Differential Effects of Sterol Inhibitors on Growth, Cell Membrane Permeability, and Ultrastructure of Two Target Fungi

H. Dahmen, H. C. Hoch, and T. Staub

First and third authors, Agricultural Division, Ciba-Geigy Ltd., 4002 Basle, Switzerland; and second author, Department of Plant Pathology, New York State Agricultural Experiment Station, Cornell University, Geneva 14456.
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ABSTRACT

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The time course of effects of propiconazole on germ tube elongation and ultrastructure of *Puccinia graminis* f. sp. *tritici* were studied by using video time-lapse microscopy and electron microscopy. In addition, the effect of the fungicide on cell electrolyte leakage was determined. At 1 and 10 μg/ml, germ tube elongation was inhibited after a lag time of 15 and 20 min, although major changes in the cells' ultrastructures were not noted nor was there a significant increase in electrolyte leakage from the cells. Many of the germ tube tips eventually ruptured 2–4 hr after initiation of the treatments. At 25 and 50 μg/ml, rapid cessation of germ tube elongation was accompanied by increased electrolyte leakage. Ultrastructural damage to cell membranes became apparent. The germ tube tips did not rupture at these fungicide concentrations. The effects noted for *P. graminis* were also confirmed with another fungus, *Monilinia fructicola*. The effects of the different sterol inhibitors fenpropimorph, imazalil, flutriafol, triadimenol, propiconazole, and penconazole on growth and on cell electrolyte leakage were compared. Penconazole caused a greater electrolyte leakage in *M. fructicola* than propiconazole, even though a five times high concentration was needed to observe an effect on mycelial growth. Of the other fungicides tested, only imazalil caused little electrolyte leakage, at concentrations as high as 50 μg/ml. The EC_{50} values for growth inhibition for *M. fructicola* ranged from 0.01 to 0.75 μg/ml for these fungicides. However, fungicides showed strong growth inhibition without any effects on electrolyte leakage. The results reported indicate that the direct action on fungal cell membranes may be a second mechanism of action for propiconazole and penconazole. Because this effect is only seen at concentrations substantially above those required for growth inhibition, it remains to be seen if it plays a role in practical disease control.

Inhibitors of sterol biosynthesis (SBI) are an important group of antifungal agents used both in plant protection and in the medical field. The compounds used in plant protection come from diverse chemical groups. For triazole, imidazole, pyrimidine, and piperazine derivatives, the primary mode of action is the inhibition of C14 demethylation (DMI) in sterol biosynthesis (14,16). Alternately, the morpholines can interfere with the $\Delta 14$ reductase and/or the $\Delta 8 \rightarrow \Delta 7$ isomerase (2). It is presumed that the depletion of functional sterols and the accumulation of sterol intermediates lead to a disruption of membrane functions and to growth inhibition (11,14,16). One secondary effect repeatedly described is the irregular and excessive chitin deposition at the hyphal tips and at the septae of various target fungi (7,8,10,12). Among the other possible primary effects of these compounds, the direct action on cell membranes has received considerable attention. Especially for miconazole, this effect seems quite pronounced and could contribute to the toxic effect of this compound used against dermatophytes (1,3,15).

For the sterol inhibitors used in plant protection, it is not clear if direct effects on membranes play a major role or if effects on leakage are a consequence of sterol inhibition (13). This study was undertaken to investigate this question specifically for propiconazole and other sterol inhibitors by using electron microscopy, video time-lapse light microscopy, and electrolyte leakage studies. Special emphasis was placed on establishing a time course of early effects after fungicide treatments in relation to fungicide concentration.

MATERIALS AND METHODS

Antifungal agents. The fungicides used in this study are listed in Table 1, together with the formulations and the chemical structures. All concentrations are given in μg/ml of active ingredient. A blank containing the ingredients used in the formulation of penconazole (EC-100) served as control in the leakage experiments with *Monilinia fructicola* (Wint.) Honey ATCC 9962. In all other experiments, an untreated check served as control.

Fungi and their cultivation. *M. fructicola* was maintained on 2% potato, 2% carrot agar. To obtain heavy sporulation, the fungus was grown on apricot puree. Canned apricot halves were pureed and autoclaved (15 min at 120 C) before being distributed in petri dishes. A sterile layer of filter paper (Macherey-Nagel, Dueren, W. Germany) was placed over the surface of the apricot puree. Plates inoculated with *M. fructicola* were incubated for 3–4 days in the dark at 22 C. Urediospores of *Puccinia graminis* Pers. f. sp. *tritici* were produced on wheat plants (cv. Farnese) under greenhouse conditions.

In vitro fungitoxicity tests with *P. graminis*. To assess the activity of propiconazole on germ tube elongation of *P. graminis*, urediospores were germinated in a tissue culture chamber filled with distilled water (Greiner, 7440 Nuertingen, W. Germany) for 4 hr. Subsequently, the germinating spores were treated by replacing the water with the fungicide solutions. At treatment time the germ tubes were about 190 μm in length and still nondifferentiated. Two hr after the treatments the elongation of the germ tubes was compared with that of nontreated urediospores.

The dynamic response of individual germ tubes to the propiconazole treatments was recorded (up to 9 hr after treatments) with a video time-lapse recorder. The effects of each treatment was recorded for at least 10 germ tubes.

The video time-lapse system consisted of the following components: Zeiss Photomicroscope III equipped with Nomarski/Interference contrast optics (a light gate limited germinating urediospores to a minimum time of exposure to light); Panasonic black-and-white TV camera, 1"Pasecon, model, AVT-1855 7 II; Sony monitor, model PVM 1371; U-Matic videorecorder, model CR-6650 JVC; time-lapse controller FLE 8 JVC with recording modes of 20, 12, 8, 6, and 2 images per min; remote control unit RM 70 JVC for all function systems, which allows analysis of selected images; video-type units FORA VTW 250; and video-timer FORA, model VTG 33. This system made it possible to study microscopically the time course of the effects of propiconazole on spore germination of *P. graminis*. The sequences of images were recorded at 20 images per min. In addition, an

image-analyzing computer (PC-Kontron) system was used to measure the length of the fungal hyphae on the video screen by tracing them on an electronic tablet.

Preparation for electron microscopy. For germination, the urediospores were dusted onto pieces of aluminum foil (2×2 mm) that were inverted on drops of water. The spores were then germinated for 75 min before chemical treatments. For chemical treatment excess water was gently shaken off the foil holders, which were subsequently transferred to water drops containing propiconazole. For control treatments, similar urediospore-bearing aluminum foil pieces were transferred to fresh drops of water. Freezing of the germlings of *P. graminis* without any pretreatment was done either by plunging them into liquid propane at −180 C or by slam-freezing them onto a copper block cooled with liquid helium (4,9). For both freezing methods, samples were substituted with anhydrous acetone containing 2% OsO_4 (EMS 1780) and 0.05% uranium acetate (Merck 8473) precooled to −80 C. The germlings were subsequently embedded, sectioned, and examined in a Philips 300 electron microscope according to published protocol (4).

