

## Papilla Formation: Timing and Significance during Penetration of Barley Coleoptiles by *Erysiphe graminis hordei*

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### ABSTRACT

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Because *Erysiphe graminis* penetration pegs sometimes fail to penetrate host papillae, it has been suggested that the papillae can prevent haustoria from forming. We sought evidence for this suggestion by using interference contrast microscopy to monitor timing of papilla formation in relation to growth of *E. graminis hordei* penetration pegs into living barley (*Hordeum vulgare*) coleoptile cells. Papillae were formed at 85% of the 151 encounter sites studied. A substantial proportion (34%) of the papillae was formed long after pegs were initiated and was correlated with a high (84%) parasite penetration efficiency. Relatively few (12%) of the papillae were formed well in advance of pegs and these were

correlated with a low (27%) penetration efficiency; this suggests that papilla formation is a disease-resistance mechanism. However, the papillae that formed before pegs did not delay development of the pegs or haustoria which subsequently formed. Furthermore, some penetration attempts failed even in the absence of papilla deposition, which suggests that other failures, associated with papillae, may also have occurred for reasons unrelated to papilla formation. Further experiments are required to show whether penetration failures are caused by papillae or by a deficiency in the potential of the fungus to complete penetration.

*Additional key words:* cytology, host-parasite interactions, host responses.

Papillae are host wound appositions formed in response to fungal attack and have been implicated in resistance of plants to fungi (1). Several workers found correlations (3, 5, 6, 9) which suggested that papillae may prevent successful host penetration by some *Erysiphe graminis* penetration pegs. Stanbridge et al. (9) showed that 50-70% of *E. graminis hordei* appressoria failed to produce haustoria in the presence of papillae in cells of a compatible barley host. Bushnell and Bergquist (3) estimated that papillae prevented 20% of selected appressoria of *E. graminis hordei* from producing haustoria. However, much critical information was not provided by their study because (i) a maximum of only 28 appressoria was studied for any one parasite-host combination, (ii) important specific correlations could have been masked because detailed analyses were made on pooled data from several different parasite-host pairings, and (iii) the timing of papilla formation relative to that of penetration pegs was not established.

The primary purpose of this study was to examine the postulated role of barley papillae in resisting ingress by *E. graminis*. Specifically, we were interested in determining when, in relation to penetration peg initiation, papillae would be initiated and whether or not there would be a correlation between early papilla initiation and penetration failures. A secondary purpose was to establish, with an appropriately sized data base, the overall time courses of visible events associated with penetration.

### MATERIALS AND METHODS

A single parasite-host system was used throughout this study. *Erysiphe graminis* DC. f. sp. *hordei* Em. Marchal (race Ao) was maintained in an environmental control chamber on a highly compatible host, *Hordeum vulgare* L. 'Proctor' (9). During the 16-hr photoperiods the plants were maintained at 22 C, 65% RH and were provided with 19,000-30,000 lux of mixed incandescent - fluorescent light before inoculation and 3,600-8,900 lux after inoculation. During the dark periods the temperature was

