Infection and Colonization of Tobacco Callus by *Peronospora tabacina*

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ABSTRACT

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The infection and colonization of susceptible tobacco callus inoculated with sporangia of *Peronospora tabacina* was studied by using light-, scanning-, and transmission-electron microscopy. Germ tubes typically exceeded 50 μm in length and often grew in contact with the host cell wall for considerable distances. Prior to cellular penetration, the apex of most germ tubes expanded to form a well-defined, bulbous, appressoriumlike structure capable of producing intercellular hyphae, intracellular hyphae, and haustoria. Host cells were invaded by either intracellular hyphae or haustoria. Intracellular hyphae, 8–9 μm in diameter, exhibited indeterminate growth and were surrounded by an electron-dense matrix. Haustoria, frequently observed in the same host cell as intracellular hyphae, grew determinately and were ~3 μm in diameter. Haustoria were also surrounded by an electron-dense matrix but, in addition, were encased in calloselike material. Haustoria in intact leaf cells were similar in dimensions and morphology to haustoria in callus cells. A scanty mycelium developed on the surface of the callus cultures after 10 days, but then declined so that after 20 days only a few hyphal cells remained. Sporangiophores and sporangia were not produced.

Additional key words: blue mold, *Nicotiana tabacum*, ultrastructure.

Blue mold caused by *Peronospora tabacina* Adam on tobacco is an economically important disease in the United States (9). Disease resistant cultivars have provided some control (14), but the obligate nature of the fungus and difficulty in obtaining inoculum have slowed breeding efforts. Izard et al (7) reported that callus tissue from a susceptible tobacco hybrid supported the growth and sporulation of the pathogen, whereas callus from *N. debneyi* L., a resistant host species, only supported restricted fungal growth and limited sporulation. *Phytophthora parasitica* Dast. var. *nicotianae* (Breda de Hann) Tucker, a nonobligate pathogen, also has been grown in vitro on host callus, and field tests of regenerated plants have shown that accurate disease resistance assessment can be obtained (4). Recently, deZoeten et al (2) demonstrated that the ultrastructure of tobacco callus cells infected with *P. parasitica* var. *nicotianae* was similar to that of infected tobacco root cells (3). Adoption of in vitro techniques for blue mold disease may allow for an easier method for maintaining the pathogen and may also provide a method for screening genotypes for resistance.

Preliminary to development of an in vitro resistance screening program for blue mold, it is important that basic similarities and differences in the host-pathogen relationship of the callus system be compared with that of intact plants. The infection (5,11) and colonization (17–20) of susceptible tobacco leaves has been previously studied using light and electron microscopy. Sporangia on the adaxial surface of tobacco leaves produce short germ tubes and penetrate the periclinal wall of epidermal cells after forming appressoria. A 10- to 15-μm-diameter vesicle is formed immediately after penetration of the epidermal wall. Intracellular hyphae of indeterminate growth originating from the vesicle may infect adjacent epidermal cells and/or exit the epidermal cell into intercellular spaces. Intercellular hyphae ramify throughout leaf mesophyll tissue producing haustoria of determinate length. Izard et al (7) described a method for growing *P. tabacina* on tobacco callus, but did not examine the infection process or the host-pathogen relationship.

In this paper, we describe the infection and colonization of tobacco callus by *P. tabacina* and make comparisons with the same processes in intact plants.

MATERIALS AND METHODS

Callus tissue. Callus was grown from pith explants of two susceptible tobacco (*Nicotiana tabacum* L.) cultivars, Speight G-28 and Hicks, on Murashige and Skoog (12) (MS) medium supplemented after autoclaving with filter-sterilized indoleacetic acid (IAA) and kinetin. Final concentrations of IAA and kinetin were 11.5 μM and 1.0 μM, respectively. Stock cultures of callus were maintained on MS medium in darkness at 25 C and transferred at 2-wk intervals. Five days prior to inoculation with sporangia of *P. tabacina*, callus cultures were transferred to 100× 25-mm-diameter petri dishes containing a medium (MI) described by Izard et al (7), which was modified as follows. Each liter of MI medium contained 5% D-glucose, MS levels of vitamins, myoinositol and micronutrients, and 166 ml of a stock solution containing (per liter): $Ca(CO_3)_2 \cdot 4H_2O$, 0.8 g; KNO_3, 0.2 g; and KH_2PO_4, 0.2 g. The pH of the medium was adjusted to 5.7 before autoclaving, and filter-sterilized IAA and kinetin were added to a final concentration of 0.5 μM each. Callus was also transferred to MI-coated slides and maintained according to the method of Hock (6). All cultures were incubated in darkness at 25 C.

