Mode of Parasitism of Alternaria brassicae by Nectria inventa

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ABSTRACT

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The *Verticillium* state of *Nectria inventa* is a destructive mycoparasite of *Alternaria brassicae*. Parasitism occurs either by means of contact without penetration or by penetration of the host. The parasitic hyphae induce abnormal responses in host cells upon contact. A reaction zone that consists largely of an electron-transparent matrix with dispersed tubule-like electron-dense material develops between the cell wall and the invaginated plasma membrane. The tubule-like elements subsequently aggregate to form electron-dense deposits below the cell wall. The affected cell forms a septal plug, accumulates membranes in some cases, and finally degenerates. *Nectria inventa* hyphae penetrate mainly the conidial cells of *A. brassicae*. This process appears to be primarily chemical in nature. The cytoplasm of the penetrated cell becomes progressively less dense and the cell eventually appears to be empty.

Alternaria brassicae, the causal fungus of blackspot of rapeseed causes substantial reduction in crop yield and in quality of oil (11). According to recent disease surveys in Alberta, however, the incidence of this disease has been variable. In addition to abiotic factors, the phylloplane microflora of rapeseed might contribute to this fluctuation in disease incidence. Therefore, phylloplane fungi were isolated from rapeseed leaves throughout the period of plant growth, and the fungi were tested for reaction to *A. brassicae* in culture. Among the isolates tested, the *Verticillium* state of *Nectria inventa* was strongly parasitic to *A. brassicae,* and this study was conducted to determine the mode of this mycoparasitism. Mycoparasitism is defined as the parasitism of one fungus on another.

MATERIALS AND METHODS

The mycoparasite used in this study was isolated from a leaf surface of rapeseed grown in Edmonton, Alberta, and was identified by the Commonwealth Mycological Institute (I M I 189322) as *Nectria inventa* Pethybridge. The conidial state of *N. inventa,* which is the type species of the genus *Verticillium,* variously has been referred to as *Acrostalagmus cinnabarinus, V. cinnabarinum, V. tenerum,* and *V. latericium* (19). Because of some uncertainty in nomenclature, we decided to use the name of the perfect stage of the fungus in this paper.

Alternaria brassicae (Berk.) Sacc. was isolated from a blackspot stem lesion on rapeseed grown in Edmonton, Alberta. Identification of this isolate was confirmed by G. A. Petrie, Canada Department of Agriculture Research Station, Saskatoon.

The cultures of *N. inventa* and *A. brassicae* were maintained on Difco potato-dextrose agar (PDA) and V-8 juice agar (V-8 juice, 200 ml; $CaCO_3$, 3 g; rose bengal, 50 mg; agar, 20 g per liter of distilled water), respectively, at 25 C in the dark.

Conidia of both fungi were collected by adding sterile distilled water to a 10- to 14-day-old culture and scraping the culture surface with an inoculation needle. The conidia were washed in a Büchner funnel and suspended in sterile distilled water. The spore density was adjusted to about 5×10^5 spores/ml for *A. brassicae* and to 10^8 spores/ml for *N. inventa*. Equal volumes of each spore suspension were mixed, and 0.5 ml of the mixture was spread evenly on a cellophane membrane disk (8-cm diameter) that was placed on 2% water agar in a plastic petri dish. The culture was incubated at 20 C in the dark for 24-72 hours.

For observation with a light microscope, after incubation, the cellophane membrane was cut into strips of appropriate sizes, and mounted in water or in cotton blue-lactophenol on a microscope slide.

For transmission electron microscopy, the mycelia grown on the cellophane membrane were gently brushed off with a paint brush into 3% glutaraldehyde in 0.1M Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0). The liquid containing the mycelia was passed through a Millipore filter (pore size 0.45μm). The mycelia retained on the filter were fixed overnight in glutaraldehyde, washed with buffer, and postfixed in 2% OsO_4 in the same buffer for 3-5 hours. The material was dehydrated in an ethanol series, taken to propylene oxide, and embedded in Araldite 502. Gold and silver sections were cut on a Reichert Model Om-U2 ultratome with a glass or a diamond knife. The thin sections were stained with aqueous 2% uranyl acetate for 2 hours followed by aqueous 0.2% lead citrate for 5 minutes. Electron micrographs were taken in Philips EM-300 and EM-200 electron microscopes.

For scanning electron microscopy, a thin layer of water agar was made on a cover slip (15-mm diameter) and a drop of the mixed spore suspension was smeared over it. These spores were incubated for 24-72 hours in a plastic

petri dish lined with moist filter paper. The mycelia that developed on the agar layer were fixed by the same procedures as for transmission electron microscopy. The fixed mycelia on agar were frozen quickly in Freon 22, stored briefly in liquid nitrogen and freeze-dried in vacuum at ~ −70 C (24). The cover slips with freeze-dried material were stuck to stubs with conductive glue and coated with carbon and gold in a vacuum evaporator. Micrographs were taken in a Cambridge Stereoscan S_4 scanning electron microscope.

