Light- and Scanning Electron Microscopy of Cucumber and Barley Powdery Mildew on Host and Nonhost Plants

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ABSTRACT

The development of *Erysiphe graminis* and *E. cichoracearum* on their respective nonhost plants, cucumber and barley, was studied and compared to normal development on their host plants. On cucumber leaves, total germination and rate of germination of *E. graminis* were only half as high as on barley. Nevertheless, the germinating spores formed mature appressoria and penetrated the epidermal cells of the cucumber leaves. The invaded cells responded with a hypersensitive reaction before branching of the haustorial initials. On barley, *E. cichoracearum* conidia germinated normally, but the germ tubes were unable to dissolve the epicuticular wax crystals and penetration of the epidermal cells was prevented.

After the fungal structures were removed, scanning electron microscopy revealed the imprints of germ tubes, appressoria, and hyphae on the cuticular surfaces of both the host and nonhost plants. On barley leaves, the epicuticular wax crystals in contact with *E. graminis* had been dissolved. On cucumber, both fungi left traces, consisting probably, at least in part, of fungal secretions, on the cuticular surface. The absence of tearing around the penetration holes suggested the involvement of enzymes in cuticular penetration. The penetration holes of *E. graminis* on both barley and cucumber were usually in the center of appressorial imprints, whereas those of *E. cichoracearum* on cucumber also occurred along the center of hyphal traces. These observations indicate that germ tubes and hyphae of powdery mildew fungi may share in some functions previously attributed only to appressoria.

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The development of plant pathogenic fungi on nonhost plants has received relatively little attention in the past. Although most teachers of plant pathology make it clear to their students that any one plant species is attacked by only a small number of fungal species, and that as a rule plants are immune to plant pathogens, we know very little about the mechanisms by which this rule is enforced in nature. Possibly a better knowledge of immunity mechanisms in plants may teach us new ways of controlling plant diseases.

The thickness of the cuticle has been implicated as a factor in the immunity of plants against foliar pathogens (2, 12). In his review of the role of the cuticle in the defense against plant diseases, Martin (11) presents evidence both for and against the involvement of the physical toughness of cuticles in the protection of plants against fungal pathogens.

Matta (12), in his discussion of premunity of plants, focused primarily on the consequences of the changes elicited by uncongenial microorganisms on the subsequent infection by congenial pathogens. He discussed some aspects of general plant immunity, and concluded that varietal resistance and immunity of uncongenial hosts are physiologically indistinguishable. However, it seems that our understanding of the mechanisms responsible for the immunity of plants against uncongenial pathogens is rather incomplete to make such a comparison.

The few earlier studies on the development of powdery mildew fungi on nonhost plants were discussed, and in part confirmed, by White and Baker (21) who reported that the development of *Erysiphe graminis* f. sp. *hordei* on oats, wheat, and rye was halted only after the formation of appressoria but before the formation of haustoria. It was not always clear whether the fungus had perforated the cuticle and the epidermal cell wall of the nonhost plants. On only one wheat cultivar had the pathogen clearly penetrated the epidermal cells and formed haustoria and some mycelium.

Studies of fungi which enter through stomates, indicate that appressorium formation and invasion of the substomatal chamber are not host-specific. Heath (6) compared the reactions of highly resistant cowpeas and of beans to *Uromyces phaseoli* var. *vignae*. On the resistant host variety fungal development was stopped after haustorium formation, whereas on the nonhost plant only infection hyphae but no haustoria were formed. Similarly, Leath and Rowell (8) showed that the development of *Puccinia graminis* on the nonhost corn plants was halted after infection hyphae had formed. As early as 1863, DeBary had described many cases in which rust fungi invaded the substomatal chambers of nonhost plants where their development was stopped (3). The cessation of fungal growth in the substomatal chambers of nonhost plants is usually accompanied by a browning of the parenchyma cells in contact with the infection hyphae (6, 8).

Among plant pathogens, powdery mildew fungi seem to offer particular advantages for the study of fungal development on nonhost plants. Being exobiontic parasites, their spore germination, appressorium and haustorium formation, as well as hyphal growth and certain host reactions, can be conveniently observed under a light microscope (1, 4, 14, 20, 21) or a scanning electron microscope (16, 19).

In most of the studies discussed above, the nonhost plants are related to the host plants of the fungi used. Our study was undertaken to investigate the development of powdery mildew fungi on nonhost plants quite unrelated to their normal hosts. Using light- and scanning electron microscopy, we attempted to determine at which stage and how the development of *Erysiphe cichoracearum* and *E. graminis* is halted on the uncongenial hosts barley and cucumber respectively. For reference, the infection processes of the two fungi on their host plants were also studied.

MATERIALS AND METHODS.—Cucumber (*Cucumis sativus* L. 'Verbesserte Chinesische Schlange') and barley (*Hordeum vulgare* L. 'Herta') were grown in a greenhouse in 8-cm diam plastic pots containing a mixture (7:1, v/v) of a standard soil (type TKS I, Torfstreuverband GmbH, Oldenburg) and sand at about 24 C. Seven- to 10-day-old plants were used for the experiments which were conducted in growth chambers at 20 C, 70% relative humidity (RH), and light intensity of 15,000 lux for 16-h/day.

Erysiphe cichoracearum DC. and *E. graminis* DC. f. sp. *hordei* Em. Marchal were maintained on 1- to 3-wk-old host plants at 20 C. For the experiments, only freshly formed conidia from heavily infected leaves were used; the older conidia had been tapped off and discarded 16 h prior to the utilization of the infected leaves as an inoculum source. Portions of abaxial surfaces of barley primary leaves and of cucumber cotyledons were inoculated by placing a glass cylinder 9 cm high and 1.5 cm in diam vertically on the horizontally arranged leaves, putting heavily infected leaves, infected side down, over the upper end of the cylinder and tapping off the conidia. By this inoculation method single conidia were evenly distributed over the leaf surface.

