Light and Electron Microscopy of Phytophthora Rot in Soybeans Treated With Metalaxyl

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ABSTRACT

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Hypocotyls of soybean seedlings were inoculated with suspensions of zoospores of *Phytophthora megasperma* var. *sojae* after overnight immersion of the roots in a solution (2 μg/ml) of the systemic fungicide metalaxyl or water. Lesions were excised after 12 or 24 hr of incubation and examined by light and electron microscopy. Treatment with metalaxyl greatly decreased hyphal branching and spread in the host; however, relative numbers of haustoria were not reduced significantly. In contrast to controls, cytoplasm in hyphae in treated seedlings usually did not stain with toluidine blue. Changes in fungal ultrastructure included: increased convolution of the plasmalemma; separation of the plasmalemma from the hyphal wall, leaving protrusions and vesicles in the intervening space; and disintegration of cristae in mitochondria. The cytoplasm near hyphal tips in treated seedlings appeared to remain healthy and the fungus continued to grow slowly between 12 and 24 hr as indicated by increased numbers of penetrated host cells. In both treated and control seedlings, haustoria usually were encased by extrahaustorial matrix only. Structure of wall appositions was not influenced by treatment with metalaxyl. Most host cells in lesions were necrotic regardless of treatment. It appears that metalaxyl acts primarily on the fungus and that necrosis and glyceollin production associated with metalaxyl treatment is a secondary effect.

There have been relatively few ultrastructural studies of the effects of fungicides on plant disease development. These have included: oxycarboxin on bean rust (17), chrysanthemum rust (12), and oat crown rust (19); benomyl on oat crown rust (19); benomyl and fenarimol on apple scab (11); and metalaxyl on pea downy mildew (10). The most frequently reported effects were development of encasements around haustoria, swelling and disruption of mitochondria, and vacuolation of fungal cytoplasm.

In this paper, the interaction between soybeans (*Glycine max* L. Merr.) and *Phytophthora megasperma* Drechs. var. *sojae* Hildeb. (*Pms*) following treatment of seedlings with the systemic fungicide metalaxyl (*N*-[2,6-dimethylphenyl]-*N*-[methoxyacetyl]alanine methyl ester) is examined by light and electron microscopy. Application of metalaxyl to roots of seedlings prevents disease development in hypocotyls inoculated with zoospores of *Pms* (22). Instead of typical susceptible water-soaked lesions, restricted brown necrotic lesions resembling those in interactions with incompatible races of *Pms* developed in treated seedlings. Production of the phytoalexin, glyceollin, also increased to levels usually associated with incompatible interactions. Similar effects have been described in the treatment of other diseases with some systemic fungicides (5–7,11,14,15). It is possible that in these cases normal defense responses are activated and that these contribute to inhibition of the pathogen (4,22).

MATERIALS AND METHODS

Seeds of soybean cultivar Altona and the culture of Pms race 6 (compatible) were kindly supplied by R. I. Buzzell, Agriculture Canada, Harrow, Ontario. Procedures for the growth of 6-day-old etiolated soybean seedlings have been described previously (23). In the growth chamber, seedlings were treated prior to inoculation by immersing the roots overnight (~15 hr) in a solution of metalaxyl in distilled water (2 μg/ml). Controls received distilled water only. Hypocotyls were inoculated by placing 10-μl droplets of zoospore suspension (1×10^5 zoospores per milliliter) on the uppermost 2–3 cm, where typical cultivar-race-specific reactions occur (16), and were incubated at 25 C in the dark for 12 and 24 hr, respectively.

For light microscopy, lesions of infected hypocotyls were excised with a razor blade, cut in half and processed according to methods given in detail earlier (20). For electron microscopy, the infected tissues were dissected into ~1-mm pieces and subsequently fixed, embedded, sectioned, and stained as described elsewhere (21). Sections were examined in a Jeol JEM-100 S transmission microscope operated at 60 kV.