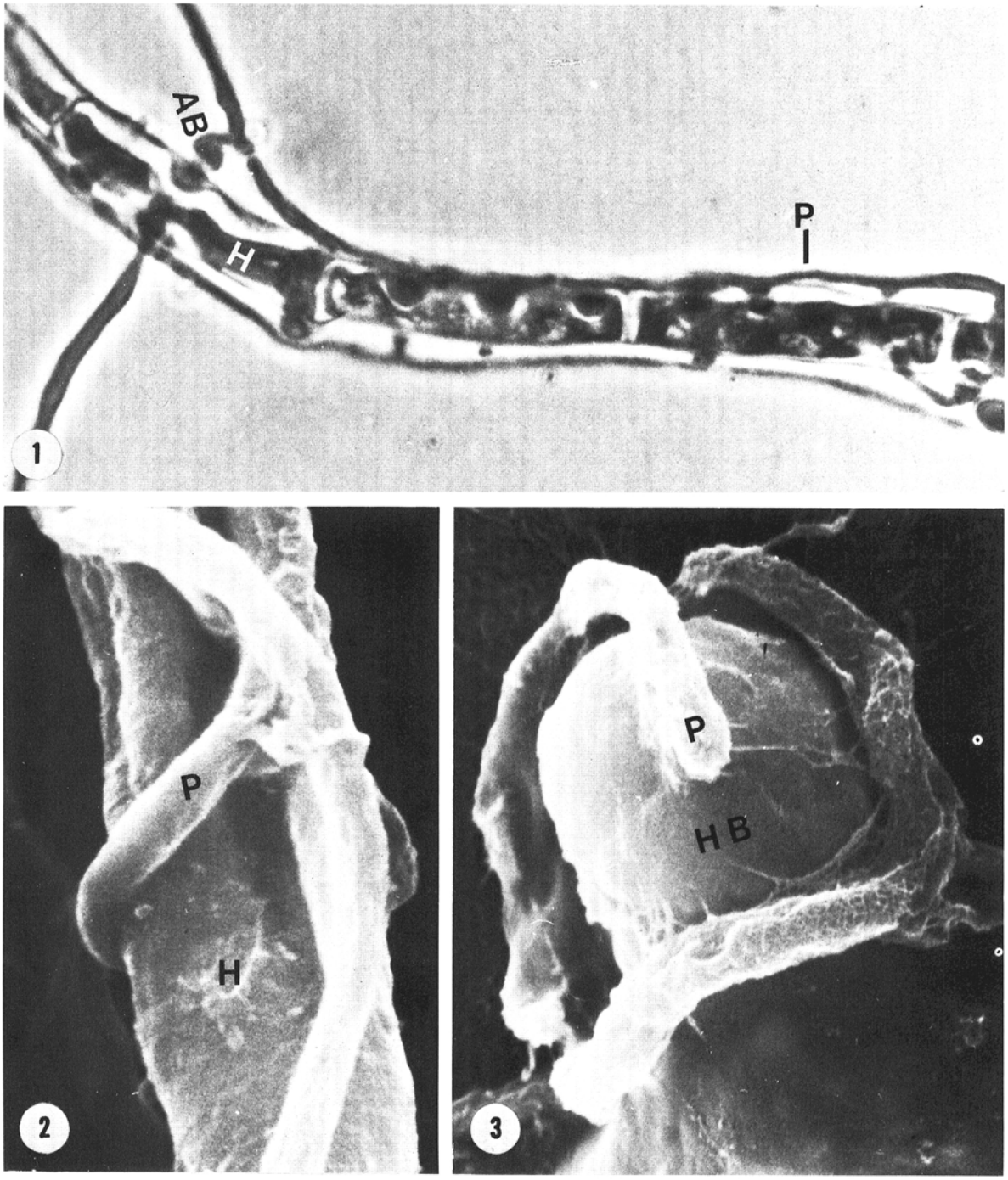


Fig. 1-3. Hyphae of *Alternaria brassicae* parasitized by *Nectria inventa*. **1)** Light micrograph of parasite hyphae parallel to a host hypha. Note the swollen appressorium-like body of the parasite (× 4,300). **2, 3)** Scanning electron micrographs of the parasite coiling around host hyphae (× 15,000). Legend: P = parasite; H = host; AB = appressorium-like body of parasite; and HB = hyphal branch of the host.

RESULTS

The average diameters of hyphae of *N. inventa* and *A. brassicae* grown on water agar were 1.0 μm and 3.5 μm, respectively. The hyphae and conidia of *A. brassicae* had a thick superficial electron-dense layer that either was absent or inconspicuous in *N. inventa*. The presence of lomasomes in *Nectria* hyphae and their absence, except in some parasitized cells of *A. brassicae,* was another characteristic that facilitated identification of the two fungi in thin sections.