Preparations for light microscopy.—Cleared leaves were used to determine germination, and epidermal strips were used to observe germ tubes, penetration sites, and haustoria in more detail. The cleared leaf preparations proved superior to the epidermal strips for germination counts, because many spores were lost during the preparations of the epidermal strips.

For germination counts, leaves were removed from the plants at various intervals after inoculation and cut into pieces 3 × 3 mm. The leaf pieces were placed in a watch glass with just enough water to allow moistening of their edges. The watch glass was set on an inverted petri plate in a glass jar containing a small amount of absolute isopropanol. The jar was sealed for 24 h to allow the alcohol vapors to dehydrate and clear the leaf pieces. The cleared pieces were transferred to glass slides and stained with cotton blue in lactophenol (1%, w/v).

For more detailed observation of the infection process, epidermal strips were removed from the leaves at various intervals after inoculation and stained with cotton blue. For some observations on epidermal strips, the staining solution used for cleared leaf pieces was diluted 1:1 with water to prevent shrinking of the protoplasts (Fig. 14).

Preparations for scanning electron microscopy.—Scanning electron microscopy (SEM) observations were made on the early stages of fungal development on host and nonhost plants. In addition, alterations of the leaf surfaces around the penetration site were examined. To remove the fungal structures, gelatine (20% aqueous, w/v) at 35 C was poured over the inoculated leaf surface and removed after it had solidified. Among several methods tried, the gelatine method caused the least distortion of the cuticular wax lattice.

The leaf portions to be observed with SEM were cut into pieces of 2 × 2 mm. Quick freezing in liquid nitrogen with subsequent lyophilization proved to be the most suitable method for fixation of this material. The leaf

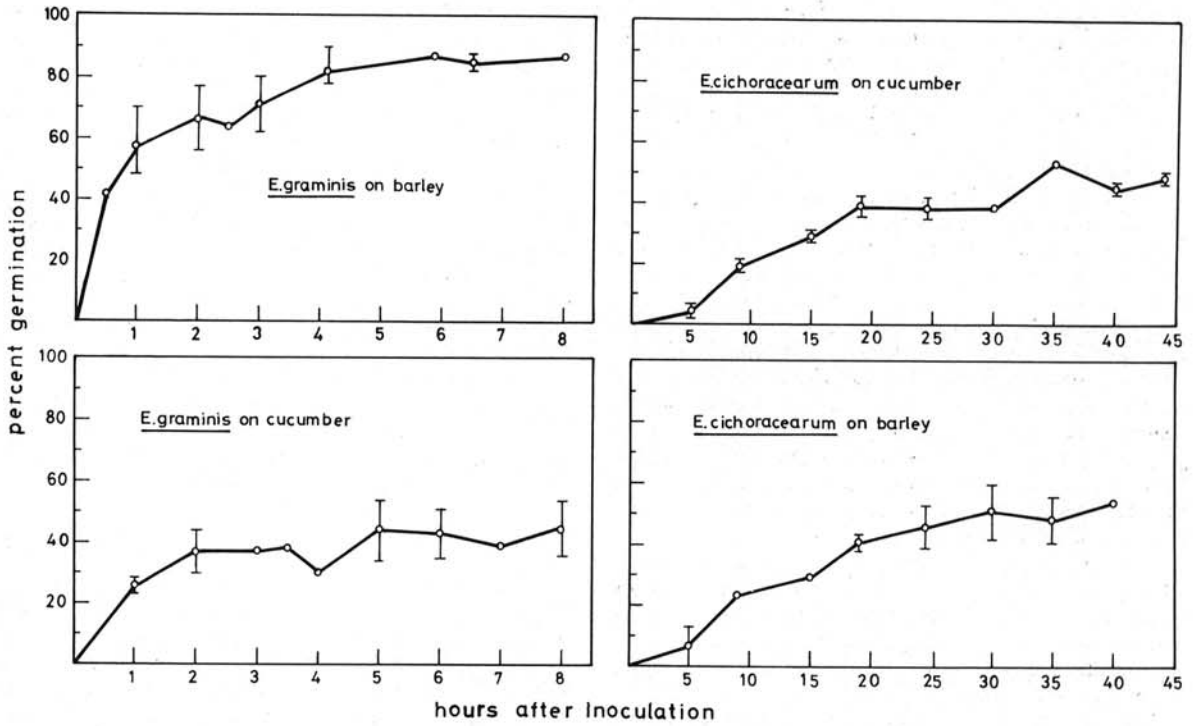


Fig. 1. Germination of *Erysiphe graminis* and *E. cichoracearum* on barley and cucumber leaves. Each point is a mean of germination counts from one to three experiments. The vertical lines indicate the range of the average germination counts in the different experiments.

pieces were submerged in melting nitrogen, that had been solidified at reduced pressure. This mixture of solid and liquid nitrogen at −210 C was more favorable for fixation than normal liquid nitrogen at −196 C since boiling did not occur around the freezing specimen. The frozen leaf pieces were quickly transferred to a freeze-drier (Modell L2, WKF, Darmstadt) set at −40 C and dried for at least 24 h.

The dried leaf pieces were affixed on specimen holders with mounting silver, coated with gold, and viewed with a Stereoscan Mk II scanning electron microscope (Cambridge Instruments Company). In some cases, fresh leaf portions were coated directly with gold for comparison.

