

Viability of *Venturia inaequalis* in Chlorotic Flecks Resulting from Fungicide Application to Infected *Malus* Leaves

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ABSTRACT

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Discrete, nonexpanding, and nonsporulating chlorotic flecks developed on apple leaves infected with *Venturia inaequalis* when treated with certain fungicides (benomyl, phenylmercuric acetate, dodine, and fenarimol) at time periods beyond those that completely prevent scab symptom development but at times prior to which they would have no control. The viability of the subcuticular *V. inaequalis* hyphae in fleck areas was determined on a nutrient medium. At 100 hr after inoculation, only

fenarimol completely eradicated the pathogen. The effect of the fungicides on the pathogen at various periods after the treatments was studied ultrastructurally and found to correlate with the isolation techniques. Subthreshold treatments caused localized necrotic reactions, whereas fully effective treatments caused complete necrosis of *V. inaequalis* hyphae. Initial responses of the fungal cell organelles included swollen mitochondria and cysterneae of the endoplasmic reticulum and the nuclear envelope.

Apple scab, caused by *Venturia inaequalis* (Cke.) Wint., is controlled primarily by protective and postinfection eradication fungicide applications. The latter approach to scab control relies primarily on fungicides that can move transcuticularly to reach and eradicate *V. inaequalis* after cuticle penetration. Certain benzimidazole, guanidine, and pyrimidine related fungicides, as well as the older proscribed organomercuric fungicides, show such activity. Individually, they have various time limits for after-infection activity and are correlated with environmental factors; eg, moisture and temperature. The timing and rate of application can have at least three clearly discernible results on scab development: (i) complete eradication of *V. inaequalis* without any symptomatic expression, (ii) incomplete eradication with subsequent lesion development and conidial sporulation on the leaf surface, and (iii) questionable eradication in which discrete, nonexpanding, and nonsporulating chlorotic flecks develop. This latter phenomenon occurs when certain fungicides are applied at rates lower than those that completely prevent scab symptoms or when they are applied at times beyond the infection period in which they would normally control scab development but at times before they would have no

control. Similar chlorotic fleck symptoms have been observed with various fungicides in other host-parasite relationships (16,17). In apple scab, it would be desirable to know the state of the fungus, living or dead, in leaves showing such symptoms. Such information could provide a basis for lower rates of application or longer intervals between the infection period and application of the fungicide. However, if the fungicides simply prevented further development of the fungus, then it might begin saprophytic growth after leaf drop in the autumn and serve as ascospore inoculum the following spring.

Attempts to isolate the fungus on growth media from leaf segments with chlorotic fleck symptoms might be inconclusive because fungicide residues might remain tenaciously on leaf surfaces as well as within leaf tissues. Therefore, the approach used in this study was to correlate the cytological appearance of the pathogen's cytoplasm with results of isolation attempts.

MATERIALS AND METHODS

The intact first four leaves on untreated, actively growing shoot apices of *Malus sylvestris* (Mill.) cultivars Niagara and Rome Beauty were used in this study. The trees (5-8 years old) were

grown in a container in a 21 C greenhouse after dormant storage (1C) and were maintained as single shoots by severe pruning at the end of each growing season (6,8). The apices were inoculated with conidia of *V. inaequalis* at about 10^5 spores per milliliter of water by atomization and incubated at 18 C for infection periods of 18–30 hr in lighted mist chambers. After the infection period, the trees were returned to the greenhouse and subjected to the following treatments: (i) dodine (*n*-dodecylguanidine acetate), 65 WP, at 0.45 g/L of water, applied 25 or 50 hr after inoculation; (ii) benomyl [methyl 1-(butylcarbamoyl)-2 benzimidazolecarbamate], 50 WP, at 0.58 g/L of water, applied 100 hr after inoculation, (iii) phenylmercuric acetate (PMA), 10%, at 0.5 ml/L of water, applied 50 or 100 hr after inoculation; (iv) fenarimol [α -(2-chlorophenyl)- α -(4-chlorophenyl)-5-pyrimidinemethanol], 12.5%, at 0.37 ml/L of water, applied 100 hr after inoculation; or (v) control (with water only), no fungicide treatment.

Fungicides were applied by momentarily dipping the leaves in the appropriate solutions. Young abaxial leaf surfaces were extremely pubescent and consequently hydrophobic; thus, in reality, only the astomatous adaxial leaf surface was treated.

Several 8-mm discs were removed with a cork borer from each leaf 1, 24, and 48 hr and 3 wk after fungicide application. Subsequently discs were processed for electron microscopy or were placed on a growth medium to determine the viability of *V. inaequalis* in the leaf samples. For electron microscopy, leaf segments, 1–3 mm square, cut from the discs were processed using a previously reported schedule (7) modified with the use of 0.08 M PO_4 (K^+) buffer. The flat, embedded leaf segments were sectioned either transversely or parallel to the leaf surface. In the first method of sectioning, the embedded segments were examined by light microscopy and oriented so sections could be parallel to specific subcuticular hyphae of *V. inaequalis*. In the latter instance, 10–24 μm thick sections of the entire leaf segment were made using a clinical microtome. They were subsequently reembedded between two glass plates (5 \times 7.5 cm) previously treated with fluorocarbon

