Mode of Penetration of Epidermal Cell Walls of *Vicia faba* by *Botrytis cinerea*

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ABSTRACT

Infection of *Vicia faba* leaves by *Botrytis cinerea,* a facultative parasite, was studied by means of light and electron microscopy, and histochemical staining. The first indication that infection was about to occur was the turning down of the tip of the germ tube until it contacted the cuticle. The tip of the germ tube was held firmly against the cuticle by means of mucilage which spread some distance (10-15 μm) around the germ tube. Then a pore developed in the fungal wall at the center of the contacting tip of the germ tube. The plasmalemma which covered the infection peg was pressed against the host cuticle and covered the blunt infection peg as it progressed through the cuticle. The cuticle appeared to be enzymatically dissolved rather than mechanically broken because a sharp clean pore without curled edges was made through the cuticle, and the infection peg did not cause an indentation of the cuticle or epidermal wall during penetration. Immediately after passage through the cuticle the epidermal wall began to degrade, split into two or more layers, and the cuticle was pushed upwards and sometimes separated from the epidermal wall. Esterase activity was histochemically detected at the tip of germ tubes 7 h after inoculation (the time of cuticle penetration) but not from 16-23 h after inoculation.

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Additional key words: chemical infection, mechanical infection

The mechanism of infection of intact surfaces, by *Botrytis cinerea,* Pers. ex Fr., a facultative plant parasitic fungus, has been considered for a long time. According to Brown (3), De Bary, Nordhausen and Ward believed the infection process was enzymatic. Brown and his colleagues (2, 3, 4, 5) in a series of experiments published from 1915 onwards, described infection with *B. cinerea,* and he (6) concluded that the principles which governed degradation of host tissue at a later stage in parasitism played no part in penetration, and that chemical degradation of the cuticle by *B. cinerea* played no significant part in penetration of the cuticle.

The trend in plant pathology has been to consider penetration of the cuticle strictly mechanical and to accept the classical work of Brown and his colleagues. In 1960, Wood (17) stated that penetration of the cuticle was mechanical, and did not depend on substances produced by the hyphae of invading organisms. Marks et al. (13) after examining the infection of leaves of *Populus tremuloides* by *Colletotrichum gloeosporioides* stated, in 1965, that penetration was mechanical and that chemical erosion of cuticle was unimportant. More recently Goodman et al. (11) stated that penetration through the cell wall is regarded as a mechanical process.

Little attention has been given to the form and characteristics of the infection peg during the initial moments of penetration into the host cuticle. However, Brown and Harvey (7) considered the infection peg a specialized organelle that is driven through the cuticle mechanically by growth forces, which are now called "elongation growth" (10). Conversely Dickinson (8) concluded that the infection peg is a plastic structure that is simply pressed into the host tissue by osmotic forces within the appressorium.

I investigated infection of broad bean (*Vicia faba* L.) leaves by *B. cinerea,* a facultative parasite, because of my desire to compare it with the infection process of barley leaves by *Erysiphe graminis* (15), an obligate parasite, and because electron microscopy studies on the infection process by *B. cinerea* were lacking.

I wished to determine whether the mechanism of penetration was mechanical or chemical. There was the possibility that the cuticle was enzymatically dissolved, or that channels and ectodesmata were present which might serve as portals for mechanical entry. In order to determine the possibility of enzymatic dissolution of the cuticle, esterase activity in conidial germ tubes was tested.

MATERIALS AND METHODS.—The culture of *Botrytis cinerea* used in these studies was No. 790 from the Plant Sciences Departmental culture collection, and proved to be pathogenic when inoculated in turnip juice on young healthy broad bean (*Vicia faba* L.) leaves which were obtained from greenhouse-grown plants.

Light and electron microscopy.—Seven to 10 days after the inoculation of corn meal agar with *B. cinerea* conidia, 10 ml of autoclaved turnip juice (2) was poured on a sporulating petri-dish culture. The conidia were liberated by gently rubbing the culture with the end of a blunt smooth glass rod. Then for inoculation, young healthy *V. faba* leaves were detached and placed upside down, for about 5 s, on the turnip juice containing the *B. cinerea* conidia before placing them right side up on moist filter paper in petri dishes which were kept in an incubator maintained at 21 C. At hourly intervals between 4-12 h after inoculation, epidermal strips with conidia were taken from broad bean leaves and placed in lactophenol containing 0.01% cotton blue on a microscope slide for light microscope examination.