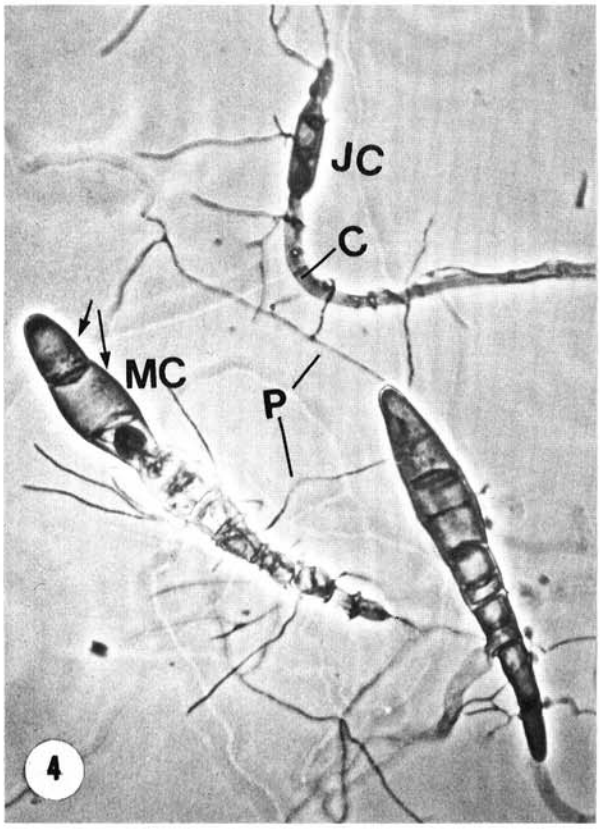
Parasitism was evident after incubation for 24 hours, and by 96 hours, the majority of hyphae and conidia of *A. brassicae* were heavily parasitized. Parasitism occurred either by means of contact of the respective hyphae without penetration or by penetration of *A. brassicae* by *N. inventa*.

Parasitism through contact.—Growth of *N. inventa* hyphae was more profuse in the vicinity of *A. brassicae* hyphae, and was particularly intense around conidia. *N. inventa* hyphae often grew parallel or sometimes coiled around the *Alternaria* hyphae and appeared to come in contact with them either by being appressed to them or, more commonly, by means of appressorium-like bodies that became attached to the host hyphae (Fig. 1-3). When *Nectria* hyphae were in the vicinity of *Alternaria* hyphae, short branches arose from the parasitic hyphae and became swollen at the tips where they had contacted the host cells, forming appressorium-like bodies (Fig. 1).

Conidiophores of *A. brassicae* were parasitized in the same way as its vegetative hyphae.

Conidia of *A. brassicae* in all stages of development also were entwined by a profuse growth of parasitic hyphae that eventually caused them to collapse (Fig. 4, 5). Formation of appressorium-like bodies on the conidium surface occurred frequently.

An abnormal response of the host cell usually was evident at the point of contact by the parasitic hypha, and especially was evident in the cells beneath the swollen appressorium-like bodies of *N. inventa*. In those areas the plasma membrane of the host cell had an invaginated outline (Fig. 6). The reaction zone (the region between the cell wall and the invaginated plasma membrane) was largely electron-transparent, and usually contained dispersed electron-dense material often organized into tubule-like structures (Fig. 6-9). Early stages of infection generally showed electron-dense material largely as tubules (Fig. 8), whereas in the later stages it appeared as irregular deposits (Fig. 10). Unusual accumulation of membranous material sometimes occurred in the affected



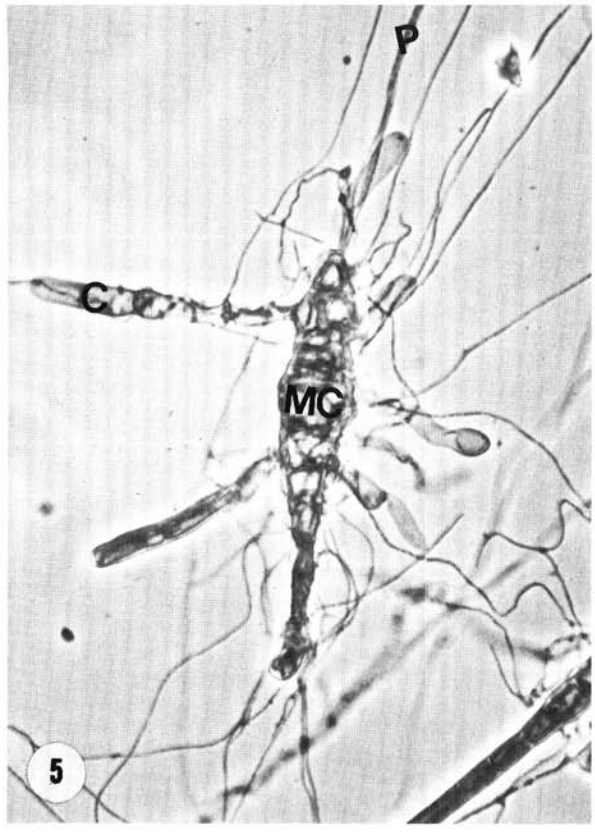


Fig. 4-5. Phase-contrast light micrographs of the conidia of *Alternaria brassicae* parasitized by *Nectria inventa*. **4)** Healthy-appearing conidium and infected mature and juvenile conidia. Note noninfected cells (arrows) in the heavily infected conidium (× 1,200). **5)** Profuse growth of parasitic hyphae around a host conidium (× 1,200). Legend: P = parasite; C = host conidiophore; MC = mature host conidium; and JC = juvenile host conidium.

cell (Fig. 7). A septal plug often was formed between an invaded cell and an adjacent cell (Fig. 6, 7). In a few cases, lomasomes were present below the reaction zone (Fig. 9).