RESULTS

Light microscopy. Examination of cross sections from several lesions in a number of hypocotyls indicated that fungal growth was substantially reduced in metalaxyl-treated seedlings. In control seedlings, without fungicide, hyphae were found 300–350 μm below the epidermis 12 hr after inoculation, and up to about 800 μm 24 hr after inoculation with abundant growth up to 450 μm. In contrast the fungus in treated plants grew only to a depth of 30–90 μm in the first 12 hr and not more than to 150 μm by 24 hr. Hyphal branching also was reduced. The total numbers of hyphae and haustoria per section were much smaller in metalaxyl-treated than in control seedlings (Table 1). However, the proportion of intracellular to intercellular hyphae either was not consistently altered by the treatment or was only slightly decreased. At 12 hr after inoculation only a few host cells were penetrated both in treated and control seedlings.

Numerous hyphae in control seedlings stained blue with toluidine blue and showed distinct vacuoles of varying size and other details. Cytoplasm in hyphae in metalaxyl-treated seedlings was usually unstained or only slightly stained and appeared to be granular; vacuoles rarely were seen. The majority of host cells in lesions in both treated and untreated seedlings were necrotic. Some of the necrotic cells in treated seedlings stained greenish blue.

Electron microscopy. In metalaxyl-treated hypocotyls the fungal plasmalemma was abnormally convoluted by 12 hr after inoculation (Fig. 1). Frequently it was detached from the hyphal wall and the space between was filled with vesicles (Fig. 2) or with long narrow protrusions from the plasmalemma (Fig. 3). Cristae in fungal mitochondria often were barely detectable or had