In vitro growth inhibition of *M. fructicola* by SBI-compounds. To assess the effect of SBI-compounds on mycelial growth of *M. fructicola*, the following procedure was carried out: *M. fructicola* was grown in 100-ml Erlenmeyer flasks, each containing 20 ml of 5% Sabouraud maltose broth (Difco), pH 5.7, into which the test fungicides had been incorporated. The cultures were incubated in the dark at 22 C for 2 wk, after which the resulting mycelial mats were harvested, freeze-dried, and weighed. The EC_{50} values were determined on the basis of the reduction of dry weight compared with the controls. The test was repeated twice.

Membrane permeability tests, electrolyte leakage. Tests for plasma membrane permeability of the respective test fungi were conducted in 100-ml Erlenmeyer flasks on a rotary shaker (90 rpm) at 22 C in the dark. The electrolyte conductivity of the ambient solution was measured by transferring 200-μl aliquots at 5- to 10-min intervals to mini wells equipped with two platinum

TABLE 1. List of ergosterol inhibiting compounds, their formulation, and chemical structure

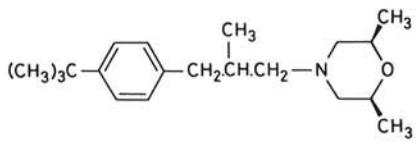
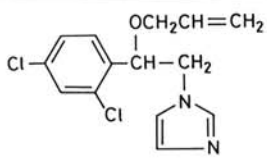
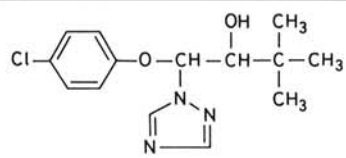
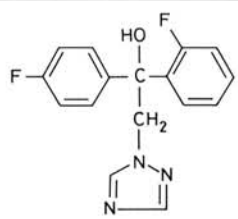
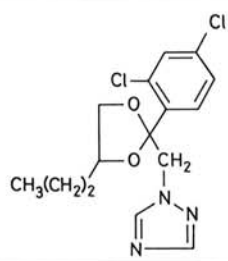
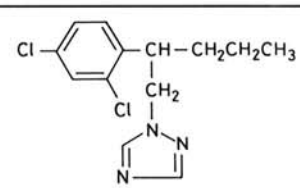
Common name and Formulation	Chemical Structure
Fenpropimorph EC-750	
Imazalil EC-250	
Triadimenol active material	
Flutriafol WP 25	
Propiconazole EC-250	
Penconazole EC-100	

TABLE 2. In vitro activity of propiconazole on germ tube elongation, leakage, and morphological and cytological changes in the hyphal tips of *Puccinia graminis* f. sp. *tritici*

Concentration μg/ml[a]	Germ tube length 2 hr after treatment μm	Morphological and cytological effects: Vacuolation %	Morphological and cytological effects: Bursting tips % per hr after treatment	Percent[b]
Untreated	124	0	0	0
1	58	0	80/4 hr	0
10	32	50	50/2 hr	0
25	2	100	0	40
50	2	100	0	100
75	...	...	...	210
100	...	...	...	260

[a] Active ingredient.
[b] Percent increase of conductivity 60 min after treatment relative to nontreated controls.

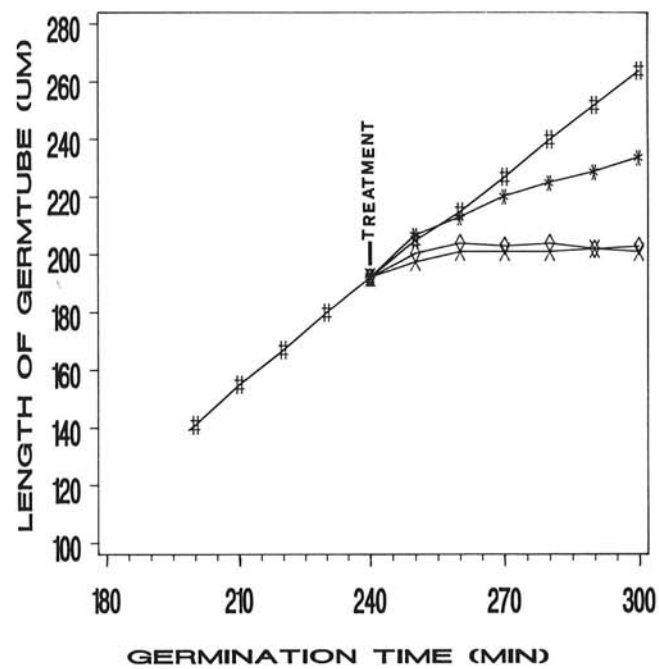


Fig. 1. The effect of propiconazole on germ tube growth of *Puccinia graminis* f. sp. *tritici* 1 hr after initiation of the treatment. # = untreated; * = 1 μg/ml; ◇ = 10 μg/ml; and × = 25 μg/ml. (The time course for 50 μg/ml was virtually identical to that of 25 μg/ml.)

electrodes (Ciba-Geigy, Messtechnik, and Automation). Conductivity, recorded with a Philips conductivity meter, model PW 9501/01, was related to a standard curve established with known concentrations of NaCl.

Both *P. graminis* and *M. fructicola* were studied for induced plasma membrane electrolyte leakage caused by SBI-compounds. For *P. graminis*, urediospores were used in which germination was initiated on water agar for 30 min. The hydrated spores were then transferred to Erlenmeyer flasks containing 20 ml of water, and the spore density was adjusted to 2×10^5 spores per milliliter. After an additional 45 min, the test concentrations of propiconazole were added. At this time the average length of the germ tubes was about 70 μm.

For *M. fructicola*, conidia were suspended in a 0.5% sucrose solution (Fluka 84110) adjusted to pH 6 and incubated for 3 hr on a rotary shaker. At this time, germ tube length was approximately 25 μm. The germinated conidia were lightly pelleted by centrifugation, and the sucrose solution was replaced by a similar solution but amended with the various fungicides (see Table 1). Final conidial density was adjusted to 3×10^6 spores per milliliter. The experiment was repeated at least three times.

RESULTS

In vitro effects of propiconazole on germ tube growth of *P. graminis*. The observations made with light microscopy are summarized in Table 2. A concentration of 1 μg/ml of propiconazole caused germ tube elongation to be reduced by 50%. Ten μg/ml treatments inhibited germ tube growth by up to 75%, and 25 and 50 μg/ml completely stopped germ tube elongation. Bursting of the germ tube tip was observed in about 80% of the germlings 4 hr after initiation of the 1 μg/ml treatment. With 10 μg/ml, about one-half of the germ tubes ruptured at around 2 hr, whereas no bursting was observed at higher concentrations.

