

Effects of Metalaxyl on *Peronospora tabacina* Infecting Tobacco

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ABSTRACT

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Soil drenches of metalaxyl applied to potted tobacco plants did not affect the germination of sporangia and subsequent penetration of leaf epidermal cells by *Peronospora tabacina*. Development of the pathogen in plants treated with fungicide before inoculation was limited to infection structures within epidermal cells; no intercellular hyphae or haustoria were formed. When metalaxyl was applied by soil drenches to blue mold-infected plants, within 48 hr, sporulation by *P. tabacina* was suppressed 96%, relative to the controls. Sporangioophores that developed on treated plants were incompletely formed and some sporangia showed abnormal morphology. Ultrastructural changes in the fungus were evident 24 hr after treatment; intercellular hyphae and haustoria were vacuolated and nuclei were condensed. Forty-eight hours after treatment, 52% of the haustoria and

Additional key words: *Nicotiana tabacum*.

77% of intercellular hyphae were either necrotic or extensively vacuolated. Ninety-four percent of the haustoria from infected, untreated plants were encased in a single layer of amorphous, moderately electron-dense appositional material and 6% were encased in two layers of appositional material. The inner layer of encasement material was composed of amorphous material, whereas the outer layer consisted of both host wall-like and membranelike portions that stained more densely than the inner layer. The frequency of haustoria encased by bilayered appositions was four times greater in metalaxyl-treated plants than in control plants. Appositional material surrounding haustoria from both control and treated plants stained positively for callose and cellulose but not for lignin.

Blue mold of tobacco, caused by *Peronospora tabacina* Adam, was epidemic in the United States in 1979 and caused an estimated crop loss of \$240 million (15). Blue mold has been controlled primarily by protective chemical sprays (2). Metalaxyl (Ridomil), an acylalanine systemic fungicide, has selective activity toward

oomyceteous fungi at low rates of application as either a soil drench or a foliar spray (29). Metalaxyl has been used to control parasites that cause downy mildews of hops (12), grapes (24), and tobacco (9) as well as diseases caused by *Phytophthora* spp. (1,3,4,7).

The mechanism of action of metalaxyl is not completely understood, but recent evidence indicated that metalaxyl inhibited nucleic acid synthesis (particularly RNA synthesis [6]) and protein synthesis in these fungi (8). Another study, however, indicated that protein synthesis was unaffected (14). Little information is available concerning the effects of metalaxyl on the fine structure of the host-pathogen relationship. Stossel et al (25) reported that metalaxyl affected the plasmalemma and mitochondrial cristae of *Phytophthora megasperma* Drechs. var. *sojae* Hildeb. infecting

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soybean. In a study of pea infected by *Peronospora pisi* Syd., effects of metalaxyl were described as more extensive fungal vacuolation and, more frequently, encased haustoria (11). In this study, we examined the effects of metalaxyl treatments on three aspects of the *P. tabacina*-*Nicotiana tabacum* L. host-parasite relationship: 1) germination, penetration, and early colonization of the tobacco leaf by *P. tabacina*; 2) sporulation by the pathogen; and 3) ultrastructure of the host-parasite interface.

MATERIALS AND METHODS

Inoculum production. The OPT 1 isolate of *P. tabacina* (22) was used for all experiments. The pathogen was maintained on Speight G-28 flue-cured tobacco (*N. tabacum*) grown under greenhouse conditions. Sporangia were collected in 50 ml of cold (4 C) distilled water and washed in an equal volume of cold distilled water before adjusting the final concentration of sporangia for individual experiments.

Influence of metalaxyl on germination, penetration, and colonization. Speight G-28 tobacco plants were grown under greenhouse conditions in 10-cm-diameter plastic pots containing a 3:1:1 (v/v) mixture of soil, sand, and peat. Ten plants in the four- to five-leaf stage of development were each treated with a 75-ml soil drench containing 42 μg a.i. of metalaxyl 2E per milliliter, whereas 10 additional plants each received 75 ml of distilled water only. Twenty-four hours after treatment, eight 1-cm² areas on the adaxial surface of the third leaf were inoculated with sporangia of the *P. tabacina* isolate in cold (4 C) distilled water. Sporangia were applied using a quantitative inoculator described by Schein (19). Four target areas on each leaf received 92 ± 8 sporangia each, whereas the other four areas received 69 ± 5 sporangia each. Ten metalaxyl-treated plants and 10 untreated plants were inoculated in a similar manner. In addition, five slides coated with 1% water agar in petri dishes were inoculated for each sporangium concentration. Inoculated plants and slides were maintained in a dark dew chamber at 21 C. Sporangial germination and subsequent infection of tobacco leaves were determined 24 hr postinoculation by excising the inoculated areas from five plants for each treatment. Leaf tissue was cleared and stained in hot (80 C) 50% (w/w) chloral hydrate-0.1% aniline blue and examined microscopically for sporangia, germinated sporangia, and ingress of the pathogen. The same procedure was used for the inoculated areas on the remaining five plants from each treatment group 48 hr after inoculation.