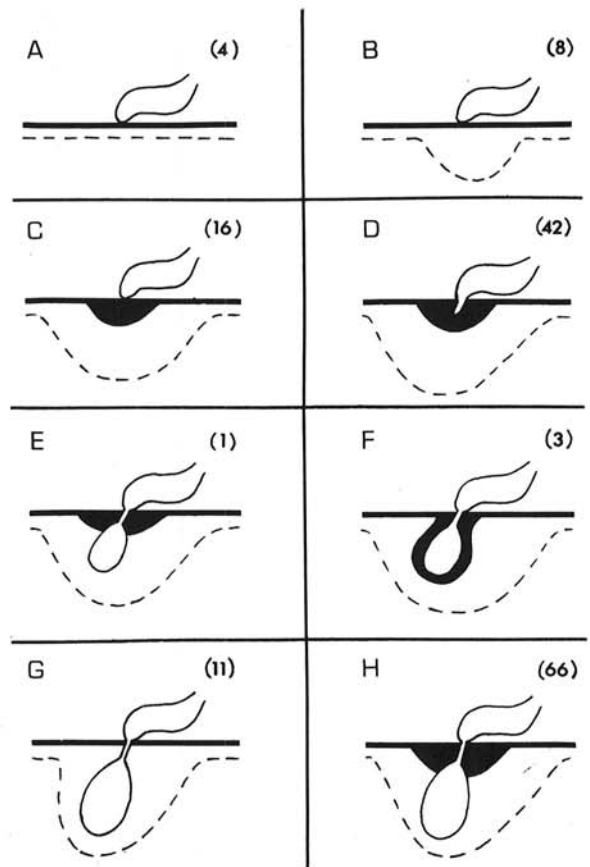
18 C. Conidia for experimental purposes were collected at the end of a photoperiod 12 hr after removal of old conidia from the plants by tapping of the infected leaves. The fresh conidia were collected only once from each set of plants 8 days after inoculation and were deposited directly into large test tubes which had been coated on the inside with Nybar (William F. Nye, Inc., New Bedford, Mass.) to reduce adherence of the conidia to the glass. Within 30 min, the collected conidia were inoculated in a spore settling tower (3) onto partially dissected 7-day-old coleoptiles (4). The coleoptiles had been previously mounted between two plastic wafers (4) modified to permit Koehler illumination with standard Zeiss optics.

The inoculated, mounted coleoptiles were then incubated on small .01 M  $\text{Ca}(\text{NO}_3)_2$ -containing reservoirs (inverted vial caps made of polyethylene) in petri dish moist chambers in the dark at 18 C. An RH of 65% was maintained in the chambers by addition of a 75% aqueous solution of glycerin to the filter paper in the bottoms of the chambers.

In separate, preliminary, replicated experiments, all penetrations from primary hooks which were mature at 10 hr after inoculation occurred between 10 and 18 hr after inoculation. Thus, the coleoptiles used in the present study were examined briefly at a low magnification 9.5 hr after inoculation, and those with at least five suitable appressoria were used for further work. Fresh  $\text{Ca}(\text{NO}_3)_2$  solution then was added to the shallow milled recess (18-mm diameter) on the underside of the lower plastic wafer of each coleoptile mount and a coverslip was applied to the recess. Selection of test appressoria preceded any visible signs of penetration or host reactions, and only apparently viable, mature appressoria with primary hooks near, but not over, anticlinal host walls (Fig. 2, 3) were chosen. Appressoria that formed hooks over host cells which were in contact with other appressorial hooks were not selected; i.e., only those host cells attacked by one appressorium were studied.