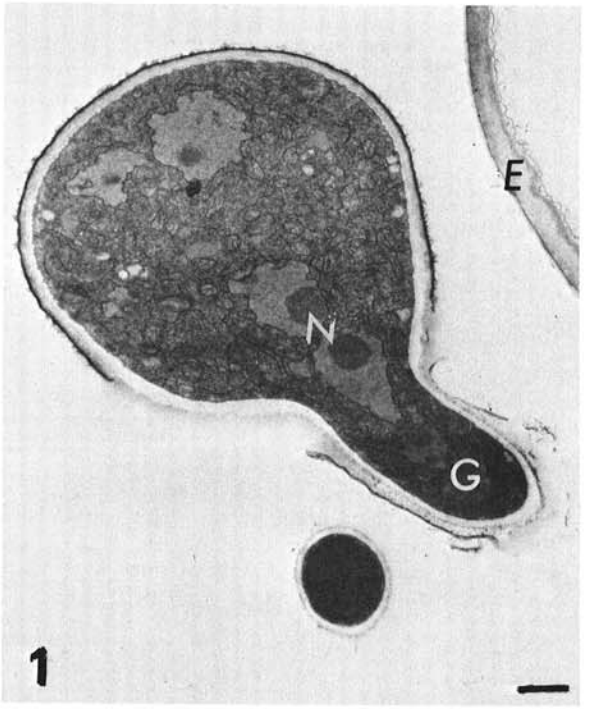
For electron microscopy, leaf disks supporting conidia, 6, 7 and 8 h after inoculation, were excised, in glutaraldehyde-osmium tetroxide, with the tip of a No. 17 hypodermic needle sharpened to resemble a cork borer. Leaf disks were placed in glutaraldehyde-osmium tetroxide which was made by mixing ice-cold stock solutions of glutaraldehyde (4%) and osmium tetroxide (2%), both buffered to a pH of 6.8 with 0.1 M sodium cacodylate, and used immediately (9). The material was fixed for 20 min at 5 C before being rinsed in cold 0.1 M sodium cacodylate buffer followed by postfixation in buffered 2% osmium tetroxide for 5 min. Then it was rinsed in distilled water and warmed to ambient temp before being stained for 20 min in 5% magnesium uranyl acetate.

After fixation, disks were dehydrated in a graded ethanol series, infiltrated with and embedded in Epon-Araldite (14) before being placed in a 60 C oven for 24 h to polymerize the plastic.

Later, blocks were trimmed with a glass knife until the face of the block was about 0.5 μm from desired material. Thin serial sections were then cut on a Porter-Blum (Sorvall MT-1) ultramicrotome with a diamond knife, mounted on 200-mesh copper grids, and stained with lead citrate. Sections were examined in a Philips EM200 using 60 kV and an objective aperture of 40 μm.

Histochemical detection of esterase activity with indoxyl acetate.—Fresh solutions of indoxyl acetate (Sigma Chemical Co., Dekalb St., St. Louis, Missouri) were prepared for assay according to Barrnett and Seligman (1). Because self hydrolysis of indoxyl acetate occurs, substrate solutions were prepared fresh for each assay. Epidermal strips were removed from broad bean leaves 3, 4, 5, 6, 7 and 8 h after inoculation with *B. cinerea* conidia, immersed directly in the substrate solution on glass slides and examined after 5 min for the presence of crystals of indigo blue. Some conidia were also allowed to