In advanced stages of parasitism the plasma membrane became disorganized and eventually disappeared (Fig. 6). The cellular organelles were disrupted and only remnants of some membranous elements and scattered ribosomes remained (Fig. 10).

Nectria inventa hyphae apparently were incapable of inducing these abnormal responses in host cells at a distance.

Parasitism by means of penetration.—Penetration of *Alternaria* hyphae by *Nectria* hyphae was rare, but penetration of conidia was common. The walls between conidial cells are the areas most frequently penetrated (Fig. 12, 13).

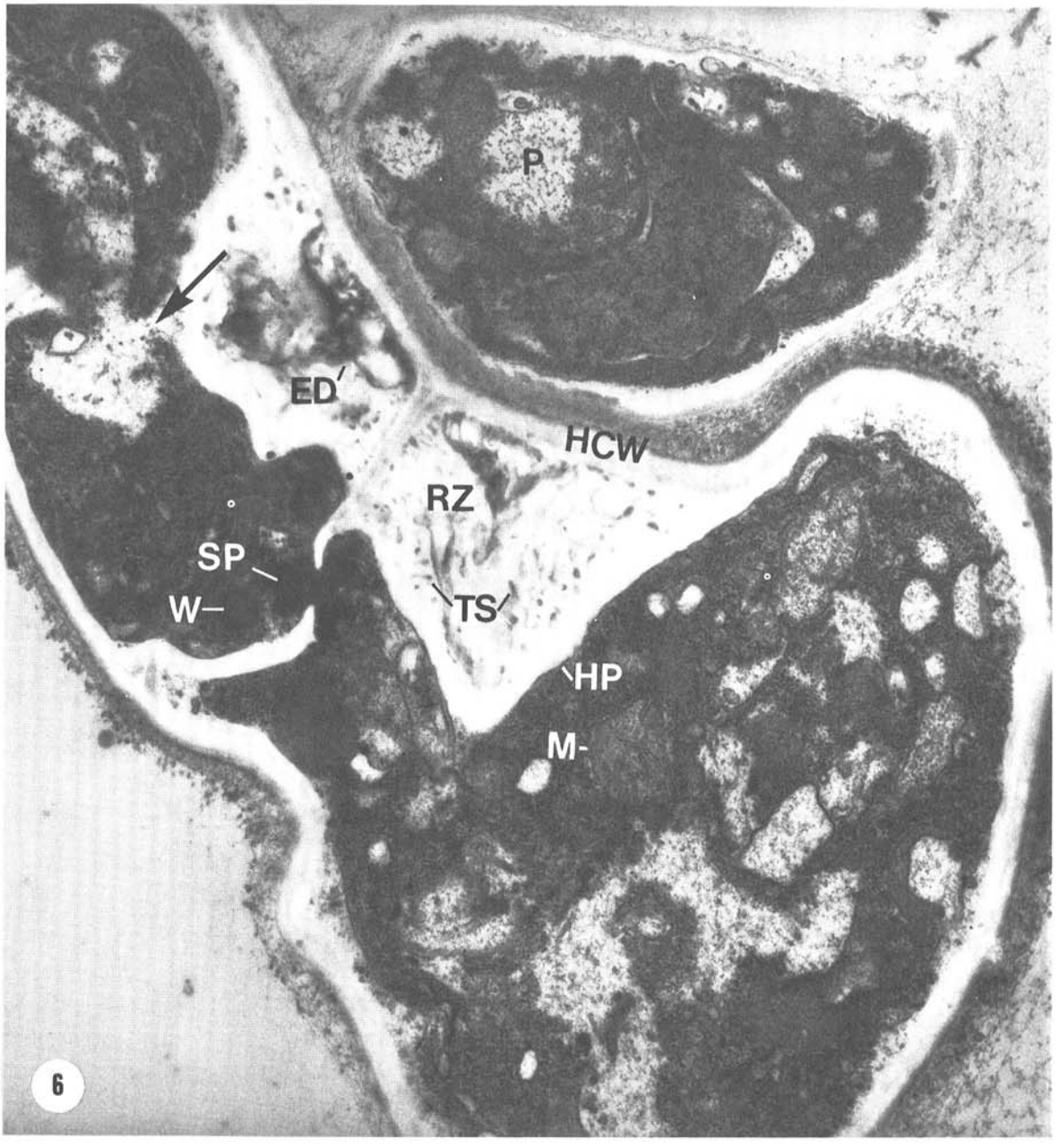


Fig. 6. Transmission electron micrograph of cells of *Alternaria brassicae* parasitized by *Nectria inventa* through contact. Development of reaction zone in host hyphal cells in response to contact by the parasite. Note invagination of host plasma membrane, electron-dense tubule-like structures and deposits below the host cell wall, presence of septal plug, and lack of recognizable host plasma membrane (arrow) in the middle of the upper host cell (×31,500). Legend: P = parasite; ED = electron-dense deposit; HCW = host cell wall; M = mitochondrion; HP = host plasma membrane; RZ = reaction zone; SP = septal plug; TS = tubule-like structures; and W = Woronin body.