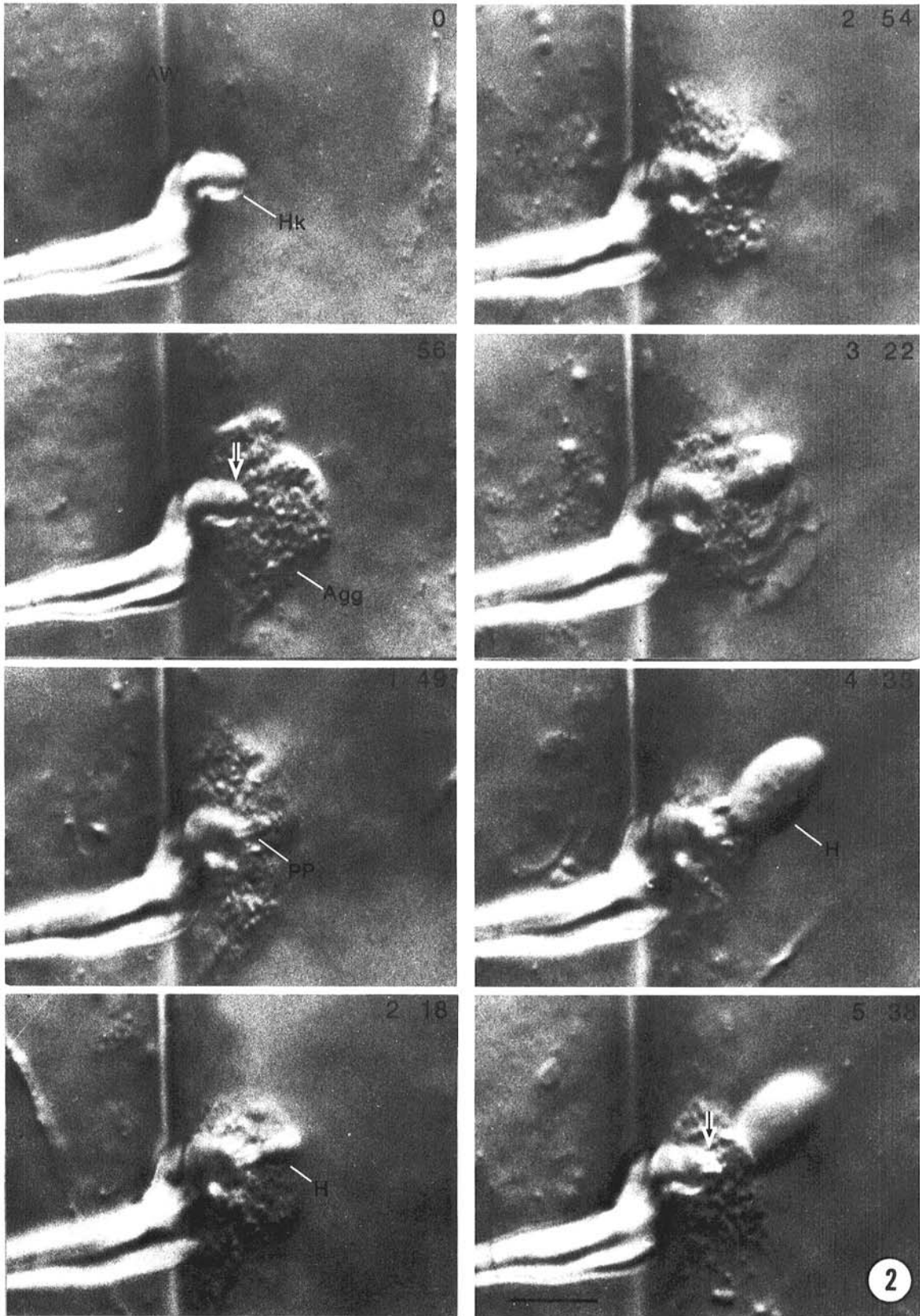
For observations, a small piece of No. 1 coverslip was placed over the central observation port in the upper wafer of each coleoptile mount and the encounter sites were viewed *in vivo* (a heat-reflecting filter was used at all times) at  $\times 800$ -1,000 magnifications with interference contrast optics. The microscope room was maintained at  $18 \pm 0.5$  C. For each coleoptile, five to ten encounter sites were each observed at 10-min intervals, and the times of initiation and termination of host cytoplasmic aggregates and initiation of papillae, penetration pegs, and haustoria were recorded. Data were accumulated over a period of several months. Some penetration pegs which failed to produce haustoria were difficult to identify *in vivo*

because they were embedded in papillae. In such cases identity of the pegs was verified 18 hr after inoculation; the coleoptiles were mounted in lactophenol-cotton blue,



**Fig. 1-(A to H).** Diagrammatic representation of interaction types in encounters between inner epidermal cells of *Hordeum vulgare* coleoptiles and appressoria of *Erysiphe graminis hordei*. These are based on the same data as are the time courses in Fig. 4. The number of sites of each type is given in parentheses. Thick solid lines represent the host wall, dashed lines represent the host tonoplast, solid black areas represent papillae, and solid white areas above the tonoplast, host cytoplasm. Some appressoria **A, B**) did not induce papillae or penetrate, some **C**) induced papillae but did not penetrate and some **D-F**) induced papillae and traversed the host walls but did not produce fully formed haustorial central bodies. Several appressoria **G**) penetrated without inducing papillae, and many **H**), about 44%, penetrated successfully and induced papillae.