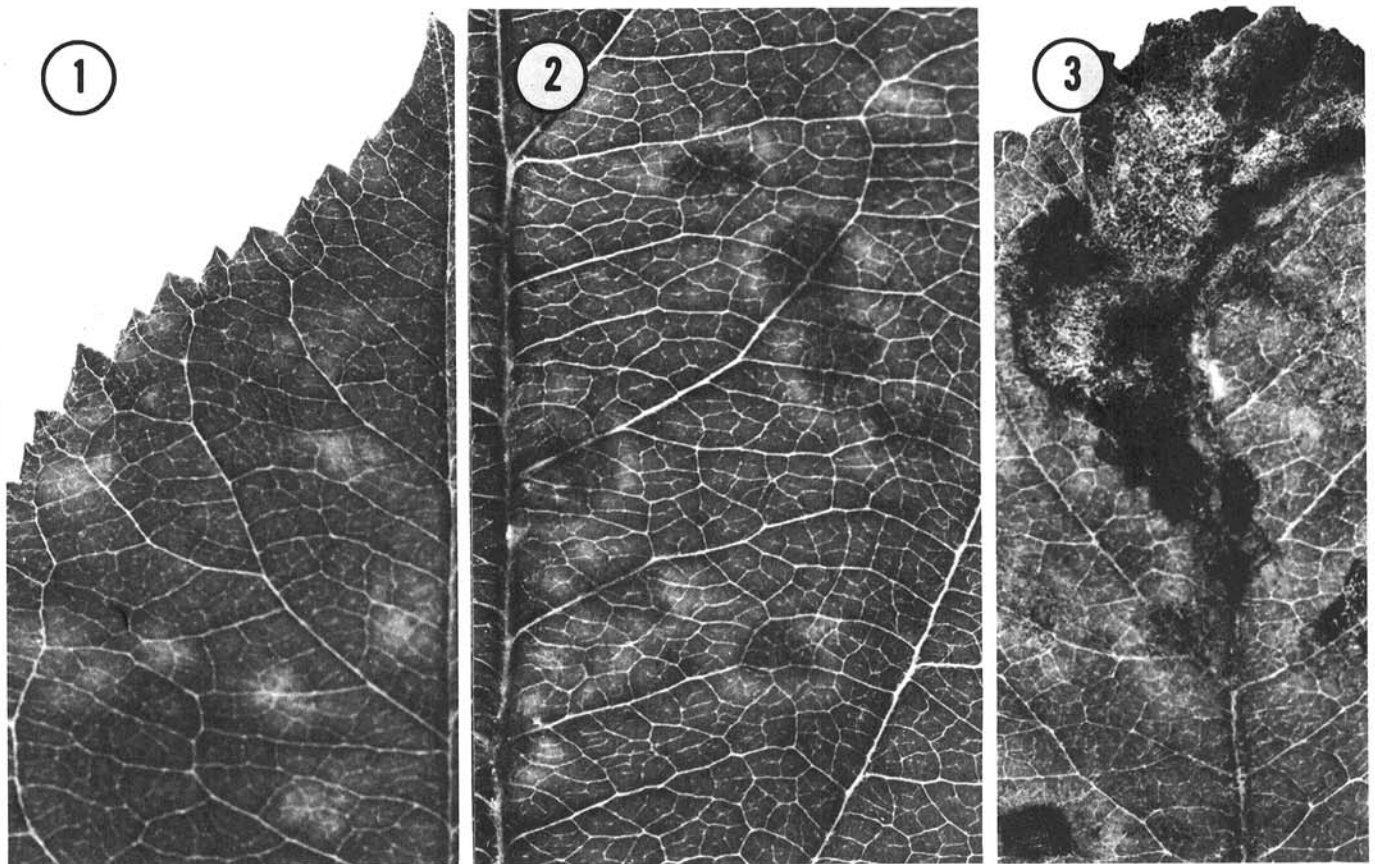
(9) or to which Teflon-coated aluminum foil (Lamast Corp., Clifton, NJ 07000) had been attached. The polymerized plastic, slightly thicker than the sections, was easily separated from the glass plates. The embedded thick sections were examined by phase-contrast light microscopy and selected for ultrathin sectioning by remounting onto blank specimen blocks with epoxy cement. This latter procedure, using reembedded thick sections, allowed exact location of the parasite in leaf segments normally too opaque to be examined by light microscopy. In most instances, five or more sample sites containing *V. inaequalis* were examined ultrastructurally. Thin section size (area) was as large as feasible so that the maximum number and area of fungal hyphae could be examined from any one sample.

For isolation of *V. inaequalis*, the 8-mm diameter leaf discs were washed in running water for 5 hr to remove as much residual fungicide as possible. They were then dipped momentarily in 70% ethanol, blotted with a paper towel, and cut so that each chlorotic fleck was quartered into 2-mm squares. These latter samples were placed on 3% malt-extract agar amended with 250 ppm streptomycin in petri plates and incubated at 21 C for 21 days, after which they were examined for *V. inaequalis* colonies. A total of 25 flecks (100 samples) was plated for each fungicide treatment. Nonfungicide-treated leaf discs were used to test the isolation procedure. These were sampled 6–9 days after inoculation, before *V. inaequalis* hyphae erupted through the cuticle.

RESULTS

Typical macroscopic symptoms of nonsporulating chlorotic flecks induced by the appropriate chemical treatment and sporulating scab lesions are exemplified by Figs. 1 and 2. Apple scab lesions of nonfungicide-treated control leaves often coalesced and ultimately became necrotic (Fig. 3).