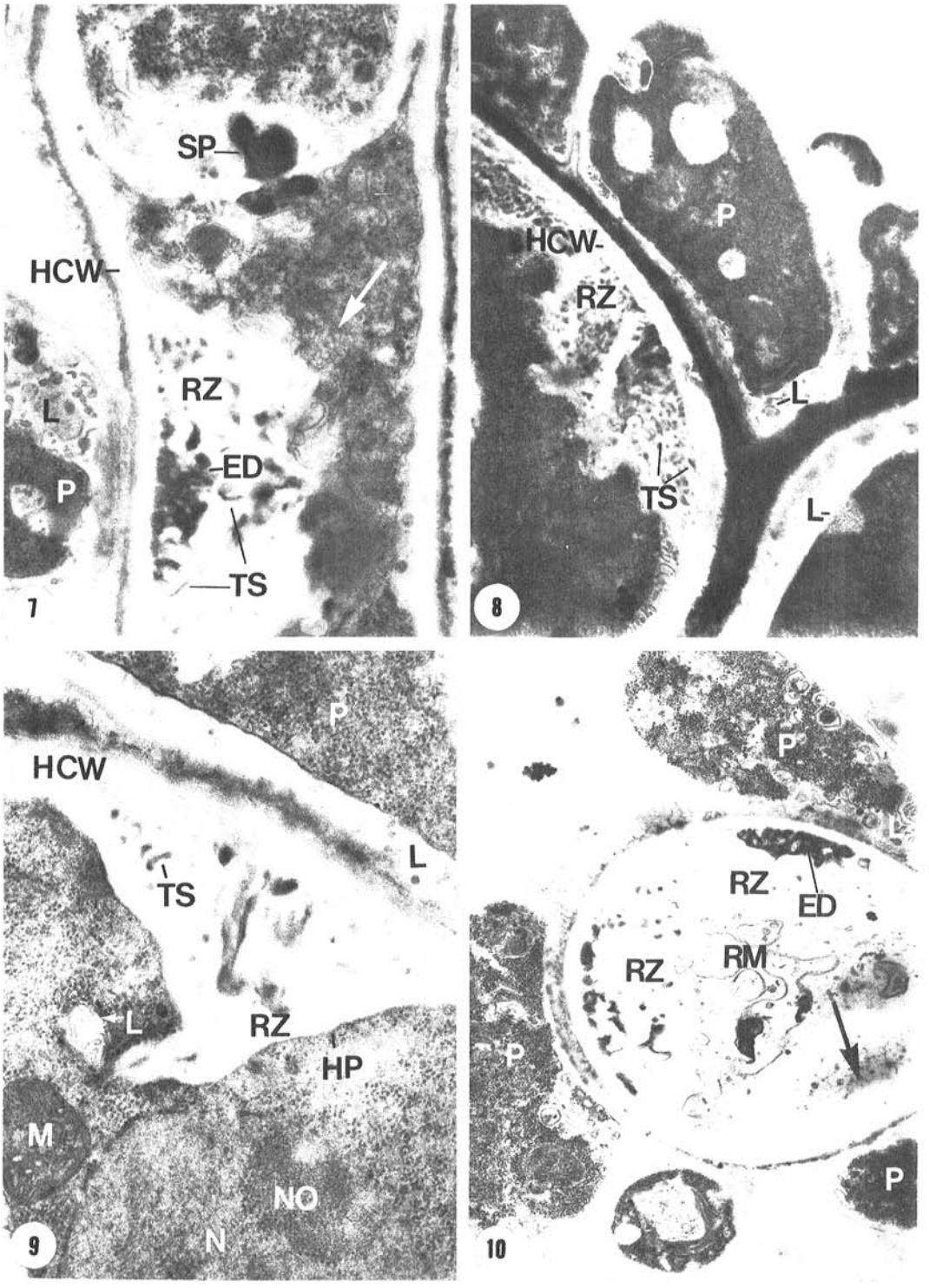


Fig. 7-10. Transmission electron micrographs of cells of *Alternaria brassicae* parasitized by *Nectria inventa* through contact. **7)** Unusual accumulation of membranes (arrow) in an infected host hyphal cell. Also note the presence of septal plug (× 38,000). **8)** Initial development of a reaction zone in host conidial cells. Note the extensive development of tubule-like structures in the reaction zone (× 22,000). **9)** Lomasomes in the area adjacent to the reaction zone in a host conidial cell (arrow). Note the presence of lomasomes in the parasite (× 43,000). **10)** Advanced stage of parasitism. Note lack of electron-dense deposit in one reaction zone (arrow) and disintegrated cytoplasm of the host hyphal cell (× 22,000). Legend: P = parasite; ED = electron-dense deposit; HCW = host cell wall; L = lomasomes; RM = remnants of membranous material; M = mitochondrion; N = nucleus; NO = nucleolus; HP = host plasma membrane; RZ = reaction zone; SP = septal plug; TS = tubule-like structures.