**Fig. 2.** A time-lapse series of interference contrast photomicrographs showing progressive events at a single encounter site during penetration of a living *Hordeum vulgare* coleoptile cell from an appressorium of *Erysiphe graminis hordei*. The elapsed time (in hours and minutes) after the first frame is shown in the upper right corner of each frame. The first visible event was aggregation of host cytoplasm at the encounter site (frame 56). At this time the penetration peg was emerging from the inner surface of the host wall (arrow, frame 56). The peg extended fully into the aggregate (frame 1 49) before enlarging at the tip (frame 2 18) to initiate the haustorium. During the next 2 hr (frames 2 54-4 33) the haustorial central body grew to full size. A minute, refractive collar-shaped papilla (arrow, frame 5 38) was deposited onto the haustorial neck after the haustorium formed. Legend: Agg = cytoplasmic aggregate; AW = anticlinal wall; H = haustorium; Hk = appressorial hook; and PP = penetration peg. Magnification:  $\times 1,500$ . Scale bar calibration: 10  $\mu\text{m}$ .





which stains penetration pegs more intensely than the papillae, and the same pegs were observed again at  $\times 1,250$ - $2,500$  using interference contrast optics. Light micrographs were taken at  $\times 256$  on Kodak Tri-X film in a Zeiss Photomicroscope II equipped with an electronic flash.

For the time course analysis, the 151 encounter sites studied were divided into groups defined by whether papillae were initiated  $\geq 20$  min before or after penetration pegs and whether or not penetration was successful. The 20-min interval assured accuracy in determining whether the papilla or the peg was initiated first, and probably amplified any effects papillae might have had on penetration. Differences in penetration efficiency (defined below) between groups were analyzed by the  $\chi^2$  test (8). To detect significant time differences between events within a given group, the paired *t*-test (10) was used. Differences in the time of occurrence of the same event or in the interval between events in different groups were evaluated by the nonpaired *t*-test (10). Comparisons between all observed events in all six groups in Fig. 4 were made, and those most relevant to papilla function were statistically analyzed. Because we never saw papillae encase large haustoria in this parasite-host system, we viewed successful penetration as the production of a fully formed haustorial central body (Fig. 2). Penetration efficiency (PE) was then defined as the number of fully formed haustorial central bodies divided by the number of encounter sites observed, the quantity multiplied by 100%.

## RESULTS

The optical clarity of the preparations was such that we could detect growing penetration pegs shortly after their passage through the host walls (Fig. 2) and thereby determine the order of events relative to peg formation. In a few instances (Fig. 3), the lactophenol-cotton blue method was used to corroborate difficult *in vivo* observations. Comparisons between events at encounter sites exposed continuously or periodically to the microscope light and those at encounter sites incubated continuously in the dark did not reveal large differences.

**Interaction types.**—The 151 parasite-host interactions studied had various outcomes and are grouped into 8 interaction types in Fig. 1. A few appressoria failed to induce detectable papillae or to initiate penetration (Fig. 1-A,B). Others induced papillae but did not produce detectable penetration pegs (Fig. 1-C). Still others induced papillae and produced pegs but did not initiate haustoria (Fig. 1-D). In one case, penetration did not proceed to completion even though the papilla was breached (Fig. 1-E). A few small haustorial central bodies were encased by papillae (Fig. 1-F). Although some successful penetrations were not associated with detectable papillae (Fig. 1-G), most were (Fig. 1-H).