Inoculum production and inoculation of callus. Leaves of Speight G-28 tobacco plants were inoculated with 2×10^4 sporangia per milliliter of the *P. tabacina* isolate OPT 1 described by Spurr and Todd (16) and incubated at 22 C under greenhouse conditions. After chlorotic lesions developed (7 days), the plants were placed in darkness for 2–4 hr, after which the surface of the lesions and surrounding area were lightly swabbed with 70% ethanol. Plants

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were then maintained in darkness and 100% humidity overnight to induce fungal sporulation. The average number of sporangia per square centimeter of lesion tissue was determined by collecting the sporangia from three lesions in water. Sporangia were counted by using a hemocytometer and the lesion area was measured with an automatic area meter type AAA-5 (Hayashi Denko Co., Ltd., Tokyo, Japan). Sporangia counts per square centimeter of leaf lesion tissue were approximately 5×10^4. Twenty callus cultures in petri dishes, and callus grown on 10 slides coated with MI medium in petri dishes were inoculated by using ~4 cm^2 of infected leaf tissue attached, sporangiophores downward, with 1% water agar to the inner surface of the petri dish cover (1). The dishes were not sealed to promote slight dessication of the sporangiophores and subsequent sporangial dehiscence (13). In a similar manner, four dishes with healthy leaf portions were prepared as controls. After ~18 hr, the dish covers with the leaf portions were replaced with sterile covers. Dishes were then sealed with Parafilm (American Can Co., Greenwich, CT 06830), and all cultures were incubated at 25 C in darkness.

Microscopy. Forty-eight and 96 hr after the beginning of the inoculation procedure, portions of five inoculated cultures for each tobacco cultivar were suspended in 0.1 M, pH 7.2, phosphate buffer for light microscopy, whereas for callose determinations other portions of the cultures were stained with 0.05% aniline blue in 0.03 M potassium phosphate (10). Whole mounts of infected cells were examined by using a Nikon Optiphot microscope equipped for differential interference contrast and fluorescence microscopy. Aniline blue-callose studies employed a HBO 50-Hg lamp, a 330–380-nm excitation filter and a 420-nm absorption filter. The remaining eight inoculated cultures and control cultures were not opened, but were observed with a dissecting microscope every other day over 20 days for mycelial and sporangiophore development. Inoculated slide cultures were examined directly with a compound microscope after being covered with a glass coverslip.

Samples for transmission electron microscopy (TEM) were prepared in a manner similar to the method described by Hock (6). Agar-coated slides (1%) were flooded with inoculated callus cells suspended in 0.1 M, pH 7.2, phosphate buffer. Infected cells were located using a compound microscope and covered with cooled liquid 1% water agar. Agar containing infected cells was trimmed to ~0.25 cm^2 and fixed at 4 C with 3% glutaraldehyde in 0.1 M, pH 7.2, phosphate buffer. Samples were postfixed in cold 0.1 M phosphate buffered 1% OsO_4 for 30 min, dehydrated in a graded ethanol series, and embedded in Spurr's (15) low-viscosity medium. Thin sections (700–900 Å) were cut on a LKB ultramicrotome and mounted on uncoated 200-mesh copper grids. Sections were stained at 25 C with uranyl acetate (45 min), subsequently stained at room temperature with lead citrate (8 min) and viewed on a JEOL 100S electron microscope operated at 80 kV. Speight G-28 leaf tissue infected with the OPT isolate of *P. tabacina*, embedded for previous studies (18,19), was compared to the infected callus cells.

Small samples of callus tissue from cultures inoculated 10 days previously were prepared for scanning electron microscopy (SEM) in a similar manner as for TEM except that after dehydration in ethanol, the samples were transferred through a graded freon series and critical point dried. Specimens were gold-palladium coated and viewed with a JEOL T200 scanning electron microscope operated at 25 kV.

