# Electron microscopic observation of unripe banana fruit infected with *Colletotrichum musae*

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## ABSTRACT

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Electron microscopic observation revealed that on unripe banana fruit *Colletotrichum musae* germinated after 8 hr, appressoria were formed after 16-18 hr, and infection peg was formed 24 hr after inoculation. The thickness of appressorial wall on unripe banana fruits was 0.1-0.2 μm 16-18 hr after inoculation, and 0.3-0.5 μm 45 days after inoculation. On the 45th day after inoculation, infection peg with collar and cone was observed. After 24 hr inoculation with conidial suspension, feather-like deposits were observed in the epidermal cells of 5, 10, 15, 30, 45, 60, 75, and 90-day-old unripe banana fruits after blooming. In the epidermal cells of 30 day old unripe banana fruits after blooming, these deposits were also observed 8-12 hr, 16-24 hr, 5, 10, 15, 30, 45, or 60 days after inoculation. However, those feather-like deposits were not observed in the uninoculated unripe; neither in ripe banana fruits, nor in the inoculated ripe banana fruits. We assumed that the feather-like deposits disintegrated and finally disappeared in the epidermal cells of ripening banana fruits, and the fungus penetrated into epidermal cells. We proposed that feather-like deposits were related to antifungal mechanism, and might be a category of phytoalexin produced by banana plant.

(Key words: *Colletotrichum musae,* ripe rots, appressoria, infection peg, feather-like deposits and phytoalexin)

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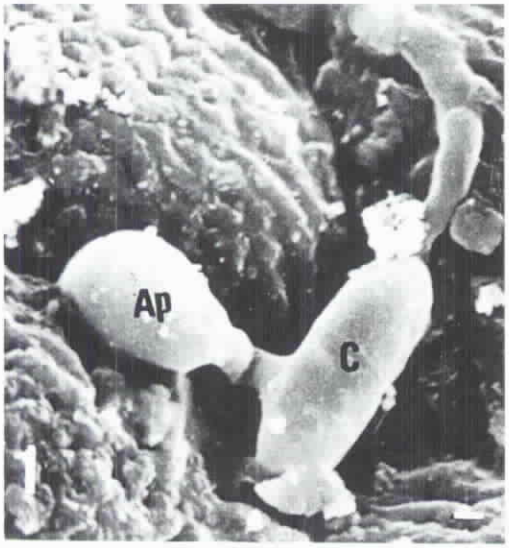
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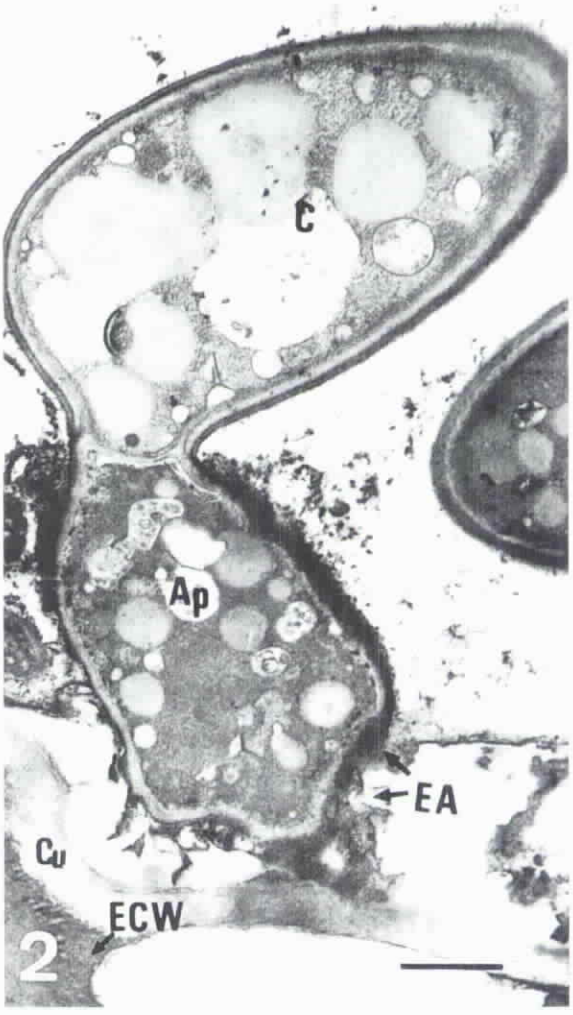
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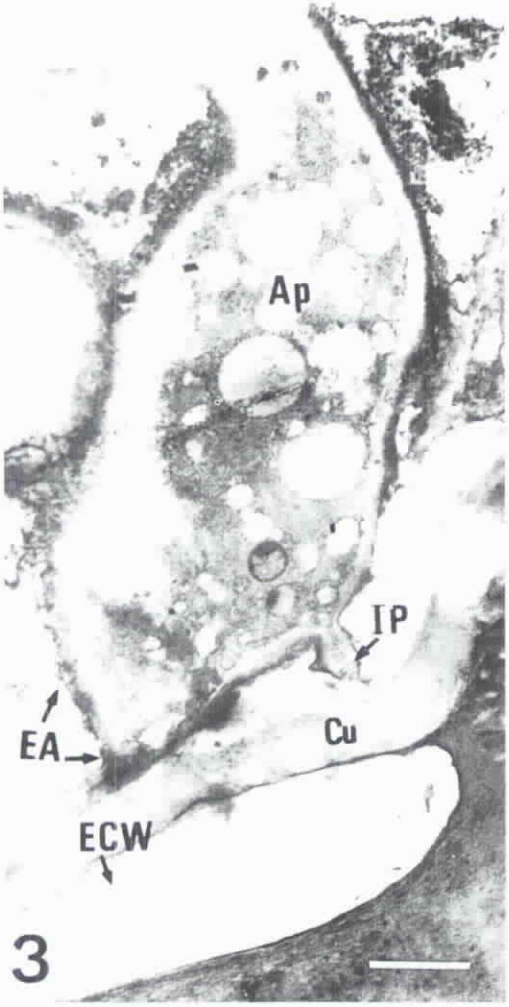
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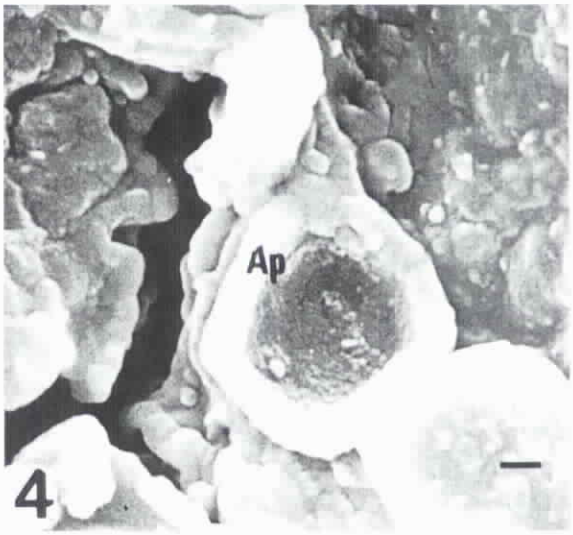
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# INTRODUCTION