Fig. 1-4. Stages of penetration of broad bean (*Vicia faba*) by *Botrytis cinerea*. The scale lines are 1 μm. **1)** An electron micrograph of a section of a germinated conidium containing five nuclei (N). The conidium is nonvacuolated and the tip of the germ tube (G) which is not contacting the bean epidermal wall (E) is electron dense. (× 5,060). **2)** An electron micrograph of a section through an infection hypha (H) in the host wall. The epidermal wall (E) is swollen and partially digested. The cuticle (C) is raised and the host anticlinal wall (A) is split. The infection hypha is filled with organelles and cytoplasm. The host nucleus (N) is adjacent to the infection site. (× 10,500). **3)** An electron micrograph of the tip of a germ tube, cuticle (C), and epidermal wall (E) of a broad bean leaf. Observe the pore in the fungal wall (F). The cuticle is unaltered. The contact area between the germ tube and the host cuticle is surprisingly small. The area at the tip of the germ tube is quite transparent and contains some vesicles. (× 25,400). **4)** An electron micrograph of an infection site. The young infection peg (P) has passed through a pore in the fungal wall (F) and has just penetrated the host cuticle (C). The host epidermal wall (E) is slightly altered. (× 25,300).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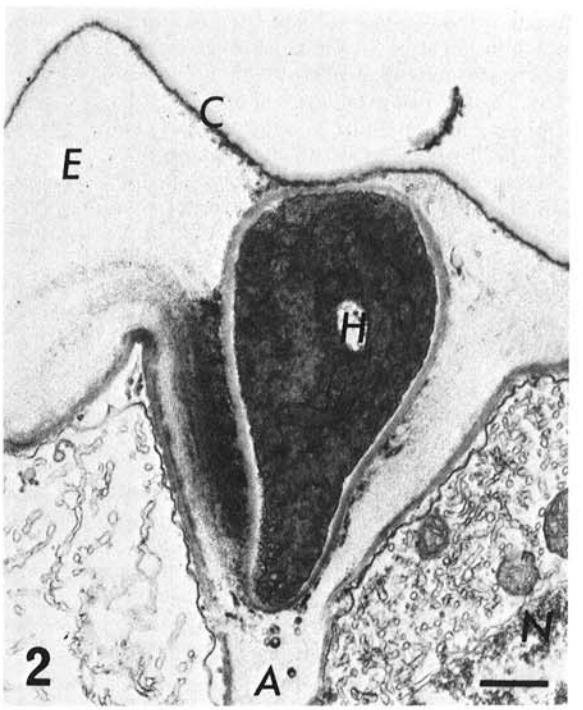
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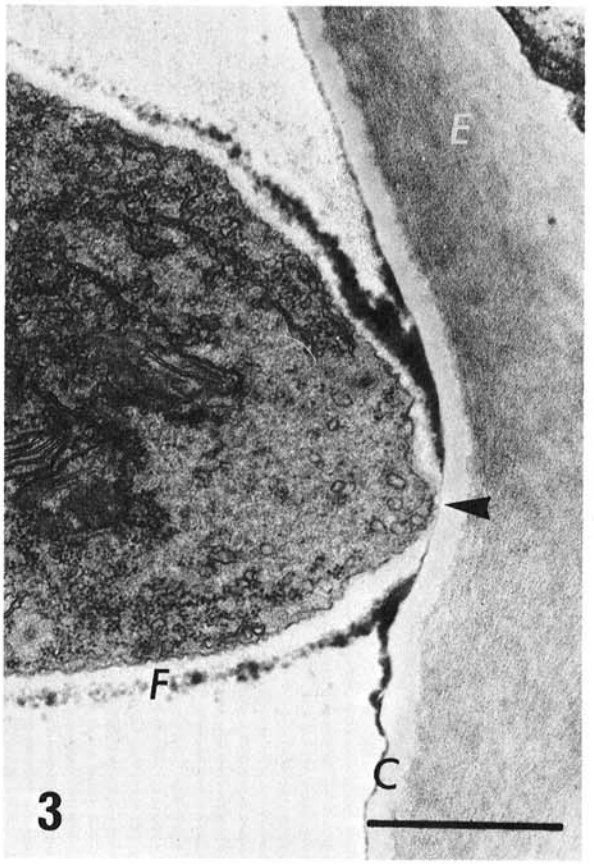
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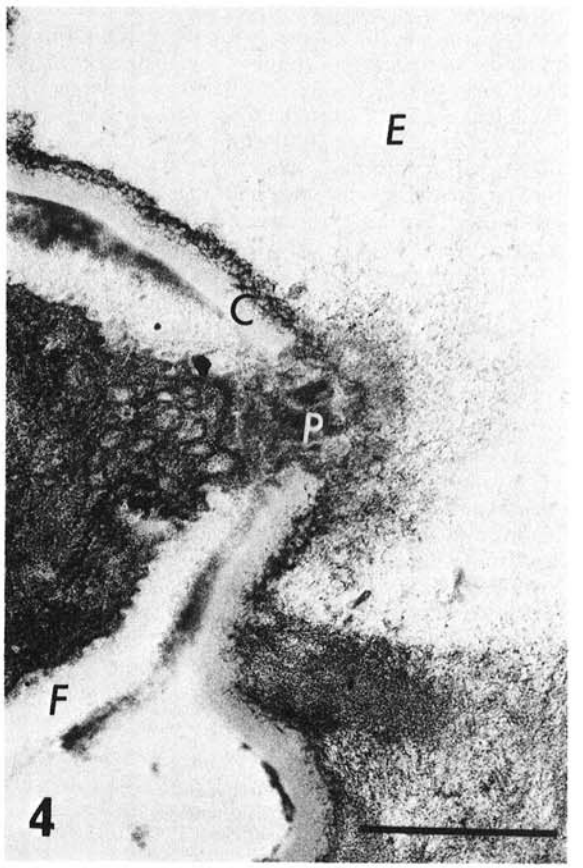
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germinate in turnip juice and tested 16-20 h after inoculation.