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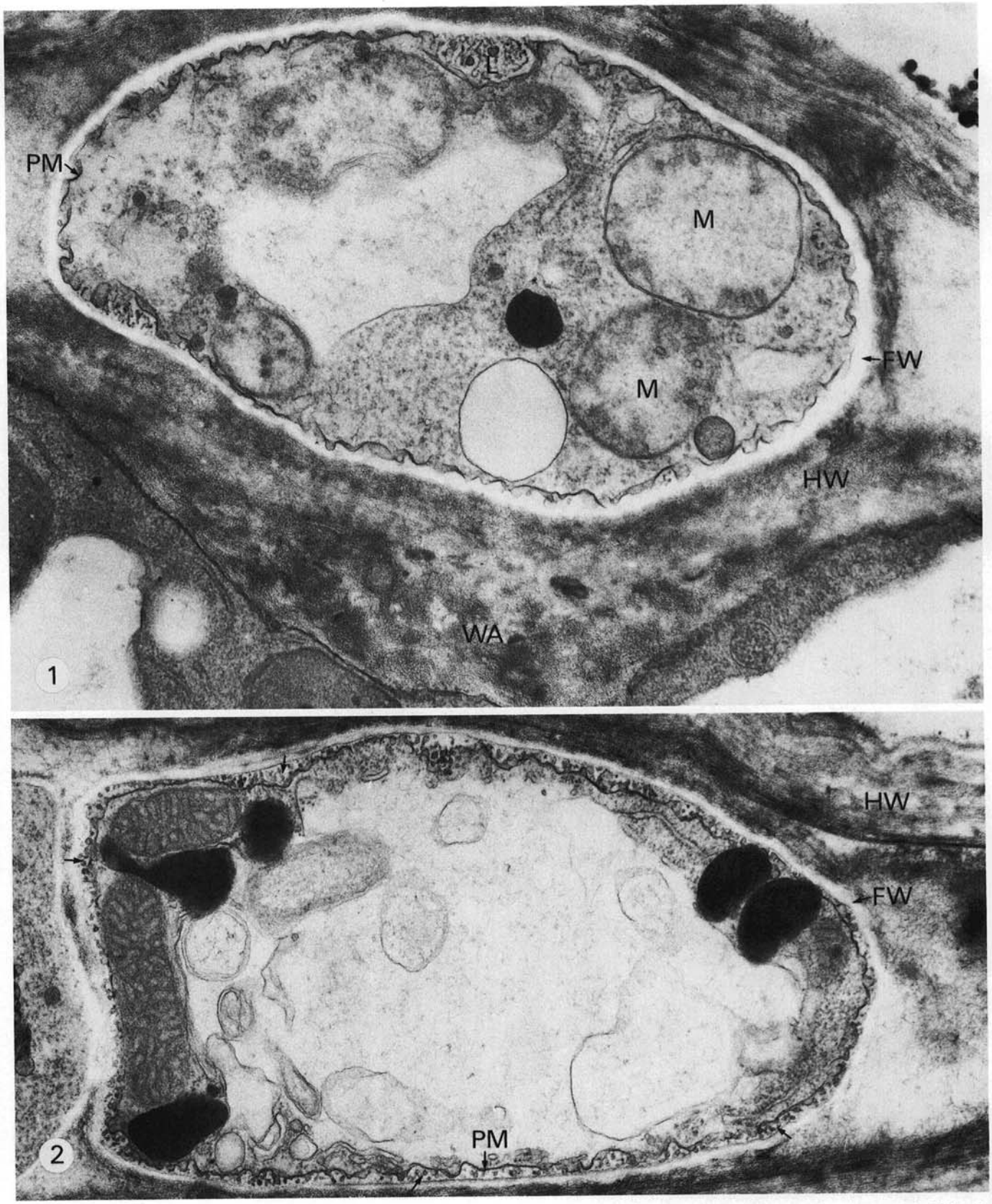