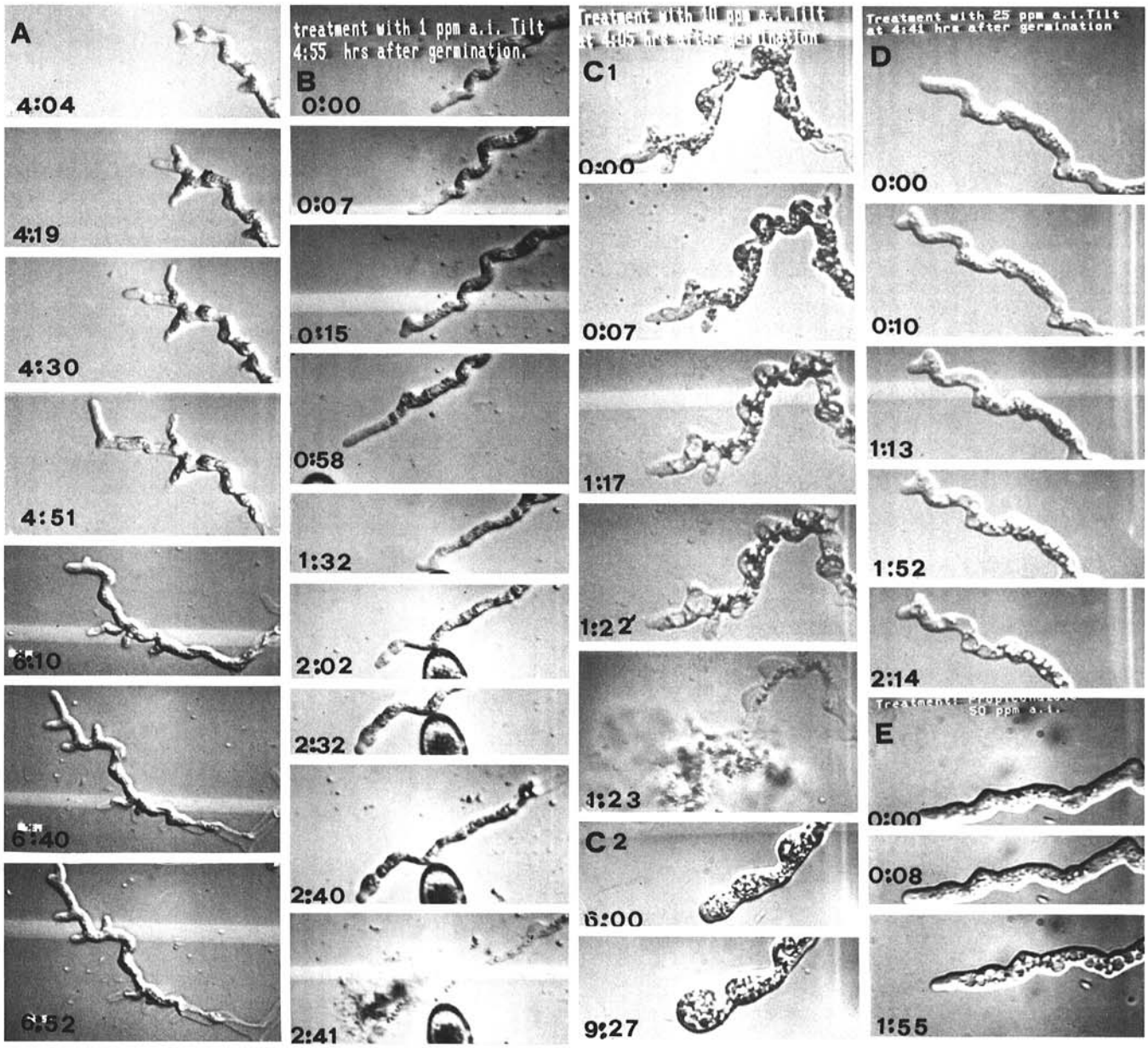


Fig. 2. Effects of propiconazole on the growth of the germ tube tip of *Puccinia graminis* f. sp. *tritici* recorded by time-lapse video microscopy. The sequence of images was taken from the monitor screen for selected images with a Minolta XE1, Macro 50 mm. The numbers in **A** represent the germination time in hr:min, and in **B**–**E** the treatment time is in hr:min. **A**, Untreated germling, germination time recorded until 6:52. Displacement of the germling itself between still pictures 4:51 and 6:10. Note the concentration of cytoplasm in the germ tube tip and the emptying of the older part of the germ tube. **B**, Treatment with 1 μg/ml. Germ tube tip growth eventually stops, and the germ tube tip ruptures. **C1**, Treatment with 10 μg/ml. Heavy vacuolation can be observed in the first part of the germ tube until it ruptures. **C2**, 10 μg/ml treatment, germ tube tip swells. **D**, Treatment with 25 μg/ml. Rapid effect on germ tube growth and vacuolation. The results with 50 μg/ml treatments (**E**) are similar to those for 25 μg/ml; the sequence of events develops sooner.

Furthermore, vacuolation was observed in 50% of the germ tube tips treated with 10 μg/ml. At higher concentrations all tips showed extensive vacuolation. The time course of inhibition over the first hour at treatment is shown in Figure 1. Inhibition of germ tube growth by 1 μg/ml of propiconazole started 20–30 min after initiation of the treatment. At 10 μg/ml or higher, inhibition of growth occurred 10 min following the initiation of the treatments.

Cytoplasmic and morphological responses of *P. graminis*. The effects of propiconazole on germinating urediospores were recorded by video time-lapse microscopy. In the nontreated germling, migration of the cytoplasm was toward the germ tube tip and can clearly be seen in young germ tubes of Figure 2. The cytoplasm continued to migrate forward with the growing hyphal tip, leaving the older part of the germ tube devoid of cytoplasm (sequence A, 2:53–6:52). Following treatment with 1 μg/ml of propiconazole (sequence B), growth continued at an apparently normal rate for 20–30 min (0:00–0:58). Later this rate of growth slowed down, and at about 2 hr after initial treatment, germ tube growth ceased (1:32–2:40). However, the cytoplasm continued to migrate into the hyphal tip. Because of increased osmotic pressure or a weakened cell wall, the germ tube tip ruptured and the cytoplasm was released (2:41).

The response to treatments with 10 μg/ml of propiconazole are shown in Figure 2, sequences C1 and C2. The cytoplasm immediately started to flow backward and became markedly more granular. These events are not clearly seen on the still pictures (C1, 0:00-0:07), but are obvious on the video film. Within a few minutes the backward flow stopped and the granular particles moved by Brownian movement. Hyphal growth stopped at around 15 min, and the cytoplasm became highly disorganized and vacuolated. Often, but not always, the germ tube tip ruptured and the cytoplasm emptied into the surrounding environment (C1, 1:17–1:23). In another example with the same treatment concentration, germ tube growth stopped, as in C1, but the germ tube tip swelled and the tip did not rupture (C2, 6:00–9:27).