Isolation of *V. inaequalis* from *Malus* leaves. Recovery of *V. inaequalis*, on agar media, from *Malus* leaves postinfection-treated



Figs. 1–3. Macroscopic symptoms of *Venturia inaequalis*-infected apple leaves. 1, Typical chlorotic fleck symptoms 21 days after fungicide treatments. 2 and 3, Control leaves not treated with any fungicides 25 and 45 days, respectively, after inoculation.

with fungicides varied with the fungicide (Table 1). Infected leaves in which *V. inaequalis* had proliferated for 6–9 days and were not treated with fungicides showed localized “haze” symptoms on the upper cuticle surface. Recovery of the pathogen from these areas was nearly 100% and served as a check for the isolation procedure. Recovery of *V. inaequalis* from chlorotic flecks resulting from postinfection fungicide treatment (100 hr after inoculation) with benomyl, PMA, and fenarimol were 26, 75, and 0%, respectively. Recovery of the pathogen from chlorotic flecks resulting from postinfection fungicide treatment (50 hr after inoculation) with dodine and fenarimol were 88 and 1%, respectively.

Chlorotic flecks remained discrete from the surrounding green leaf tissues until senescence occurred. They did not increase in size. Sporulation or eruption of the cuticle surface by hyphae and conidiophores of *V. inaequalis* was not observed. Similar chlorotic flecks have likewise been observed not to support sporulation of the fungus after postinfection applications of either $\alpha(2,4$, dichlorophenyl)- α -phenyl-5-pyrimidinemethanol (triarimol) (2) or PMA (5,15).

Ultrastructure of *V. inaequalis* in control *Malus* leaves. The protoplasmic organization and structural integrity of the various cellular components of *V. inaequalis* in *Malus* leaves, not treated with fungicide, was similar to that reported previously (4,10). However, several morphological features of the pathogen are redescribed and illustrated for comparison with hyphae affected by the various fungicides. Growth of the pathogen after initial host penetration was entirely subcuticular during the stages of parasitism examined in this study. The cytoplasm of stromatic hyphae from 51 to 148 hr old infections was uniformly dense with ribosomes and ground cytoplasm (Fig. 4). Vacuoles occupied a relatively small portion of the cytoplasm. They contained membranous inclusions, many of which were invaginations of the tonoplast, and spherical electron-opaque inclusions. Mitochondria were elongated with a dense matrix and platelike cristae (Fig. 5). Nuclei contained discrete nucleoli and finely granular nucleoplasm. The nuclear envelope membranes were parallel. Woronin bodies were juxtaposed to septa, especially near the septal pores. The plasmalemma was clearly discerned and was closely appressed to the hyphal wall. Large deposits of glycogenlike rosettes were

present in the cytoplasm of *V. inaequalis* from 10 days onward, after inoculation of the host.

Effect of fungicides on *V. inaequalis* ultrastructure in *Malus* leaves. No conspicuous changes were noted in the ultrastructure of subcuticular hyphae 1 hr after application of any fungicide, but changes were noted in hyphae from subsequent sampling periods. The effects were not uniform within most stromata but ranged from necrotic to apparently unaffected protoplasm for all fungicide treatments except PMA applied 50 hr after inoculation. In this treatment, the protoplasm was necrotic and the structural integrity of the organelles was such that they were difficult to recognize (Fig. 6). Occasionally, membrane profiles and possible Woronin bodies were discerned.

PMA applied to leaves 100 hr after inoculation had varied effects in individual hyphal cells and in different hyphae of the same

TABLE 1. Isolation of *Venturia inaequalis* on malt-extract agar from *Malus sylvestris* leaves treated with various fungicides after infection^a

Treatment	Time of fungicide application after inoculation (hr) ^b	Symptoms at 21 days ^c	Isolation of <i>V. inaequalis</i> (% recovery)
Control		Haze	96
Benomyl	100	Cf	36
Phenylmercuric acetate	50	Ns	3
	100	Cf	75
Fenarimol	50	Cf	1
	100	Cf	0
Dodine	50	Cf	88

^a Isolations made 3 wk after fungicide treatment, except in controls that were isolated 6–9 days after inoculation.

^b Penetration of cuticle occurred 10–16 hr after inoculation and incubation at 18 C.

^c Haze = first detectable sign of infection before eruption of cuticle but at 7–9 days; Cf = chlorotic fleck; Ns = no symptoms.