RESULTS.—Conidia, germ tubes, appressoria, and mycelia of *B. cinerea* stained very well in dilute cotton blue solution after 5-15 min. Many conidia remained on the leaf surface and at least 99% had germinated 4 h after inoculation. Most produced a short germ tube, 10-20 μm, prior to infection while some produced a germ tube 40-100 μm before an appressorium was formed.

Because light microscope studies showed that infection had occurred by the 9th h, electron microscope studies were made at 6, 7, and 8 h after inoculation.

Electron microscope studies revealed, that by 8 h after inoculation, infection had occurred in most instances. At 7 h, about half the infection pegs had penetrated the cuticle, some were penetrating the cuticle, and many were about to initiate penetration. No penetration had occurred by 6 h.

The germinated conidium (Fig. 1) contained several nuclei, a large amount of cytoplasm, much endoplasmic reticulum, several mitochondria, and many ribosomes.

The short germ tubes which produced infection were surrounded by mucilage which spread up to 10 μm away from the germ tube (Fig. 5). The germ tube was filled with organelles and the terminal portion stained more intensely than the basal portion. However, prior to and during penetration, a small zone above the infection peg contained small vesicles about 0.02 μm in diam, and an electron-lucent area (Fig. 3) about 0.5 μm in diam.

The first indication that penetration was to be initiated was the turning down of the tip of the germ tube so that about 1 μm was in contact with the cuticle. First a thinning, and then a pore about 0.2 μm in diam appeared in the center of the fungal wall which was in contact with the cuticle (Fig. 3, 4). Then the blunt infection peg, about 0.02 μm in diam, covered by the fungal plasmalemma was found at various distances in the cuticle. The infection peg made a clean passage through the cuticle and no indentation, breaking, or tearing of the cuticle or epidermal wall could be detected (Fig. 5, 6).

After the peg passed through the cuticle a small area around and in advance of the peg appeared more electron-opaque than the healthy epidermal wall.

Immediately after the infection peg passed through the cuticle, the cellulose wall of the host began to break down, split, and swell (Fig. 2, 7), the infection hypha frequently remained in the epidermal wall for a very long time before turning down into the mesophyll. As the wall swelled, the cuticle raised around the tip of the germ tube, leaving it in a depression.

During infection the host reacted by forming many vesicles in an area below the infection site and often the host nucleus appeared in this zone (Fig. 2).

After the infection peg passed through the cuticle, the peg enlarged to about normal mycelial diam or slightly larger (Fig. 7, 8) and the infection hole increased in diam (Fig. 8). By the time the infection hypha was 5-10 μm in length the epidermal wall was degraded 30-50 μm around the infection peg and the host cell plasmalemma was withdrawn from the cell wall and the cytoplasm appeared coagulated.