Banana anthracnose caused by *Colletotrichum musae* (Berk. & Curt.) Arx., is commonly found in the banana growing area such as Jamaica [21]. Queensland [26, 27], Phillipines [2], and Taiwan [23, 30]. Unripe banana fruits infected by the fungus showed latent infection [21]. However, ripe banana fruit showed typical symptoms in the field or during transportation and storage [21, 32]. Simmonds [27] suggested that the fungus penetrated into the cuticular layer and remained latent until banana fruits were ripe. Simmonds [26] also proposed four hypotheses to explain the latency phenomenon. He proclaimed that firstly the nutritional requirements of the fungus were not favorable to the unripe fruits. Secondly, the enzyme potential necessary for invading the unripe fruits might be greater than for ripe fruits. Thirdly, a toxic substance, that might affect the invasion of fungus, could present in unripe fruits. At last, he suggested the energy requirements of the fungus could only be met when the metabolism of banana fruits had passed from unripe to ripening stage. Simmonds also concluded that latency occurred with some or all of the four hypotheses.

The morphological studies of appressoria on banana [16], red pepper [1], mango [14], and avocado [8] have been reported. Barnell [4-6] and Wang [31] cited that the carbohydrate compounds of the unripe and ripe fruits of banana were different. Chakravarty [11] reported that the unripe fruit contained toxic substance to limit the growth of *C. musae*. Mulvena *et al.* [22] isolated 3,4-dihydroxybenzaldehyde which restricted conidium germination of *C. musae*. Brown and Swinburne [9] also extracted antifungal compounds from unripe banana fruits after glucan treatment.

In this study, we reported electron microscopic observation on morphological changes of the fungus, and cytological responses in the host cells of unripe banana fruits that were infected with *C. musae*.

# MATERIALS AND METHODS

Banana (Cavendish) plants were cultivated in large plastic containers (ca. 200 liters) with enriched soil in a greenhouse for more than six months. Seventy-five-day old banana fruits after blooming were still unripe and green. Ninety to 95-day old banana fruits after blooming began to ripen and had a yellowish green to greenish yellow color. Ninety-five to 100-day old banana fruits after blooming were ripened and had a color of greenish yellow to yellow.