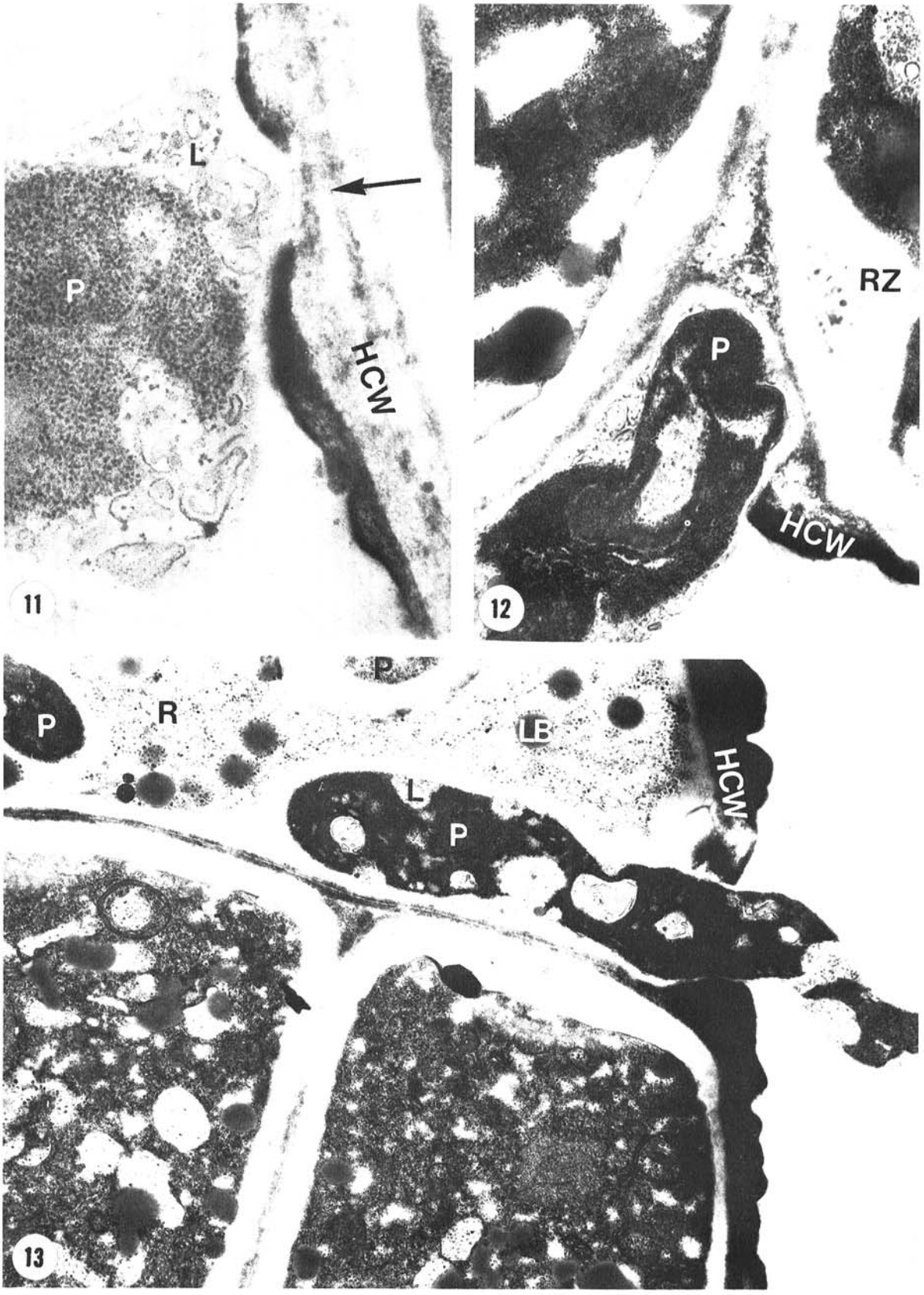


Fig. 11-13. Transmission electron micrographs of conidial cells of *Alternaria brassicae* parasitized by *Nectria inventa* through penetration. **11)** Initial stage of penetration of host cell wall by a parasitic hypha. Note dissolution of the outer cell wall material and lack of stress in the microfibrillar region of host cell wall (arrow) (× 43,000). **12)** Stage prior to penetration (× 22,000). **13)** Penetrated host conidial cell. Compare the density of cytoplasm of the penetrated and healthy cells. Note the electron-transparent area surrounding the penetrating hypha of the parasite and lack of significant indentation of the host cell wall at the site of penetration (× 19,000). Legend: P = parasite; HCW = host cell wall; LB = lipid body; L = lomasomes; R = ribosomes; and RZ = reaction zone.