Ten minutes after initiation of treatments with 25 μg/ml of propiconazole, germ tube growth stopped (Fig. 2, sequence D, 0:00–0:10). One hr later the cytoplasm became disorganized and vacuolated (Fig. 2, sequence D, 1:13–2:14). In this case, neither bursting nor swelling of the germ tube tip occurred. The sequence of events during treatment with 50 μg/ml of propiconazole was similar to that observed at 25 μg/ml. The whole sequence and especially vacuolation occurred somewhat more rapidly than that observed with 25 μg/ml treatments of the chemical (Fig. 2, sequence E, 0:00–1:55).

Effects of propiconazole on membrane permeability of *P. graminis*. Electrolyte leakage from propiconazole-treated germ tubes of *P. graminis* was monitored for 60 min (Fig. 3). At concentrations of 75 and 100 μg/ml of propiconazole, conductivity increased twofold over that of control treatments within the first 5 min and more than fourfold after 60 min of treatment. At 25 and 50 μg/ml, the induction of electrolyte leakage was less dramatic. The nontreated urediospores did not leak significant amounts of electrolytes.

Ultrastructure effects. Nontreated germlings of *P. graminis* frozen by either plunging into cooled propane (4,9) or slammed onto a copper block (4,5), then processed for substitution, had well-preserved ultrastructural features. No obvious differences were noted between the two methods of freezing (Figs. 4 and 5). The distribution of cytoplasmic organelles in the germ tube tip was similar to that described by Hoch et al (9) for *Uromyces phaseoli* An accumulation of apical vesicles and microvesicles was noted in the extreme apex of the germ tube tip. Beginning 20–30 μm from the apex, an aggregation of mitochondria was normally observed. Also, within this apical region, many Golgi bodies and considerable smooth endoplasmic reticulum were situated. Microtubules were also present.

Germlings treated for 30 min with 1 μg/ml of propiconazole did not appear ultrastructurally different than the nontreated germlings (Fig. 6). However, after 60 min of exposure to propiconazole, the apical vesicles were no longer organized in a cluster in the germ tube tip. Golgi bodies and endoplasmic reticulum were still positioned within the apical region, particularly near the mitochondria (Fig. 7).

Changes in the ultrastructure of hyphal tips treated with 10 μg/ml of propiconazole were marked. Although the effects of propiconazole on the cytoplasmic distribution of apical vesicles were not analysed statistically, at 1 min they were not observed to be organized in an apical cluster in germ tube tips (Figs. 8 and 9). They were distributed throughout this apical region, especially along the plasma membrane. At a treatment time of 10 min (Fig. 9), only a few apical vesicles were present, and after 15 min of treatment (Fig. 10) with propiconazole, apical vesicles were extremely rare. At 15 min, the cytoplasm had become granulated in appearance and was obviously very different from the cytoplasm of the nontreated germ tube. An accumulation of membranous material occurred in the hyphal tip.

Treatment of germlings with 25 μg/ml of propiconazole for 15–20 min caused the distribution of apical vesicles to be highly disorganized. In some cells a large number of apical vesicles were observed to be distributed throughout the germ tube apex (Fig. 11), whereas in other cells apical vesicles appeared to be completely absent (Fig. 12). However, in these latter cells an enormous accumulation of membranes was observed and appeared to be endoplasmic reticulum-like material (Fig. 13). The germ tube tip was very disorganized, and Golgi bodies were frequently observed near the germ tube apex (Figs. 11 and 14). Mitochondria were positioned more distally and appeared swollen. Normal-appearing endoplasmic reticulum was no longer observed. Microtubules remained present during these treatments (Figs. 11 and 14). Seventy min after treatment, 50% of the germ tubes appeared dead (Fig. 15).

The effect of 50 μg/ml of propiconazole on *P. graminis* was similar to those observed for 25 μg/ml. After 10 and 30 min of treatment, the germ tube tip appeared devoid of large organelles (Figs. 16 and 17). Frequently, electron opaque deposits were

