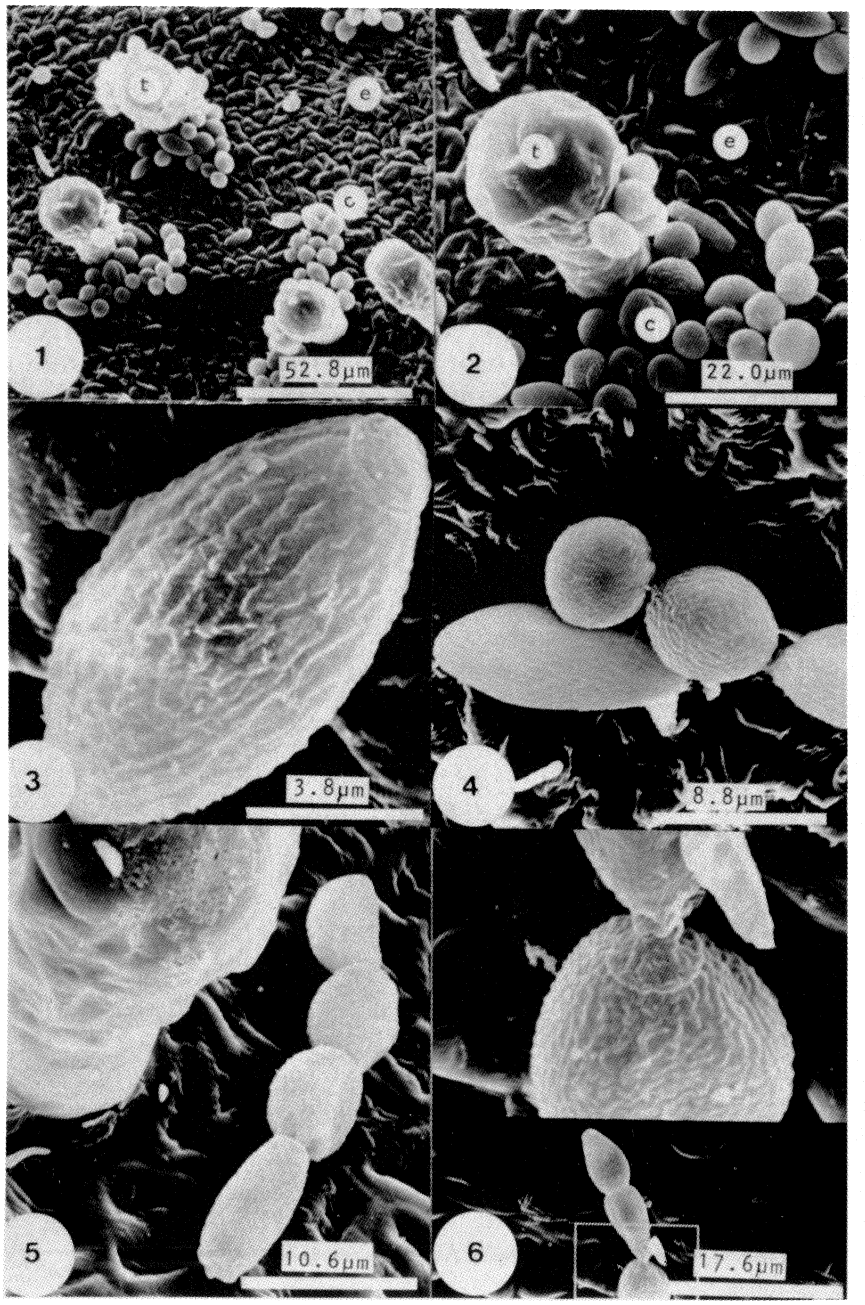


Fig. 1. Conidia (c) of *Moniliophthora roreri* on cocoa pods *(Theobroma cacao)*, (t) trichomes, (e) epidermis. Note accumulation of conidia at the base of trichomes. Fig. 2. Conidia (c) on cocoa pods, (t) trichomes, (e) epidermis. Note conidia attached to the surface of trichomes. Fig. 3. Conidium of *M. roreri*. Note the rugose surface and the germination pore at the spore's terminus. Fig. 4. Conidia of *M. roreri*. Note the variable shapes. Fig. 5. Chain of inoculated conidia of *M. roreri* on the surface of cocoa pods and on a naturally infested pod (Fig. 6).