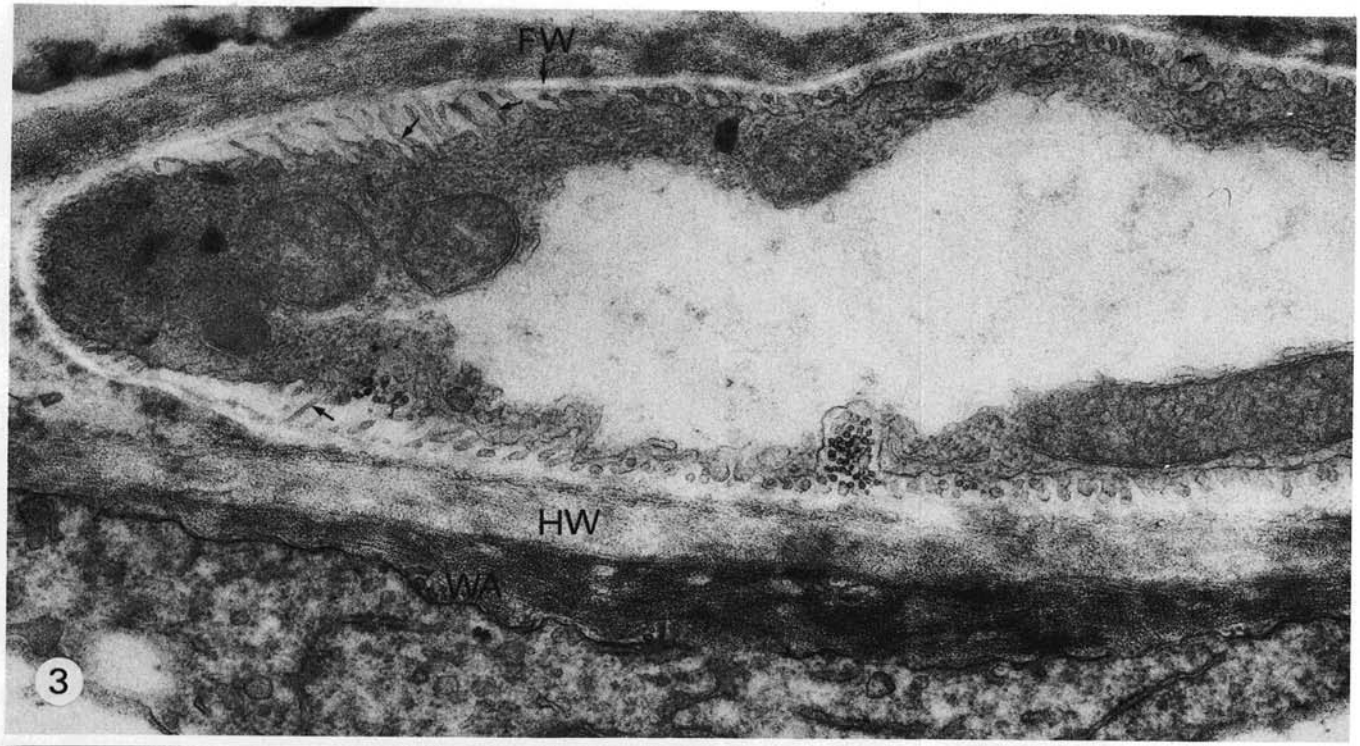
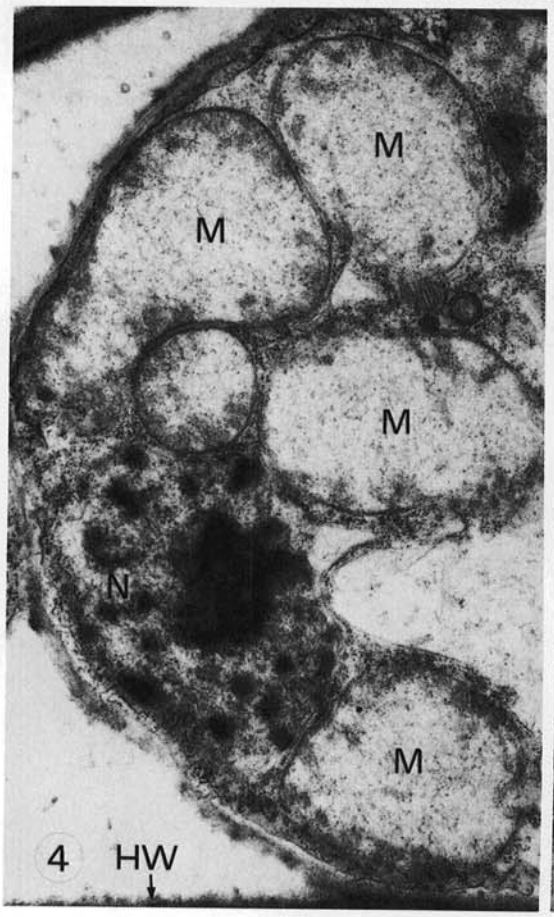
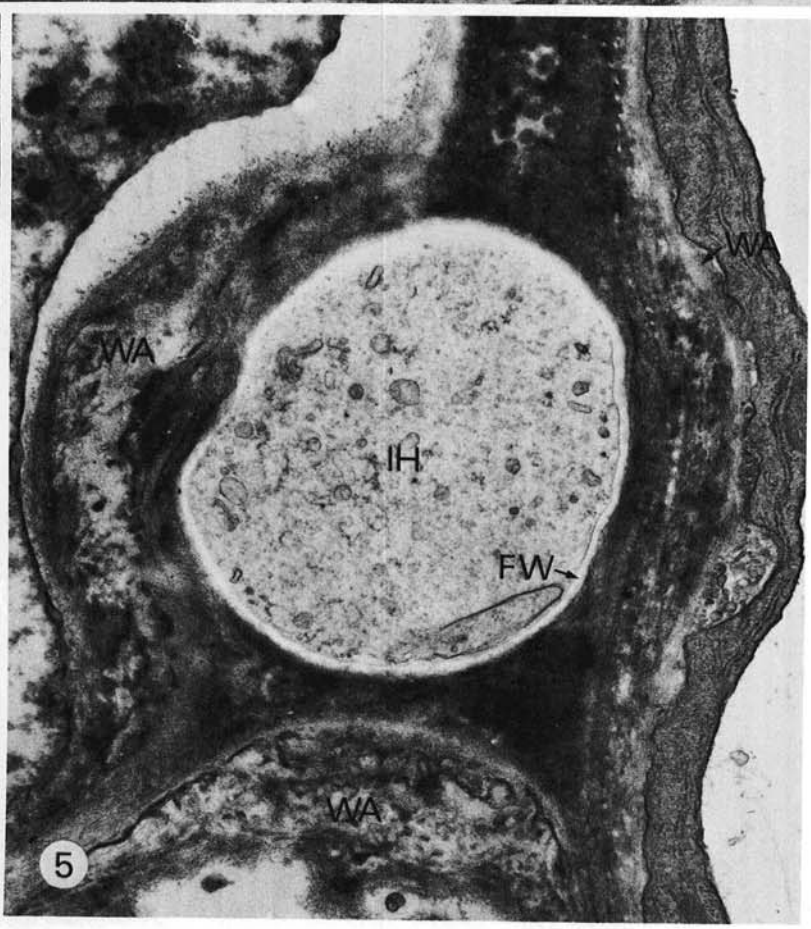