The effect of metalaxyl on *in vitro* sporangial germination was measured by pipetting 50 μl of a suspension containing 6×10^3 sporangia per milliliter into wells on sterilized glass depression slides. Equal volumes of sterile, distilled water or filter-sterilized solutions containing 10, 42, or 84 μg a.i. of metalaxyl per milliliter were added to the sporangial suspensions. The final concentrations of metalaxyl in the wells were 0, 5, 21, and 42 μg a.i./ml. Three replicates for each concentration of metalaxyl were prepared. Slides were placed in petri dishes containing moist filter paper and incubated in darkness at 21 C. Germination percentages based on 100 or more sporangia per slide were determined after 24 hr.

Influence of metalaxyl on sporulation of the pathogen. Speight G-28 tobacco plants in the four- to five-leaf stage of development were grown under conditions described previously. Plants were sprayed with a suspension of 2×10^3 sporangia per milliliter until moist, maintained in darkness under high relative humidity overnight, and subsequently moved to the greenhouse. Soil drenches consisting of 21 μg a.i. (0.5 kg/ha) or 42 μg a.i. (1 kg/ha) of metalaxyl 2E per milliliter were applied at 75 ml/plant to different groups of five plants for each rate of metalaxyl 1 day before inoculation or 3 or 7 days after inoculation. One group of five plants was inoculated but received only 75 ml of water per plant as a treatment. A randomized complete-block design with five replicates was used. Plants were maintained under greenhouse conditions (21 C) for 8 days after inoculation, then placed in 100% relative humidity overnight in darkness (21 C) to induce fungal sporulation. The next morning, sporangia produced on the third or fourth leaves of metalaxyl-treated and untreated plants were collected in 4 ml of water and counted with a hemacytometer.

Lesion area (cm²) was determined with an automatic area meter type AAA-5 (Hayashi Denko Co., Ltd., Tokyo, Japan). Ten 5-mm-diameter leaf samples from each group of plants were cleared and stained in a 50% (w/w) chloral hydrate-0.1% aniline blue solution. These samples were examined microscopically for the presence and condition of the fungus.

Ultrastructural effects of metalaxyl. Speight G-28 tobacco plants in the six- or seven-leaf stage were grown as described previously. Plants were inoculated with isolate OPT 1 as described and maintained under greenhouse conditions (21 C). Seven days after inoculation, chlorotic lesions had developed. The next day, a soil drench consisting of 75 ml/plant of 42 μg a.i. of metalaxyl 2E per milliliter was applied to plants, whereas other plants received 75 ml of distilled water. Thirty-six hours after the drench application, plants were maintained in the dark at 100% relative humidity to induce fungal sporulation. Sporulation was determined 48 and 72 hr after fungicide treatment by collecting the sporangia from six lesions on the third and fourth leaves taken from treated and untreated plants as described previously. Lesion area was determined as before. Sporulation was calculated as the average number of sporangia per square centimeter of lesion area. Samples for scanning electron microscopy (SEM) were collected from lesions on treated and untreated plants 12 hr after the fungus was induced to sporulate. Samples were fixed in 0.025 M, pH 7.2, phosphate-buffered 3% glutaraldehyde at 4 C, dehydrated in a graded ethanol series, transferred through a graded series of freon, and critical-point dried. Subsequently, samples were sputter-coated with gold-palladium and viewed with a Jeol T200 scanning electron microscope operated at 25 kV.

Samples for transmission electron microscopy (TEM) were obtained from plants treated as described for plants used for SEM, except the fungus was not induced to sporulate. Samples 3 mm in diameter, consisting of green and chlorotic tissue, were cut with a cork borer from lesion margins 6, 12, 24, 48, 96, and 168 hr after treatment with metalaxyl. Specimens were also taken from untreated plants in a similar manner. Leaf disks were immediately immersed in cold 0.025 M, pH 7.2, phosphate buffer, cut in half, and fixed in 0.025 M, pH 7.2, phosphate-buffered 3% glutaraldehyde at 4 C. Samples were postfixed in 4 C, 0.025 M, pH 7.2, phosphate-buffered 1% OsO₄ for 2 hr, dehydrated in a graded ethanol series, and embedded in Spurr's low-viscosity medium (21). Thin sections (700–900 Å) were cut with a diamond knife and mounted on 200-mesh grids. Sections were stained at room temperature (22 C) with uranyl acetate and lead citrate and viewed on a Jeol 100S electron microscope operated at 80 kV.