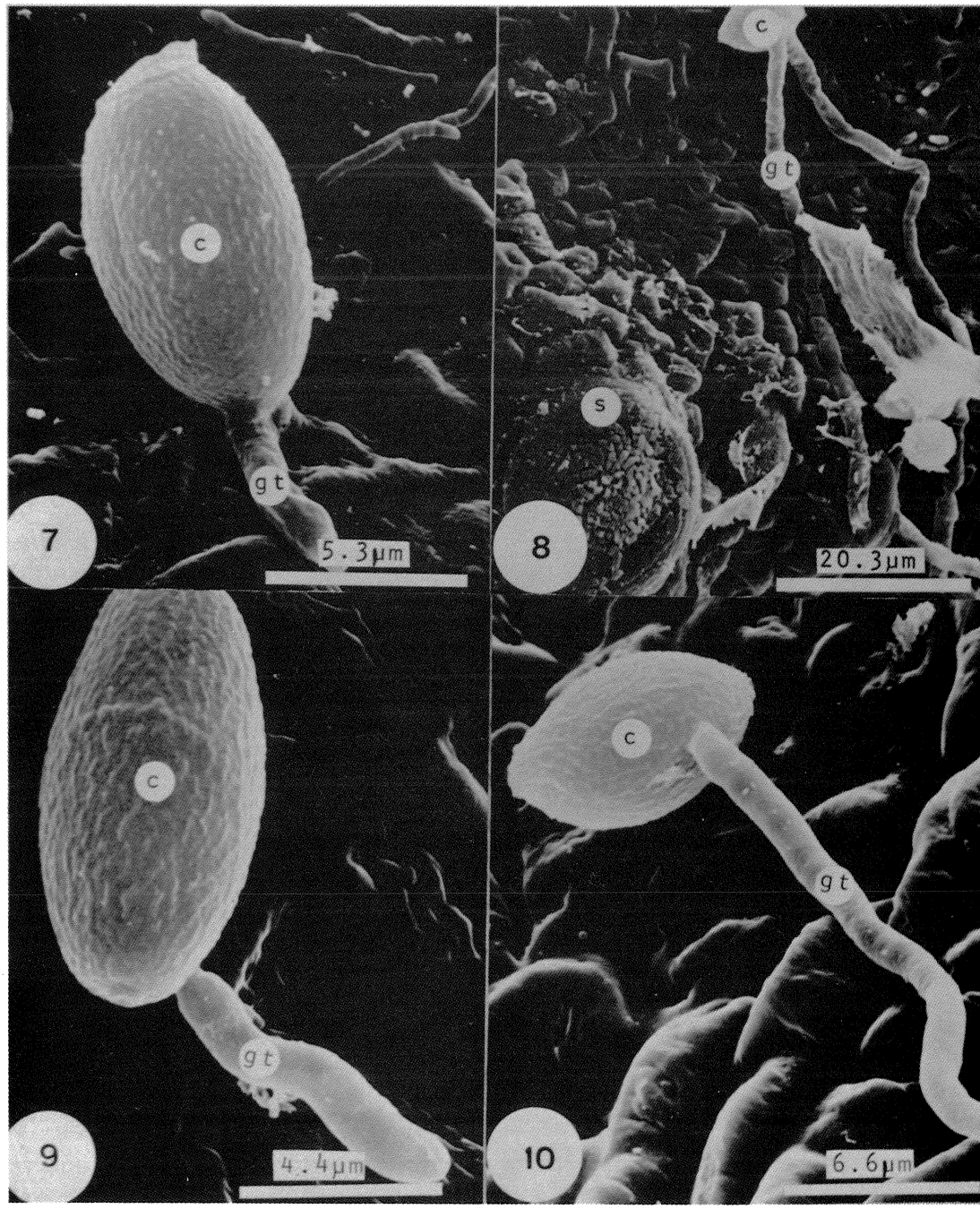


Fig. 7. Conidia of *M. roreri* showing a germination tube (g.t) emerging through the spore'pole , subterminally (Fig. 9), or laterally (Fig. 10). Fig. 8. Conidium (c) of *M. roreri* showing 2 germination tubes (g.t), (s) stoma. Infection via stoma is suggested.

