Electron Microscope Study of Race-Specific and Age-Related Resistant and Susceptible Reactions of Soybeans to *Phytophthora megasperma* var. *sojae*

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ABSTRACT

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Six-day-old soybean (*Glycine max* 'Altona') hypocotyls were inoculated with zoospores of race 6 or race 4 of *Phytophthora megasperma* var. *sojae* either at the top (youngest tissue), which is suceptible to race 6 but resistant to race 4, or at the bottom (older tissue), which is resistant to both races. In all cases a range of host cell responses was observed. These included necrosis of host cells without evidence of fungal invasion and the production of wall appositions with and without fungal invasion and necrosis. In a small number (<5%) of cells in tissue inoculated with race 6, cell invasion occurred without wall apposition formation or necrosis. These were the only instances in which the interaction between individual host cells and hyphae was compatible. Thus, compatibility is either a rare event or one of short duration. Differences between the susceptible reaction to race 6 at the top of the hypocotyl and the resistant reaction to this race at the bottom appeared to lie chiefly in the greater proportion of uninvaded, necrotic cells at the bottom, especially in the epidermis and cell layers immediately beneath. The interactions with hyphae of race 4 were similar to those with race 6 except that no instances of invasion without necrosis were observed. At the bottom of the hypocotyl, hyphae of this race were restricted to the surface cell layers. It is concluded that the majority of hypocotyl cells react in an incompatible manner to hyphae of both races; only at the top of the hypocotyl following inoculation with race 6 is the frequency of incompatible reactions sufficiently reduced or delayed that visibly susceptible symptoms develop.

A number of ultrastructural studies of pathogenesis by *Phytophthora* spp. in various host plants has been published (2–4, 7–9, 11, 16, 17) but only two deal with Phytophthora rot of soybeans (11,17). Slusher et al (17) examined only the compatible interaction in roots immersed for 3 days in zoospore suspension. Klarman and Corbett (11) examined both compatible and incompatible interactions with races of the causal fungus, *Phytophthora megasperma* var. *sojae* (*Pms*), but their study differed from ours in that hypocotyls were wound-inoculated and tissues were taken for fixation at a much later stage in disease development.

Compatible interactions of soybean cultivars with races of *Pms* are characterized by rapidly spreading water-soaked lesions; incompatible interactions are characterized by restricted necrotic lesions and considerable accumulation of the phytoalexin, glyceollin (12). Recently, we reported that in intact 6-day-old hypocotyls such characteristic race-specific responses are confined to the young tissues at the top of the hypocotyl (13). In the older tissues at the bottom of the hypocotyl all races cause an incompatible interaction with the development of typical hypersensitivity and necrotic lesions. These observations and our light-microscopic (18) and physiological studies (13,20) suggest that the incompatible response, whether to the compatible race in the older tissue or to the incompatible race in both young and older tissue, was basically the same and differed only in degree. Results of the electron microscopic study reported here provide support for these conclusions and indicate that differences between the susceptible and resistant responses are quantitative rather than qualitative.

MATERIALS AND METHODS

The growth of 6-day-old etiolated seedlings of soybean (*Glycine max* (L.) Merr. 'Altona') and the production of zoospore inoculum of races 4 (incompatible) and 6 (compatible) of *Pms* have been described (19). The hypocotyls were inoculated either at the top (1.5 cm below the cotyledons) or at the bottom (2 cm above the roots) by placing four closely spaced drops (10 μl) of zoospore suspension on the hypocotyl surface, as described previously (19).

After incubation at 25 C in the dark for 17 hr, infected areas from several seedlings were excised to a depth of 0.5–1.0 mm and dissected into 1-mm pieces under 50 mM sodium cacodylate buffer, pH 7.2. The pieces were vacuum-infiltrated in 2.5% glutaraldehyde in the same buffer. The solution was changed and fixation was continued for 1 hr at room temperature. After rinsing several times in buffer, postfixation was done with 1% OsO_4 in 50 mM sodium cacodylate buffer (pH 7.2) for 1 hr at 4 C. The material was dehydrated in ethanol and embedded in a mixture of Epon, Araldite, and dodecenylsuccinic anhydride (14).