RESULTS.—*Development of E. graminis on cucumber and barley.*—Total germination and rate of germination of *E. graminis* were roughly twice as high on barley as on cucumber (Fig. 1). On both plants, however, the average germination time was less than 1 h.

Although SEM revealed great differences in the leaf surface structures of the two plants (compare Fig. 2, 4 and 5), the early development of *E. graminis* on both was very similar. Germ tubes and mature appressoria, usually bent at the tip (Fig. 5, 8, 12, 13), and haustorial initials in the epidermal cells (Fig. 11, 14) were formed on both plants. On barley, the haustorial initials differentiated further and produced many fingerlike branches (Fig. 11). On cucumber, haustorial branching of *E. graminis* was prevented by the death of the invaded epidermal cells (Fig. 14, 15, 16). Concomitant with the cessation of haustorium development, the formation of secondary hyphae on the leaf surface was stopped (Fig. 5, 12). Only occasionally were small initials of secondary hyphae observed (Fig. 13). On barley, the secondary hyphae of *E. graminis* branched laterally from the primary appressoria (Fig. 2, 8) and clearly exhibited directed growth along the cell walls which separated epidermal cells (Fig. 9).

Circular halos, stained lightly with cotton blue, could be observed around the penetration sites of *E. graminis* on barley (Fig. 10, 11) and on cucumber leaves (Fig. 14-16). However, they were more pronounced on barley. A more distinctly stained area, presumably the penetration peg and its immediate vicinity, could be observed in the center of most of the halos (Fig. 10, 11, 15).

On both barley and cucumber, *E. graminis* altered the leaf surface structure, leaving imprints of the characteristically shaped appressoria, germ tubes, and hyphae (Fig. 3, 4, 6, 7). The imprints became clearly visible where the fungal organs were removed with gelatine. It is not clear, especially on cucumber, whether the imprints observed resulted entirely from alterations in the leaf surface structures, or whether they consisted partially of fungal secretions.

On both plants, the perforations in the cuticle through which the penetration pegs of *E. graminis* had entered could be observed with SEM (Fig. 4, 6, 7). The penetration holes were round, had smooth edges and were consistently larger on cucumber (up to 0.5 μm) than on barley (up to 0.2 μm).

Development of E. cichoracearum on barley and cucumber.—Total germination and rate of germination of *E. cichoracearum* were the same on barley and cucumber (Fig. 1). The average germination time was about 14 h compared with less than 1 h for *E. graminis*. Occasionally *E. cichoracearum* produced branched germ tubes (Fig. 17) which were observed more frequently on barley than on cucumber. Hyphal growth of *E. cichoracearum* on cucumber leaves appeared to be random (Fig. 17, 18, 23).

On barley, *E. cichoracearum* was not able to penetrate the epidermal cells. No haustorial initials could be found in the epidermal cells beneath the germ tubes, and halos, similar to those surrounding the appressoria of *E. graminis,* were extremely rare and when present they lacked a distinctly staining center. With SEM, neither imprints of germ tubes or appressoria nor cuticular perforations could be observed on barley leaves from which the germinated conidia of *E. cichoracearum* had been removed with gelatine up to 84 h after inoculation. No secondary hyphae were formed (Fig. 19, 24).

On cucumber leaves, *E. cichoracearum* penetrated the epidermal cells and formed characteristic haustoria. The haustorial initials were surrounded by a sheath (Fig. 26) which expanded with maturation of the haustorium and eventually became spherical and filled tightly with numerous haustorial branches coiled around the central body (Fig. 27, 28). The halos around the penetration sites of *E. cichoracearum* were similar to those around the penetration sites of *E. graminis* on cucumber (compare Fig. 15, 16, and 25). The margins and the centers of these halos appeared to have greater affinity for stain than the areas in between.

Where germ tubes, appressoria, and hyphae of *E. cichoracearum* had been removed from the cucumber leaves, SEM revealed similar traces of these fungal structures on the cuticular surface (Fig. 20-22). In the traces the cuticular surface seemed slightly altered as though some material had been deposited along their edges (Fig. 21, 22). It was not clear whether these deposits consisted of altered cuticular material or fungal secretions.

Cuticular perforations 0.2 to 0.7 μm in diam were observed, not only in the center of appressorial imprints, but also along the center of traces from germ tubes and hyphae where *E. cichoracearum* had penetrated the cuticle of the cucumber leaves (Fig. 20, 21). Like those made by *E. graminis,* these penetration holes were round with smooth edges and showed no signs of physical stress or tearing (Fig. 21, 22).

Reactions by the uncongenial plants.—On barley inoculated with *E. cichoracearum,* no reactions of the epidermal cells beneath the germ tubes could be observed, except the very occasional halos in epidermal cell walls mentioned above. This is not surprising since the fungus apparently did not penetrate the epidermal cells.

E. graminis on cucumber penetrated the epidermal cells; however, its haustorial development was stopped by a hypersensitive reaction of the invaded epidermal cells before branching occurred. The cells which were penetrated showed different staining properties and their protoplasts failed to shrink in the full strength cotton blue solution as did those of adjacent cells (Fig. 14, 15). The mesophyll cells beneath the invaded epidermal cells were also examined, and they showed no reaction.