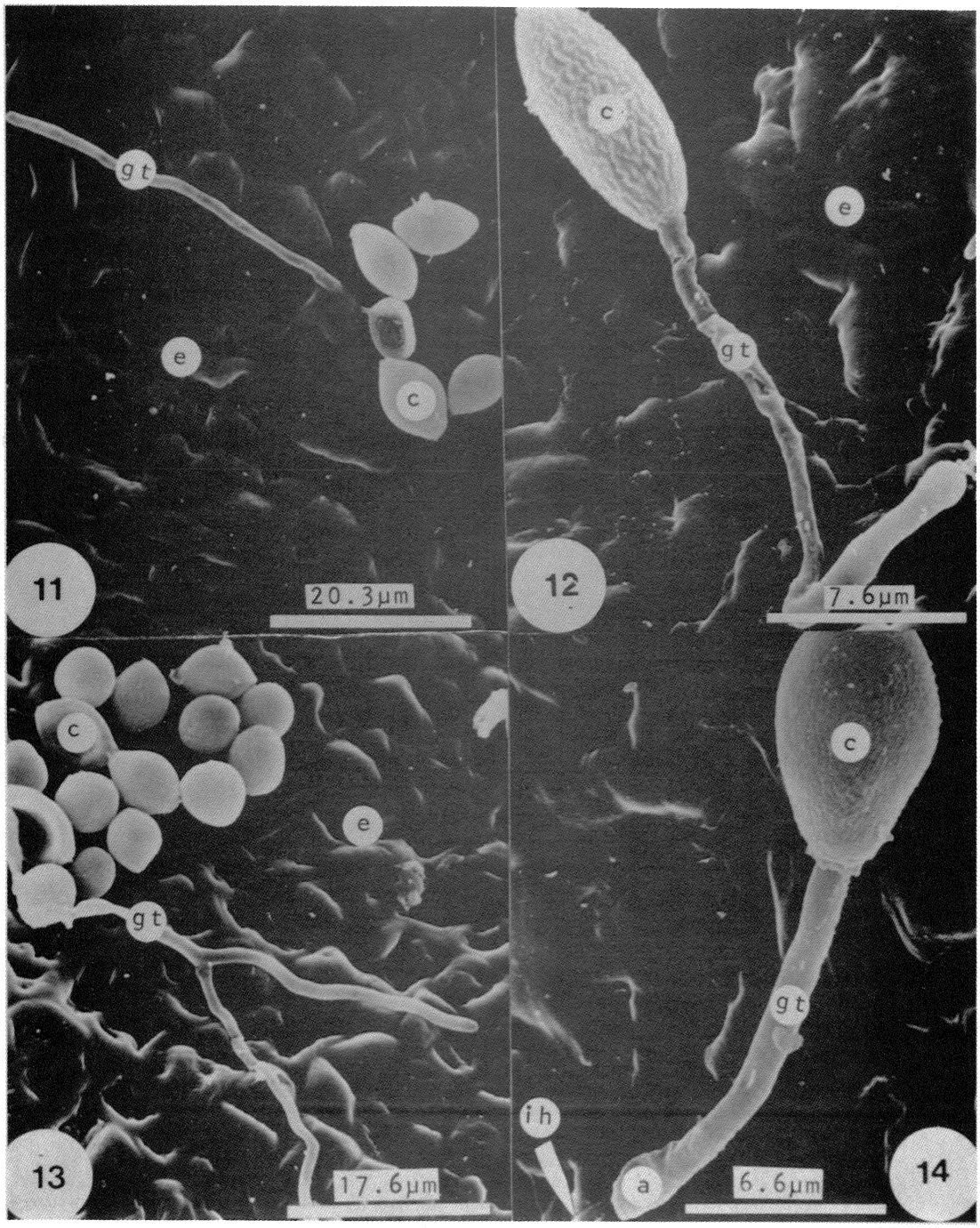


**Fig. 11. Collapse conidium (c), (g.t) germination tube, (e) epidermis. Fig. 12. A germination tube (g.t) following the contour of the epidermis (e), (c) conidium. Fig. 13. Bifurcated germination tube (g.t), (c) conidium, (e) epidermis. Fig. 14. Appressorium-like structure (a) at the tip of a germination tube (g.t), (c) conidium, (e) epidermi infecting hypha (i.h).**