Samples for light microscopy and histochemical studies of the host-pathogen interface were fixed in formalin-aceto-alcohol (FAA), then embedded in Paraplast Plus (Sherwood Medical, St. Louis, MO) using a Fisher Histomatic Tissue Processor Model 166. Fifteen-micrometer sections were obtained and stained for 5 sec in safranin (in 50% ethanol), washed in water, and either flooded with 0.05% aniline blue in 0.03 M potassium phosphate (16) or 0.05% calcofluor in water. Sections were examined with a Nikon Optiphot microscope (HBO 50-Hg lamp) using an excitation filter (330–380 nm) and a 420-nm absorption filter for aniline blue-callose studies or an excitation filter (395–425 nm) with a 470-nm absorption filter for calcofluor-cellulose studies. Sections mounted in water only were examined for autofluorescence. Additional sections, not previously stained with safranin, were stained with toluidine blue (18) or phloroglucinol-HCl (13) for detecting lignin.

RESULTS

Sporangial germination on 1% water agar was 47 and 43% for sporangial concentrations of 92 and 69 sporangia per square centimeter, respectively. Metalaxyl did not affect germination of sporangia *in vitro*. Average germination percentages were 51.5, 52.1, 55.8, and 50.6 for metalaxyl concentrations of 0, 5, 21, and 42 μg a.i./ml, respectively. Sporangial germination on treated and untreated leaf surfaces was substantially lower, averaging about 1%. Sporangial germination was not affected by metalaxyl treatments. Penetration of epidermal cells was apparently

uninhibited by metalaxyl, but subsequent growth was limited to intracellular hyphae or vesicles (infection structures) within the epidermal cells. Intercellular hyphae and haustoria were observed in the leaf mesophyll of untreated plants 24 hr after inoculation. Similar results for both fungicide-treated and untreated plants were obtained 48 hr after inoculation (Table 1).

Tobacco plants treated with soil drenches (75 ml/plant) of 21 or 42 μg a.i. of metalaxyl per milliliter 1 day preinoculation or 3 days postinoculation did not show disease symptoms and fungal sporulation was not observed up to 10 days postinoculation. Microscopic examination of leaf tissue showed that no intercellular hyphae or haustoria were present. Plants treated 7 days postinoculation with 75 ml of 42 μg a.i. of metalaxyl per milliliter developed typical chlorotic lesions but fungal sporulation did not occur. Plants treated with 75 ml of 21 μg a.i./ml 7 days postinoculation and untreated plants both had sporulating lesions 10 days after inoculation. Lesions from untreated plants and plants receiving 21 and 42 μg a.i./ml averaged 3.00, 1.87, and 1.18 cm^2 , respectively. Lesions from untreated plants produced an average of 2.7×10^4 sporangia per square centimeter, whereas lesions from plants treated with 21 μg a.i./ml produced an average of 9.2×10^2 sporangia per square centimeter.

In another experiment, plants treated with 75 ml of 42 μg a.i. of metalaxyl per milliliter after disease symptoms had developed produced an average of 1.2×10^3 sporangia per square centimeter of lesion area 48 hr after treatment, whereas lesions from untreated plants produced an average of 2.85×10^4 sporangia per square centimeter. No sporulation was noted on treated plants 72 hr after application of the fungicide, whereas sporulation continued on untreated plants but at a reduced rate of 4.9×10^3 sporangia per square centimeter of lesion area.

When metalaxyl was applied to plants after lesions appeared, abundant sporangiophores were located on the abaxial surface of lesions 48 hr after treatment (Fig. 1). The sporangiophores were incompletely formed (Fig. 2) and sporangia that were produced were abnormally shaped (Fig. 3) compared with sporangia produced on untreated plants (Fig. 4).

Haustoria in untreated plants were encased with calloselike material and a densely staining extrahaustorial matrix occurred between the haustorial wall and the extrahaustorial membrane. All haustorial encasements fluoresced intensely after staining with aniline blue or calcofluor. The cytoplasm of haustoria as well as intercellular hyphae were vacuolated but contained nuclei and mitochondria (Fig. 5).

Ultrastructural effects of metalaxyl on *P. tabacina* were not seen until 24 hr after metalaxyl treatment. At this time, intercellular hyphae and haustoria appeared to be highly vacuolated and the nuclei were condensed. Mitochondria appeared unaffected (Fig. 6). Forty-eight hours after fungicide application, the cytoplasm of

some intercellular hyphae and haustoria was necrotic and organelles were absent except for condensed nuclei and dictyosomes (Fig. 7) (Table 2).

Forty-eight hours after fungicide treatment, haustoria, with one exception, were encased, and the encasements were of two types (Table 2). Seventy-three percent of the haustoria were encased by a single layer of amorphous appositional material occurring between the extrahaustorial matrix and the host plasmalemma (Fig. 6). The other haustoria were surrounded by a bilayered encasement (Fig. 7). The inner layer, juxtaposed to the extrahaustorial matrix, was similar to the material of the single-layered encasement observed surrounding haustoria in untreated tissue. The outer layer was separated from the inner layer by a membranelike structure (Fig. 8). The outer encasement was composed of host wall-like portions as well as membranelike portions (Fig. 8). All encasements fluoresced intensely after staining with aniline blue (callose) or calcofluor (cellulose or callose), and some stained positively for cellulose after staining with toluidine blue. The toluidine blue and phloroglucinol-HCl tests for lignin were negative.