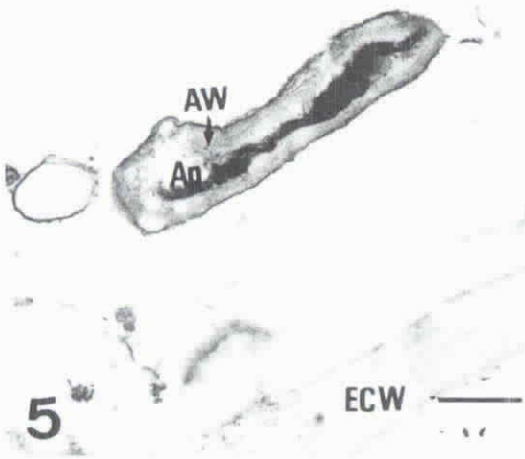
The samples for the observation by electron microscopy were prepared by three different inoculations. Banana fruits (5, 10, 15, 30, 45, 60, 75, 90-95, and 95-100 days after blooming) were inoculated *in situ* with conidial suspension (4-5 x $10^6$ conidia/ml) of

Fig. 1. Appressorium (Ap) formed from the germinated conidium(C) on unripe banana fruit 16-18 hr after inoculation.

Fig. 2. An erosion area (EA) appeared between appressorium (Ap) and cuticular layer of unripe fruit 24 hr after inoculation. (ECW: epidermal cell wall)

Fig. 3. Appressorium (Ap) produced infection peg (IP) 24 hr after inoculation.

Fig. 4. Appressorium (Ap) collapsed in the tissue of unripe banana fruit 15-60 days after inoculation.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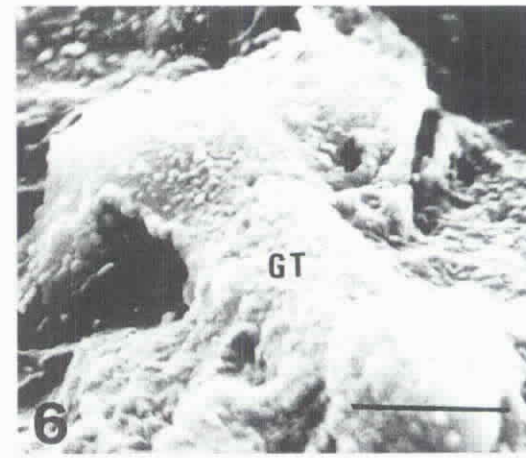
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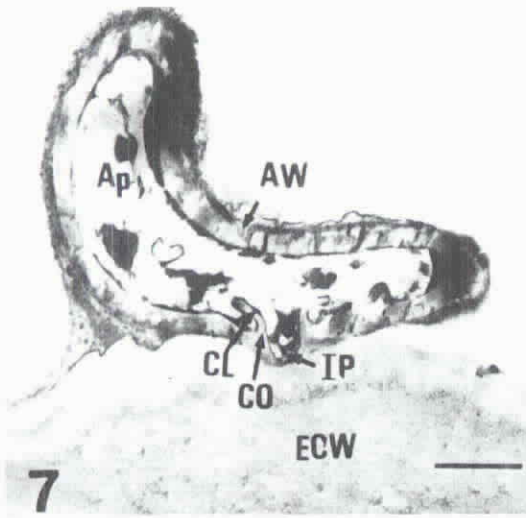
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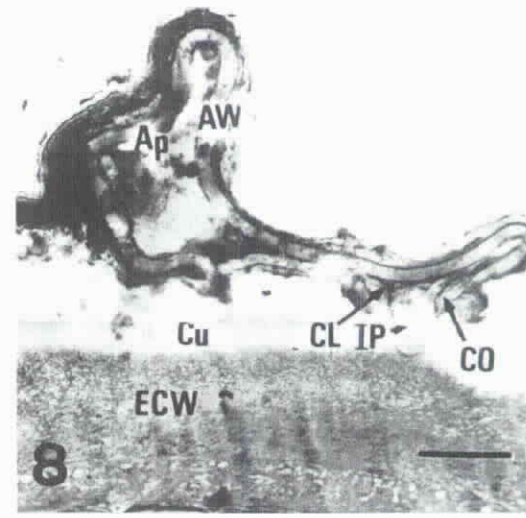
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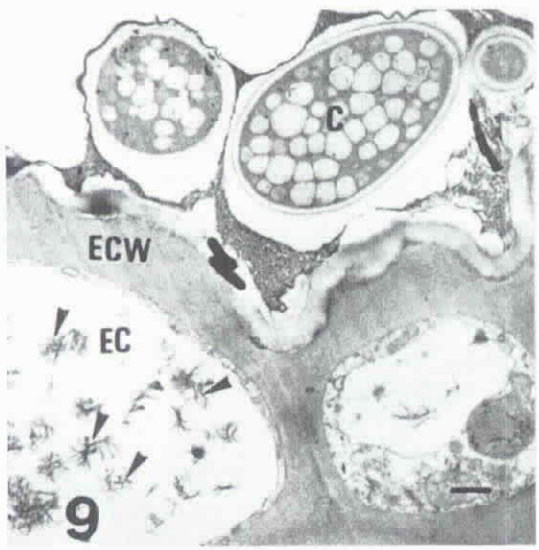
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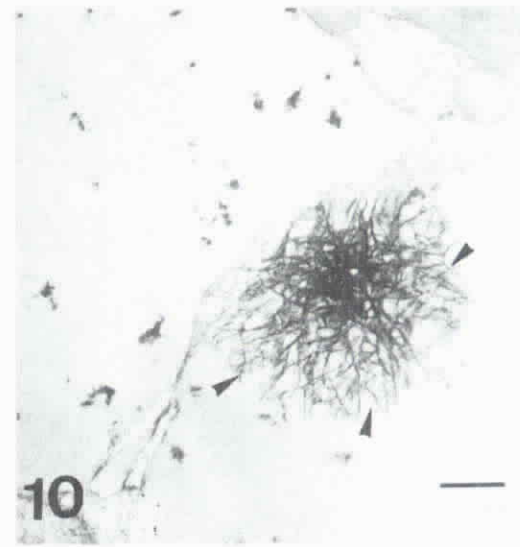
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*C. musae* from fungal culture, which was collected and isolated from banana plants in Nan-Hein area, Taiwan. The inoculated banana fruits were kept in saturated relative humidity at 22-28 °C for 24 hr. Then the banana fruits were sliced and prepared for electron microscopy [12]. Thirty-day-old unripe banana fruits after blooming were inoculated *in situ* with conidial suspension. The samples were sliced 8, 12, 16, 20, 24 hr, and 5, 10, 15, 30, 45, 60 days (still unripe), 60-65 days (beginning to ripen) and 65-70 days (ripe) after inoculation, respectively. The sliced samples from the inoculated and uninoculated fruit peels were prepared for scanning and transmission electron microscopies.