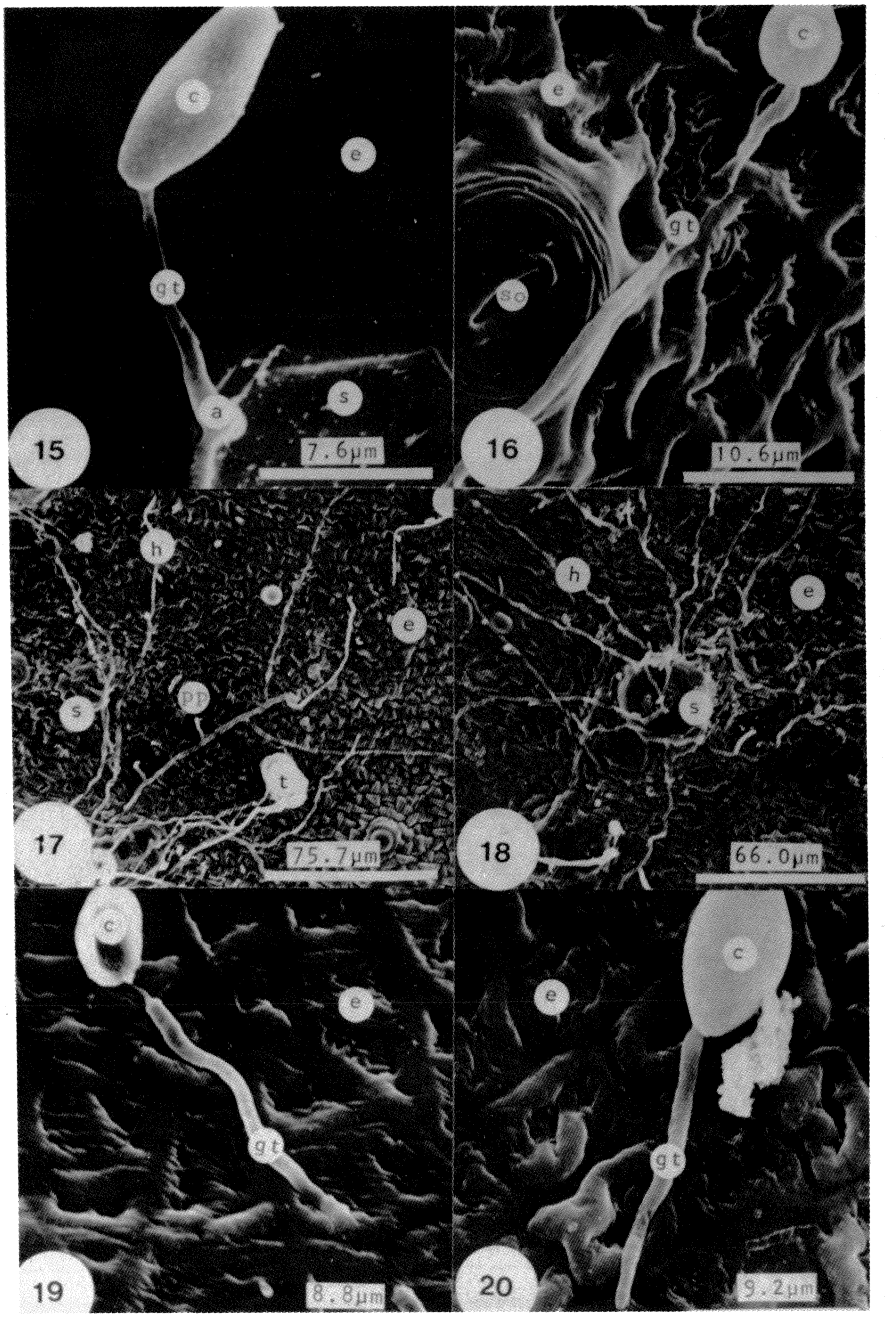


Fig. 15. Appressorium-like structure (a) at the tip of a germination tube (g.t), (c) conidium, (e) epidermis. Note a swelling at the tip of a germination tube which is similar to a penetration peg, (s) stomata. Fig. 16. Germination tube (g.t) near a stoma without infecting it, (s.o) stomatal ostiole, (c) conidium, (e) epidermis. Fig. 17. Infection of a stoma (s) by hyphae (h) growing from infected trichomes (t). Note swellings similar to penetration pegs (pp). Fig. 18. Hyphae (h) emerging through a stoma (s), (e) epidermis. Figs. 19 and 20. Germination tubes (g.t) that have penetrated the cuticle, (e) epidermis.

thinner and longer hyphae appeared to emerge from the trichomes (Fig. 22). This growth did not appear to be linked to the spore but rather to the trichomes or/and subjacent infected tissue where the mycelium can obtain fruit substrates. Hyphae grew on the surface of the fruit, from trichome to trichome (Fig. 24), from trichome to stoma (Fig. 23) and from trichome to injuries in the epidermis (Fig. 25). Although infection by the germtubes appears to occur via epidermis, infection of trichomes and further growth and emergence of new infecting hyphae would allow for a greater number of possible infection sites on the fruit surface.