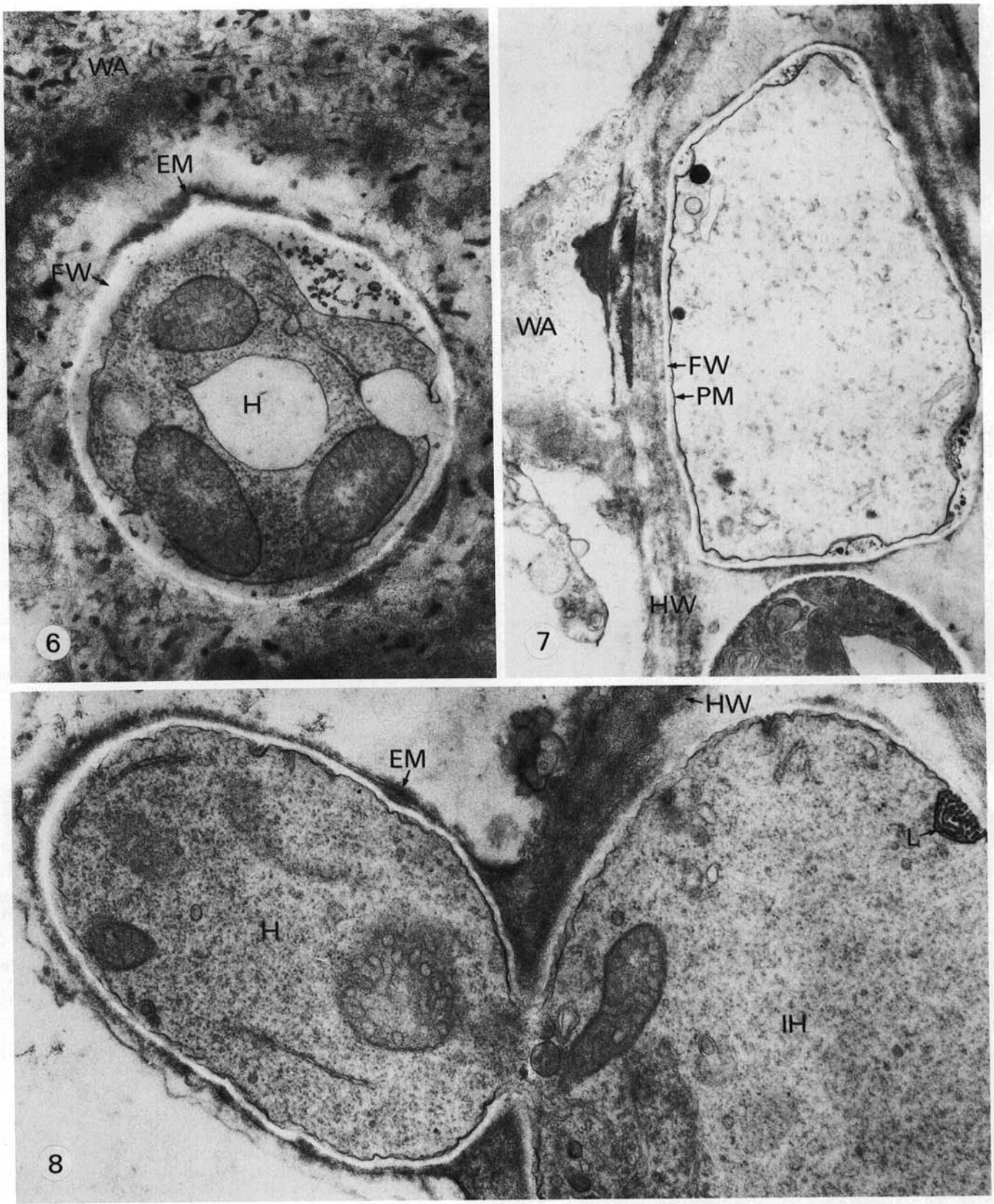
Figs. 1 and 2. Electron micrographs of intercellular hyphae of *Phytophthora megasperma* var. *sojae* in metalaxyl-treated soybean hypocotyls 12 hr after inoculation. **1,** The fungal plasmalemma is convoluted unusually and slightly detached from the hyphal wall (×28,000). **2,** Fungal plasmalemma detached from hyphal wall; the separation zone contains numerous vesicles which are possibly protrusions of the plasmalemma (arrows) (26,000). FW = fungal wall, HW = host cell wall, L = lomasome, M = mitochondria, PM = plasmalemma, and WA = wall apposition.