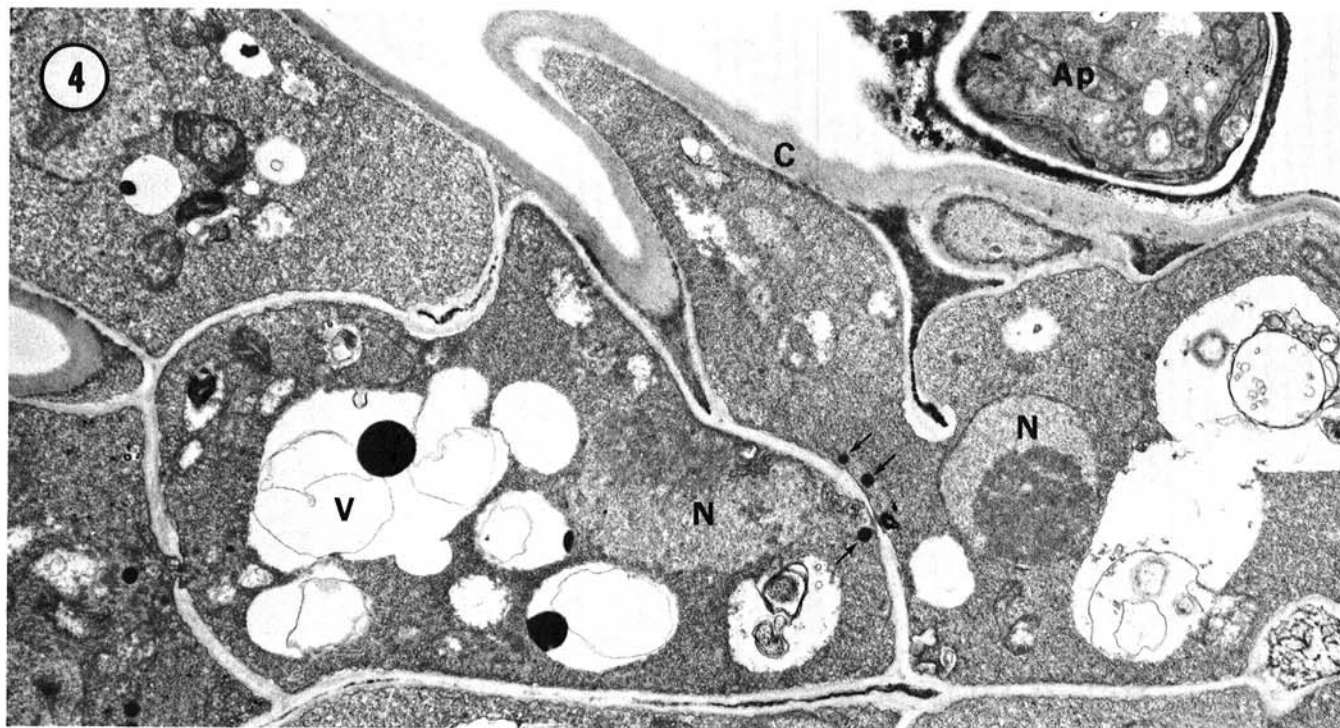


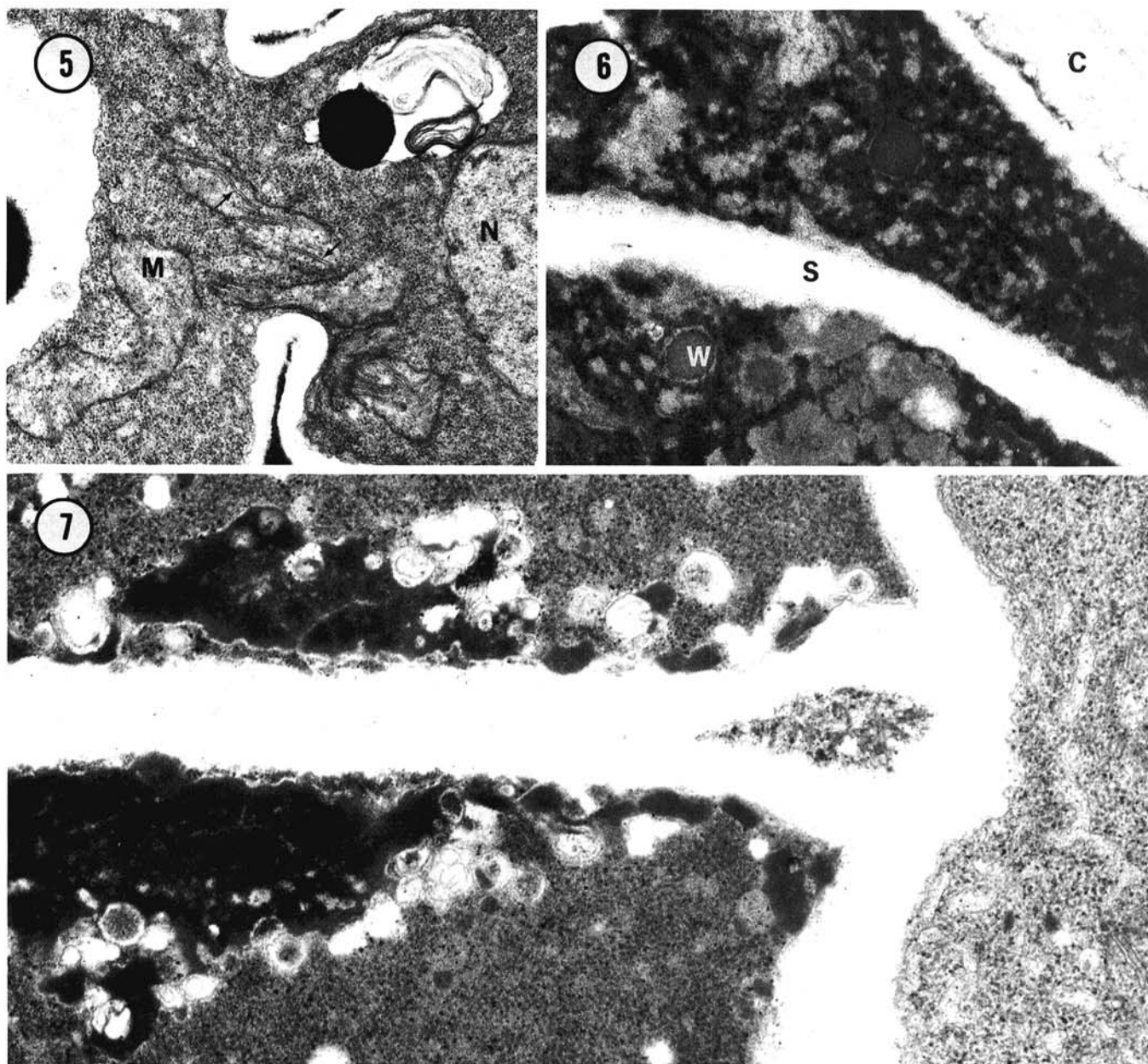
Fig. 4. Cytological features of *Venturia inaequalis* cytoplasm not affected with fungicides. Stroma of *V. inaequalis* sectioned parallel to the leaf surface shows morphology and distribution of various fungal organelles. Ap = appressorium, C = leaf cuticle, N = nuclei, V = vacuoles, arrows = Woronin bodies ($\times 6,900$).