Indigo blue crystals were observed in the conidia prior to germination, and in the tip of many of the germ tubes 5-7 h after inoculation. No indigo blue crystals were found in tips of germ tubes 16-20 h after inoculation in turnip juice.

DISCUSSION.—Infection with *B. cinerea* frequently occurred while the germ tube was short, 10-20 μm, without the formation of an appressorium. Blackman and Welsford made similar observations (2).

Mucilage around the germ tube obviously securely fastened the germ tube to the cuticle because it was not dislodged during electron microscopy preparatory procedures.

It was surprising to find that about 50% of the infection pegs had passed through the epidermal wall 7 h after inoculation because Brown (3) reported that infection took place between 12 and 24 h.

Wood (18) believed that *B. cinerea* penetrated the cuticle in a matter of hours but my investigation shows that the cuticle is penetrated quickly (in a few minutes) because no infection pegs were present by 6 h after inoculation and half had penetrated the cuticle by 7 h after inoculation.

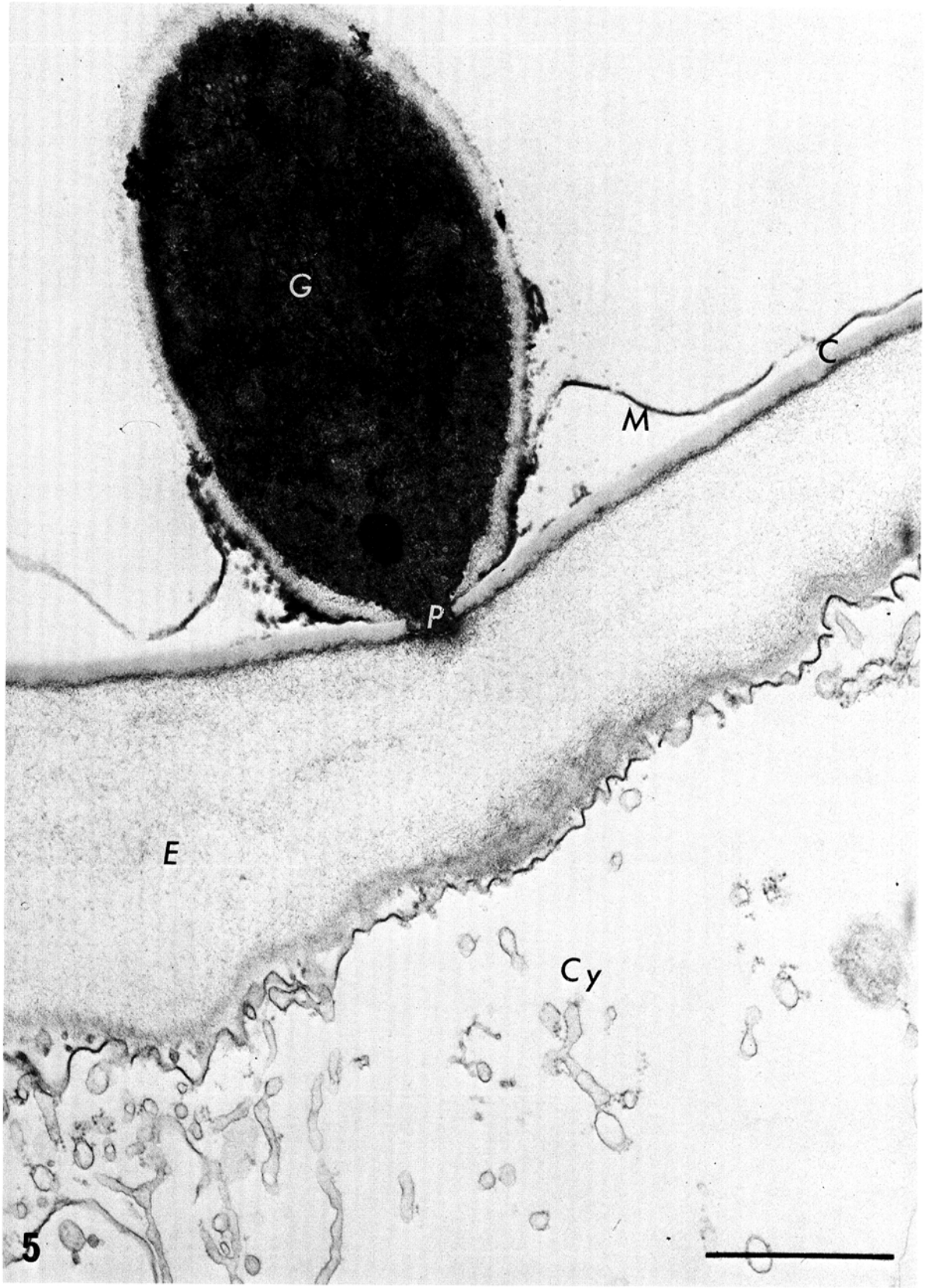
Blackman and Welsford (2) detected a slight indentation of the cuticle and wall prior to penetration, but I never found a depression below the infection peg prior to penetration. I frequently found the infection peg was in a depression of the cuticle after infection and I believe that this results from a swelling of the epidermal wall after infection and a pushing out of the cuticle around the tip of the germ tube.