Thirty-day-old banana fruits after blooming were inoculated *in situ* with conidial suspension. The samples were sliced 8, 12, 16, 20, 24 hr and 5, 10, 15, 30, 45, 60 days (still unripe), 60-65 days (beginning to ripen) and 65-70 days (ripe) after inoculation, respectively. The sliced samples from the inoculated and uninoculated fruit peels were prepared for scanning and transmission electron microscopy.

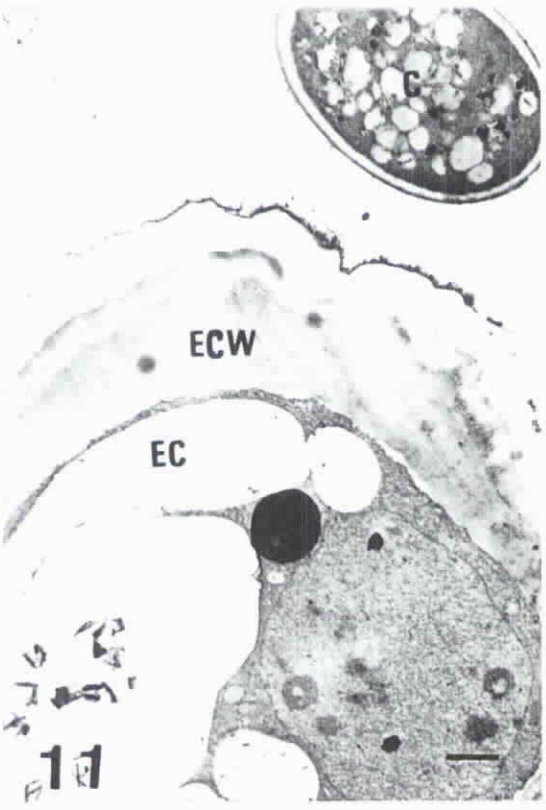
For a control study, ten to fifteen days old unripe banana fruits after blooming were injected with 5 ml of sterilized distilled water in the pulp of each finger. Then, the samples from pulp and peel were sliced at one-week intervals for 2 months, and they were prepared for electron microscopy.

For transmission electron microscopy (TEM), samples were prepared with 3% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.0) at 4°C for 4 hr, and postfixed with 1% osmium tetroxide in the same buffer at 4°C for 4 hr. Then, samples were dehydrated through a graduated series of ethyl alcohol and embedded in Spurr's plastic resin. The thin sections were stained with 6% uranyl acetate and 2% lead citrate, and observed under a Hitachi H-300 electron microscope. For scanning electron microscopy (SEM), samples were fixed with 2% osmium tetroxide at 4°C for 6 hr, and dehydrated with ethyl alcohol series, then dried in a Ladd critical point dryer and coated in an Eiko IB-2 ion coater with gold. Samples were observed under a Hitachi S-410 electron microscope.