During the initial stages of penetration, the conidial wall dissolved at the point of contact with the hyphal tip of *N. inventa* without showing any evidence of deformation of the fibrillar structure of the cell wall (Fig. 11). Invagination of the plasma membrane and deposition of electron-dense material occurred in the host cells (Fig. 12). However, sometimes host cells appeared to have been penetrated without developing a reaction zone. An electron-transparent zone surrounded the internal parasitic hypha (Fig. 13). The internal hypha penetrated from cell to cell by the production of a swollen structure and a constriction at the site of passage through the host cell wall (Fig. 14). The cytoplasm of penetrated host cells became progressively less dense. Later, only some lipid bodies, disorganized membranes, and scattered ribosomes remained, and eventually the host cell appeared to be empty.

DISCUSSION

There are many reports on the mode of parasitism of the mycoparasites *Piptocephalis* spp. (1, 5, 6, 13, 22), *Trichoderma* spp. (7, 21, 29) and *Gliocladium roseum* (2, 26). Several species of *Verticillium* also have been reported as mycoparasites. *Verticillium malthousei* and *V. psalliotae* are parasites of the commercial mushroom (17, 28). *Verticillium psalliotae* also parasitizes *Rhopalomyces elegans* (10). Barron and Fletcher (3) demonstrated that *V. dahliae* and *V. albo-atrum,* two important soil-borne pathogens of higher plants, parasitized *R. elegans*. Both parasites readily penetrated conidiophores of the host, but no evidence was presented that the vegetative hyphae were infected. The mode of parasitism of *N. inventa* is somewhat similar to that of *G. roseum* (perfect stage, *N. gliocladioides*), although *G. roseum* seldom penetrates living cells (2, 26). *Gliocladium roseum* parasitizes other fungi by sending out short branches that contact and coil around the host mycelium. Neither *N. inventa* nor *G. roseum* disintegrates host cells at a distance as do *Trichoderma* spp. (21, 29) and *Scytalidium album* (20). We observed no significant inhibition of the hyphal growth of *A. brassicae* by *N. inventa* prior to their contact. However, *N. inventa,* when in close proximity, induced cellular changes in *A. brassicae*. It would appear, therefore, that *N. inventa* does not produce a diffusible substance that affects host cells at any appreciable distance.

Only a few reports are available concerning the host-parasite interface in mycoparasitism (1, 22). Manocha and Lee (22) observed that the parasite, *Piptocephalis*