stroma. Infected leaves sampled 24 hr after PMA treatment revealed localized moribund areas in the cytoplasm of *V. inaequalis* (Fig. 7). These areas were more electron-opaque than the surrounding cytoplasm. Numerous vesicles concentrated in and around these areas and possibly contributed to their formation. In other areas, entire cells were necrotic (Fig. 8). Often, cells adjacent to necrotic cells of the same hypha, separated by septa, were less affected (Fig. 8), if at all. A gradation of effects, separated by septa, could be seen in long profiles of individual hyphae. Considerable organelle deformation occurred. Most mitochondria were swollen, and their normally flattened cristae were inflated and appeared tubular. Cisternae of nuclear envelopes were occasionally inflated. In addition, electron-opaque spherical inclusions were distributed throughout the cytoplasm and the nucleoplasm. Although Woronin bodies appeared more electron-opaque than normal, they were not confused with the often larger bodies.

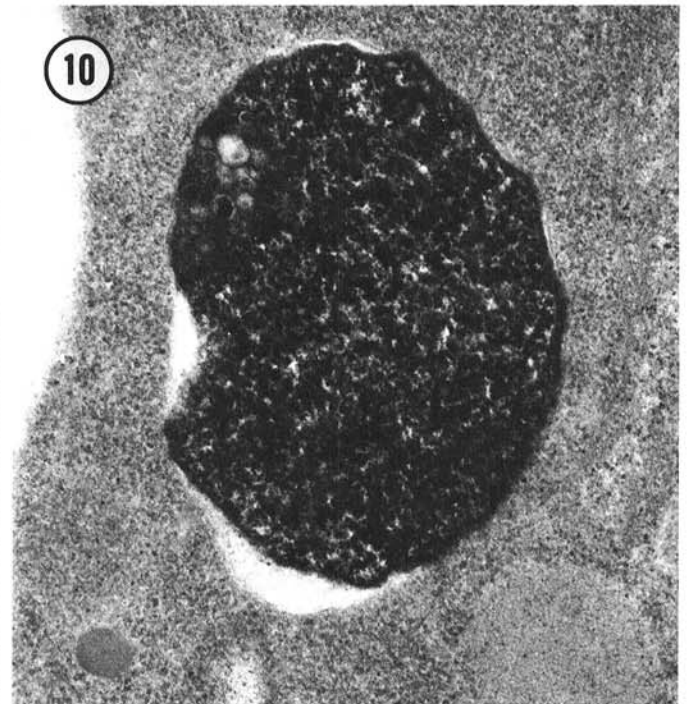
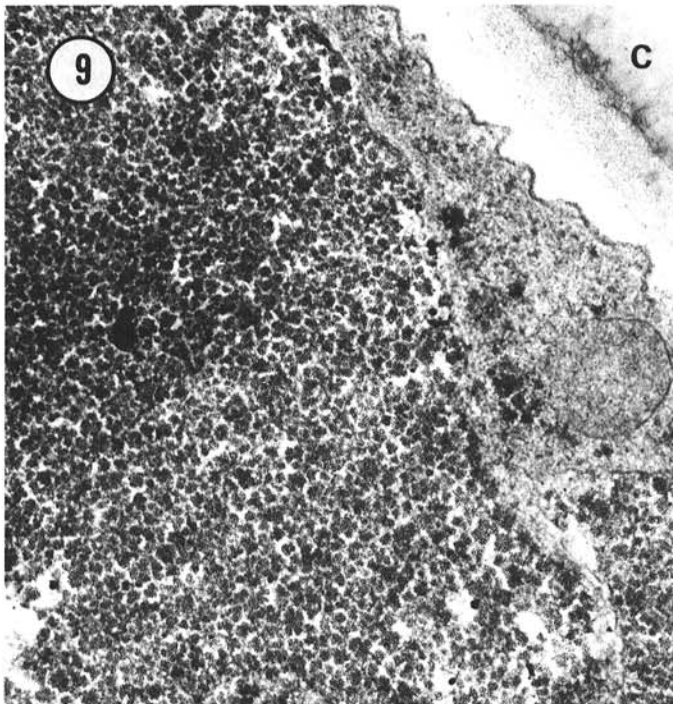
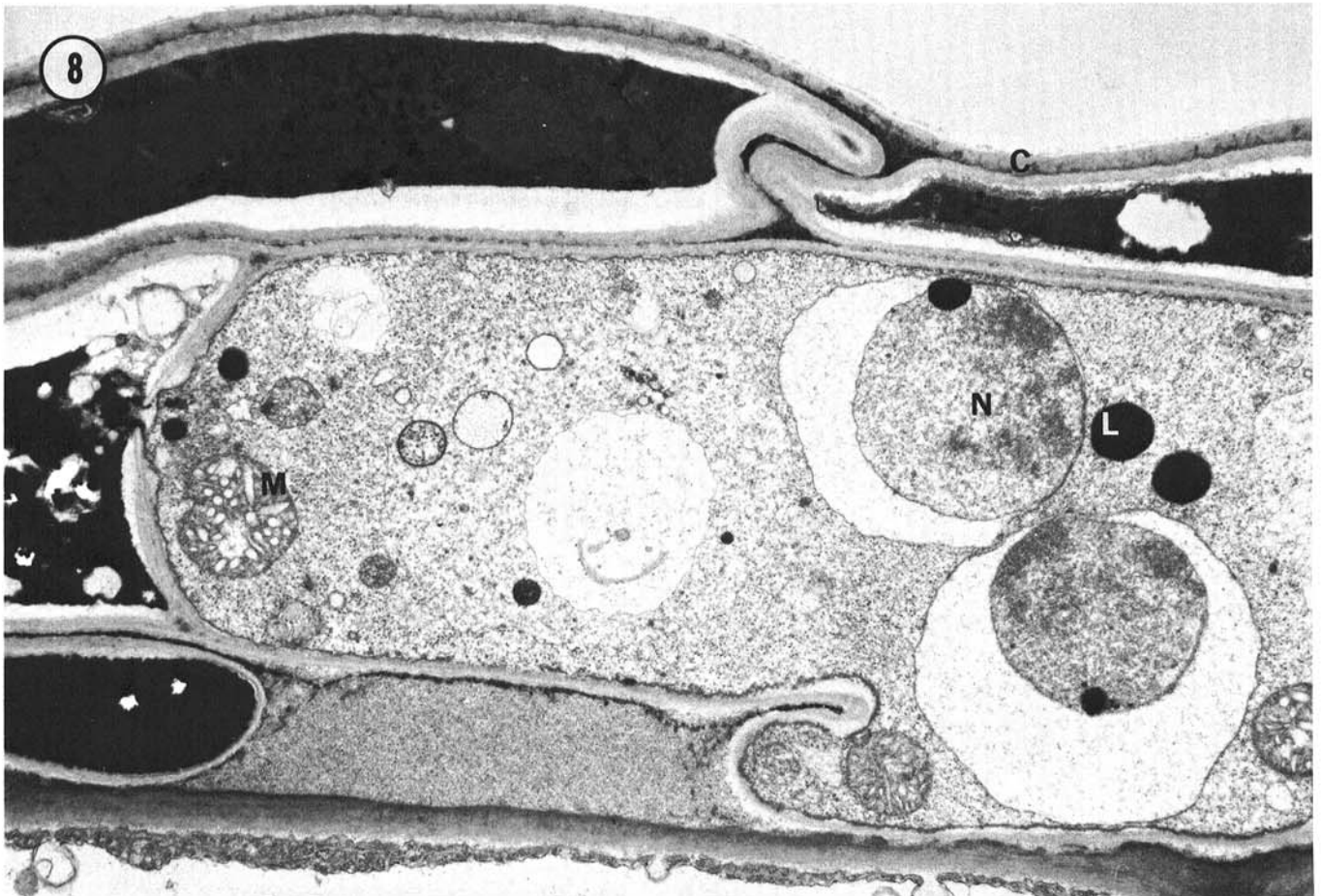
Material sampled 48 hr after fungicide application had a similar

pattern of effects in which only some of the cells and hyphae were affected.