## RESULTS

Conidia germinated 8 hr after inoculation. An Ellipse-shaped appressorium was formed 16-18 hr after inoculation on unripe banana fruits (Fig. 1). An erosion-like area appeared between appressorium and cuticular layer of host tissue (Figs. 2, 3). Thickness of the appressorial wall was 0. 1-0.2 μm, and the same with that of conidial cell wall in this stage (Fig. 2). Appressorium

Fig. 5. Appressorial wall (AW) was thick in the tissue of unripe banana fruit 15-60 days after inoculation.
Fig. 6. Germ tube (GT) was observed on unripe banana fruit surface.
Fig. 7. Infection peg (IP), cone (CO) and collar (CL) in unripe banana fruit 45 days after inoculation.
Fig. 8. Infection peg (IP) penetrated in cuticular layer (Cu) of host tissue of unripe banana fruit 60 days after inoculation.
Fig. 9. Feather-like deposits (arrows) were observed 24 hr after inoculation in the epidermal cell (EC) of 30-day-old unripe fruit after blooming.
Fig. 10. A cluster of feather-like deposits (arrow).

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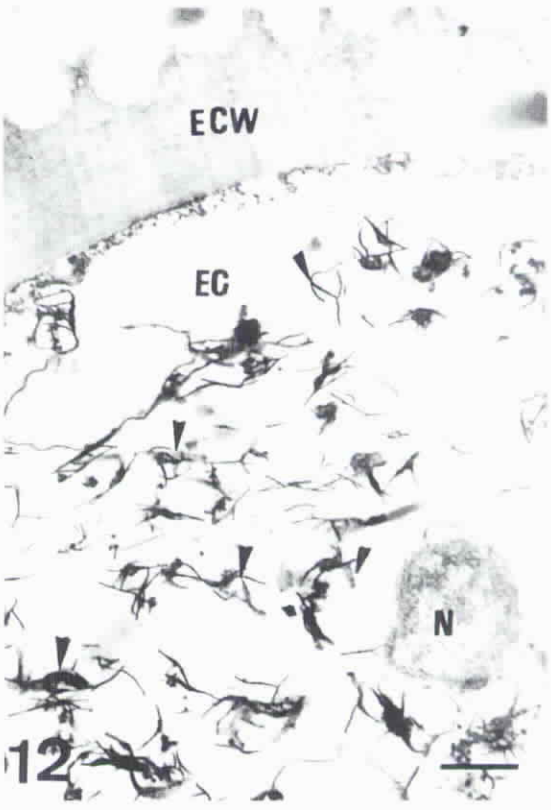


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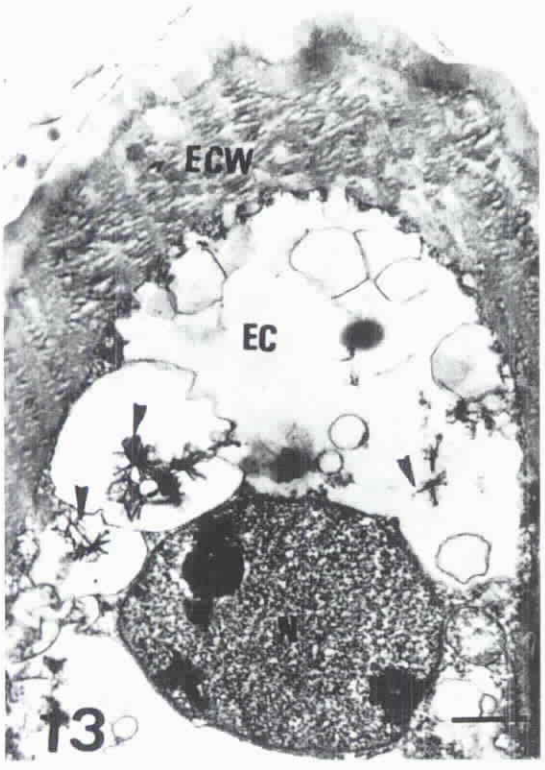


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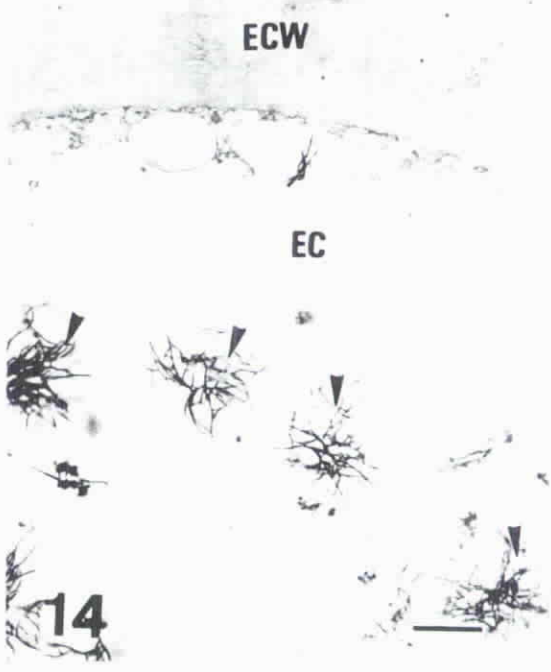


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collapsed in the tissue of unripe fruit 15-60 day after inoculation (Figs. 4, 5). Occasionally, germination tube and appressorium were observed in the cuticle of host tissue (Fig. 6).