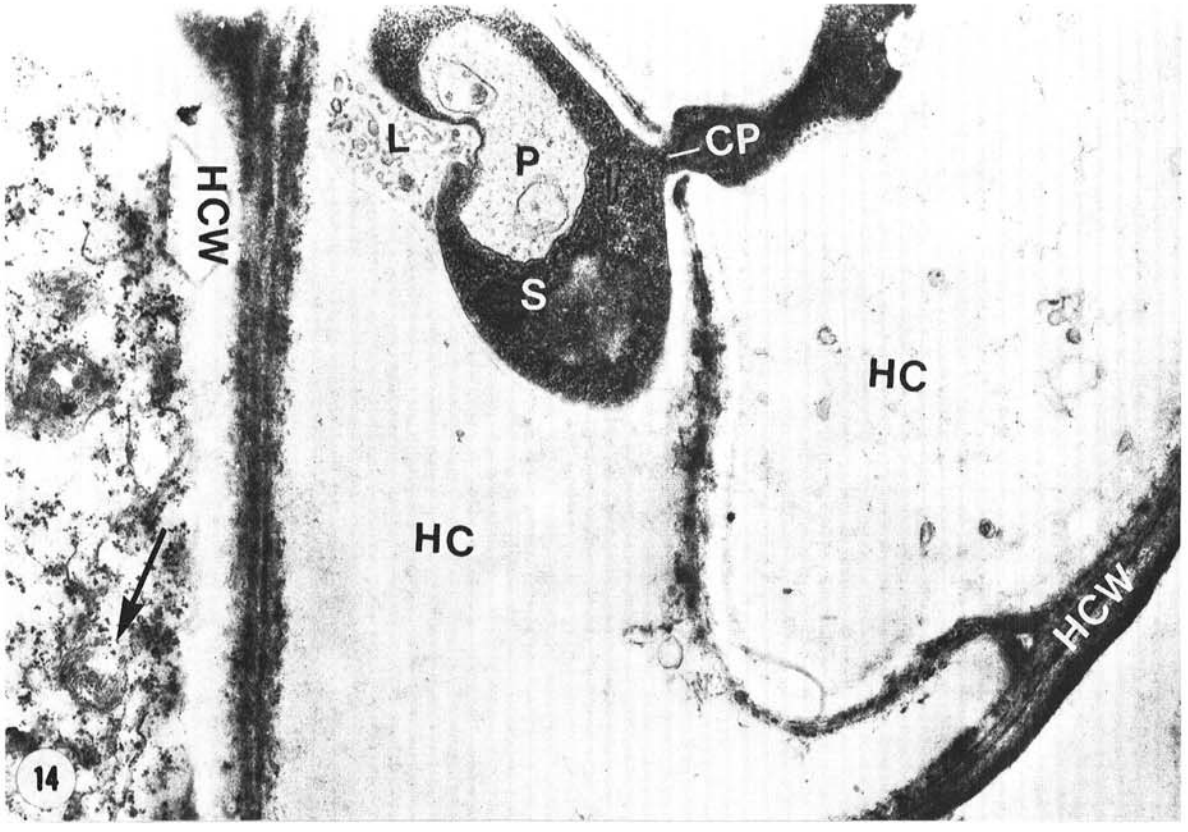


Fig. 14. Transmission electron micrograph of internal parasitic hyphae in several cells of a host conidium. Note the swollen and constricted parasite hypha at the site of penetration. Also note empty-appearing host cells and a partially degraded cell containing ruptured mitochondrion (arrow), remnants of other membranous organelles and ribosomes (× 20,000). Legend: P = parasite; CP = constriction in the parasitic hypha; HC = host conidial cell; HCW = host cell wall; L = lomasomes; and S = swollen structure in the parasitic hypha.

virginiana produced a haustorium in the host cell of *Choanephora cucurbitarum*. A collar formed around the haustorial neck, the endoplasmic reticulum proliferated, and an electron-dense sheath formed around the mature haustorium. This kind of balanced host-parasite relationship is common between vascular plants and obligate fungal parasites. Enrichment of host cytoplasm with ribosomes, dictyosomes, mitochondria, and endoplasmic reticulum is thought to be a characteristic host response to obligate fungal parasites (4, 15).

The host-parasite relationship between *N. inventa* and *A. brassicae* is not balanced; instead it is destructive. The appearance of the reaction zone that usually develops in the host cells in response to contact by a *N. inventa* hypha is somewhat similar to a papilla. Papillae are commonly formed in cells of vascular plants in response to penetration by pathogenic fungi such as *Erysiphe* sp. (14, 23). Edwards and Allen (14) described papillae as electron-dense with membranous material embedded in an amorphous matrix. Bushnell and Bergquist (8) suggested that papillae are significant components of generalized host resistance to powdery mildew fungi. Butler (9) observed deposition of wall-like material around infection hyphae of *Rhizoctonia solani* in invaded cells of some phycomycetous fungi such as *Rhizopus nigricans*. Manocha and Lee (22) reported that papillae were not detected in cells of *C. cucurbitarum* penetrated by *P. virginiana*. Recently, Swart (27) demonstrated that wall thickenings were induced in cells of *Phycomyces blakesleeanus* and *Aspergillus clavatus* as a result of parasitism by *V. dahliae*. The term 'callosity' was used for the wall thickenings. Formation of callosity was confined to sporangiophores or conidiophores of the host fungi and was not detected in the vegetative hyphae.

We suspect that the electron-dense amorphous material deposited in invaded cells of *A. brassicae* may consist of degradation products from tubule-like structures, that often accumulate beneath the host cell wall in the early stages of infection (Fig. 8), possibly by the action of enzymes or substances released by the parasite. The amorphous deposits contain materials synthesized by the host cells in response to infection by *N. inventa,* because the electron-dense deposits apparently are not produced after the host cell is killed (Fig. 10).

The chemical nature of these deposits is not known. Fluorescence microscopy combined with histochemistry (12, 16) failed to demonstrate callose in the reaction zone in the cells of parasitized *Alternaria* (Tsuneda and Skoropad, *unpublished*). Therefore, the term 'callosity' may not be suitable for the reaction zone in *Alternaria*. The apparent difference in electron density between the amorphous deposits and the cell wall of *Alternaria* hyphae (Fig. 6, 7) would indicate that there is a difference in their chemical composition.

Abnormal membrane production in response to infection by pathogenic fungi is a common phenomenon in vascular plants (14, 18, 25). According to Whaley et al. (30), unusual membrane systems also can be induced in onion cells by abiotic stresses such as mechanical injury. The abnormal membrane production that is evident sometimes in invaded cells of *Alternaria* can be regarded as a host response to *Nectria*. This response, as well as the formation of electron-dense irregular deposits, in some way may be resistant reactions of the host cells to penetration by *N. inventa* hyphae.

The function of lomasomes, often observed in cells of the parasite at the host-parasite interface, is not well understood; they may participate in absorptive or secretory functions of the parasitic hyphae (25).

Formation of septal plugs generally regarded as a protective mechanism may be a host resistance response.

Although Manocha and Lee (22) suggested that penetration of *C. cucurbitarum* by *P. virginiana* primarily may involve a mechanical process, this may not be a major mechanism for penetration of *A. brassicae* cells by *N. inventa* hyphae. The micrograph showing initial stages of penetration (Fig. 11) indicates that there is little or no stress in the microfibrillar portion of the host cell wall beneath the tip of a parasitic hypha, and there is no significant indentation of host cell wall at the site of penetration (Fig. 12, 13). Therefore, penetration by *N. inventa* may be primarily chemical in nature.

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