DISCUSSION

Many sporangia were lost during clearing treatments of leaves; therefore, the germination rate was based on the calculated number of sporangia applied per square centimeter of leaf (Table 1). Fewer sporangia were recovered from samples receiving two clearing treatments, but the germination rate was similar to those of samples receiving only one clearing treatment. Because the germination rates are similar for both samples, it is apparent that clearing treatments do not affect the recovery of germinated sporangia; only ungerminated sporangia are lost.

The cuticular wax of *N. tabacum* leaves contains two macrocyclic diterpenes, α and β isomers of 4,8,13,-duvatrienne-1,3 diol, which inhibit the germination of *P. tabacina* sporangia (5). Therefore, it is not surprising that germination of sporangia on leaf surfaces was much lower than on water agar. Metalaxyl had no measurable effect on sporangial germination. The low germination rate of *P. tabacina* sporangia on tobacco leaf surfaces of both metalaxyl treated and untreated plants in this experiment was similar to the rates reported by Shepherd and Mandryk (20).

Growth of *P. tabacina* in plants treated with metalaxyl before inoculation was limited to the formation of vesicles or intracellular hyphae (infection structures) in penetrated epidermal cells; no intercellular hyphae or haustoria were observed. Staub and Young (24) reported that metalaxyl did not prevent penetration of tobacco roots by *Phytophthora parasitica* but subsequently inhibited fungal colonization of tissue. Similar results were obtained with *P. infestans* infecting tomato and *Plasmopara viticola* infecting grapes (23). Development of *P. tabacina* in untreated plants was

TABLE 1. Influence of metalaxyl on penetration and colonization of tobacco leaves by *Peronospora tabacina*^a

Treatment ^b	Incubation period (hr)	Calculated sporangium number/cm ² on leaf	Sporangia on leaf surface after clearing (%)	Sporangial germination ^c (%)	Progress of infection (% of germinated sporangia)		
					Germ tube only	Penetration of epidermal cell	Intercellular hyphae and haustoria present
Water	24	92	15.2	1.2	18	27	55
		69	24.6	1.5	10	70	20
Metalaxyl	24	92	15.9	0.9	50	50	0
		69	23.9	1.2	25	75	0
Water	48	92	5.5 ^d	0.9	25	25	50
		69	8.3 ^d	1.0	0	29	81
Metalaxyl	48	92	7.4 ^d	1.3	33	67	0
		69	5.1 ^d	1.0	63	37	0
Water agar	...	92	...	47.1 ^e
Water agar	...	69	...	42.6 ^e

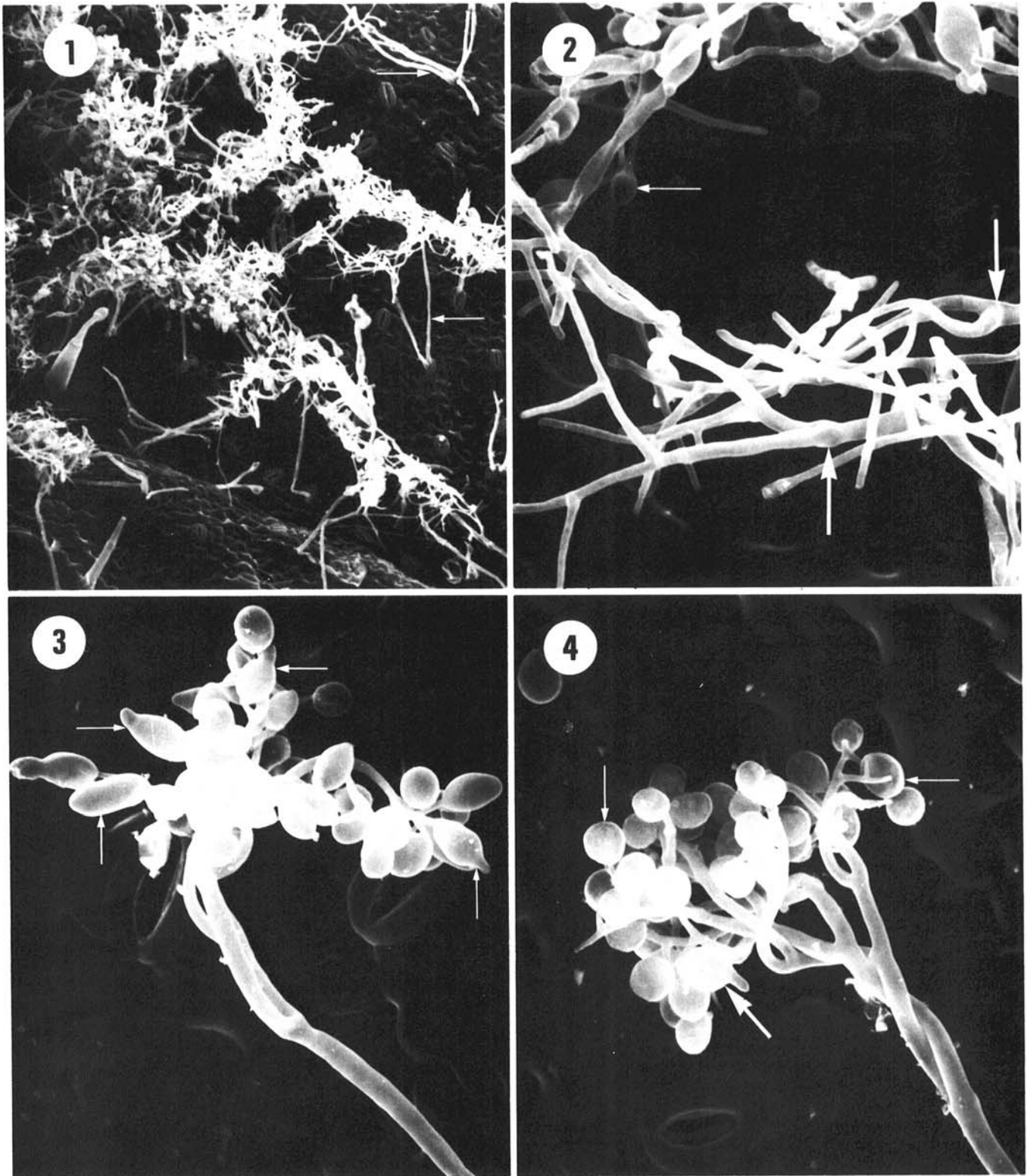
^a Based on five replicates of four samples for each sporangium concentration and time period.