RESULTS

Eighteen of 20 callus tissue cultures inoculated with *P. tabacina* and all control cultures remained free from contaminating fungi and bacteria throughout the experimental period of 20 days as determined by examination with a dissecting microscope. The remainder became contaminated and were discarded.

Eighteen hours after inoculation, many sporangia had been deposited onto the surface of callus cells and ~40% had germinated. Germ tubes often exceeded 50 μm and often were in contact with the host cell wall for considerable distances (approximately one-half cell length) before penetrating (Fig. 1). Germ tubes formed on agar-coated slide cultures were of similar length or longer, and some grew over cells without penetrating. Prior to penetration, most germ tube apices on callus cells expanded into well-defined, bulbous appressorialike structures of ~13–17 μm diameter (Figs. 1 and 2). Some germ tubes that formed these structures did not produce a penetration peg and failed to penetrate the host cell wall, whereas others penetrated and produced either haustoria (Figs. 2 and 7) or intracellular hyphae (Figs. 3 and 5). Short lengths of intercellular hyphae, which often grew from appressorialike structures, were apparent after 48 hr (Fig. 2), and by 96 hr longer segments of hyphae from either appressorialike structures or from intracellular hyphae which had exited host cells (Figs. 3 and 4) had developed into a scanty surface mycelium. The status of the mycelium declined over the following 15 days so that only a few hyphae remained after 20 days. Sporangiophores and sporangia were not formed.

Thirty-five intracellular hyphae observed in Speight G-28 and Hicks callus cells were of indeterminate growth and averaged 8–9 μm in diameter. Intracellular hyphae were never encased with callose as demonstrated by the lack of fluorescence after staining with aniline blue or examination with TEM. The hyphal wall, which consisted of electron-dense and electron-lucent areas, was ~0.25 μm thick. An electron-opaque matrix of variable thickness was located between the hyphal wall and the host plasmalemma (Fig. 6).

Haustoria within Speight G-28 and Hicks callus cells (Figs. 7–9) were of determinate growth and were ~3–4 μm in diameter. The haustorial wall was ~0.15 μm thick and consisted of a single layer. Haustoria were surrounded by an electron-dense extrahaustorial matrix, which was bounded by an extrahaustorial membrane. The haustoria in all 47 observations were encased, and the encasements stained positively for callose with aniline blue (Figs. 7 and 8). Haustoria from intact leaves (Fig. 10) were surrounded by an electron-dense extrahaustorial matrix and were also encased with callose-like material. An extrahaustorial membrane was located between the extrahaustorial matrix and encasement. Haustoria from intact leaves were similar to haustoria from callus culture in size and morphology.

Many infected cells had necrotic areas (Fig. 3), especially around penetration sites. However, similar necrotic areas also developed in cells grown on MI medium that had not been inoculated. Callus grown on MS medium did not produce necrotic cells. The host cytoplasm of most infected cells was plasmolyzed.