Embedded pieces were examined by light microscope and three samples with 6–12 penetrating hyphae per 1–2 mm^2 of epidermal surface were selected for each of the four interactions. Ultrathin sections were cut parallel to the epidermis with glass knives on a Sorvall Ultramicrotome MT 5000. Sectioning was continued to the limit of fungal colonization (up to 350 μm beneath the epidermis, depending on the interaction [18]). Sections were mounted on uncoated 74-μm (200-mesh) copper grids, stained with 1% uranyl acetate in distilled water and with lead citrate (15), and examined in a Jeol JEM-100S transmission electron microscope operated at 60 kV.

RESULTS

***Pms* race 6 (compatible).** At the top of the hypocotyl, race 6 rarely invaded epidermal cells. Wall appositions were seen frequently both in collapsed cells and also in apparently healthy uninvaded cells in the epidermis and other tissues (Figs. 1 and 2), suggesting that attempted penetrations may have aborted at an early stage. Wall deposits sometimes were composed of several layers (Fig. 2): an electron-dense region on the wall, termed the penetration matrix by Hickey and Coffey (5); electron-translucent material, interspersed with denser material, probably remnants of cytoplasm (7); and marginal electron-opaque material. The complexity of the wall appositions varied considerably (Figs. 1,2,4,6). In other cells only the penetration matrix had developed

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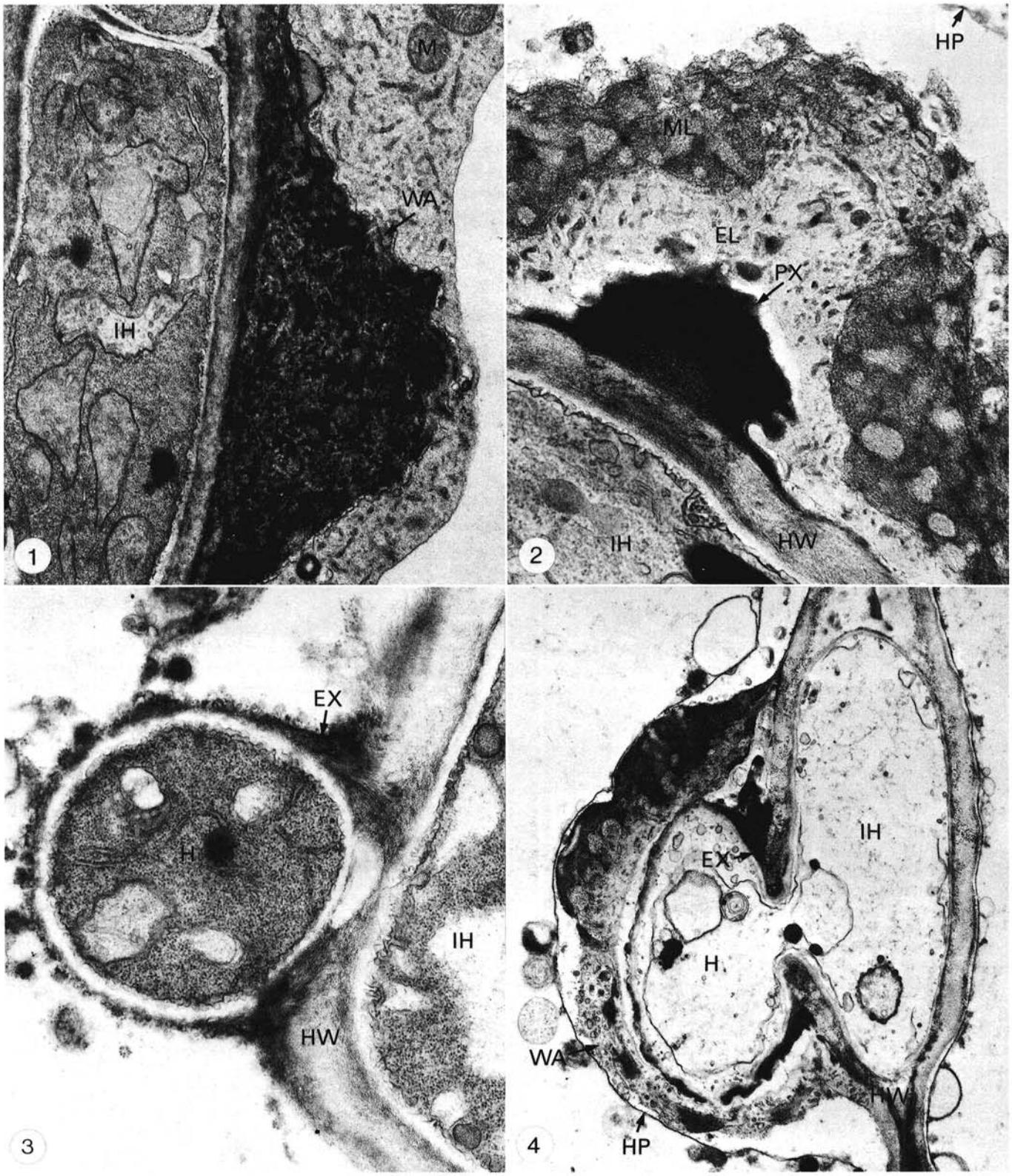
prior to disintegration of the host cytoplasm.