The swelling of the epidermal wall and its degradation is very rapid after the infection peg passes through the cuticle. The increase in the thickness of the epidermal wall was extremely great and the wide separation of epidermal wall layers and the pushing up of the cuticle must have been caused by degeneration products from the host wall.

The turning down of the germ tube tip to contact the cuticle, and the development of a pore in the fungal wall in the center of the contact area, are the first indications of infection. The fact that the infection peg is blunt, about 0.2 μm in diam, covered only by a plasma membrane and makes a clean passage through the cuticle rather than a hole surrounded by a cuticular flange brings into question the theory of mechanical penetration as postulated by Brown (6). It is difficult to visualize how the blunt infection peg could pass through the host cuticle by "elongation growth" mentioned by Frey-Wyssling (10) or by means of osmotic pressure as propounded by Dickinson (8). If infection were the result of only mechanical pressure surely portions of the cuticle would be driven down into the epidermal wall.

Micrographs of the infection peg in the nonindented

Fig. 5. Infection of broad bean (*Vicia faba*) by *Botrytis cinerea*. An electron micrograph of a section through the tip of a germ tube (G), an infection peg (P), host wall (E), and cytoplasm (Cy). The scale line is 1 μm. The peg is almost through the cuticle (C). The cuticle is not indented and the area of contact between the germ tube and the cuticle is relatively small. A considerable amount of mucilage (M) is present on both sides of the germ tube. The epidermal wall (E) is not altered except for a small area in advance of the peg. (× 32,600).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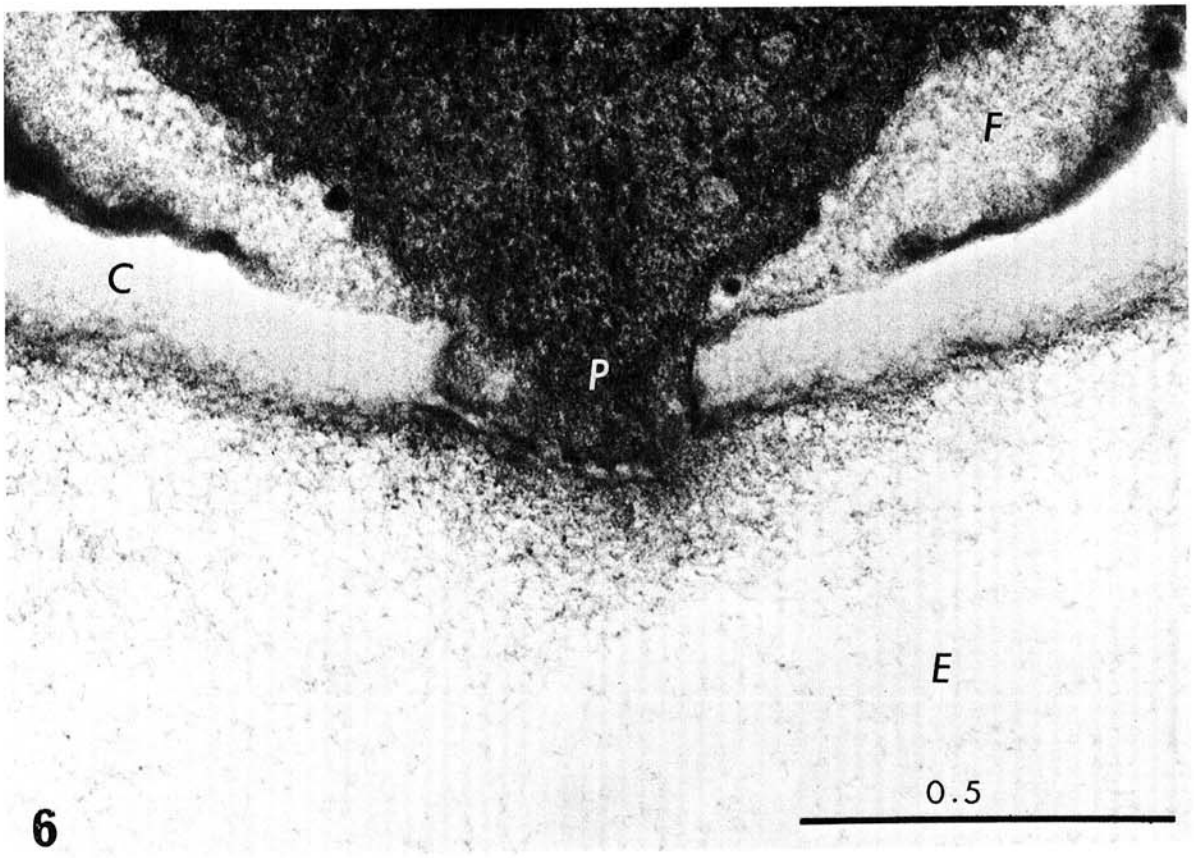