SEM revealed that cucumber epidermal cells invaded by *E. graminis* reacted quite differently than the

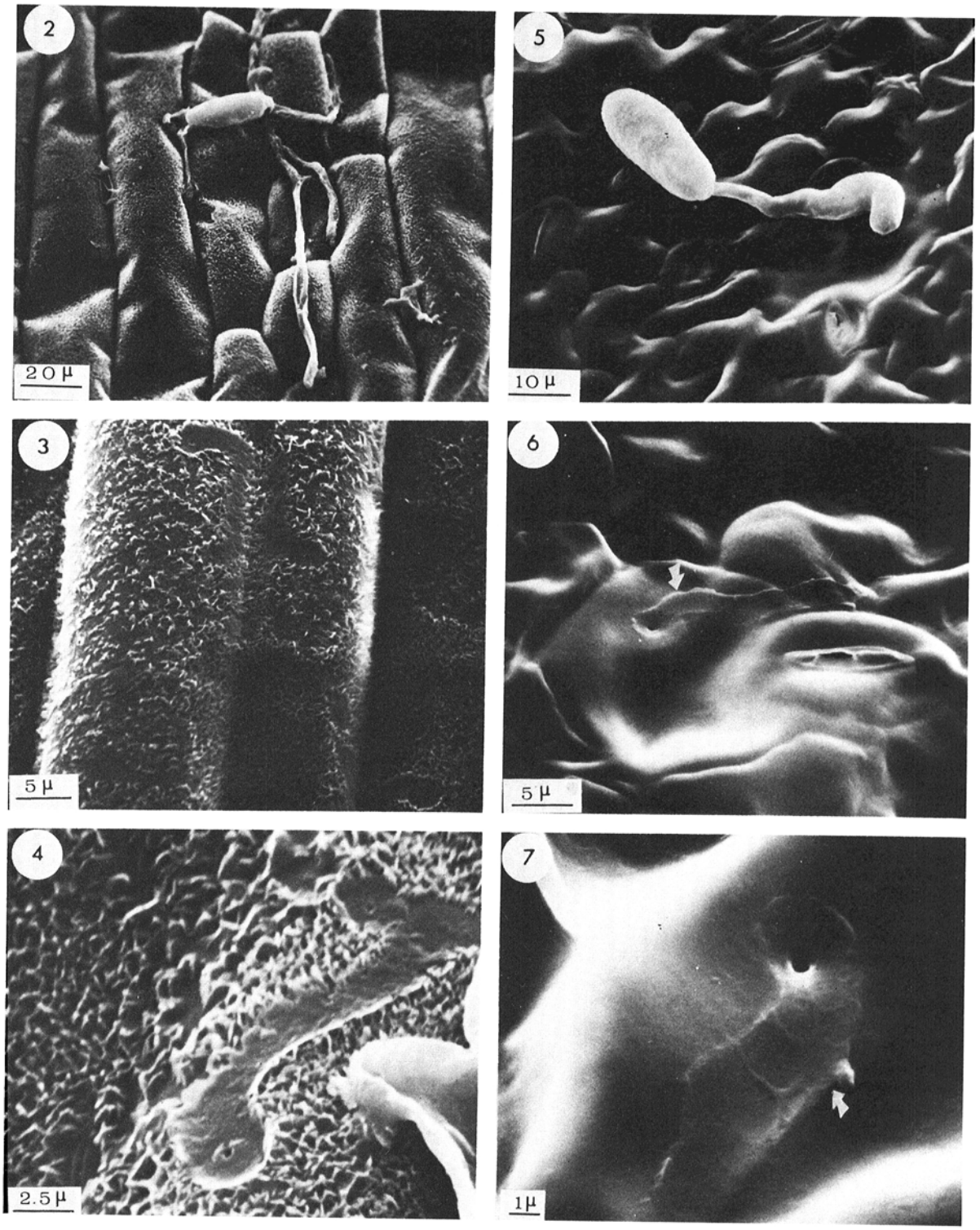


Fig. 2-7. Scanning electron micrographs of *Erysiphe graminis* and its traces on barley leaves **(2-4)** and on cucumber cotyledons **(5-7). 2, 5** *E. graminis* **2)** on barley 40 h and **5)** on cucumber 64 h after inoculation. **3, 4)** Imprints and cuticular perforations of *E. graminis* in barley leaf surface 40 h after inoculation. Note dissolution of wax crystals under germ tubes, hyphae, and appressoria. **6, 7)** Traces and cuticular perforations of *E. graminis* on cucumber cotyledons 64 h after inoculation. Note deposits (arrows) along the edges of the traces.