DISCUSSION

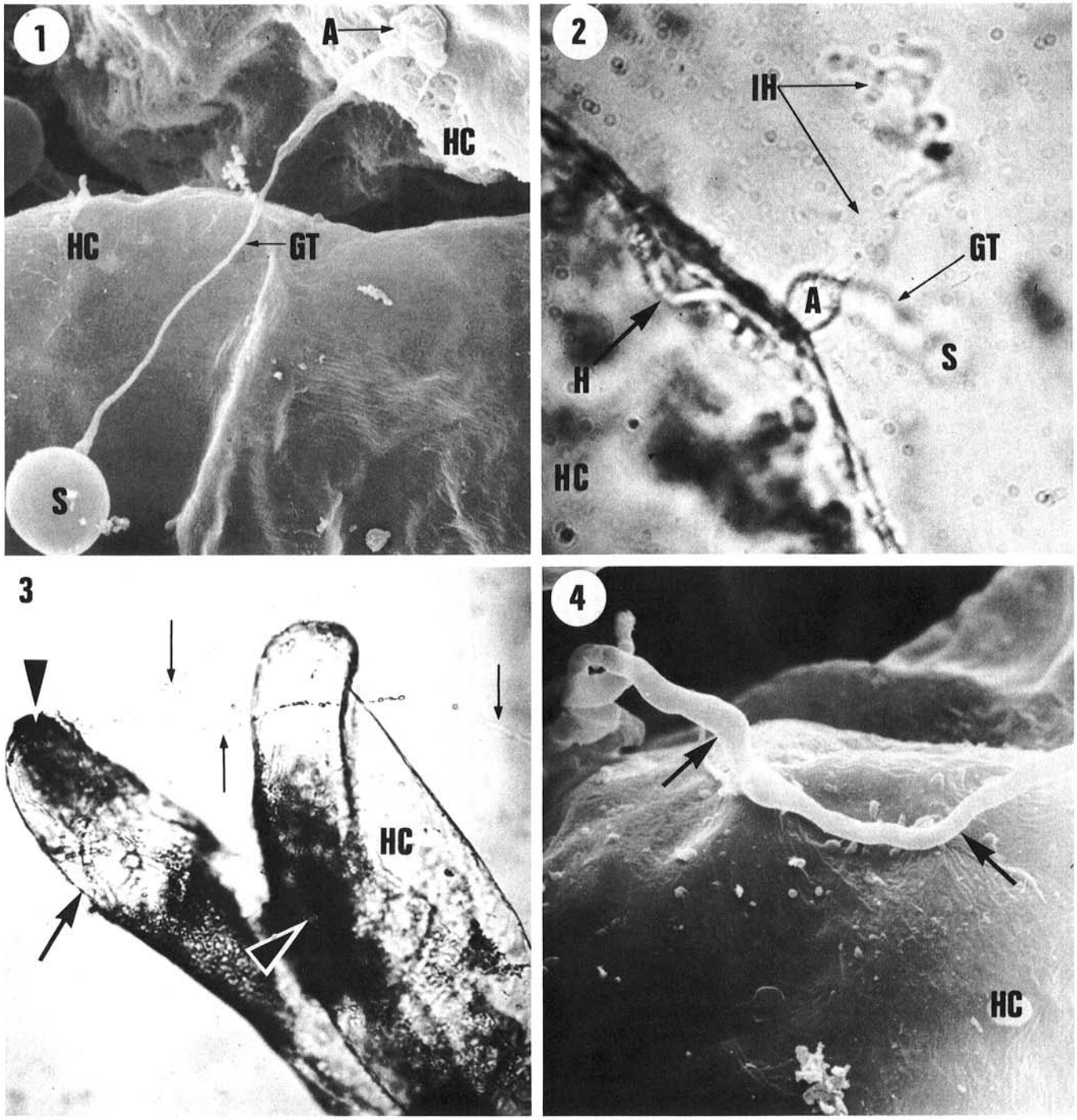
Germ tubes of sporangia of *P. tabacina* usually exceeded 50 μm in length and often grew appressed to host cells for considerable distances prior to penetration. Henderson (5) and McKeen and Svircev (11) found that germ tubes were relatively short on leaf surfaces, rarely exceeding 10 μm in length before appressorial formation and subsequent penetration. Ostensibly, germ tubes in callus cultures penetrate cells at random locations, which is similar to the growth of intercellular hyphae and the random development of haustoria (penetration of cells) in leaf mesophyll (17–19). These observations suggest that the relatively restricted growth of germ tubes prior to appressorial formation on leaf surfaces may be mediated by epidermal factors not found on callus cells.

Prior to penetration of host cells, many germ tubes apices expanded into well-defined, bulbous, appressorialike structures that measured ~13–17 μm in diameter. Also, intercellular hyphae often originated from these structures. These results differ from appressoria formed on intact leaves. Appressoria on leaves have been described as being a "slight swelling" or "almost imperceptible" by Henderson (5). Other studies (10,18) support the observations made by Henderson. Additionally, hyphal growth from appressoria has not been reported in previous studies of intact plants. In this study, we found that intracellular hyphae were formed after penetration of some host walls. These differ from the sequence of fungal structures formed after the penetration of intact epidermal cells. McKeen and Svircev (11) reported that a vesicle, ~10–15 μm in diameter, was formed after penetration and then an intracellular hyphae grew from this vesicle. The appressoriumlike

structure in our study is similar to the vesicle in size and in that additional fungal growth may originate from it. Therefore, it is possible that the appressorialike structures formed from germ tubes in tissue culture are homologous to vesicles formed after epidermal cells are penetrated. If the appressorialike structures we describe here are homologous to the vesicle in intact plants, then the fungal structures formed in callus culture are only similar to those structures formed after cell penetration in intact plants, and appressoria are lacking.