Many cells beneath the epidermis contained haustoria. Wall appositions, however, occurred less frequently in these cells, which were usually necrotic, than in cells in which penetrations were not observed (Table 1). Haustoria in necrotic cells, either with or without wall appositions, were always surrounded by an electron-opaque extrahaustorial matrix (Figs. 3 and 4). There were relatively few penetrated cells that were not necrotic (Table 1, Figs. 5 and 6). In these cells haustoria were separated from the intact cytoplasm by extrahaustorial matrix, invaginated plasmalemma, and little or no



Figs. 1–4. Electron micrographs of susceptible tissues from soybean hypocotyls inoculated at the top with *Phytophthora megasperma* var. *sojae* race 6 (compatible). **1,** Wall apposition in host cell with intact cytoplasm (×14,800). **2,** Wall apposition with electron-translucent and marginal electron-opaque layer in a necrotic cell (×28,500). **3,** Necrotic host cell with a haustorium surrounded only by extrahaustorial matrix and remnants of host cytoplasm (×25,000). **4,** Haustorium in necrotic cell fully enclosed by wall apposition (×12,000). EL = electron-translucent layer of wall apposition. EX = extrahaustorial matrix, H = haustorium, HP = host plasmalemma, HW = host cell wall, IH = intercellular hypha, M = mitochondrion, PX = penetration matrix (5), WA = wall apposition.

wall apposition (Fig. 5), or they were completely encased by wall apposition (Fig. 6). Although many cells without evident penetration were necrotic, other cells in the same lesion, frequently with closely associated hyphae, remained unharmed (Fig. 1).

In the interaction with the compatible race 6 at the bottom of the hypocotyl, most cells in the epidermis and the cell layer beneath were necrotic. Haustoria in these cells were surrounded only by an extrahaustorial matrix and the invaginated host plasmalemma or its remnants (Fig. 7). Some invaded cells remained intact, and the haustorium was encased in a wall apposition. Deeper in the tissue (approximately 100 μm) many cells were seen with intact cytoplasm and wall appositions adjacent to intercellular hyphae. As at the top of the hypocotyl, in a few cells the cytoplasm appeared normal despite the presence of haustoria encased only by an