In a separate study of host cells attacked by more than one appressorium, the different interaction types were evenly distributed among the host cell population; both successful and unsuccessful penetration attempts often occurred simultaneously on the same host cell and were often less than  $40 \mu\text{m}$  apart. Similarly, in another study in

which postinoculation incubations longer than 24 hr were used to determine when all possible penetrations from primary hooks were completed, we found that secondary hooks may form haustoria in the same host cells that the primary hooks had previously failed to penetrate.

**Time-course studies.**—Initiation of a cytoplasmic aggregate (a seething accumulation of host cytoplasm) and its termination were always the first and last events, respectively. Interactions at two encounter sites are illustrated in detail. At one site (Fig. 2), a large cytoplasmic aggregate formed at about the same time the penetration peg became detectable (frame 56). At this stage, the peg appeared as a bright circle with an eccentrically located dark spot (arrow, frame 56) and was in the same focal plane as the host wall; i.e., the host wall had just been or was being breached. The peg then elongated into the cytoplasmic aggregate where it was clearly visible as a slightly curved tube (frame 149). A swelling developed at the end of the peg (frame 218) and grew into a fully enlarged haustorial central body (frame 254 - 433). Eventually a small papilla in the shape of a collar was formed on the haustorial neck near the host wall (arrow, frame 538); the cytoplasmic aggregate had almost completely dispersed by this time.

At the other site (Fig. 3), a papilla was deposited (frame 100) shortly after the cytoplasm aggregated (frame 50). The papilla enlarged for up to 30 min (frame 130) before a penetration peg (indistinctly shown) began to grow into it. Later (frame 750), the peg was more clearly visible, and examination of the same site after lactophenol-cotton blue staining (frame 800) confirmed the presence of a penetration peg in the papilla.