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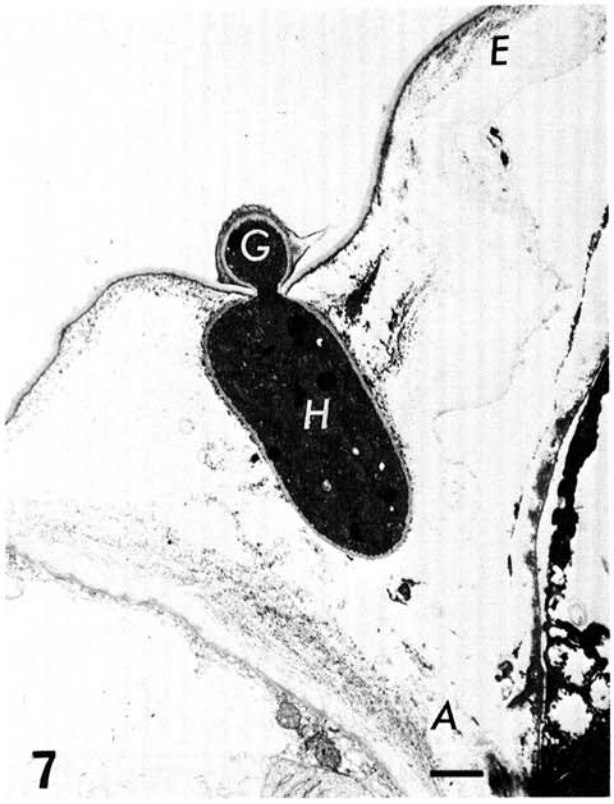