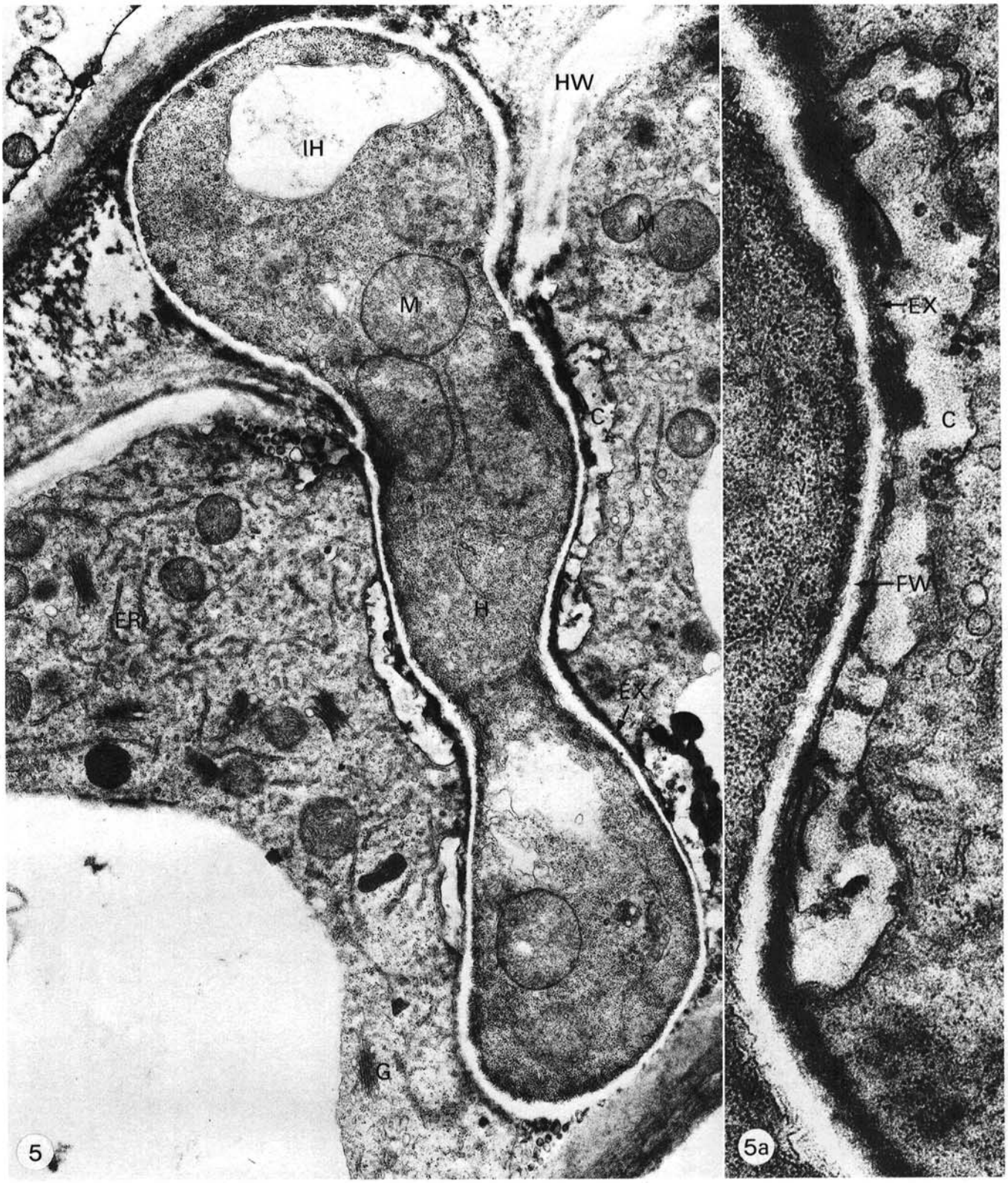