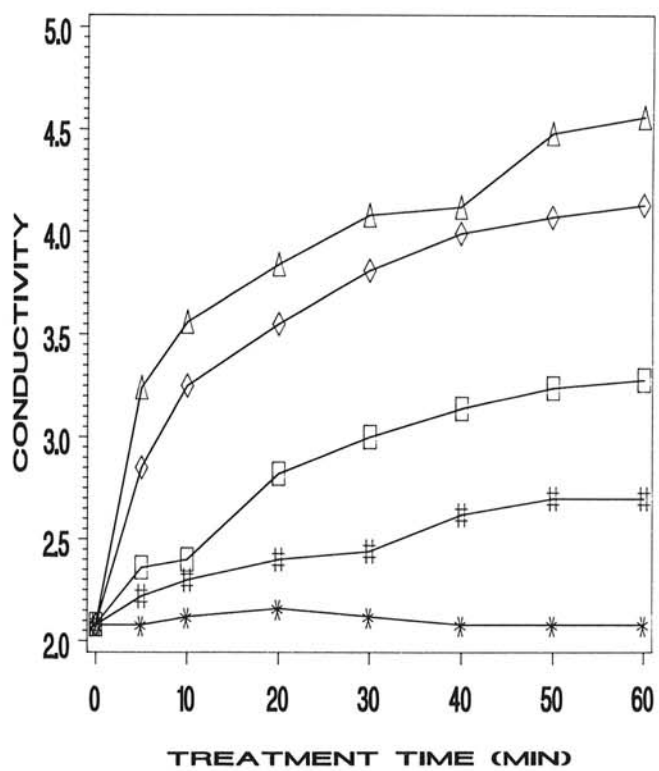
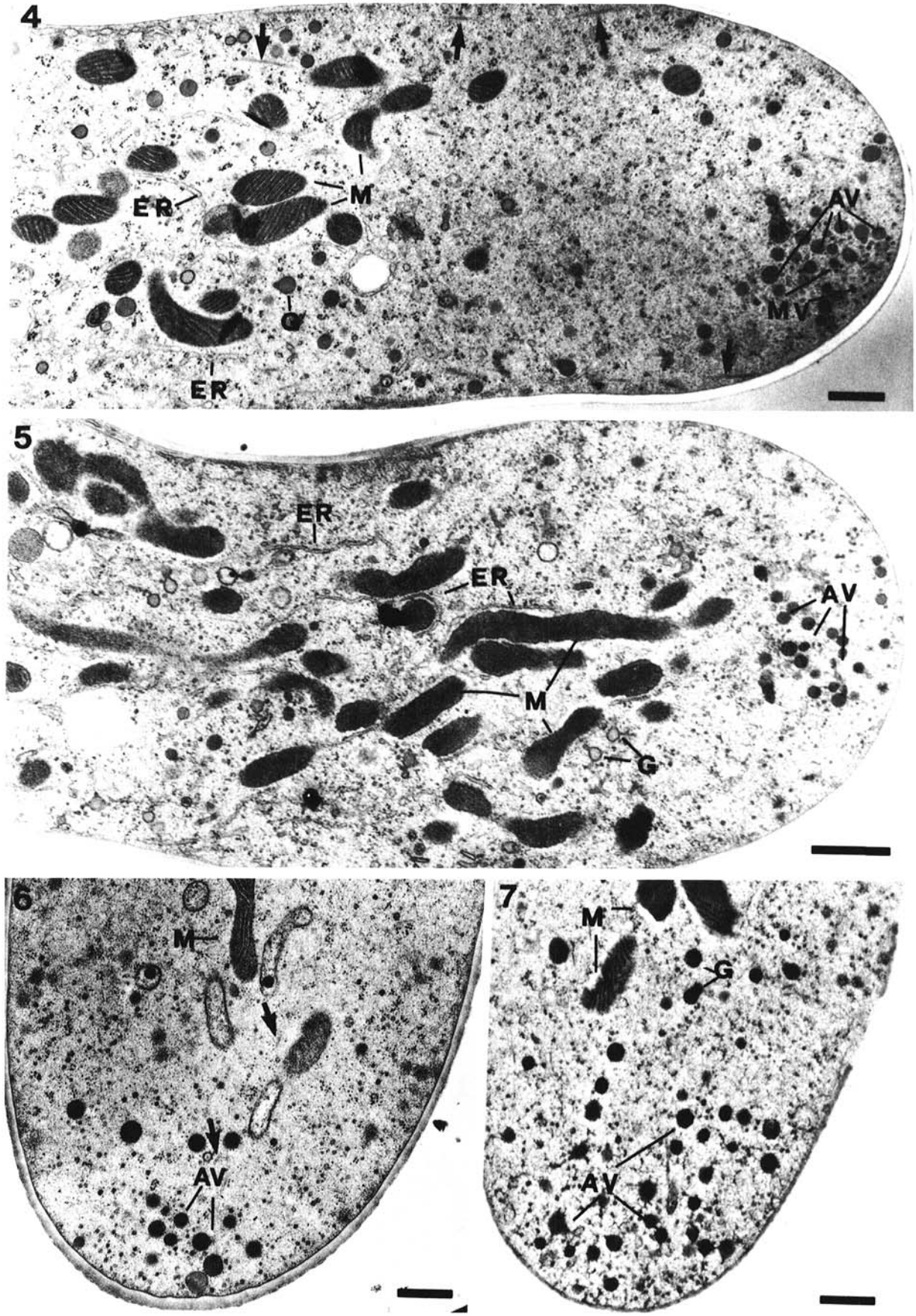
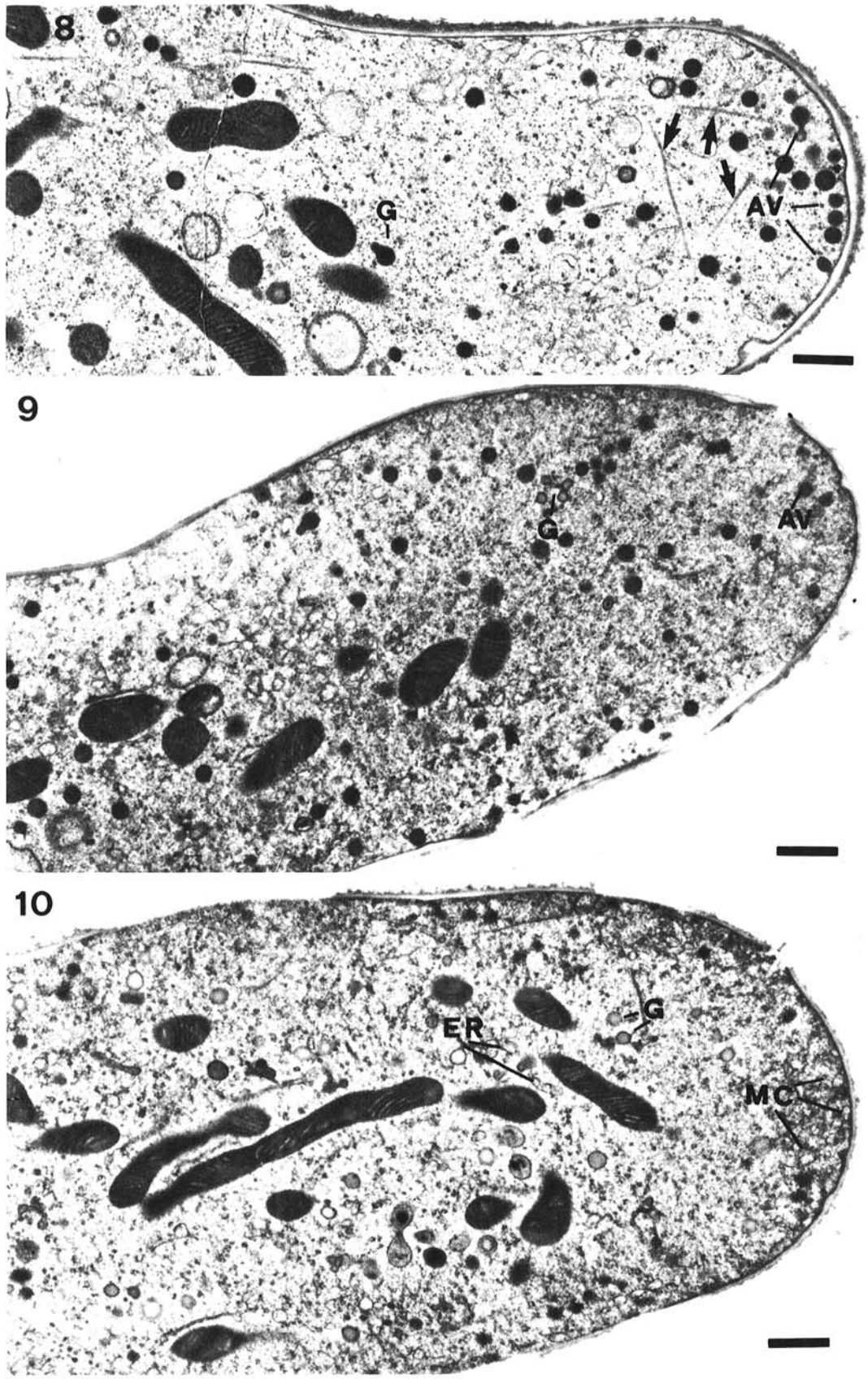