^b Treatment of potted plants with either 75 ml of water or a 75-ml soil drench consisting of 0.42 μg a.i. of metalaxyl per milliliter 24 hr before inoculation.

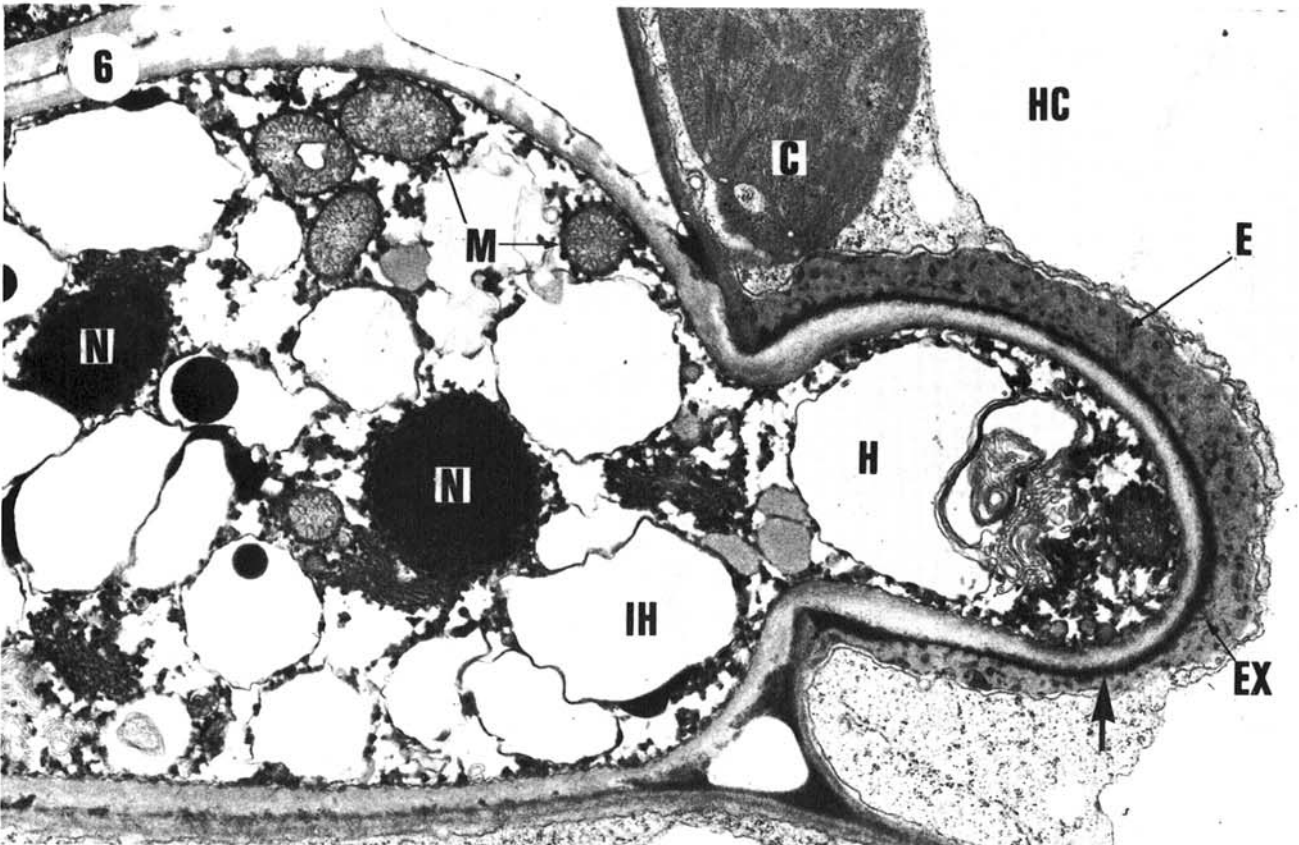
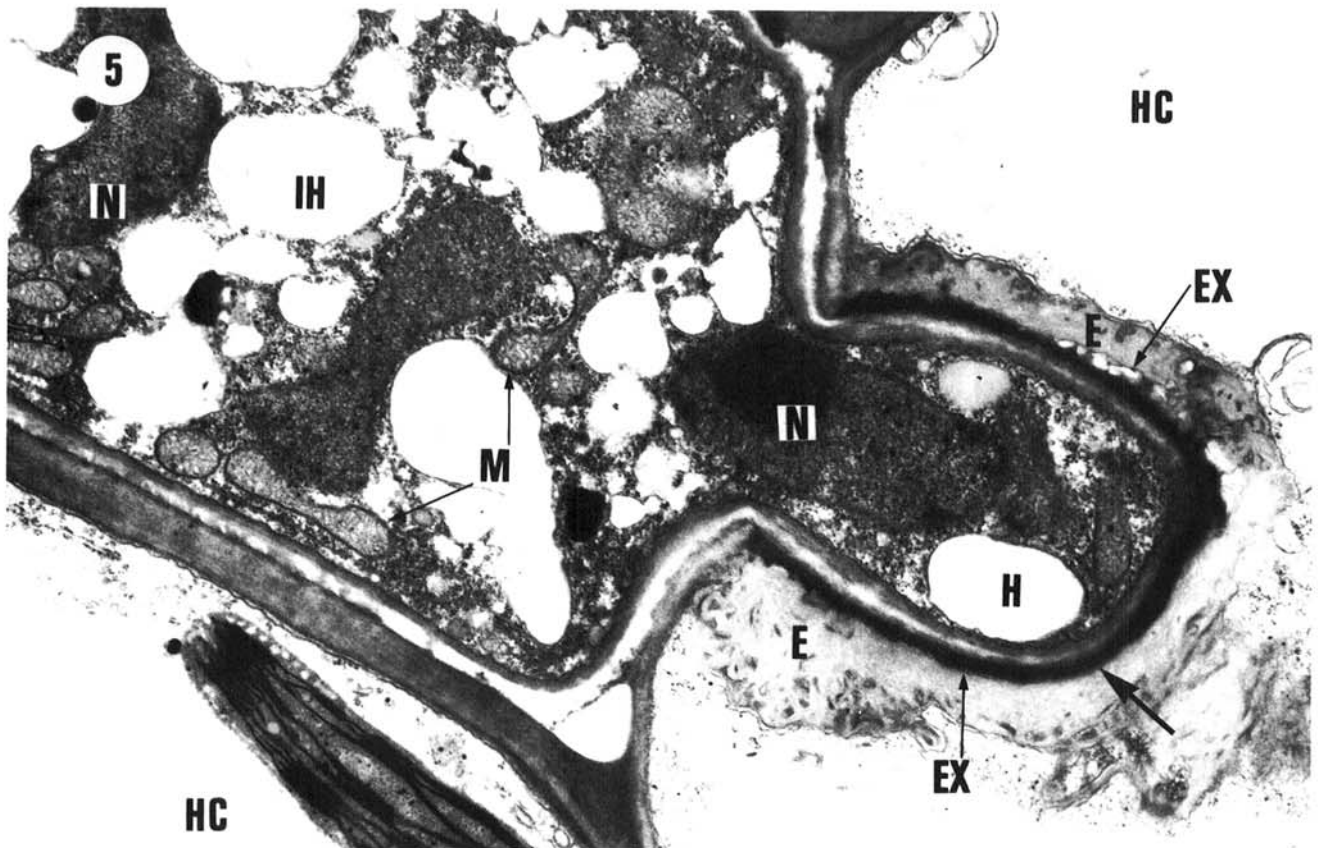
^c Based on calculated number of sporangia.

^d Received two clearing treatments.