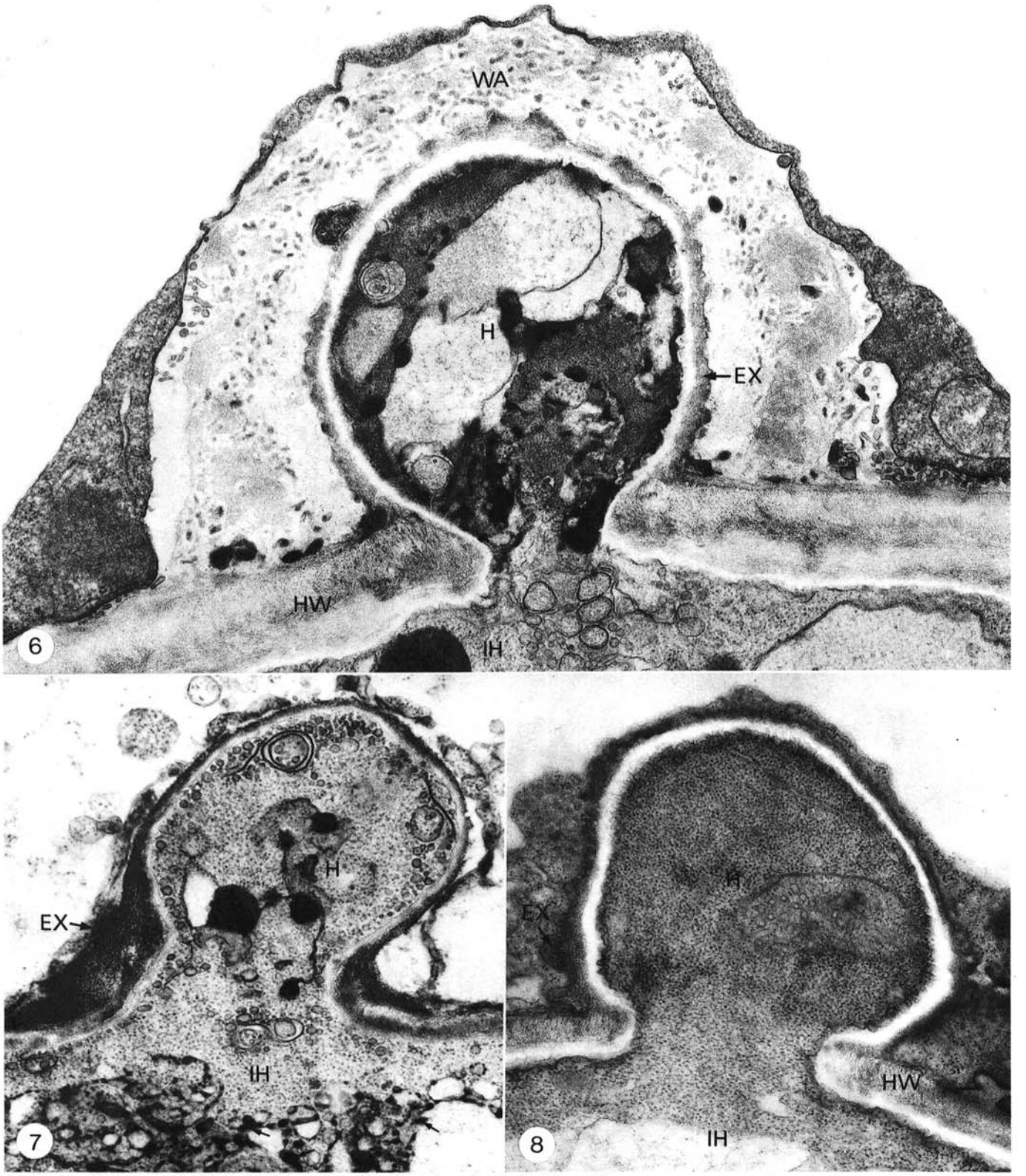