Haustoria from callus cells were similar in size and shape to haustoria from intact leaf cells. The haustoria from both types of tissue were encased in amorphous material, which stained positively for callose, and both had an electron-dense extrahaustorial matrix located between the haustorial wall and the encasement. These observations agree well with the studies of Svircev and McKeen (17) and Trigiano et al (19,20) on infection and colonization of intact leaves. Our studies indicate that under the cultural conditions used, callus cells respond to infection in a manner similar to cells in susceptible intact leaves.

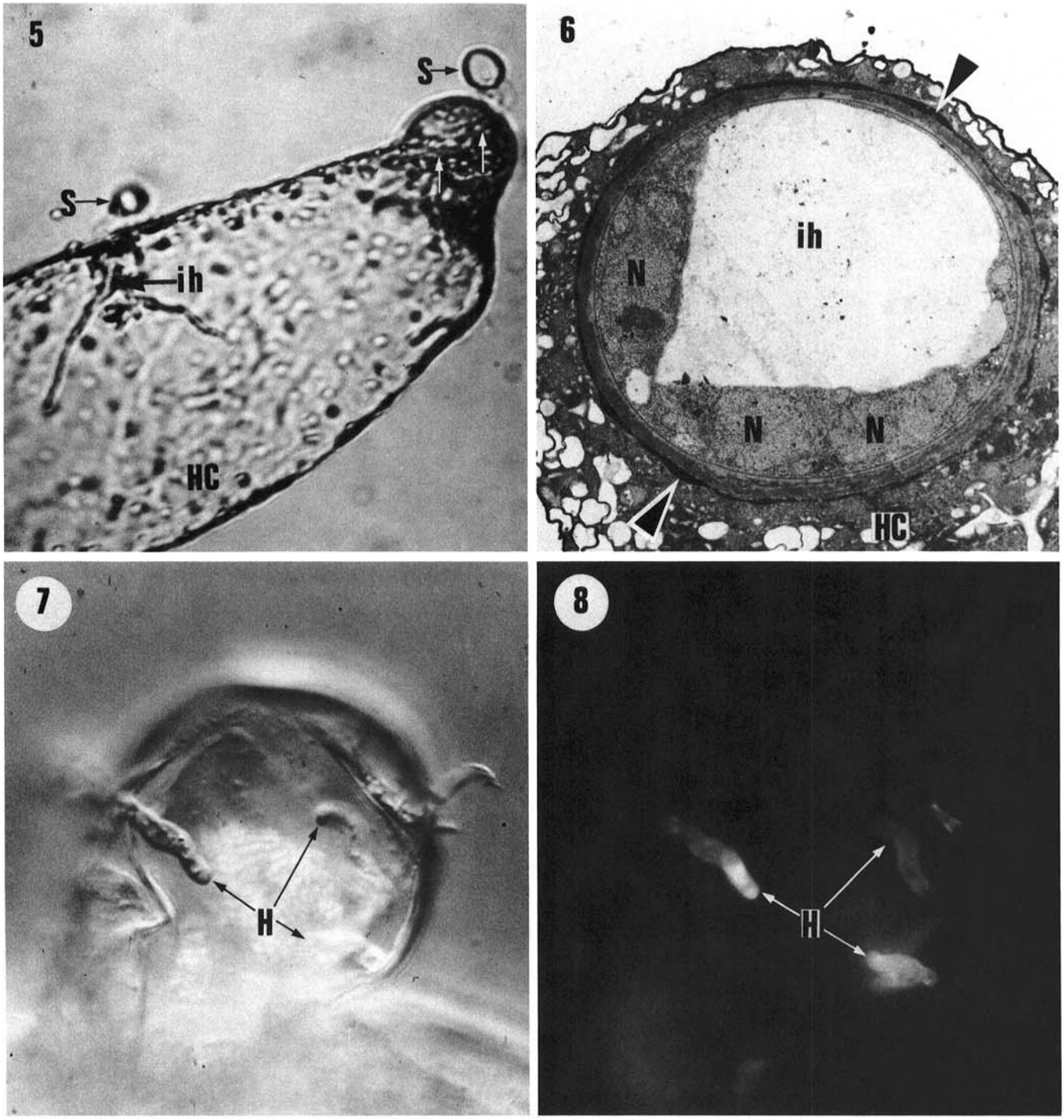
Infected host cells were usually plasmolyzed. We feel that this is not a consequence of fungus-host interactions, but rather the result of tissue preparation techniques. Furthermore, uninoculated callus