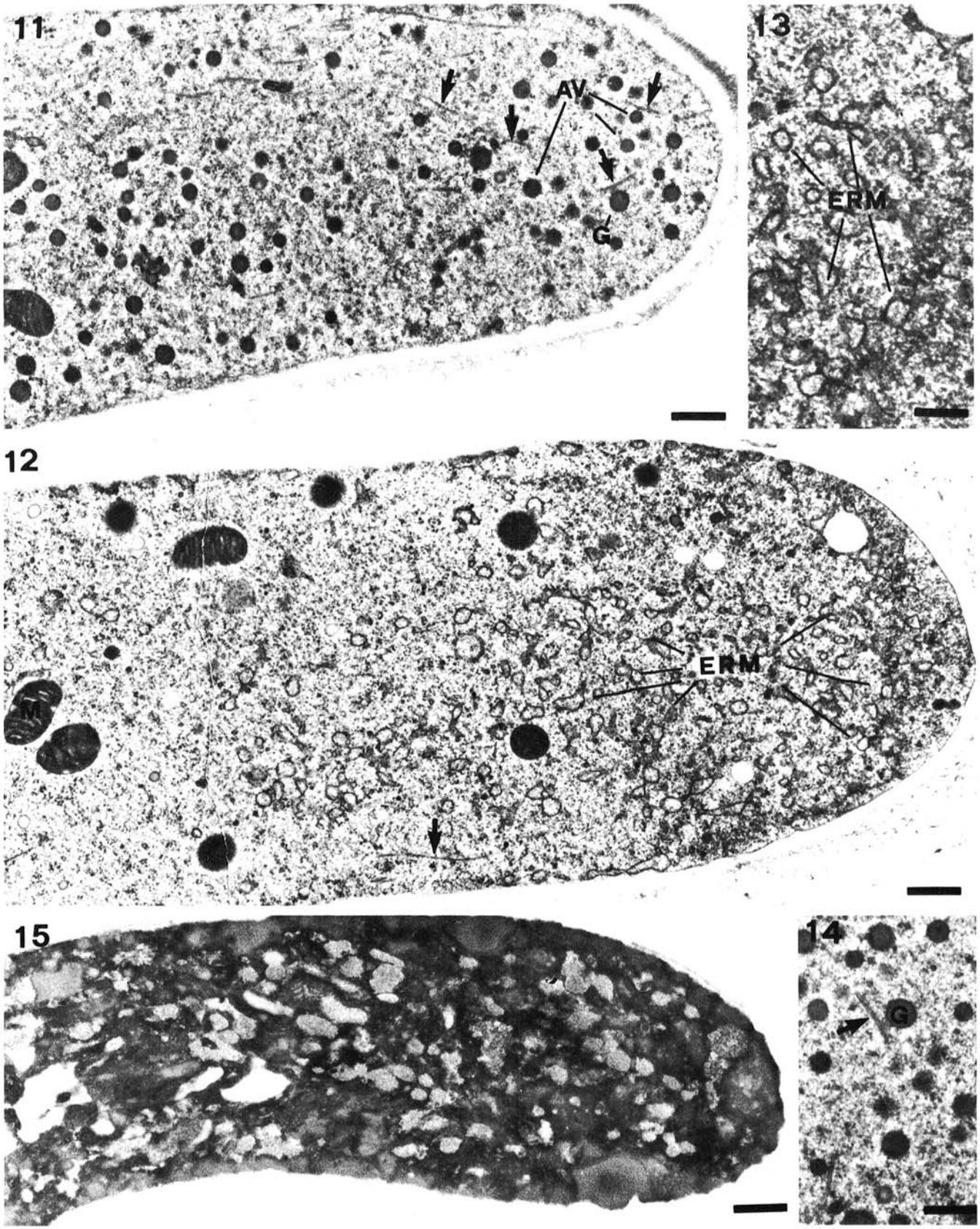
Fig. 3. The effect of propiconazole on membrane permeability of germinating urediospores of *Puccinia graminis* f. sp. *tritici*. Δ = 100 μg/ml; ◇ = 75 μg/ml; □ = 50 μg/ml; # = 25 μg/ml; and * = untreated. The measured values were corrected for the conductivity contribution by the fungicides. Conductivity is expressed in μg equivalents of NaCl in 200 μl of distilled water. Each point represents four measurements.



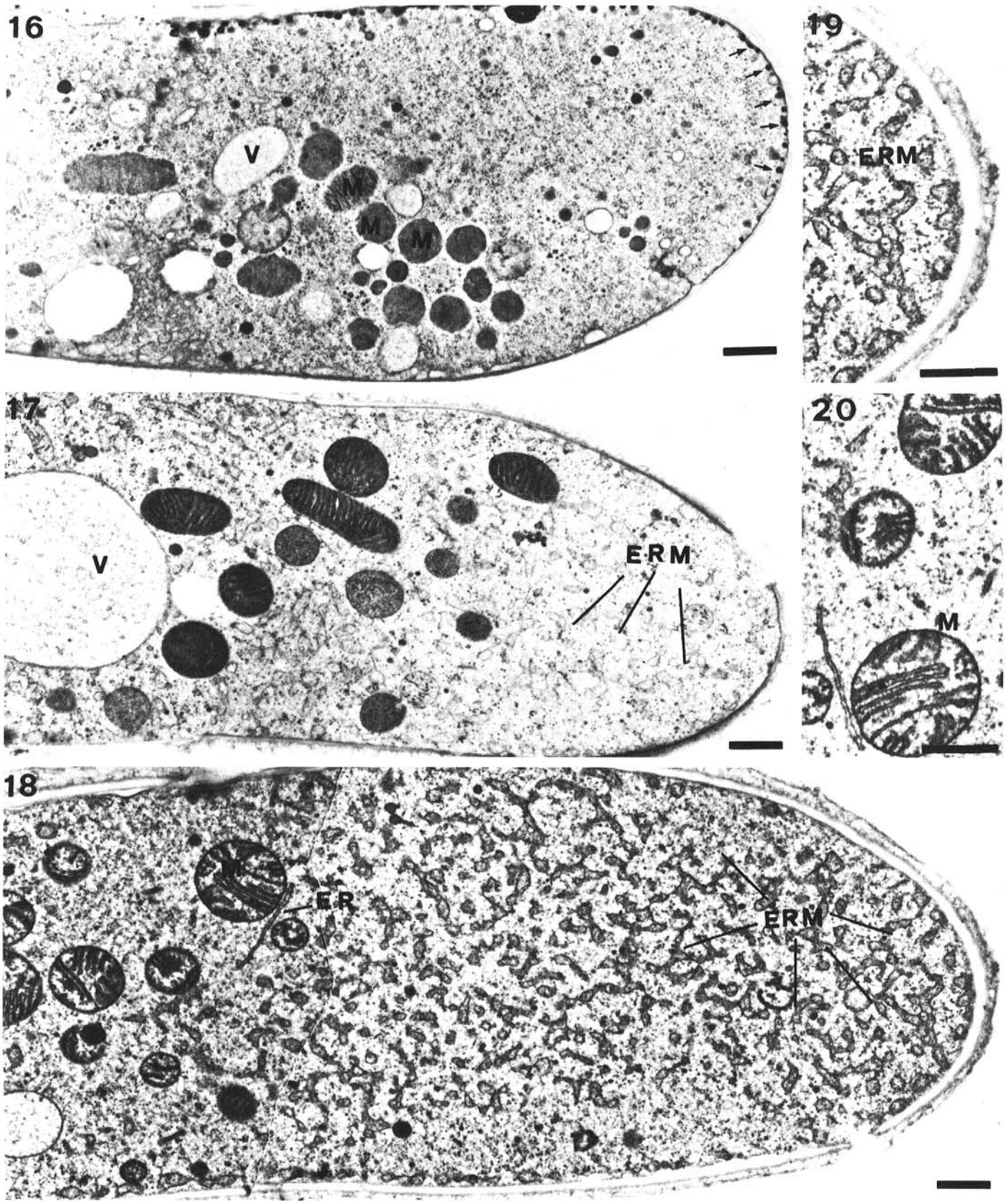
Figs. 4–7. Ultrastructural effects of propiconazole on *Puccinia graminis* f. sp. *tritici* urediospore germ tubes. **4** and **5**, Nontreated germ tube tips frozen in propane or on a helium-cooled copper block, respectively. The cluster of apical vesicles and microvesicles, the positioning of mitochondria, Golgi bodies, endoplasmic reticulum, and the microtubules are typical for *P. graminis*. **6** and **7**, Treatment with 1 μg/ml of propiconazole for 30 and 60 min, respectively. After 30 min of treatment the distribution pattern of the organelles is the same as that in nontreated germ tubes. After 60 min of treatment the cluster of apical vesicles is no longer observed. AV, apical vesicles; MV, microvesicles; M, mitochondria; microtubules (arrows); G, Golgi bodies; ER, endoplasmic reticulum. Bar = 0.5 μm.