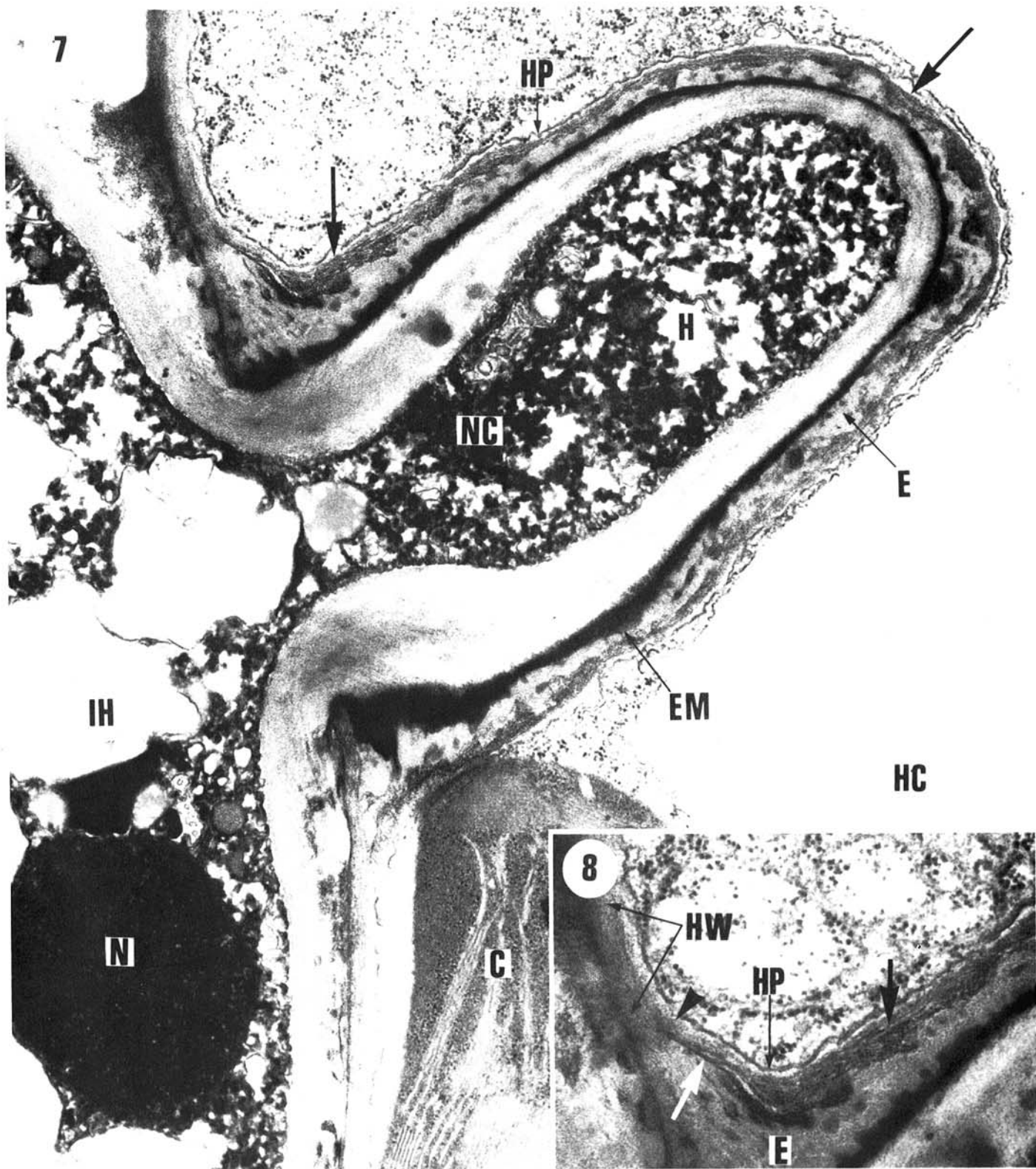
^e Mean of five replicates.



Figs. 1-4. Scanning electron micrographs of sporulation of *Peronospora tabacina* on metalaxyl-treated and untreated plants. **1.** Abaxial surface of a leaf 48 hr after metalaxyl treatment. Few sporangiophores (arrows) are visible ($\times 75$). **2.** Enlargement of an area shown in Fig. 1. Sporangiophores (large arrows) are incompletely formed. A sporangium (small arrow) is present ($\times 450$). **3.** Sporangiophore produced on a metalaxyl-treated plant. Note abnormally shaped sporangia (arrows) ($\times 385$). **4.** Two sporangiophores produced on an untreated tobacco plant. Small arrows point to sporangia. Large white arrow points to a sporangium with a tip of the sporangiophore attached ($\times 450$).



Figs. 5 and 6. Transmission electron micrographs of the *Peronospora tabacina*-*Nicotiana tabacum* interface. **5**, Longitudinal section through an intercellular hypha (IH) and a haustorium (H) in a host mesophyll cell (HC) from a plant 9 days after inoculation but not treated with metalaxyl. The haustorium is surrounded by an electron-dense extrahaustorial matrix (large arrow). An extrahaustorial membrane (EX) lies between the matrix and a single-layered encasement (E). The cytoplasm contains numerous ribosomes, mitochondria (M), and several nuclei (N) ($\times 16,7500$). **6**, Longitudinal section through an intercellular hypha (IH) and a haustorium (H) in a host mesophyll cell (HC) in a tobacco plant 9 days after inoculation and 24 hr after metalaxyl treatment. The haustorium is surrounded by an electron-opaque extrahaustorial matrix (large arrow) and a single-layered encasement (E). Both the intercellular hypha and haustorium are highly vacuolated and the nuclei (N) in the intercellular hypha are condensed. Membranes of the mitochondria (M) are intact. C = chloroplast and EX = extrahaustorial membrane ($\times 14,260$).