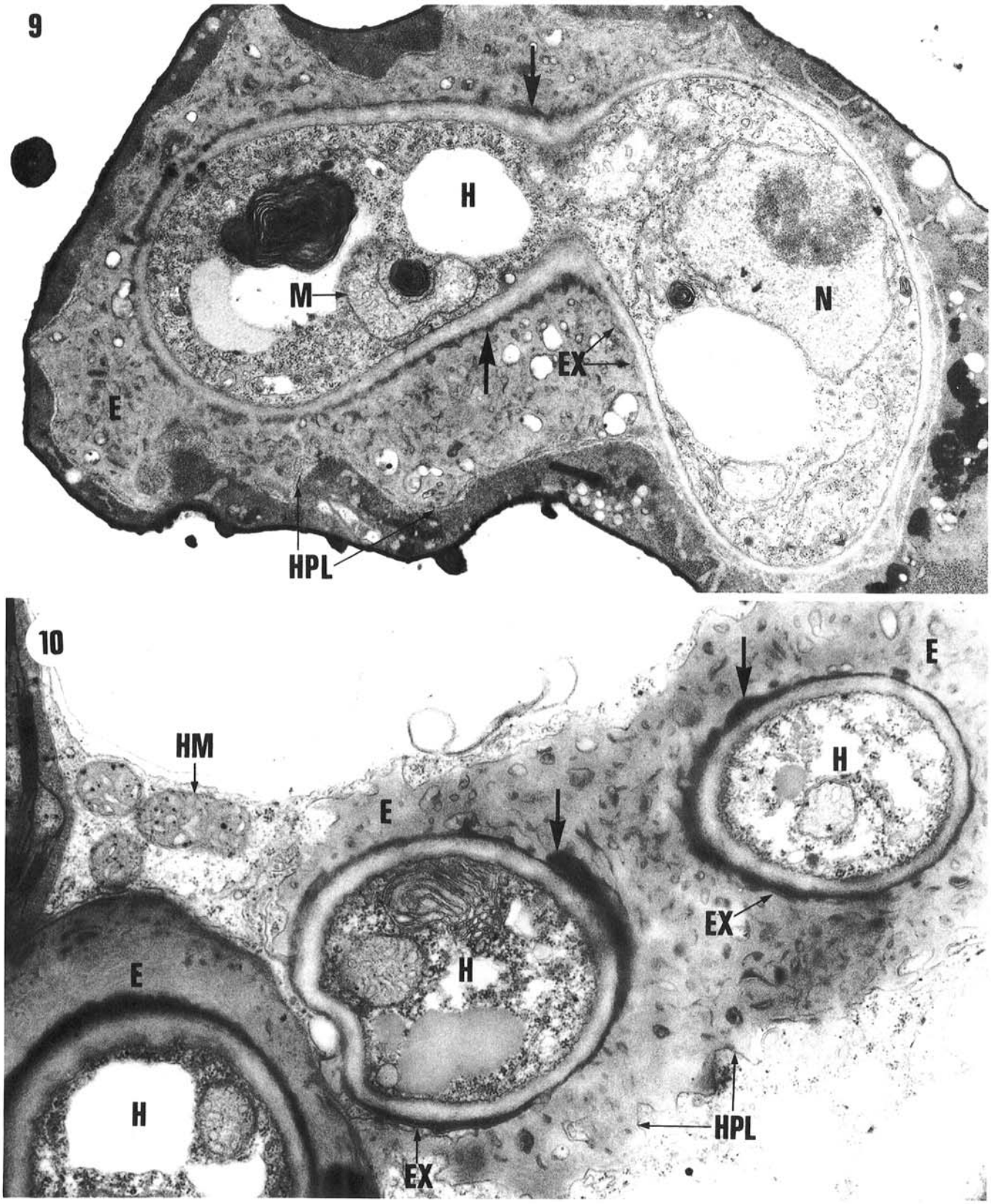
Figs. 1–4. *Peronospora tabacina* in and on tobacco callus cells. **1,** Scanning electron microscopy (SEM) of a germinated sporangium (S), germ tube (GT) and an appressoriumlike structure (A) attached to a host cell (HC) (×1,100). **2,** Whole mount of a sporangium (S), germ tube (GT) (below focal plane), appressoriumlike structure (A) and subsequent external hyphal development (IH) (below focal plane) from the appressoriumlike structure. A haustorium (H) is shown within the host cell (HC). The haustorial encasement stained positively for callose with aniline blue (×500). **3,** Whole mount of host cell (HC) with external hyphae (small black arrows). Intensive fungal growth within one host cell (large arrow) and localized necrotic areas (white bordered arrowheads) on both host cells (×230). **4,** SEM of an intercellular hypha (arrows) growing on callus (×1,050).