Fig. 5. Electron micrograph of a haustorium of *Phytophthora megasperma* var. *sojae* race 6 (compatible) in a host cell with intact cytoplasm, from susceptible tissue at the top of the soybean hypocotyl (×15,000). **5a,** The insert shows the haustorial encasement, consisting of a thin layer of extrahaustorial matrix and an irregularly shaped collar (×48,000). C = collar, FR = endoplasmic reticulum, EX = extrahaustorial matrix, FW = fungal wall, G = Golgi apparatus, H = haustorium, HW = host cell wall, IH = intercellular hypha, M = mitochondrion.

extrahaustorial matrix (Fig. 8). The fungal cytoplasm in such cells usually was healthy, but both here and at the top of the hypocotyl the cytoplasm in hyphae in or adjacent to necrotic cells frequently was electron-dense or contained a large number of electron-dense droplets of variable size and shape associated with membranes (Fig. 7).

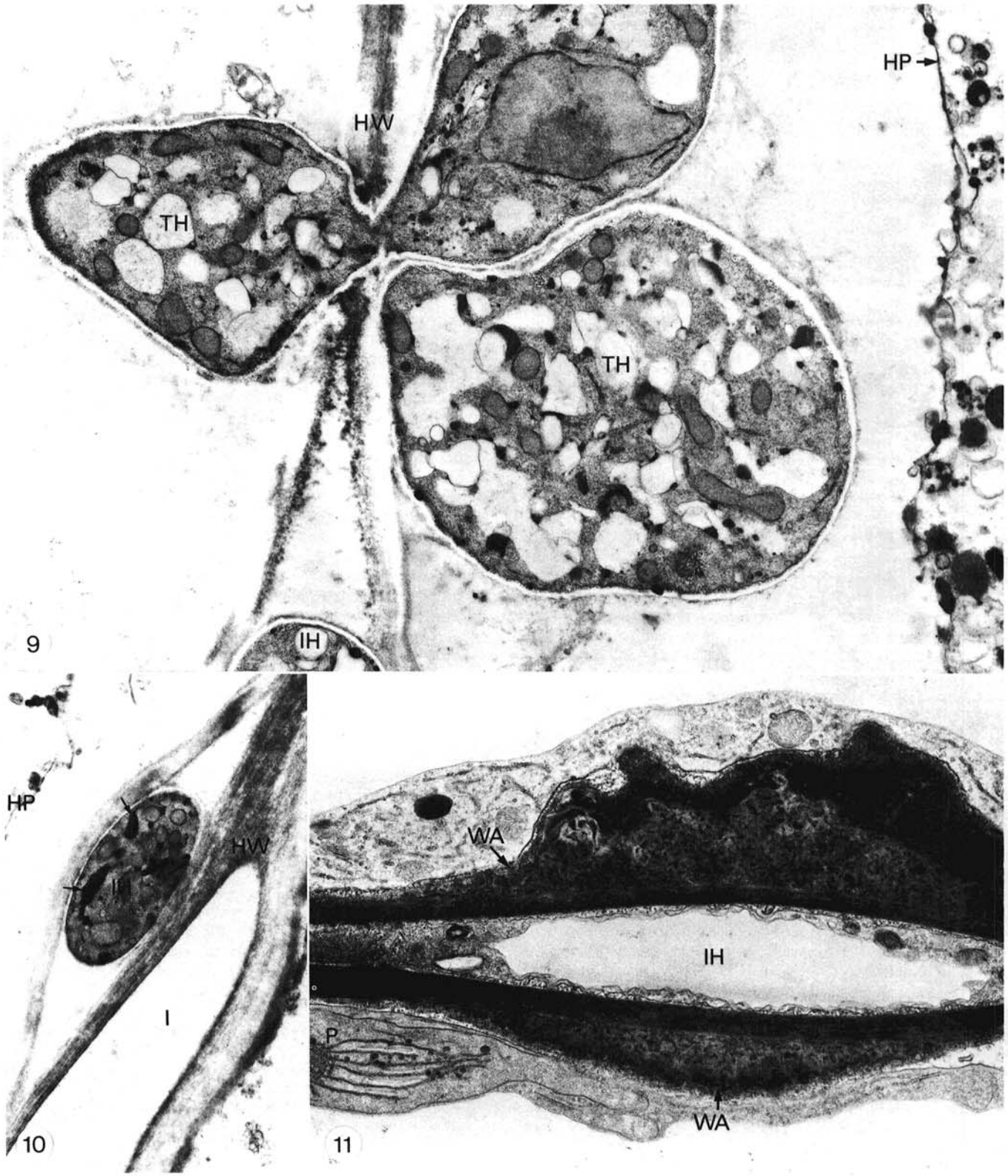
***Pms* race 4 (incompatible).** At the top of the hypocotyl, invaded and uninvaded cells were necrotic down to about the seventh cell layer beneath the epidermis, approximately 200 μm from the surface. Wall appositions developed in many cells that did not appear to be invaded, but none of these contained the marginal electron-opaque layer often seen in appositions in cells at the top of