In later steps of disease progress, coinciding with the so called "brown lesions", new mycelium appeared, emerging on the surface of the fruit (Fig. 26). All the observations made applied as well to cvs 'Pound-7' and 'UF-296'.

## DISCUSSION

No major anatomical differences were found on the surface of the cacao cv 'UF-296' and 'Pound-7' (Flores, Ramírez and Galindo, this isssue). A series of sequential observations and photographs were made of the fruit surfaces after inoculation with spores of *M. roreri*. Abundant anomocitic stomata on the surface of the fruits of these cultivars did not appear to be the main target for infection by emerging germtubes. Although germtube or appressorium were observed, in very few instances, penetrating the stomata, these structures were not the usual site of entry as is for rust fungi (Hoch & Staples 1987). Germtubes of *M. roreri* appear to infect the fruit by direct penetration of the epidermis either on epidermal cells or on trichomes, and appressorium-like structures were not apparently necessary for this to occur. It was not possible to observe infection of trichomes since the close contact of spores and trichomes could have prevented the visualization of an infecting germtube. Nonetheless, appressorium-like structures were associated with a small infecting hyphae. Althought sequential tissue sections of the fruits inoculated with conidia remain the conclusive approach to validate this interpretation, seemingly sequential photographs and observations of the surface of the fruits that were taken during the infection period of the fungus on the fruits failed to reveal other infection modes; massive infection of stomata or direct epidermal cuticle penetration followed by a collapse of the epidermis supports our interpretation. Futhermore, the observed structures coincided with the known sequence of events that leads to infection. For example, that the hyphae observed emerging from trichomes in later stages, 45 hr after inoculation, of the infection process are far larger and more slender than the germtubes, shorter and thicker, formed in earlier stages after inoculation. By the same token, photographs taken shortly after the inoculation took place did no show longer hyphae. Additionally, appressorium-like structures were seen in germtubes about the time infection was taking place, 20 hr after inoculation. It appears that initial infection of the fruit is limited by the small number of germinated spores and by the small fraction of infecting germtubes. The implication of these observations are discussed bellow.

The thigmotatic response of the germ tube to produce appressoria becomes extremely important in rust fungi since infection occurs via stomata (Hoch and Staples 1987). An enlargement of the tip of the hyphae has been associated in other fungi with the accummulation of enzymes responsible for the infection of the fungus (Corlett and Chong 1977, Rijkenberg *et al* 1980, Kunoh 1982). However, since appressoria formation seems to be influenced by environmental conditions (Hoch & Staples 1987), the possibility can not be ruled out that these structures can still be formed in a larger percentage of the germinating tubes under another set of conditions.

Fungal penetration of the cuticle can be either mechanical, enzymatic or both (Yoder and Gillian 1985). The observation on the penetration of germtubes of *M. roreri* suggests a mechanical piercing of the cuticle with no apparent enzymes involved since the sites of entry on the epidermis did not show a halo of maceration or tissue collapse, as has been observed in pathogens like *Bothrytis cinerea* infecting tomato fruits (Rijkenberg *et al* 1980). Infection by *M. roreri* does not appear to require the formation of these structures, nor is the collapse of epidermal cells a prerequisite