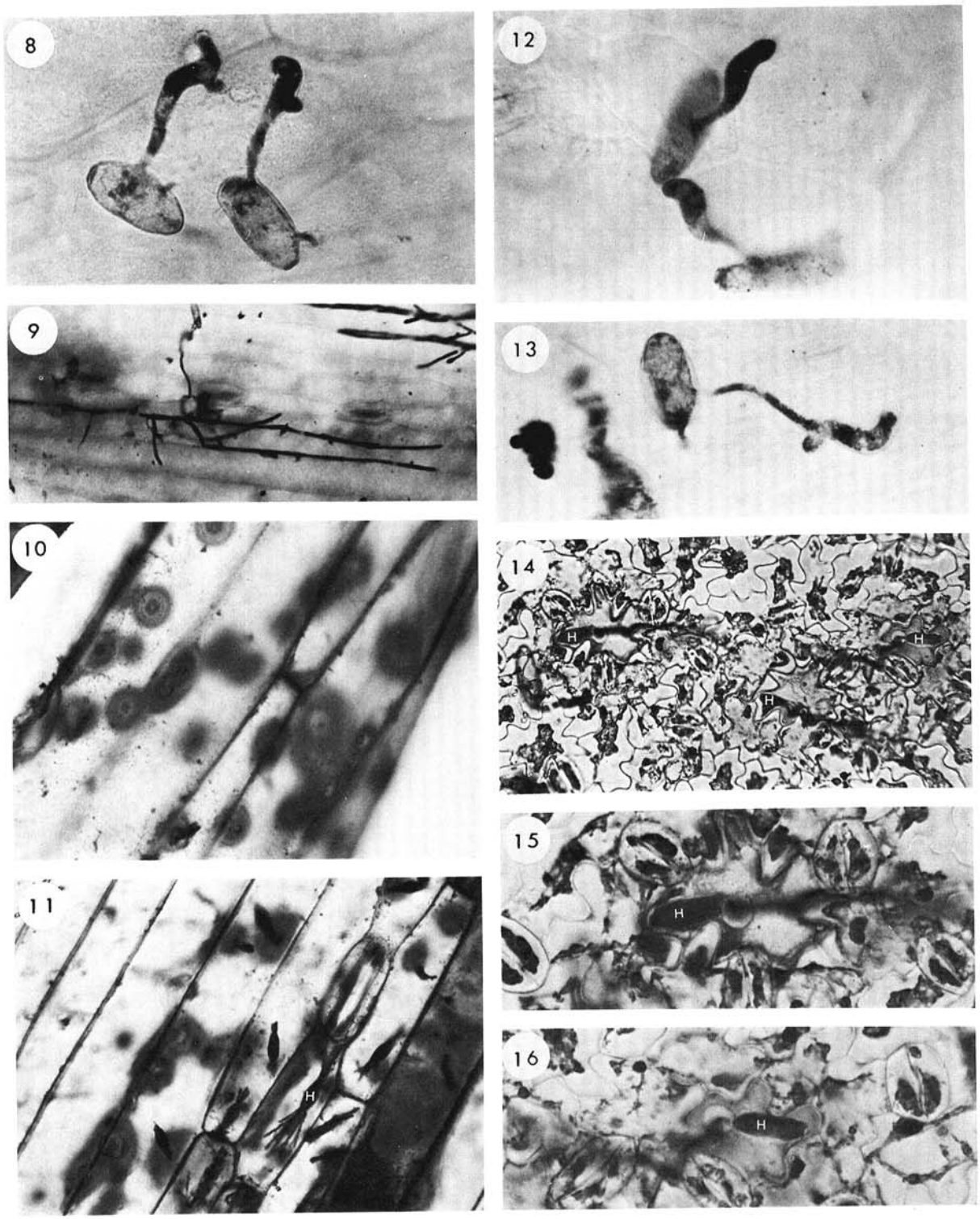


Fig. 8-16. Light micrographs of *Erysiphe graminis* on barley leaves (8-11) and on cucumber cotyledons (12-16); preparations stained with cotton blue. **8)** *E. graminis* on barley 24 h (×1,500), and **9)** 64 h after inoculation (×375). **10)** Barley epidermal strip with halos where appressoria of *E. graminis* had been removed (×750). **11)** Mature primary haustorium of *E. graminis* (H) in barley epidermal cell surrounded by several secondary haustoria at various stages of development 88 h after inoculation (×750). **12)** *E. graminis* on cucumber 15 h and **13)** 64 h after inoculation (×1,500). Note that after normal germ tube development the formation of secondary hyphae is prevented. **14)** Three haustorial initials (H) of *E. graminis* in cucumber epidermal cells which had reacted to fungal invasion. Note that the protoplasts of the invaded cells did not shrink as did those of the surrounding cells (×750). **15, 16)** Details from Fig. 14 showing the halos around the penetration sites and the haustorial initials (H) (×1,500).