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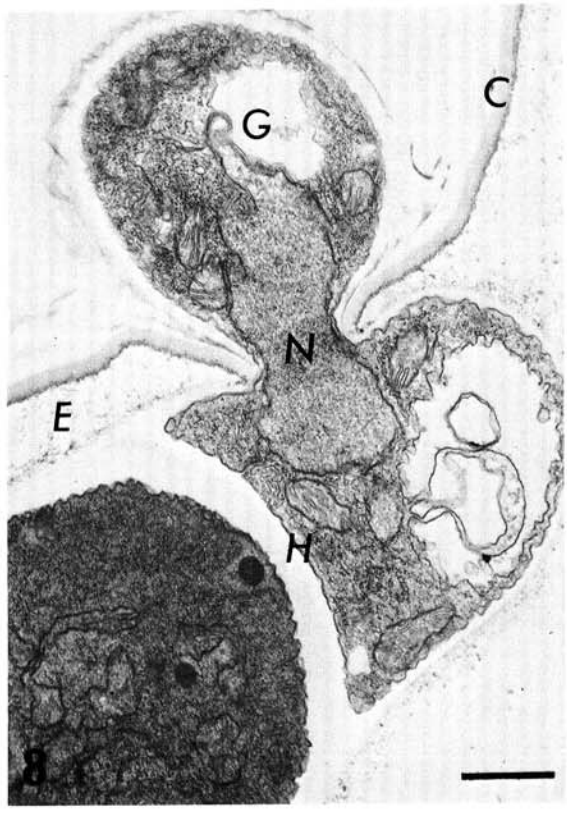

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Fig. 6-8. Stages of infection of broad bean (*Vicia faba*) with *Botrytis cinerea*. The scale line is 0.5 μm in Fig. 6 and 1 μm in Fig. 7,8. **6)** An electron micrograph (part of Fig. 5) of a section through an infection peg (P) which is through the cuticle (C) except for a portion on the left side. There is a plasma membrane but no fungal wall (F) around the infection peg which has a blunt tip. A clean passage is made through the cuticle. Observe the relatively small area of the tip of the germ tube which is in contact with the host cuticle. The epidermal wall (E) is slightly altered. (× 100,000). **7)** An electron micrograph of a section through the tip of a germ tube (G), infection peg, young infection hypha (H), epidermal wall (E), and cytoplasm. The infection peg has enlarged after it passed through the cuticle. The cuticle has been pushed outwards by the swollen epidermal wall. Note that the infection site is above an anticlinal wall (A). (× 6,750). **8)** An electron micrograph of a section through the tip of a germ tube (G), an infection peg and a septate infection hypha (H) about 1 h after penetration of the cuticle. Mucilage is present on both sides of the germ tube. A nucleus (N) is through the infection pore. The cuticle (C) is turned down around the infection peg which is 1 μm in diam. The epidermal wall (E) around the infection hypha is almost completely degraded. (× 12,700).

cuticle (Fig. 6) indicate chemical degradation at the tip of the blunt infection peg and show that the fungal plasmalemma is firmly pressed against the cuticle. The presence of indigo blue crystals in the tip of the germ tubes until penetration, gives support to the enzymatic theory.

Purely mechanical penetration of the cuticle would seem to be ruled out, also, because the area of contact between the tip of the germ tube and the cuticle is not sufficiently large to reasonably withstand the back thrust which would be required to force a blunt peg of relatively great diam through the cuticle.

Brown (3) could not extract an enzyme from *B. cinerea* germ tubes which would degrade cutin. However, he made extracts from germ tubes 23 h after inoculation, i.e., 16 h after penetration. My histochemical tests showed that esterases were present in the tip of many of the germ tubes 7 h after inoculation but not 16 h after placement in turnip juice. The esterases could aid in plasticizing the fungal wall where the infection pore forms in the tip of the germ tube and in dissolving the host cuticle. The relatively electron-lucent area, containing tiny vesicles, immediately above the infection peg may contain cutinase. Because of its small volume, approx. 4×10^{-13} ml, and of its presence for only a short time it would be very difficult to isolate. In 1963, Linskens and Haage (12) claimed that *B. cinerea* produced cutinase. More recently Nicholson et al. demonstrated transitory esterase activity in *Venturia inaequalis* (16).

It is difficult to explain the results of the experiments in which films of undegradable substances such as gold were penetrated by infection pegs (17). Perhaps relatively little mechanical force is required or they have mechanically weak areas. This work should be reinvestigated by electron microscopy.

It seems that passage through the cuticle is not accomplished by either chemical or mechanical means alone but by the pathogen pressing against the host cuticle in a relatively small area and excreting a limited amount of cutinase for a short time through the fungal plasmalemma which covers the blunt tip of the infection peg.

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