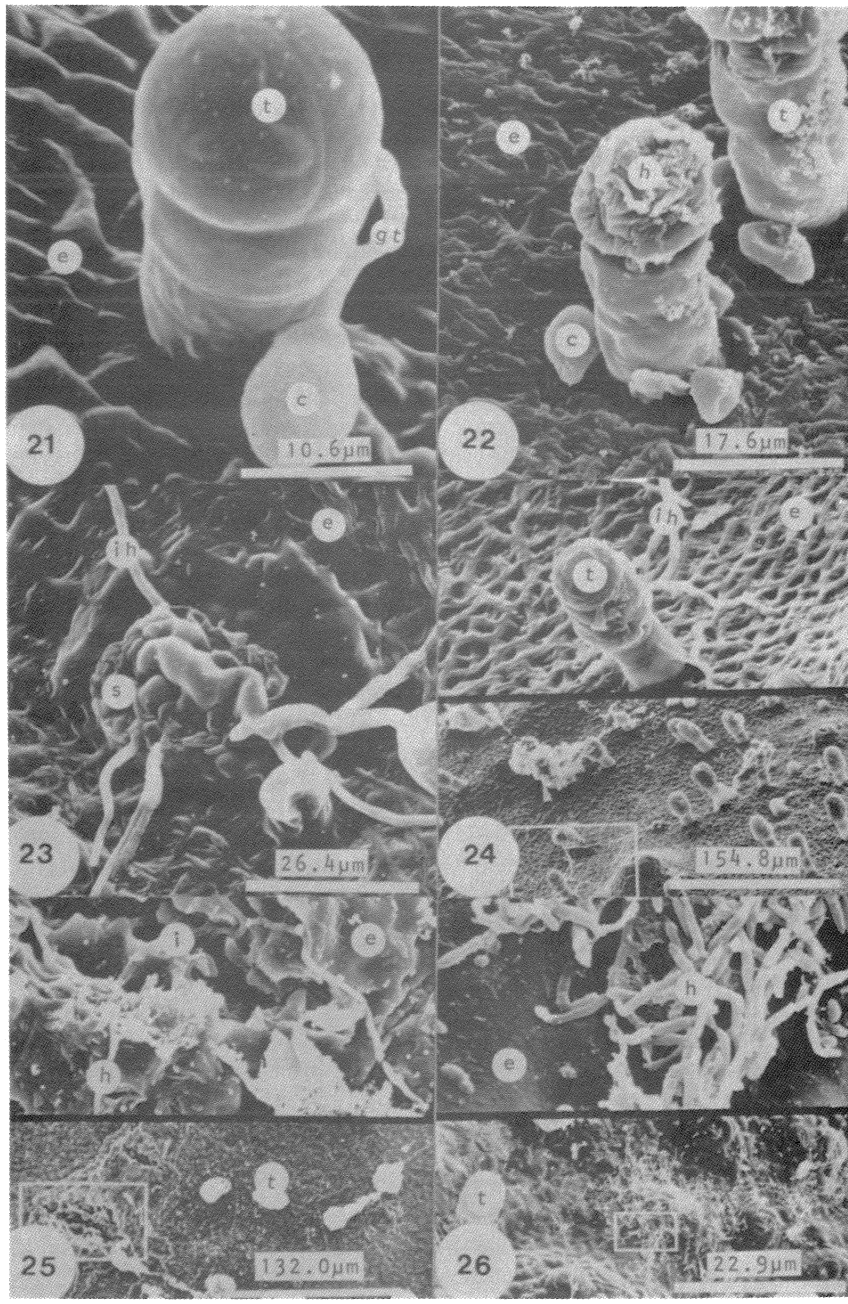


Fig. 21. Conidium (c) attached to a trichome (t) showing a germination tube (g.t), (e) epidermis. Fig. 22. Hyphae (h) emerging from infected trichomes (t). Note the loss of turgidity of the trichome, collapsed conidium (c). Fig. 23. Collapsed stoma (s) with infecting hyphae (i.h). Fig. 24. Infecting hyphae (i.h) establishing connections between trichomes (t), (e) epidermis. Fig. 25. Infection via injury (i) of hyphae (h) coming from adjacent infected trichomes (t), (e) epidermis. Fig. 26. Hyphae (h) appear on the surface of the fruit concomitantly with the appearance of typical brown-spot symptoms.

for infection as it is the case for *Sclerotium rolfsii*, which excretes oxalic acid to do so (Smith *et al* 1986). However, further studies on the penetration by *M. roreri* are necessary in order to corroborate our findings.

Indeed, no differences between the cultivars were observed in the pre-penetration activities of the fungus. If differences appear, probably they would occur as active mechanisms resulting from infection and not preventing infection *per se* (Cahill *et al* 1989).

Alternatively, infection can occur via small injuries on the epidermis, which can be caused by insects as suggested by Naundorf (1954). Hyphae were observed penetrating injuries; these were not germtubes but slender hyphae coming from infected trichomes, which were observed after initial infection by germtubes.