Figs. 3–5. Electron micrographs of intercellular hyphae of *Phytophthora megasperma* var. *sojae* in metalaxyl-treated soybean hypocotyls, 12 hr after inoculation. **3,** Fungal plasmalemma detached from the hyphal wall with numerous protrusions of the plasmalemma (arrows) into the separation zone (×35,000). **4,** Hypha with mitochondria lacking cristae and degenerating nucleus (×26,000). **5,** Hypha containing only vesicles and fragments of membranes (×19,200). FW = fungal wall, HW = host cell wall, IH = intercellular hypha, M = mitochondria, N = nucleus, and WA = wall apposition.



Figs. 6–8. 6, Electron micrograph of encased haustorium in a metalaxyl-treated hypocotyl 12 hr after inoculation with zoospores of *Phytophthora megasperma* var. *sojae* (×31,500). **7 and 8,** Electron micrographs of hypha and haustorium, respectively of *Phytophthora megasperma* var. *sojae* 24 hr after inoculation of untreated soybean hypocotyls. **7,** Note the intact fungal plasmalemma (×18,000). **8,** Haustorial encasement, consisting of extrahaustorial matrix only (×26,400). EM = estrahaustorial matrix, FW = fungal wall, H = haustorium, HW = host cell wall, IH = intercellular hypha, L = lomasome, PM = plasmalemma, and WA = wall apposition.

disappeared completely (Figs. 1 and 4), although the outer membranes were often intact (Figs. 1 and 4). In some instances, nuclear degeneration was observed (Fig. 4). In older parts of hyphae, the cytoplasm was disintegrated and only vesicles and fragments of membranes remained (Fig. 5). Hyphae in metalaxyl-treated seedlings fixed 24 hr after inoculation showed similar features, although near the tips the cytoplasm still appeared to be relatively intact. Between 12 and 24 hr the number of host cells that were penetrated increased. Most haustoria were surrounded by extrahaustorial matrix (3) only, although some were encased by wall appositions (Fig. 6). The latter varied in composition as also did those formed in apparently uninvaded cells (Figs. 1, 3, and 5). The cytoplasm of very few penetrated cells remained intact.

In untreated soybean seedlings, the fungal plasmalemma was only slightly convoluted and remained attached to the hyphal wall, even in hyphae with highly vacuolated or disintegrated cytoplasm (Fig. 7). Usually, haustoria were encased only by extrahaustorial matrix (Fig. 8). Most penetrated host cells and the majority of uninvaded host cells within the lesion were necrotic. As in metalaxyl-treated hypocotyls, wall appositions were composed of electron-translucent material with dark inclusions and/or moderately electron-dense material (Fig. 7).