Figs. 8–10. *Puccinia graminis* f. sp. *tritici* urediospore germlings treated with 10 μg/ml of propiconazole and frozen with helium. **8**, The apical vesicles are not organized in a cluster after a treatment time of 1 min. **9**, After 5-min exposure to propiconazole only a few apical vesicles are observed. **10**, At a treatment time of 15 min almost no apical vesicles are present, but a cluster of membranes in the hyphal tip is now observed. Microtubules (arrows); AV, apical vesicles; G, Golgi bodies; ER, endoplasmic reticulum; MC, cluster of membranes. Bar = 0.5 μm.



Figs. 11-15. *Puccinia graminis* f. sp. *tritici* urediospore germlings treated with 25 μg/ml of propiconazole. **11,** Treatment time, 15 min. Apical vesicles are distributed throughout the hyphal tip. Golgi bodies can be observed in the hyphal tip (Fig. 14). **12** and **13**, Treatment time, 20 min. No apical vesicles are present, but an accumulation of membranes resembling endoplasmic reticulum-like material is observed. The mitochondria are displaced distally. They appear swollen and are no longer oriented with the longitudinal axis of the cell. **15**, Treatment time, 70 min. Hyphal cell was necrotic at freezing time. G, Golgi bodies; M, mitochondria; ERM, endoplasmic reticulum-like material; microtubules, (arrows). **11**, **12**, and **15**, bar = 0.5 μm; **13** and **14**, bar = 0.25 μm.



Figs. 16-20. *Puccinia graminis* f. sp. *tritici* urediospore germlings treated with 50 μg/ml of propiconazole. **16,** Treatment time, 10 min. The hypha looks devoid of larger organelles, and the mitochondria look swollen and are no longer oriented with the longitudinal axis of the cell. Vacuolation is observed. Electron opaque deposits are observed on the plasma membrane (arrows). **17**, Treatment time, 30 min. Vacuolation is obvious, and an accumulation of membranes resembling endoplasmic reticulum-like material is observed. **18**, Treatment time, 60 min. An accumulation of membranes over the first hyphal zone is observed. These membranes look like endoplasmic reticulum-like material (Fig. 19). **20**, The christae of the swollen mitochondria disappear. V, vacuole; M, mitochondria; ER, endoplasmic reticulum; ERM, endoplasmic reticulum-like material. Bar = 0.5 μm.

observed to be associated with the plasma membrane (Fig. 16). Also at this time, considerable vacuolation in the area of the mitochondria was observed (Figs. 16 and 17). The mitochondria were swollen and disorganized. After 30 and 60 min of treatment, the accumulation of endoplasmic reticulum-like membranes appeared within the germ tube tip and distally to the region of mitochondria (Figs. 17–19). Normal-appearing endoplasmic reticulum was not observed. Sixty min after initiation of treatment with propiconazole, the mitochondria were swollen (Fig. 20). Eighty percent of the germ tubes were dead at the time that the cells were frozen.

In vitro effects of SBI-compounds on mycelial growth of *M. fructicola*. The most active compounds that inhibited mycelial growth (EC_{50} values from 0.01–0.05 μg/ml) were propiconazole, fenpropimorph, imazalil, and penconazole (Table 3). Flutriafol and triadimenol were less inhibitory (EC_{50} values of 0.5 and 0.75 μg/ml, respectively).

Effects of SBI-compounds on membrane permeability of *M. fructicola*. Table 3 summarizes the effects of all SBI-compounds tested for electrolyte leakage from *M. fructicola*. The lowest concentrations of test compounds causing significant leakage were 2.5, 10, and 50 μg/ml for penconazole, propiconazole, and imazalil, respectively. No electrolyte leakage was induced by fenpropimorph, flutriafol, or triadimenol, even at 50 μg/ml, or by the EC-100 blank formulation of penconazole.

Figure 21 represents the effects of the SBI-compounds on membrane permeability of *M. fructicola* over a 1-hr period following addition of the various fungicides. Immediate electrolyte leakage occurred in *M. fructicola* during treatment with propiconazole at 50 μg/ml. The effects of penconazole were even more pronounced than those of propiconazole and led to a 350% increase in conductivity in the ambient solution compared with the control treated with the blank formulation. Propiconazole at 50 μg/ml and penconazole at 25 μg/ml caused about the same effect on electrolyte leakage, with a 200% increase in conductivity after 60 min of treatment. At 25 μg/ml of propiconazole, the effects on electrolyte leakage were much weaker and were about the same as for imazalil at 50 μg/ml.