Figs. 7 and 8. Transmission electron micrograph of the *Peronospora tabacina*-*Nicotiana tabacum* interface 10 days after inoculation and 48 hr after metalaxyl treatment. **7**, Slightly oblique longitudinal section through an intercellular hypha (IH) and a haustorium (H) in a host cell (HC). The cytoplasm of the fungus is necrotic (NC) and a condensed nucleus (N) is present in the intercellular hypha. The haustorium is surrounded by an electron-dense extrahaustorial matrix (EM) and a bilayered encasement. E = inner layer of encasement (larger arrows point to the outer layer of the encasement) and C = chloroplast ($\times 33,500$). **8**, Enlargement of an area shown in Fig. 7. The haustorium is surrounded by a bilayered encasement. The inner layer of the encasement (E) is composed of amorphous material bounded by a membranelike structure (white arrow); the outer layer of the encasement is composed of host wall-like portions (arrowhead) and membranelike portions (black arrow). HP = host plasmalemma and HW = host wall ($\times 54,200$).

TABLE 2. Effects of metalaxyl on the ultrastructure of haustoria and intercellular hyphae of *Peronospora tabacina* infecting *Nicotiana tabacum*^a 48 hr after application^b

Treatment	Haustoria with increased vacuolation or necrosis (% of total)	Encasement type (% of total)			Intercellular hyphae with increased vacuolation or necrosis (% of total)
		Single layer	Double layer	None	
Metalaxyl ^c	52	73	25	2	77
No metalaxyl	11	94	6	0	7

^a Plants inoculated 8 days before treatment with metalaxyl.

^b Based on the observation of 70 haustoria and 81 intercellular hyphae.

^c Metalaxyl applied as a 75-ml soil drench consisting of 42 µg a.i./ml.

similar to that reported by other investigators (10,17,27,28).

Application of metalaxyl to the soils of plants showing chlorotic lesions reduced lesion expansion and sporulation of the pathogen. These results indicate that the fungicide was acropetally translocated to the leaves (3,7,24). Similar curative or therapeutic properties of metalaxyl have been reported by other investigators (4,11,24,25). Sporangiothores that formed on metalaxyl-treated plants were often incompletely developed or had altered morphology. The fungicide may affect the sporulation process directly, as suggested by Hickey and Coffey (11). Furthermore, many sporangia were morphologically atypical compared with sporangia produced on control plants. Bruck et al (4) reported reduced viability of *P. infestans* sporangia produced on metalaxyl-treated potato plants. Further research is necessary to determine whether the morphological abnormalities observed are indicative of changes in viability or virulence of the sporangia.

In plants infected several days before metalaxyl treatment, ultrastructural effects on the pathogen were seen as early as 24 hr after application of the fungicide. These changes included increased vacuolation and condensation of nuclei in intercellular hyphae and haustoria. Necrotic hyphae and haustoria were observed occasionally. Other organelles, such as mitochondria and plasmalemma, appeared unaffected. Hickey and Coffey (11) also reported that metalaxyl treatment of *Pisum sativum* L. systemically infected with *Peronospora pisi* caused increased vacuolation and necrosis of haustoria and hyphae but did not affect other fungal organelles. However, metalaxyl has been reported to affect the plasmalemma and mitochondrial cristae of *Phytophthora megasperma* var. *sojae* infecting soybeans (25). Inasmuch as metalaxyl is reported to inhibit RNA synthesis (6,8) and possibly protein synthesis, the ultrastructural changes in the pathogen induced by metalaxyl may be secondary effects.

With one exception, haustoria in metalaxyl-treated, infected plants and untreated, infected control plants were encased. Bilayered encasements of haustoria were four times more frequent in treated plants than in control plants. Hickey and Coffey (11) also observed multiple layers of appositional material surrounding haustoria of *P. pisi* infecting pea plants after treatment with metalaxyl. They suggested that necrosis of the haustorial cytoplasm was the direct result of haustorial encasement. However, the haustoria of *P. tabacina* infecting untreated tobacco plants are encased and these haustoria were not necrotic (26-28). Perhaps the fungistatic nature of metalaxyl (3,11) allowed more time for the host to deposit additional material around the haustorium. Although the different layers of appositional material are ultrastructurally dissimilar, no differences in chemical composition could be detected between encasements from untreated and treated plants using the histochemical techniques described. Encasements from treated and untreated plants were positive for cellulose and callose, but lignin was not detected.

This study demonstrates that metalaxyl provides protective and therapeutic action against *P. tabacina* infecting tobacco plants. The rapid acropetal translocation of the fungicide to the foliage quickly suppresses sporulation and thus may be epidemiologically important in limiting dissemination of airborne sporangia.

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