The infection peg protruded from an appressorium 24 hr after inoculation, and stayed in cuticle layer (Fig. 3). On the 45$^{th}$ day after inoculation, the thickness of appressorial wall was 0.3-0.5 μm on unripe fruit surface. Collar and cone were observed during this latency period (Fig. 7). Then, the infection peg advanced to the epidermal cell wall (Fig. 8). In the appressorium, electron-dense substance were observed, but organelles were not observed (Figs. 7, 8).

Feather-like deposits were observed 24 hr after inoculation in the epidermal cell of unripe fruits (5, 10, 15, 30, 45, 60 and 75 days after blooming) (Figs. 9, 10). These deposits were neither observed in the uninoculated unripe and ripening fruits (95-100 days after blooming) or in the inoculated and uninoculated ripe fruits (95-100 days). Further investigations revealed that the thread-like deposits, initial feather-like deposits appeared in the epidermal cells 8-12 hr after inoculation on the 30-day-old unripe banana fruits after blooming (Fig. 11). Typical feather-like structure appeared 16-24 hr after inoculation (Fig. 12), and still existed in the epidermal cells of unripe fruits 5, 10, 15, 30, 45 and 60 days after inoculation (Figs. 13, 14). When the fruits began to ripen, the feather-like deposits disintegrated and finally could not be observed 90-95 days after inoculation (Figs. 15-17). On ripe banana fruits, the fungus penetrated through the epidermal cells within 24 hr after inoculation, and no such deposits were observed (Fig. 18).

The pulp of ripe fruits inoculating with fungal conidial suspension, developed necrotic lesions on the site of inoculation, yet no feather-like deposits were observed in these ripen banana pulp tissues under TEM. The banana fruits injected with sterilized distilled water showed no lesion on the peel, and nor feather-like deposits were observed in these banana fruits.

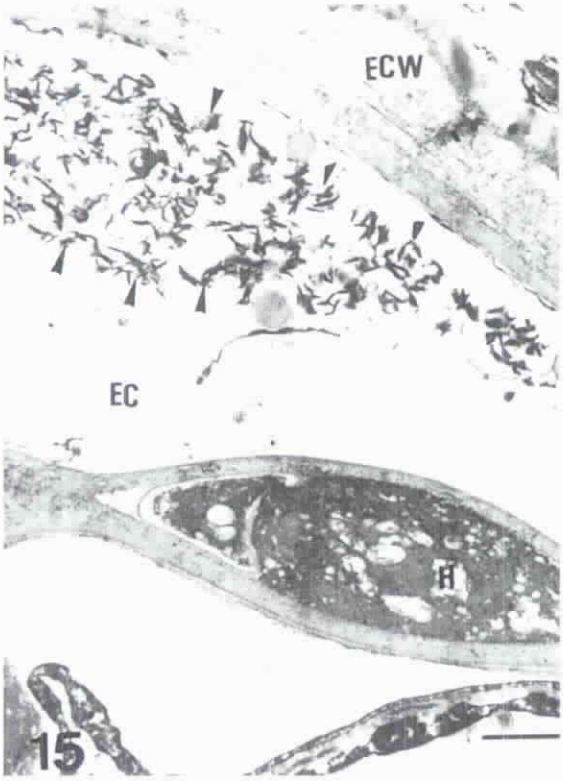
## DISCUSSION

Appressoria of *Colletotrichum musae* were formed on the green unripe banana fruits 16-18 hr after inoculation. The thickness of appressorial wall was 0.1-0.2 μm, and the same with the thickness of conidial wall [12]. The infection peg penetrated into cuticular layer of the green unripe banana fruit one day after inoculation and remained there until the fruits ripened. On the contrary, the infection peg penetrated directly into the epidermal cell wall of yellow ripe banana fruit 24 hrs after inoculation [12]. The appressoria of *C. musae* sunk or embedded in the host tissue of unripe fruit 15-60 days after inoculation. The same phenomenon was also observed on the appressoria of *C. gloeosporioides* on avocado [8] and C. *capsici* on red pepper [1]. Appressoria of *C. musae* also formed collar and cone on the

Fig. 11. Thread-like initial of feather-like deposits appeared in the vesicle 8 hr after inoculation on 30-day-old unripe fruit after blooming.