Malus leaves, treated with PMA 100 hr after inoculation with *V. inaequalis* and left attached to the trees, developed discrete chlorotic lesions within 3 wk. No eruption of the pathogen through the cuticle was seen in cross sections of leaves examined by light and electron microscopy. Hyphae of *V. inaequalis* had invaded much of the subcuticular area, but growth was held in check and did not extend beyond the lesion area. Necrotic and often collapsed hyphae were scattered throughout the stomata, but they comprised only a small percentage (about 10%) of the total hyphal mass. The remaining hyphae appeared unaffected by the fungicide treatment. These observations coincided with the recovery of the pathogen onto growth media from similar chlorotic flecks (Table 1). Enormous areas containing glycogenlike rosettes were distributed throughout the cytoplasm and within the membrane-bounded confines of vacuoles (Fig. 9). These areas comprised 20–40% of the



Figs. 5–7. Cytological features of *Venturia inaequalis*. **5**, Electron-opaque vacuolar inclusions, mitochondria (**M**) with platelike cristae (arrows), and nucleus (**N**). Not treated with fungicides ($\times 21,300$). **6**, *V. inaequalis* cytoplasm shows effects of phenylmercuric acetate (PMA). Necrotic fungal cytoplasm is electron-dense and shows considerable disorganization. PMA applied 50 hr after inoculation. **C** = leaf cuticle, **S** = hyphal septum, **W** = Woronin body ($\times 46,000$). **7**, Localized necrotic regions were evident in hyphae treated with PMA 100 hr after inoculation and were sampled 24 hr later for electron microscopy ($\times 40,700$).