cells also exhibited plasmolysis. Some infected callus cells exhibited small necrotic areas. Izard et al (7) also observed localized necrotic regions and suggested that the extent of the necrosis depended on the species of tobacco and the structure of the callus. Growth media may play a role in the type and extent of necrosis that develops. Some uninoculated cells grown on MI medium exhibited necrosis, whereas callus grown on MS medium did not. MI medium has a relatively high concentration of glucose (5%), and it is possible that increased quantities of secondary metabolites (such as phenolic compounds) were produced, resulting in localized necrotic areas (8). Furthermore, reducing the amount of sugar in the culture medium reduces the amount of necrosis observed in inoculated cells (S. M. Reed and R. J. Sloane, *unpublished*). This necrotic reaction apparently does not prevent the penetration and subsequent colonization of individual host callus cells.

Helgeson et al (4) demonstrated that under proper cultural conditions, callus tissues from resistant tobacco cultivars responded to *Phytophthora parasitica* var. *nicotianae* in a manner similar to that of intact plants. Furthermore, deZoeten et al (2) have shown that the ultrastructural relationships between *P. parasitica* var. *nicotianae* in tobacco roots and in callus cells are similar. The



Figs. 5–8. *Peronospora tabacina* infecting tobacco callus cells. **5,** Whole mount of a host cell (HC) which has been invaded by the germ tubes of two sporangia (S). Intracellular hyphae (ih) and a haustorium (white arrows), hidden by a necrotic area are present (×250). **6,** Transmission electron microscopy (transverse section) of the intracellular hypha (ih) shown in Fig. 6 inside the host cell (HC). An electron-dense matrix (arrowheads) surrounds the hypha. N = fungal nucleus (×8,270). **7,** Differential interference contrast micrograph of three haustoria (H) in a host cell (HC) (×850). **8,** Fluorescence microscopy of the same haustoria shown in Fig. 7 stained with aniline blue and viewed with ultraviolet light (×850).



Figs. 9 and 10. Transmission electron microscopy comparison of *Peronospora tabacina* infecting a callus cell and a mesophyllic cell from intact leaf. **9,** An oblique section of the same haustorium (H) in a callus cell shown in Fig. 5. The haustorium is surrounded by an extrahaustorial matrix (large arrows) and encased with callose-like material (E). The extrahaustorial matrix and encasement are separated by an extrahaustorial membrane (EX). HPL = host plasmalemma, M = fungal mitochondrion, and N = fungal nucleus (×17,690). **10,** Transverse section of haustoria (H) in a mesophyllic cell from intact leaf. The haustoria are surrounded by extrahaustorial matrices (arrows) and encasements (E). EX = extrahaustorial membrane, HPL = host plasmalemma, and HM = host mitochondrion (×22,750).

results of this study demonstrate that although the infection induced in tobacco callus cells by *P. tabacina* differs in some respects from that induced in intact leaves, the ultrastructural relationship between host cells and haustoria are very similar. We suggest that in vitro physiological studies of disease as well as evaluations of disease-resistant callus should be possible with refinements in the callus-pathogen culture technique.

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