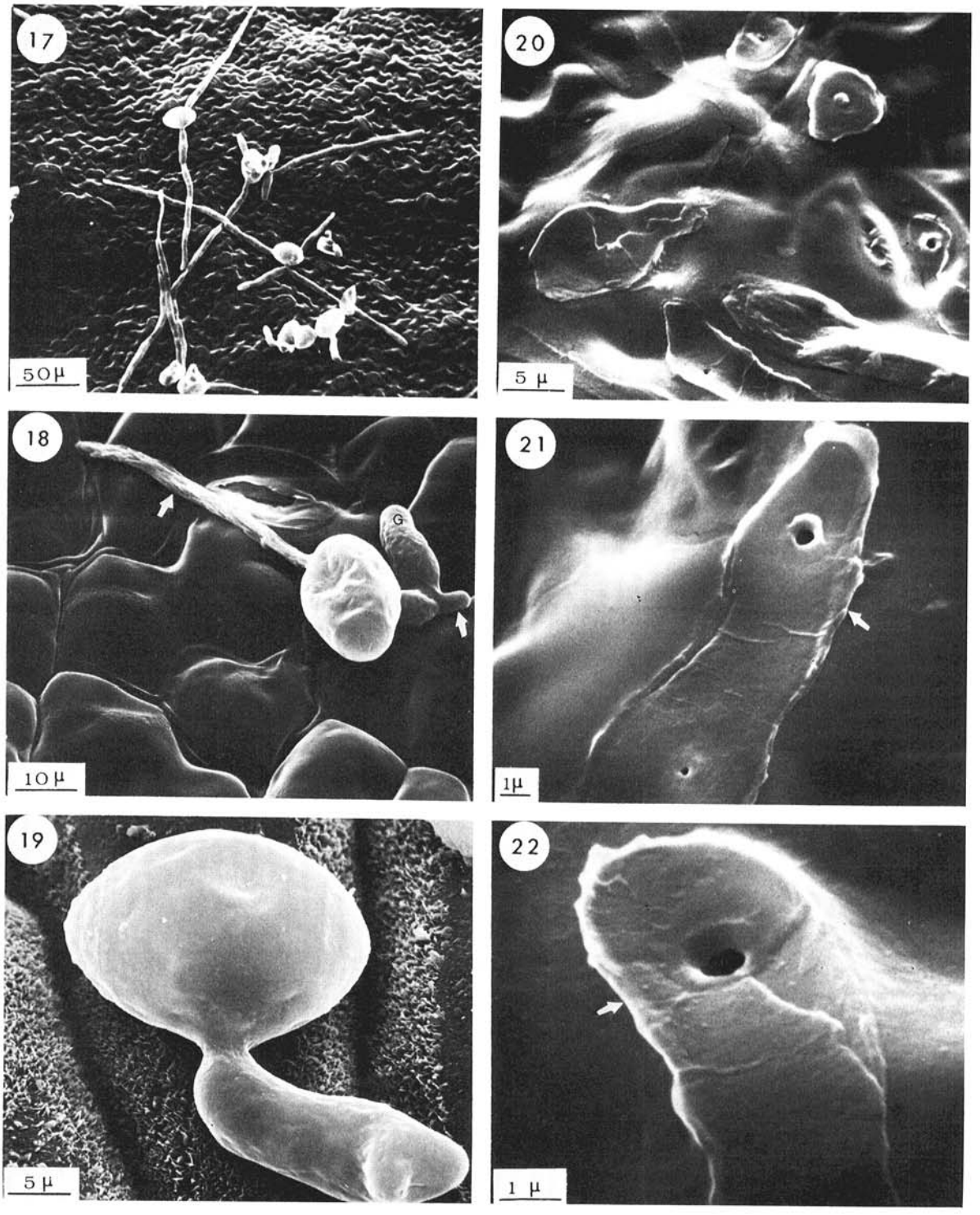


Fig. 17-22. Scanning electron micrographs of *Erysiphe cichoracearum* on cucumber cotyledons (17, 18, 20-22) and barley leaves (19). **17)** Overall view of *E. cichoracearum* on cucumber 40 h after inoculation. **18)** Close-up of germinated *E. cichoracearum* spore on cucumber with germ tube (G) and elongating secondary hyphae (arrows) 28 h after inoculation. **19)** *E. cichoracearum* spore with germ tube on barley leaf 40 h after inoculation. **20-22)** Traces and perforations in the surface of cucumber cotyledons after the removal of *E. cichoracearum* structures with gelatine 40 h after inoculation demonstrating that not only appressoria but also germ tubes and hyphae had been interacting with the cuticular surface. Note the deposits along the edges of the fungal traces (arrows) and the smooth edges of the perforations through cuticle and epidermal cell wall.