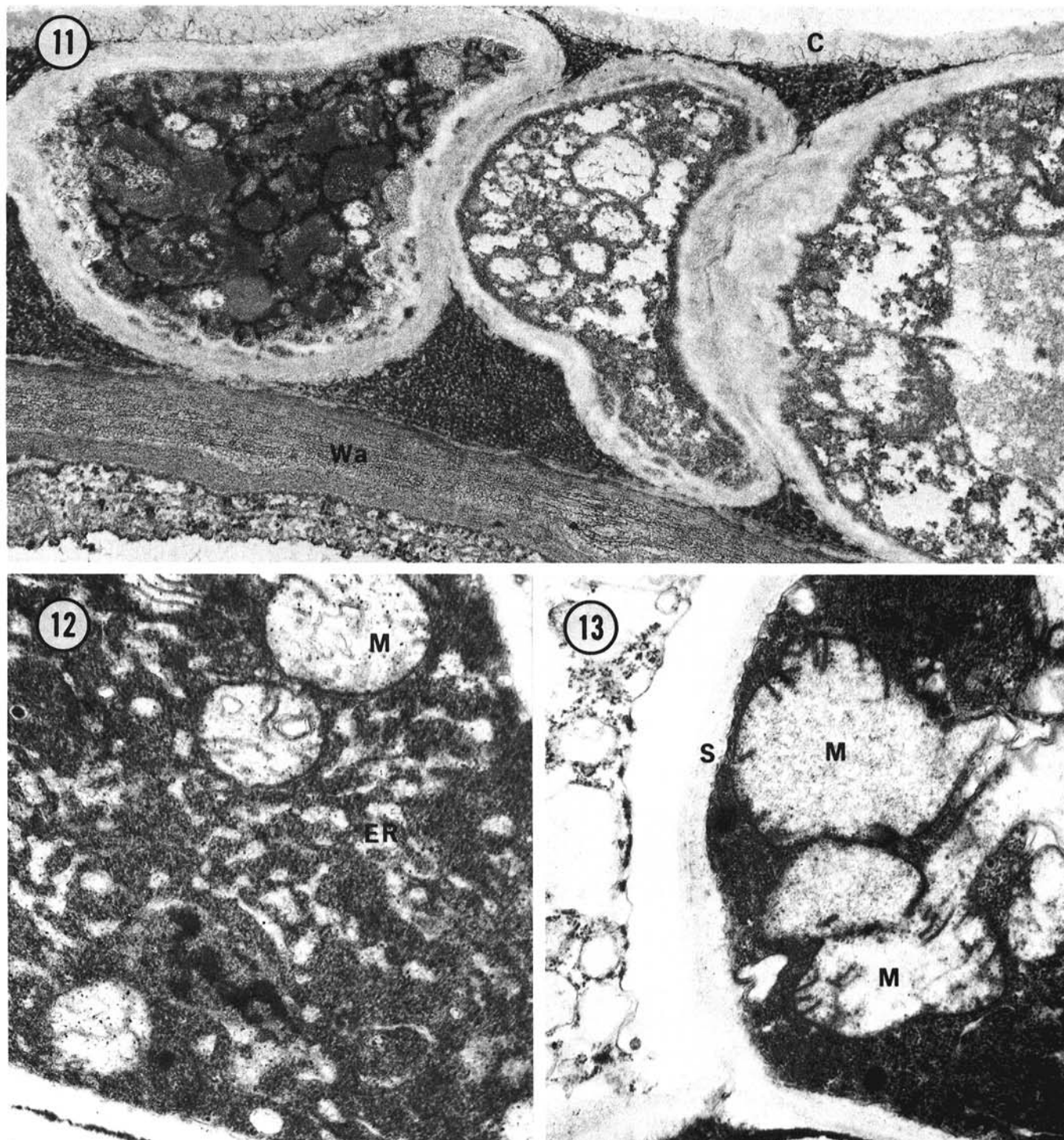
Figs. 8—10. Cytological features of *Venturia inaequalis* show effects of phenylmercuric acetate (PMA) and fenarimol. **8**, Longitudinal section through *V. inaequalis* hyphae, treated as in Fig. 9, showing varied responses of hyphal cells to PMA. Note septum separating necrotic electron-opaque cell from moribund cell. **C** = leaf cuticle, **L** = lipid, **M** = mitochondrion, **N** = nucleus ($\times 13,300$). **9**, Areas containing large amounts of glycogenlike rosettes developed in the cytoplasm and in membrane-bounded vacuoles of leaves treated 100 hr after inoculation with PMA. Sampled 3 wk after treatment. **C** = leaf cuticle ($\times 46,000$). **10**, Electron-opaque membrane-bounded areas containing vesicular bodies were common in *V. inaequalis* hyphae treated with fenarimol 50 and 100 hr after inoculation and sampled 24 hr later for electron microscopy ($\times 41,000$).

area of longitudinal profiles of sectioned hyphae.