Fig. 12. Typical feather-like deposits (arrows) appeared 16 hr after inoculation.

Figs. 13,14. Feather-like deposits (arrows) existed in the epidermal cell of unripe fruit 30 and 45 days after inoculation, respectively.

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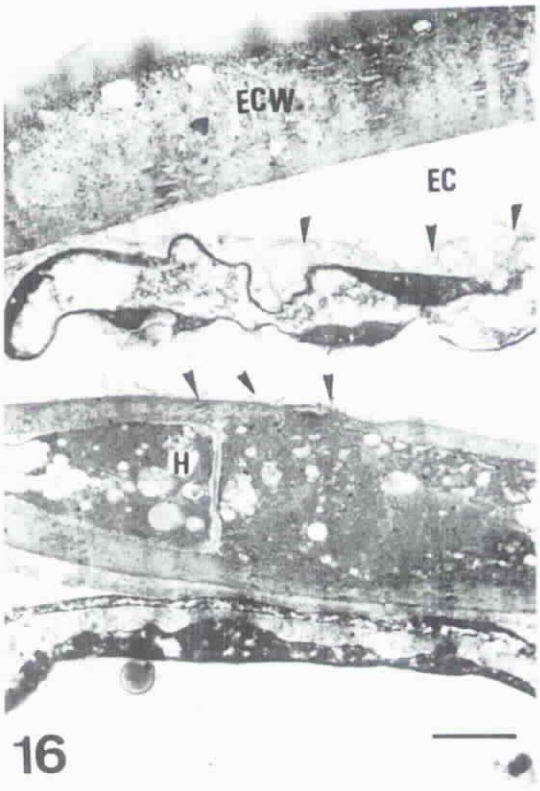


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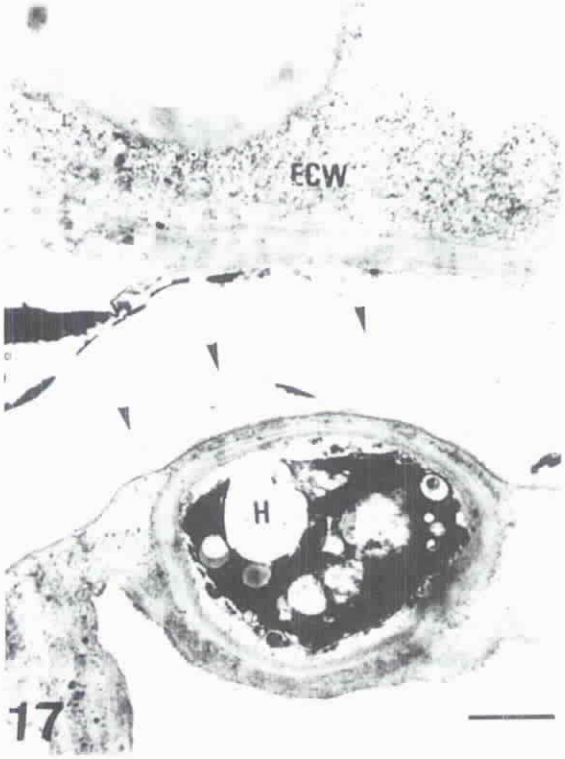


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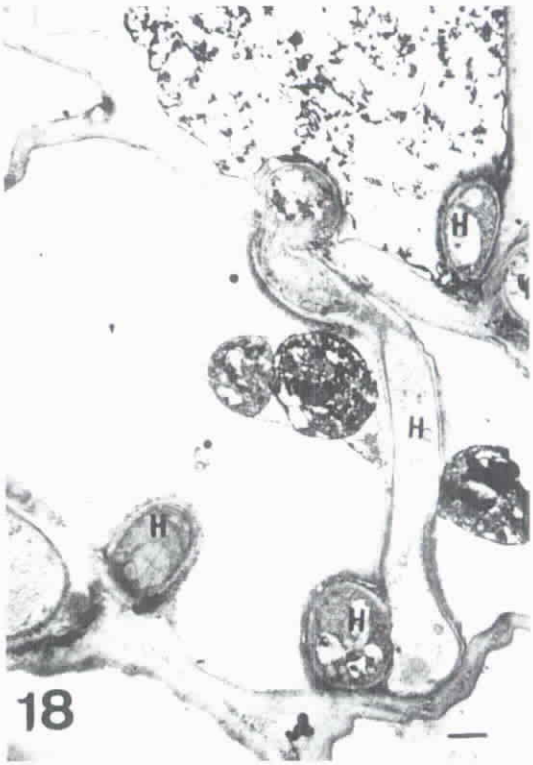


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green unripe banana fruit, which were observed on the appressoria of *C. gloeosporioides* on ripe fruits of tangerine [10] and grape[19]. The thickness of appressorial wall was 0.3-0.5 μm 45 days after inoculation. It was thought that as the fungus remained in cuticular layer of green unripe banana fruit, the morphological variations of appressoria, such as sinking and embedding, collar and cone formation and cell wall thickening, were caused.