DISCUSSION

The various methods used to study the effects of propiconazole on *P. graminis* revealed two possible mechanisms of action that can be distinguished by the dose required and by the rapidity with which they occur. The most sensitive parameter was the inhibition of the germ tube elongation. Such growth inhibition could be observed at 1 μg/ml, and it occurred with a lag time of about 20–30 min. It was not accompanied by leakage in the first hour of treatment, but it led to bursting of the hyphal tips within 2–3 hr. No ultrastructural changes could be observed in the first 30 min after treatment, and only minor changes were noted after 60 min. It can be assumed that the delayed inhibition of growth at low dosages is the major fungistatic effect caused by the inhibition of sterol synthesis common to all DMI-compounds (14). The various observations of effects on cell ultrastructure and leakage several hours after exposure to the fungicides may be indirect consequences of sterol inhibition instead of direct effects (7,8,13).

A second type of effect was observed at 25 and 50 μg/ml. It consisted of immediate cessation of germ tube elongation, accompanied by severe electrolyte leakage and obvious cytoplasmic disorganization at the ultrastructural level within a few minutes of treatments. These effects did not lead to a bursting of the germ tube tips in the video time-lapse study. Ten μg/ml of propiconazole gave an intermediate response with complete cessation of growth after a lag time of about 15 min. To discriminate between the two effects, it was essential to make early observations on both ultrastructure and leakage and to relate them to growth inhibition. Isolated ultrastructural observations, especially if they are made only several hours after the beginning of treatments, are only of limited value in mode of action studies. They merely may document the result of a long chain of events.

It appears that at low concentrations of propiconazole, the physical strength of the germ tube wall is weakened, whereas membrane permeability and possibly turgor are unchanged. This leads to the bursting of the hyphal tip in an aqueous environment of low osmotic strength. At higher concentrations, on the other hand, the rapid damage by propiconazole to the cell membranes permitted leakage of electrolytes and possibly other osmotically active molecules, thus reducing the turgor pressure and preventing bursting of the germ tube tips.

The growth inhibition and electrolyte leakage studies with *M. fructicola* confirmed the existence of two independent mechanisms of action for only some of the sterol inhibitors tested. With propiconazole and penconazole, leakage was increased significantly within 1 hr at concentrations as low as 10 and 2.5 μg/ml, respectively. Fenpropimorph, flutriafol, and triadimenol

TABLE 3. In vitro activity of SBI-compounds against *Monilinia fructicola* on mycelial growth in liquid culture and on electrolyte leakage

Compound	Percent[a] 25 μg/ml[b]	Percent[a] 50 μg/ml	Lowest concentration with leakage μg/ml	Inhibition of mycelial growth EC_{50} μg/ml
Fenpropimorph	0	0	>50	0.03
Imazalil	0	28	50	0.04
Flutriafol	0	0	>50	0.5
Triadimenol	0	0	>50	0.75
Propiconazole	65	160	10	0.01
Penconazole	148	260	2.5	0.05
Blank EC-100	0	0	>50	>50

[a] Percent increase of conductivity 60 min after treatment relative to nontreated controls.
[b] Active ingredient.

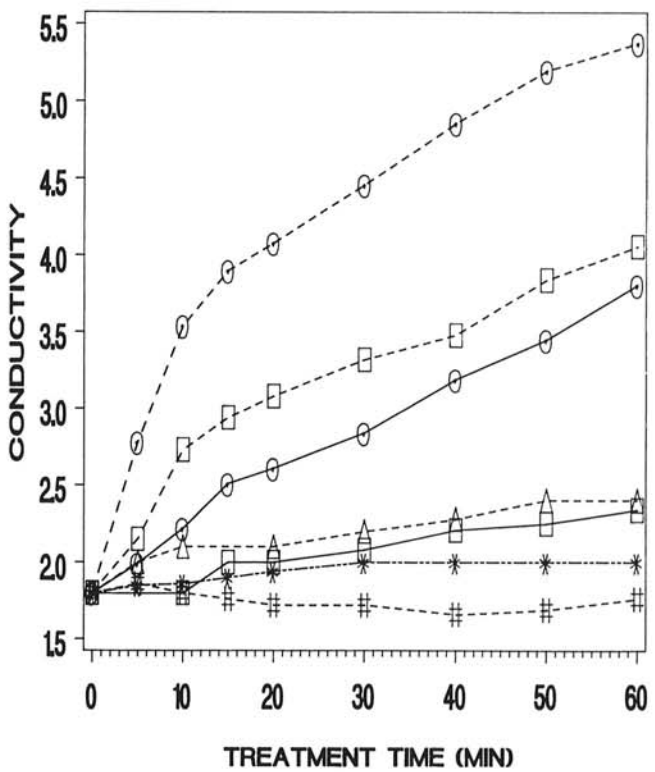


Fig. 21. The effect of sterol inhibiting-compounds on membrane permeability of germinating *Monilinia fructicola* conidia. Compounds: o = penconazole; □ = propiconazole; Δ = imazalil; # = fenpropimorph; and * = blank formulation. Concentrations: - - - -, 50 μg/ml; ——, 25 μg/ml; —· · — = check (EC-100 blank formulation of penconazole at 50 μg/ml). Fenpropimorph is an example of those compounds that showed no leakage. The measured values were corrected for the conductivity distribution of the fungicide. Conductivity is expressed in μg equivalents of NaCl in 200 μl of distilled water. Each point represents four measurements.

did not show any leakage up to 50 μg/ml, even though these compounds strongly inhibited growth at concentrations below 1 μg/ml. Imazalil was somewhat intermediate in that it showed a weak effect on leakage only at 50 μg/ml. These differential effects indicate that the rapid membrane effect is governed by a different structure activity relationship than is the effect on growth inhibition by DMI-compounds. Thus it appears that the rapid effects on leakage by propiconazole and penconazole represent a second primary mechanism of action for these compounds and that they are not a consequence of the inhibition of sterol synthesis.

This apparent specificity of the rapid membrane effects by some DMI-fungicides used in plant protection is similiar to the rapid fungicidal effects of miconazole on *Candida albicans* (3), which could not be observed for ketoconazole. In addition, direct membrane effects with miconazole could only be demonstrated when the fungal cells were actively growing. No such effects were observed when the fungal cells were in the stationary phase at treatment time.

For the antifungal compounds with dual modes of action on sterol synthesis in both plant protection and the medical field, it is not clear if the direct membrane effects contribute to the activity in practice (16). In both cases the effects on membranes are observed only at substantially higher concentrations than are the fungistatic effects on growth and the effects on sterol biosynthesis. Likewise, the observation on the increase of fatty acids in with ethaconazole-treated *Ustilago maydis* sporidia were made 5 hr after treatment (6). It would be worthwhile to pursue this question further, because it has important implications for the development of resistance in target fungi. Our results stress the importance of precise and early time course observations for an improved interpretation of leakage and ultrastructural results.

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