Figs. 6–8. Electron micrographs of tissues from soybean hypocotyls inoculated with *Phytophthora megasperma* var. *sojae* race 6 (compatible). **6,** Haustorium in a host cell from susceptible tissue of the top of a hypocotyl. Note the thick wall apposition (×28,000). **7,** Intercellular hypha with haustorium in a necrotic cell from resistant tissue at the bottom of a hypocotyl. The fungal cytoplasm shows numerous electron dense droplets (arrows) (×30,000). **8,** Invaded host cell from resistant tissue at the bottom of a hypocotyl. The haustorium is encased only by a layer of extrahaustorial matrix (×30,000). EX = extrahaustorial matrix, H = haustorium, HW = host cell wall, IH = intercellular hypha, WA = wall apposition.

hypocotyls inoculated with race 6 (Fig. 2). Haustoria and transcellular hyphae rarely were surrounded by the host plasmalemma, extrahaustorial matrix, and wall apposition as with race 6, but were in direct contact with remnants of the cytoplasm (Fig. 9). Presumably the host cells died before invasion. Hyphae frequently grew within cell walls or to one side of the middle lamella between adjoining cells rather than in the intercellular space (Fig. 10). This was in contrast to hyphae of race 6, which invariably split the middle lamella (Fig. 4). Hyphae sometimes contained electron-dense droplets (Fig. 10).

In contrast to the extensive necrosis caused by race 4 at the top of the hypocotyl, necrosis at the bottom of the hypocotyl usually was



Figs. 9–11. Electron micrographs of tissues from soybean hypocotyls inoculated with *Phytophthora megasperma* var. *sojae* race 4 (incompatible). **9,** Transcellular hyphae in tissues from the top of a hypocotyl. Note the lack of extrahaustorial matrix and wall apposition (×10,500). **10,** Intercellular hypha growing within a host cell wall in tissues from the top of the hypocotyl (×11,400). **11,** Thick wall appositions on either side of an intercellular hypha beneath the epidermis (second cell layer) at the bottom of a hypocotyl (×13,500). HP = host plasmalemma, HW = host cell wall, I = intercellular space, IH = intercellular hypha, P = plastid with prolamellar body, TH = transcellular hypha, WA = wall apposition.

TABLE 1. Host cell alterations in 6-day-old soybean (cultivar Altona) hypocotyls inoculated at the top or bottom with race 6 (compatible) or race 4 (incompatible) of *Phytophthora megasperma* var. *sojae*

Host cell alteration			Number of altered cells[y] in hypocotyls infected with:			
			Race 6		Race 4	
Necrosis	Penetration	Wall apposition	Top	Bottom	Top	Bottom
(a) Yes	No	No	Common[z]	Frequent	Very frequent	Restricted to epidermis
(b) Yes	No	Yes	19, 31, 52	7, 6, 22	9, 14, 55	0, 11, 3
(c) No	No	Yes	23, 16, 12	2, 4, 1	0, 0, 4	9, 14, 23
(d) Yes	Yes	No	21, 30, 55	2, 3, 30	7, 6, 5	0, 0, 0
(e) Yes	Yes	Yes	9, 15, 24	0, 2, 2	2, 0, 1	0, 0, 0
(f) No	Yes	Yes	2, 1, 0	1, 0, 1	0, 0, 0	0, 0, 0
(g) No	Yes	No	6, 3, 6	1, 2, 4	0, 0, 0	0, 0, 0

[y] Hypocotyls were inoculated with zoospores of race 6 (compatible) or race 4 (incompatible) below the cotyledons (top) or above the roots (bottom). Three samples of each interaction with 6–12 penetrating hyphae per 1–2 mm^2 of epidermal surface were selected randomly and sectioned parallel to the epidermis. The numbers of observations of the different combinations (a–g) of host cell observations varied widely between samples due to differences in the numbers of penetrating hyphae and in the extent of fungal growth. The data indicates overall trends rather than specific numerical differences.

[z] Numbers of necrotic cells in (a) could not be determined due to disintegration of the tissue.

restricted to the epidermis. Most of the cells in the layers immediately below were uninvaded and undamaged but contained large wall appositions (Fig. 11). Electron-dense droplets, similar to those in the hyphae of race 6 and of race 4 at the top of the hypocotyl, were not seen. Nevertheless, mycelial growth usually stopped in the third cell layer, about 30–50 μm beneath the surface.