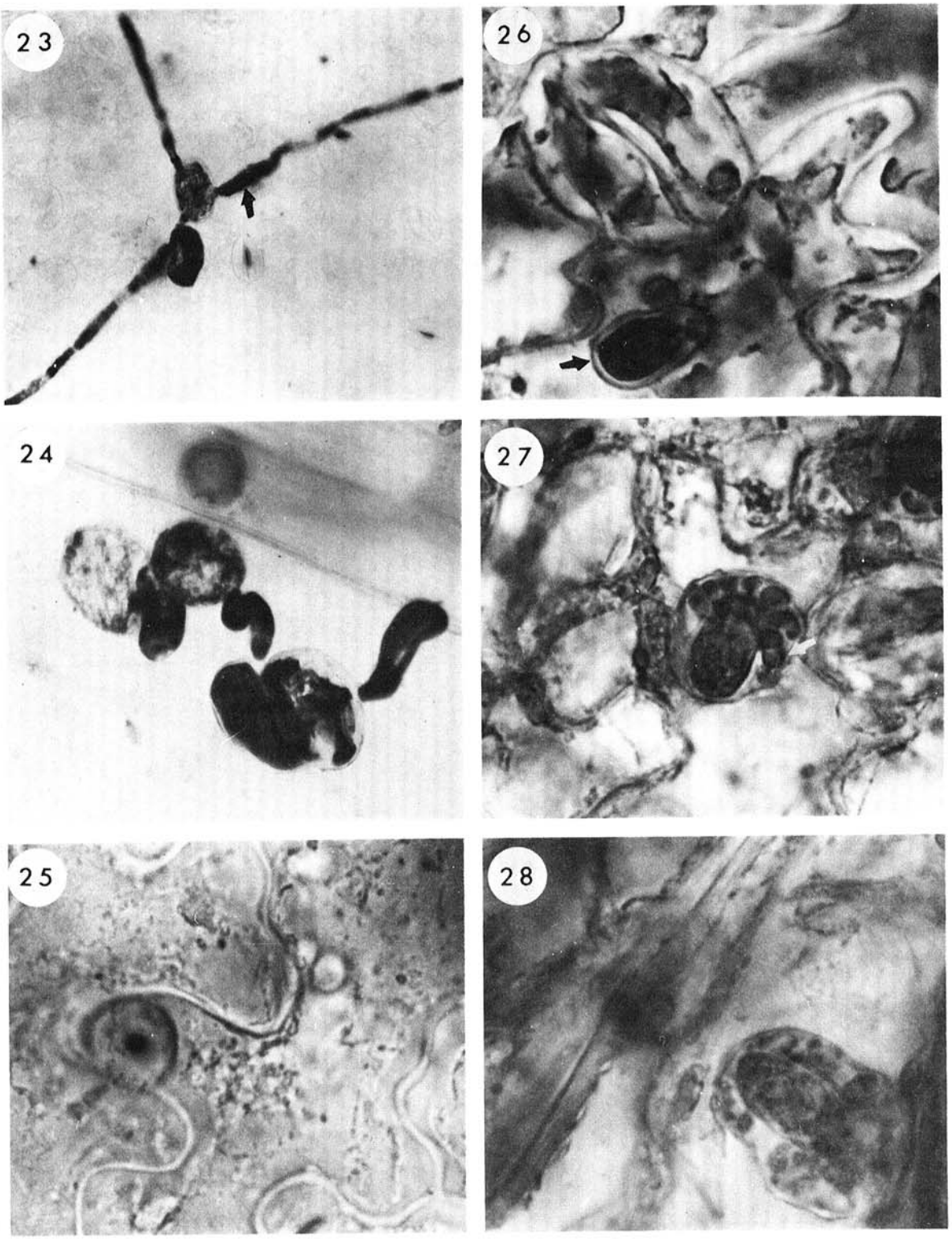


Fig. 23-28. Light micrographs of *Erysiphe cichoracearum* on cucumber cotyledons (23, 25-28) and barley leaves (24); preparations stained with cotton blue. **23)** *E. cichoracearum* spore with primary germ tube (arrow) and secondary hyphae 40 h after inoculation (×750). **24)** Germinated spores of *E. cichoracearum* on barley leaf 64 h after inoculation with only primary germ tubes and no secondary hyphae (×1,500). **25)** Halo with dark center and margin around the penetration site of *E. cichoracearum* into a cucumber epidermal cell (×3,750). **26-28)** Three stages in the haustorial development of *E. cichoracearum* in cucumber epidermal cells (×3,750). Note that the haustorial sheath (26, arrow) forms before the branches of the haustorium (27, arrow) develop. **28)** Mature haustorium with haustorial arms coiled around the central body.

surrounding cells to the stresses of the preparation methods used. In freeze-dried preparations the healthy cells had a slightly bloated appearance,whereas the dead cells could be recognized as depressions in the leaf surface (Fig. 5, 29). When fresh leaf pieces were directly coated with gold and observed under SEM, the invaded cells did not show the shrunken appearance of the rest of the leaf surface (Fig. 30). In both cases, the traces left by the germ tubes of *E. graminis* and the perforations through which the penetration pegs had entered the epidermal cells could still be seen.

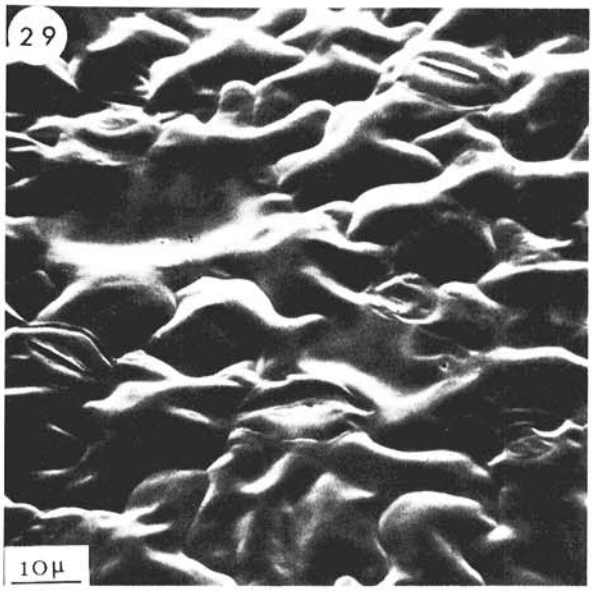
DISCUSSION.—Our studies indicate that the dissolution of the wax crystals, as was observed under the germ tubes of *E. graminis* (Fig. 3, 4), may be a critical step in the process of direct penetration of barley leaves. If the wax crystals cannot be dissolved, a close contact of the appressorium with the cuticular surface is not possible and penetration is prevented. This view is in contrast to the concept, first put forward by Melander and Craigie for barberry rust (15), and later adopted for powdery mildew and other diseases (2, 11, 12), that the cuticle thickness is the excluding factor where failure of cuticular penetration on resistant or uncongenial plants occurs.

Although our observations suggest a possible involvement of the epicuticular wax in the immunity mechanism of barley against *E. cichoracearum,* they do not indicate whether immunity is due to chemical or physical factors. In studies on varietal resistance, inhibitory substances from leaf surface waxes were implied to be factors of resistance (7, 11), and Schuett (18) correlated stimulatory substances from cuticular waxes of *Acer* and *Quercus* spp. with susceptibility to various foliar pathogens. Ellingboe (5), on the other hand, concluded that for the stimulation of mature appressoria formation of *E. graminis* on cereal leaves, the physical structure of the epicuticular wax crystals may be more important than their chemical constituents.

The hypersensitive reaction which occurred in all cucumber epidermal cells invaded by haustorial initials of *E. graminis* without any apparent alteration of the adjacent mesophyll cells, seems to be distinct from the mechanism of powdery mildew resistance reported in barley cultivars. In varietal resistance of barley the mesophyll cells under the invaded epidermal cells are also involved in the defense reaction (21), and the hypersensitive reaction occurs only in a small percentage of the invaded cells (4, 21).

The formation of mature appressoria and cuticular penetration on cucumber leaves by *E. graminis* (Fig. 5, 7, 13, 14) demonstrates that the specific chemical and physical properties of cereal cuticular waxes are not essential for the stimulation of mature and functional appressoria as was suggested by Ellingboe (5). Apparently the cuticular surface of cucumber cotyledons provides the necessary chemical and physical stimuli for appressorium formation and cuticular penetration by *E. graminis*.