The pyrimidine fungicide, fenarimol, affected subcuticular hyphae and individual cells of *V. inaequalis* in much the same way that PMA did. Progressive necrosis of the protoplasm along hyphal profiles was first visualized in the 24-hr samples following fungicide applications at 50 and 100 hr after inoculation of leaves. Membrane-confined, necrotic, electron-opaque areas within the cytoplasm were frequently observed (Fig. 10). Eventually, entire cells and hyphae became necrotic. The entire subcuticular fungal stroma in the chlorotic regions was necrotic 3 wk after application

of fenarimol (Fig. 11). This agrees with the recovery data of *V. inaequalis* (Table 1). Profiles of various organelles were more readily identified in these necrotic cells resulting from treatment with fenarimol than in those treated with PMA. Deposits of glycogenlike rosettes were not formed, indicating that the cells necrosed shortly after the fungicide treatment.

The effects of dodine and benomyl on *V. inaequalis* applied at 50 and 100 hr, respectively, after inoculation of leaves were less pronounced than those seen with PMA and fenarimol. Neither dodine nor benomyl caused extensive necrosis of *V. inaequalis* cells



Figs. 11-13. Morphological features of *Venturia inaequalis* cytoplasm showing effects of fenarimol, dodine, and benomyl. **11,** By 3 wk after the 50 and 100 hr fenarimol application, the entire fungal stroma was necrotic and exhibited a disrupted cytoplasm. **C** = leaf cuticle, **Wa** = periclinal wall of a leaf epidermal cell ($\times 27,400$) **12,** Dilated endoplasmic reticulum (**ER**) cisternae and swollen mitochondria (**M**) were features of fungal cytoplasm treated with dodine 50 hr after inoculation and prepared for electron microscopy 24 hr later ($\times 30,000$). **13,** Swollen mitochondria in benomyl-treated hyphae. **S** = hyphal septum ($\times 23,000$).

at the rates and times they were applied, but they prevented further subcuticular development of the pathogen. Furthermore, no sporulation of *V. inaequalis* occurred. Both fungicides are known to inhibit conidiation in established scab lesions (18). Dilated endoplasmic reticulum cisternae and swollen mitochondria of the pathogen were frequent in treated leaves when 24 or 48 hr samples were examined (Figs. 12 and 13). Their matrices also were less dense than those not treated. The mitochondria cristae remained platelike. Limited amounts of glycogenlike deposits were dispersed in the cytoplasm with both dodine and benomyl at 3 wk after treatment.

Protoplasmic abnormalities due to the fungicides were not observed in the host cells. Large electron-opaque vacuolar inclusions similar to those reported by Nusbaum and Keitt (12) and Maeda (10) in late stages of parasitism or in hypersensitive reactions by the host, however, were formed in the palisade cells of the chlorotic fleck areas.

DISCUSSION

Conditions that promote chlorotic fleck development in *V. inaequalis*-infected leaves treated with certain fungicides do not necessarily eradicate the pathogen. Instead, they must be considered fungistatic under some of the conditions tested. The fungus was viable in the flecks of leaves treated with benomyl, PMA, and dodine when isolated onto nutrient media. *V. inaequalis* generally was not viable in leaves treated with fenarimol. Those results correlate well with the ultrastructural appearance of the fungus in similarly treated leaves. Also, the fact that portions of subcuticular fungal stroma appeared ultrastructurally normal even though other portions of the same stroma were distinctly moribund or necrotic can be correlated to the varied isolation data of Table 1. Thus, the information obtained from the ultrastructural approach indicates that data obtained by the isolation procedure is reliable, contrary to our original concern.

The effect of the fungicides, applied at times and rates that result in chlorotic flecks, on the ultrastructure of *V. inaequalis* often revealed localized cytoplasmic reactions. Such reactions were either an accumulation of cytoplasmic vesicles (as in Fig. 7) or membrane-delimited necrotic areas (as in Fig. 10). The accumulation of vesicles to form wall-like appositions is similar to cytoplasmic responses toward toxic chemicals (1). Both types of responses may represent a cytoplasmic compartmentalization of the fungicides. The electron-opaque bodies in the cytoplasm (Fig. 8) were probably lipid bodies that appeared more electron-opaque than normal; however, the nature and occurrence of the nuclear inclusions could not be explained. Williams and Pugh (19) reported similar inclusions in vacuoles of a mercury-tolerant isolate of *Chrysosporium pannorum* treated with an organomercuric fungicide. They suggested that the inclusions represent sites of mercury binding.

Although the pathogen in the chlorotic flecks was held in check from further subcuticular growth and from producing conidia that could provide secondary inoculum, nothing is known of its potential for saprophytic growth in the leaf after leaf senescence. Thus, although more desirable preharvest spray timings could be used to prevent secondary inoculum, the still viable pathogen conceivably could provide primary ascospore inoculum the following spring. Such evidence needs to be determined, however, by overwintering leaves with chlorotic flecks and analyzing them

for ascospore discharge the following spring. In related studies, Burchill (3), Ross and Newbery (14), and others (11,13) showed that benzimidazole and pyrimidine fungicides, applied as postharvest sprays in the autumn, inhibited perithecial formation and ascospore production in apple leaves. Spray applications to the leaf litter in the spring appeared to increase ascospore production. Organomercuric fungicides were equally effective in preventing perithecial formation and ascospore production when applied in either autumn or spring.

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