DISCUSSION

In general, the ultrastructural features described in this study are comparable to those reported for other diseases caused by members of the Peronosporales (eg, 5–7,17). The present study examined host-parasite interfaces in typical race-specific responses and compared these with interfaces in older tissues in which race specificity was not expressed. The race-specific susceptible response was provided by inoculation with the compatible race at the top of the hypocotyl. Resistant responses were of three kinds: an age-related, but not race-specific, response to the compatible race at the bottom of the hypocotyl; a race-specific response to the incompatible race 4 at the top of the hypocotyl; and a response to race 4 at the bottom of the hypocotyl. Results of previous studies suggest that the three resistant responses differ in degree but are generally similar and hence that specificity in the *Pms*-soybean interaction is associated with the compatible race (13).

To simplify comparisons between the four responses, individual cell interactions were categorized according to cell invasion, wall apposition formation, and necrosis (summarized in Table 1). Because of variations in numbers and extent of growth of penetrating hyphae, and difficulty in determining with absolute certainty that apparently unpenetrated cells are not penetrated at some other point, the data should be considered to indicate overall trends rather than specific numerical differences. The observed interactions are listed in order of increasing compatibility (a–g in Table 1), based on the assumption that cell necrosis without invasion is the most incompatible (hypersensitive) response and invasion without host cell necrosis and wall apposition formation is the most compatible. It is evident that the four interactions embrace a range of intermediate responses between the extremes of susceptibility and resistance and all four interactions have some types of response in common. However, interactions in which there was cell invasion but neither necrosis nor wall apposition formation (g in Table 1) occurred only with race 6. These appear to be the only instances where a truly compatible relationship existed. The small number of these compatible interactions and the possibility that they could represent an early stage of other interactions in which cell invasion occurred (d,e,f in Table 1) indicate that compatibility either exists in a very small proportion of host cell-pathogen encounters or is only a transient phase.

Wall appositions were produced frequently, both in susceptible and resistant responses. This contrasts, for example, with potato tubers infected with *Phytophthora infestans* in which wall appositions developed only in resistant cells, although a collar occasionally surrounded the base of the haustorium in susceptible cells (7). In sunflowers infected with *Plasmopara halstedii*, a collar was all that remained of the penetrated papilla in the susceptible hosts; resistant cells responded hypersensitively prior to penetration (21). According to Aist (1), evidence that wall appositions play a role in resistance is inconclusive, although they do appear to be a general response to injury. Our observations suggest that in soybean-*Pms* interactions such appositions are an expression of incompatibility. Appositions occurred with high frequency in the most incompatible interaction (race 4 at the bottom of the hypocotyl) often without evidence of cell invasion or necrosis (b and c in Table 1). It is improbable that at this highly resistant site the production of wall appositions represents an early stage in the development of other responses. Certainly, where wall appositions occurred in uninvaded necrotic cells, they were associated with incompatibility. Since most interactions with race 6 in the susceptible region at the top of the hypocotyl were either of this type (b in Table 1) or displayed host-cell necrosis together with fungal invasion with or without apposition formation (d and e in Table 1), it is questionable whether the term compatible, in the strict sense, is appropriate for this interaction. Suggestions that soybeans are susceptible to *Pms* because they fail to react to or recognize the compatible race (10) are not supported by our observations.

The three incompatible combinations had many features in common and differed from the susceptible response to race 6, chiefly in a tendency for host cell necrosis to occur more frequently without invasion (a in Table 1). Examples of this type were not counted because of widespread tissue damage. The interaction with race 4 at the bottom of the hypocotyl was the most incompatible (Table 1); no invaded host cells were observed. Although the bottom of the hypocotyl is resistant to race 6 (13), some individual interactions were as compatible as in the susceptible region at the top of the hypocotyl (Fig. 8). In general, however, it appears that the most common interactions between individual cells and hyphae of either race of *Pms* are incompatible in nature. Only in the limited region at the top of the hypocotyl is the host response suppressed or delayed for sufficient time or in enough cells for susceptibility to develop.

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