Simmonds[27] stated four hypotheses to explain the resistance of unripe banana fruits. They were the limitations of the fungus penetration by the shortage of nutrients, the failure in degradation of cell wall components, the inhibition of fungal growth by toxins, and the metabolic changes in unripe banana fruit. Swinburne [29] reported that latency may be caused by the production of antifungal compounds, phytoalexins. Mulvena *et al.*[22] isolated a fungistatic substance, 3,4-dihydroxybenzaldehyde, from the outer skin of green unripe Cavendish bananas. This substance inhibited the growth of *C. musae*. Brown and Swinburne [9] also treated banana fruit with glucan and detected the accumulation of two antifungal compounds, which can not be detected on progressive anthracnose lesions on ripe fruits.

Chen and Wu[13] observed feather-like deposits in the rice blast lesions on highly resistant, moderately resistant and moderately susceptible cultivar by artificial inoculation of *Pyricularia oryzae*. They suggested the feather-like deposits might be phytoalexin secreted in rice leaves. Phytoalexins were reported as part of induced defense reactions in plants[7, 17].

Feather-like deposits observed exclusively in the epidermal cells of green unripe banana fruits. These deposits degraded and disappeared when the green unripe banana ripened, and the pathogen penetrated into the epidermal cells and then into the tissues. No feather-like deposits were observed on ripe banana fruits, in which the fungus could penetrate and colonize [12]. From these results, it was thought that the deposits restricted the growth of fungus on the green unripe banana fruits. Interestingly, the antimicrobial compounds, phytoalexin maackiain and its pterocarpan relatives, were oxidized by *C. gloeosporioides* isolated from the tropical forage legume *Stylosanthes* spp.[28]. The oxidation might be required for efficient excretion or carbon source scavenging. These findings supported the facts that the hyphae of *C. musae* penetrated and expanded into banana fruits after ripening. The same phenomena were also reported in grape [19] and ripe banana fruit [12]. We proposed that feather-like deposits found in unripe banana fruits might be phytoalexin, and could have antifungal activities as mentioned in previous studies[3, 15, 18, 20, 24, 25, 33]. However, characterization for the phytoalexin from unripe banana fruits remains unknown.

Figs. 15-17. The form of feather-like deposits (arrows) changed (might degenerate) gradually in epidermal cells of ripening fruit (90-95 days after inoculation), and the hyphae colonized in the host cells.

Fig. 18. Feather-like deposits were not observed in the epidermal cells of ripe banana fruit 24 hr after inoculation. The hyphae penetrated through the epidermal cell.

## ACKNOWLEDGEMENT

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## 摘 要

張淳文[1]*、Robert L. Rountree[2]、呂理燊[3] **2000** 蕉炭疽病菌在未成熟香蕉果皮上之電子顯微鏡觀察 植保會刊 42：135－146（1.台中縣 行政院農業委員會農業藥物毒物試驗所，前研究助理；2. 現職 Division of Neurotoxicology, National Center for Toxicilogical Research, FDA, Jefferson, AR 72079, USA；3. 台中縣 行政院農業委員會農業藥物毒物試驗所，已退休。）

香蕉炭疽病菌(*Colletotrichum musae*)分生孢子接種於未成熟香蕉果皮上八小時之後開始發芽，十六至十八小時之後發生附著器(appressoria)，二十四小時之後形成貫穿絲(infecton peg)，接種四十五日之後，附著器細胞壁由 0.1-0.2μm 增至 0.3-0.5μm。接種分生孢子於未成熟香蕉果實(開花後五至九十日)之果皮上二十四小時之後，可見羽狀沈著物(feather-like deposits)。爲觀察分生孢子於未成熟香蕉果皮上之發芽情形，另外接種分生孢子於開花後三十日之未成熟香蕉果皮上，於接種後八至十二小時，十六至二十四小時，五日至六十日，均於果皮上見到羽狀沈著物。在未接種之未成熟香蕉果皮上，未接種之成熟果皮上，或在已接種之成熟果皮上，均無法見到羽狀沈著物，作者認爲當香蕉果實成熟，羽狀沈著物消失，病菌才能侵害香蕉果實之外果皮表皮細胞，該羽狀沈著物可能和香蕉果皮之抗菌機制有關，可能爲香蕉植株所產生的自然抗菌素(phytoalexin)。

（關鍵詞：*Colletotrichum musae*、炭疽病、附著器、貫穿絲、羽狀沈著物、自然抗菌素）

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