DISCUSSION

Limited necrotic lesions and glyceollin levels similar to those produced in incompatible interactions develop in metalaxyl-treated seedlings 12 hr after inoculation with *Pms* (22). The observations reported here indicate that growth is not stopped at this stage, but continues slowly and cytoplasm in hyphal tips appears to remain alive for at least 24 hr. This is consistent with the in vitro fungistatic (rather than fungicidal) action of metalaxyl against *Pms* (*unpublished*) and other fungi (2). In incompatible interactions also, individual intercellular hyphae continue to spread even after the resistant response appears to be well established (20). However, in comparison to compatible controls, relative numbers of haustoria are not reduced in metalaxyl-treated tissue as they were in the incompatible interaction (20); hence, metalaxyl treatment does not appear to duplicate the normal resistant response.

The most conspicuous effect of metalaxyl treatment on the ultrastructure of the fungus was the development of irregular convolutions in the plasmalemma and the detachment of the plasmalemma from the hyphal wall, leaving numerous protrusions and vesicles (possibly protrusions in cross section) in the separation zone. Occasional breakdown of the plasmalemma was observed previously (21, Fig. 7) associated with age-related resistance in soybean hypocotyls. Possibly, glyceollin or other inhibitory factors of host origin could have contributed to the effects observed here. Some of the other changes such as disintegration of cristae have been described in oxycarboxin-treated bean rust and oat crown rust (17,19). Benomyl also caused damage to fungal mitochondria and nuclear membranes in both the oat crown rust (19) and apple scab (11) pathogens. In view of the widely different biochemical modes of action of oxycarboxin (24), benomyl (9), and (presumably) metalaxyl, which unlike the other two is active against the oomycetes (18), disintegration of mitochondria may be an early and general response to fungitoxic materials. Changes in haustorial encasements described by Hickey and Coffey (10) in metalaxyl-treated peas infected with *Peronospora pisi* were not observed in this study.

Most host cells in lesions in metalaxyl-treated and control seedlings were necrotic. Some of the cells in metalaxyl-treated seedlings apparently reacted hypersensitively as in race-specific incompatible interactions, staining greenish blue with toluidine blue (20). However, metalaxyl did not appear to cause changes in the ultrastructure of infected or uninfected host cells and presumably it acts directly on the fungus. Presumably, necrosis and associated production of glyceollin are secondary effects possibly due to release of elicitors from damaged hyphae (1,13), failure to produce a race specific suppressor or compatibility factor (8), or simply due to a reduction in growth rate thus allowing host cells sufficient time to develop a resistant-type response. Previous observations provide some support for the last possibility, for compatibility between individual host cells and hyphae appears to be at the most a transitory phase only (21).

TABLE 1. Comparison of intercellular and intracellular growth of *Phytophthora megasperma* var. *sojae* race 6 (compatible) in 6-day old metalaxyl-treated and untreated soybean hypocotyls of cultivar Altona 24 hr after inoculation

Treatment[b]	Lesion	Mean number[a] of: Intercellular hyphae	Haustoria and intracellular hyphae	Intercell. hyphae: haustoria and intracell. hyphae[c]
Metalaxyl	1	18.6 ± 5	4.8 ± 2	
	2	12.0 ± 5	1.4 ± 1	5.3 ± 2.8:1
	3	19.2 ± 6	5.4 ± 3	
None	1	77.0 ± 24	29.8 ± 7	
	2	56.0 ± 15	20.0 ± 7	2.9 ± 0.4:1
	3	233.0 ± 38	70.4 ± 11	

[a] The values are the means and standard deviations of the numbers of intercellular hyphae (or of intracellular hyphae and haustoria) in five randomly selected cross sections in each lesion examined. The hypocotyls were inoculated by placing 10-μl droplets of zoospores suspension (1×10^5 spores per milliliter) on a 2-cm-long segment below the cotyledons.

[b] Roots of hypocotyls receiving fungicide treatment were immersed in 2 μg metalaxyl per milliliter of distilled water for 15 hr prior to inoculation; roots of hypocotyls used as control were immersed in distilled water only.

[c] Averages for the three lesions of the ratios of the numbers of intercellular hyphae to intracellular hyphae and haustoria, with the number of the latter adjusted to 1.

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