The deposition of conidia at the base of trichomes seem not to be fortuitous and has epidemiological implications. Conidia were sprayed on the fruit as a suspension to the point of dripping. The wet fruit, located in the trunk and stems of cocoa trees, temporarily had a continuous layer of water. This water either dripped off from the fruit or evaporated even inside the humid chamber. In either case, those areas of the fruit surface less likely to lose water would favor the formation of a water film and thus spore germination, since conidia require free water in order to germinate (Merchan 1981, Campuzano 1981). Conidia in suspension possibly retracted along the drying water film as a result of capillary forces; thus they would tend to accumulate in the drenched areas of the fruit surface, more likely on the alveolar, rough surface where abundant trichomes exist.

Alternatively, trichomes may themselves facilitate evaporation. If this indeed happens, a water deficit would be established between the surface of the fruit and the tip of the trichomes, resulting in a water movement from the surface to the trichomes, a process that would permit the accumulation of spores at their base. The observations on inoculated fruits may be held in nature since the fruits would be exposed to frequent cycles of wetting and drying in the cocoa plantations, usually located in very humid areas.

Thus, spores that are airborne within the plantation would reach the surface of the uninfected fruits where they will show movement and distribution similar to the one just described. In fact observations made on fruits brought from the field, where moniliasis is endemic, confirmed this suggestion. Mechanisms in which polysacharides may be involved in facilitating the adsorption of spores either at the base or on the surface of trichomes (Nicholson 1984) can not be excluded, since spores were seen on top of trichomes and even hanging out laterally. At any rate, conidia location possibly would facilitate trichome infection since more conidia would be in contact with these structures. This infection, which was not clearly observed, appears to be concomitant with the infection of other sites on the epidermis.

On the epidermis of the pod, germination as well as infection by all the germinating conidia probably is initially scanty since numerous conidia did not germinate and of those that germinated relatively few managed to infect the fruit. This would appear to be an adaptation of the fungus since germination of all the spores at once can be ecologically unfit (Ram *et al*, 1988).

The possible role of trichome excretions on both adsorption and infection remains to be determined. However, an important role for trichomes in consolidating infection of the pod by *M. roreri* is suggested by the radial emergence of hyphae from them much later after inoculation and infection, which reached other infection sites. The observed mycelium emerged from the trichomes to spread infection to other sites on the surface of the fruit. This could well be a very effective infection strategy of the fungus since a single germtube infecting a trichome can then multiply *in planta*, using fruit reserves rather than spores, to yield several hyphae that emerge radially from it. These hyphae easily outnumber and outreach the germtubes, thus increasing infection, since more infection courts would be reached than during the initial stages of spore germination. The infection of stomata, suggested by Jorgensen (1975), of other trichomes and of small breaches in the epidermis also occurred by secondary hyphae, at a later stage, after the initial infection process. These suggestions await conclusive experimental validation.

As the number of both trichomes and stomata appears to be determined early during

fruit development (O'Brien and McCully 1969), the distance between them would increase with age because of a drop in density, a factor that may increase resistance to the fungus provided the above mechanism holds true.

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## RESUMEN

El hongo *Moniliophthora roreri* ( Cif y Park) Evans *et al* infecta los frutos del cacao (*Theobroma cacao* L.) y causa grandes pérdidas. Se llevó a cabo un estudio de las relaciones patógeno-hospedero en los frutos para estudiar la distribución, germinación, la formación de tubos germinativos e infección de la espora del hongo por medio de la microscopía electrónica de rastreo (MER). Frutos de 60 días de edad, tanto inoculados como no inoculados del cultivar Pound-7 (susceptible) y del UF-296 (moderadamente resistente) fueron colectados de dos sitios del trópico húmedo bajo de Costa Rica, La Lola y Turrialba. Se encontraron conidios principalmente en la base de los tricomas. Las esporas germinaron en forma similar en ambos cultivares, la germinación ocurrió a través de un poro germinativo ubicado en un extremo, o alternativamente a través de la pared lateral de la espora después de 7-8 horas de efectuarse la inoculación, el número de esporas germinadas fue bajo. La infección de los frutos causada por el tubo germinativo a través de los estomas no fue común, sino, a través de la penetración directa de células epidérmicas y tricomas. Las hifas emergieron por los tricomas y se esparcieron e infectaron otros sitios en la epidermis, incluyendo tricomas, estomas, heridas y otras células epidérmicas. Ocasionalmente se observaron abultamientos terminales en los tubos germinativos similares a estructuras apresoriales.

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