In contrast to the currently accepted concept that the close contact of powdery mildew fungi with the leaf surface is limited to appressoria (5, p. 402), this study showed that both germ tubes and hyphae were also in close contact and interacting with the cuticular surface in all three fungus-plant combinations where cuticular imprints were observed (Fig. 3, 4, 6, 7, 20, 21). An examination of the fungal tracks revealed that the association with the cuticular surface was basically the same for appressoria, hyphae, and germ tubes. This suggested that in the powdery mildews, hyphae, and germ tubes may share in some functions previously attributed



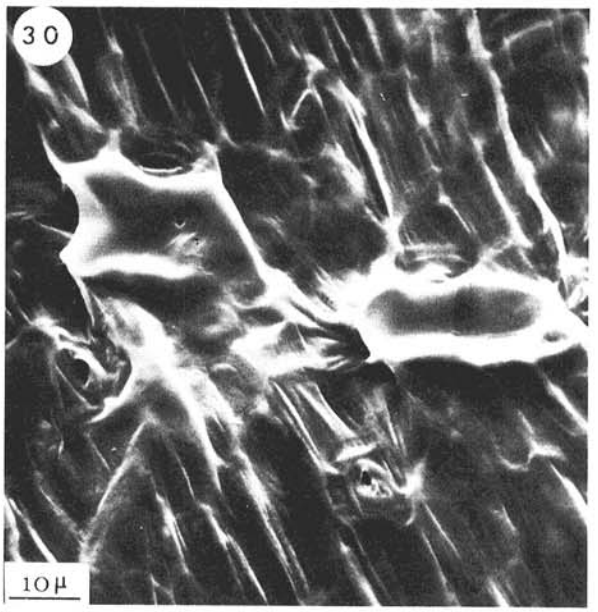


Fig. 29, 30. Surface of cucumber cotyledons with epidermal cells that had reacted to invasion by *Erysiphe graminis.* **29)** Leaf segment fixed by quick freezing in liquid nitrogen and subsequent freeze-drying: the invaded epidermal cells can be recognized as depressions in the leaf surface. **30)** Fresh leaf segment coated directly with gold: the two invaded epidermal cells do not show the shrunken appearance of the surrounding healthy cells. In both fig. the appressorial imprints and the cuticular perforations at the penetration sites can be recognized.

only to appressoria; e.g., adhesion to the leaf surface. Moreover, the cuticular perforations along the center of hyphal imprints of *E. cichoracearum* on cucumber cotyledons (Fig. 20, 21) demonstrate that, at least for this powdery mildew fungus, well-differentiated appressoria are not a prerequisite for cuticular penetration.

On barley leaves, the fungal traces apparently resulted from a dissolution (or utilization) of the wax crystals in contact with germ tubes, appressoria, or hyphae of *E. graminis*. Dissolution of the wax lattice of barley leaves under the appressoria of *E. graminis* was reported by Schwinn and Dahmen (19), and a SEM picture published by Locci and Quaroni (10, his Plate VI/3) appears to show the same phenomenon under appressoria of *Helminthosporium maydis* on corn leaves. McBride (13) described alterations in the epicuticular wax structures on larch leaves by the epiphytic yeast *Sporobolomyces roseus* and mentioned the possibility that the wax could have served as an energy source for the fungus. On cucumber leaves, which lack epicuticular wax crystals, the nature of the traces produced by *E. graminis* (Fig. 6, 7) and *E. cichoracearum* (Fig. 20-22) is less evident. They may consist of altered cuticular material and/or of fungal secretions, the role of which is unknown.

The close contact of germ tubes and hyphae with the cuticular surface may be necessary for directed growth. Contact may also enable the powdery mildew fungi to search the cuticular surface for favorable penetration sites. Evidence is accumulating that the cuticle may contain special polar pathways, possibly corresponding with ectodesmata in the epidermal cell walls (17). It is conceivable that such pathways could serve as entry ports for fungal penetration pegs. Close contact with leaf wax crystals and directed hyphal growth were also reported by Lewis and Day (9) for *Puccinia graminis* on wheat leaves. They accidentally lifted a hypha from the wheat leaf surface, and observed traces without wax crystals, similar to the traces associated with *E. graminis* on barley leaves in this study. Lewis and Day did not mention the possibility that the wax lattice could have been dissolved by the hypha, instead they attributed the trace to the adherence of the wax crystals to the hypha.

The smooth, rounded edges around the penetration holes observed on barley (Fig. 4) and cucumber leaves (Fig. 7, 21, 22) suggested that the process of penetration was aided by enzymes. A penetration process effected purely by physical pressure might be expected to leave signs of tearing around the penetration holes.

The reduced total germination of *E. graminis* on cucumber, together with the unaffected mean germination time (Fig. 1) indicated that the spore population used was not homogeneous. About 50% of the viable spores of *E. graminis* germinated normally on cucumber leaves; the rest did not germinate at all. Ellingboe (5) reported a similar physiological heterogeneity of an *E. graminis* spore population.

After analyzing the results of our study, we suggest that three different mechanisms of immunity may be operating in the two uncongenial host-parasite combinations studied: (i) the inhibition of spore germination of part of the *E. graminis* population on cucumber; (ii) the hypersensitive reaction by cucumber epidermal cells to that part of the *E. graminis* population which penetrated cucumber leaves; and (iii) the prevention of close contact between germ tubes of *E. cichoracearum* and the cuticular surface of barley leaves by the epicuticular wax lattice.

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