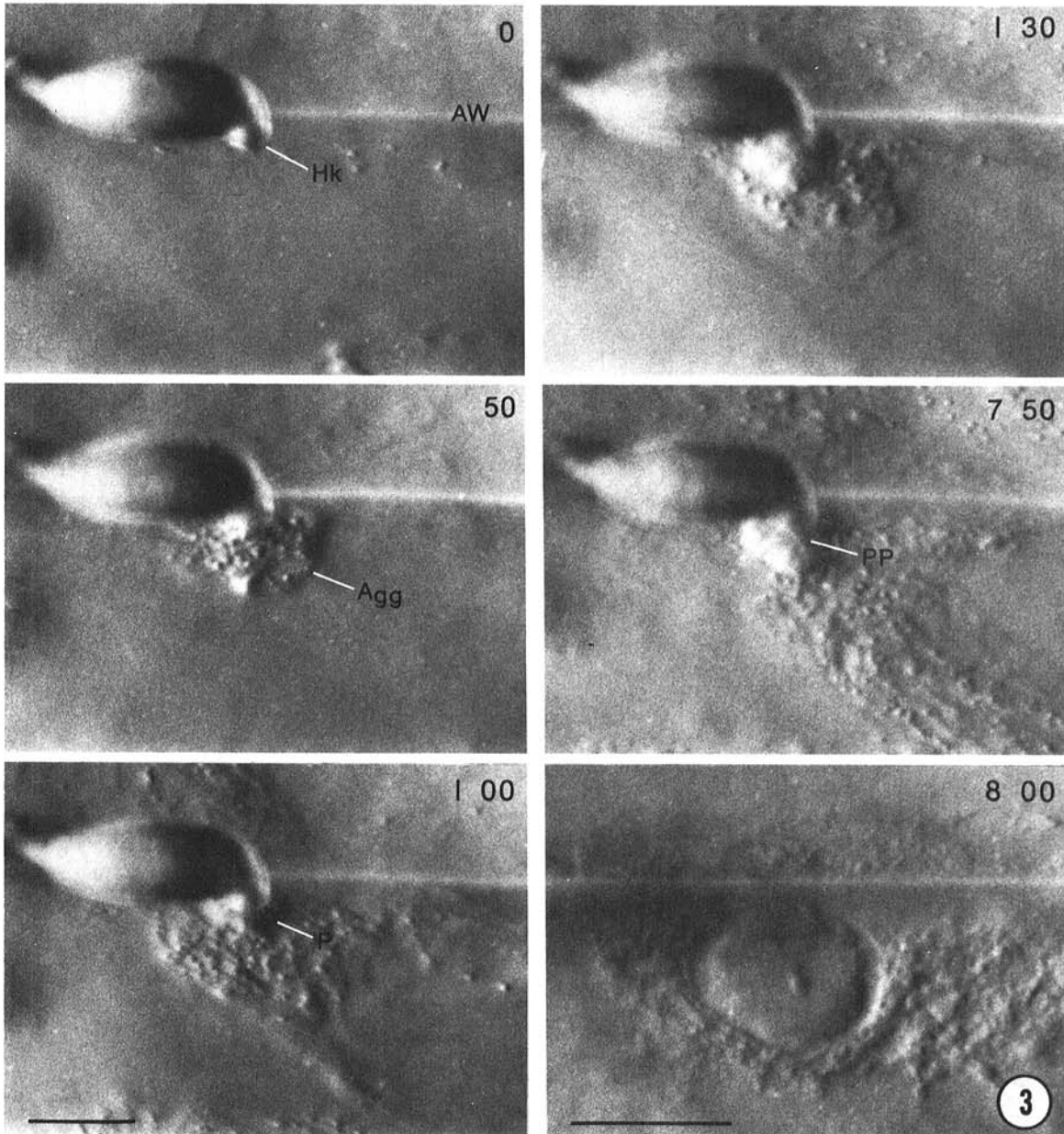
To test the possibility that papillae, such as the one shown in Fig. 3, function in disease resistance, we divided the 151 encounter sites into groups. The time courses of penetration events for the groups pertinent to this study are shown in Fig. 4. Although papillae formed at 85% of the sites (Fig. 1), only about 12% (Fig. 4, lines 4, 5) of the growing peg apices [Fig. 1-(D to H)] encountered papillae which had formed at least 20 min before host walls were breached. Where papillae did precede penetration pegs, there was a significant ( $P = 0.05$ ) reduction of 57% in PE [84% (36/43) when pegs preceded papillae (Fig. 4, lines 2, 3) minus 27% (4/15) when the reverse (Fig. 4, lines 4, 5) occurred]. At these latter sites the interval from peg initiation to haustorium initiation was also significantly ( $P = .05$ ) reduced. Although the interval from initiation of papillae to that of pegs was greater when penetration failed (Fig. 4, line 5) than when it succeeded (Fig. 4, line 4), the difference was not significant ( $P = .30$ ). Papillae were formed soon after aggregates where papillae preceded pegs (Fig. 4, lines 4, 5) but were delayed in this respect where pegs preceded papillae (Fig. 4, lines 2, 3). In all possible comparisons, aggregates terminated significantly ( $P = .10$ -.001) later in groups in which penetration failed (Fig. 4, lines 3, 5, 6) than in those in which it succeeded (Fig. 4, lines 1, 2, 4). Penetration pegs were initiated at about the same absolute time in all groups except the one in which papillae preceded pegs and penetration failed (Fig. 4, line 5); these pegs were initiated significantly ( $P = .10$ -.001) later ( $\sim 100$ - $120$  min) than were those in all the other groups. In all groups in which penetration succeeded (Fig. 4, lines 1, 2, 4),

haustoria were initiated at about the same absolute time (differences not significant).

Encasement of haustoria (Fig. 1-F) resulted only from small haustoria being overgrown by papillae [illustrated in (1)]; haustoria were never initiated within papillae.

#### DISCUSSION

In this parasite-host system, penetration failures and successes commonly occurred simultaneously within 40  $\mu\text{m}$  of each other on the same host cells. Furthermore,



**Fig. 3.** A time-lapse series of interference contrast photomicrographs showing progressive events at a single encounter site during penetration of a living *Hordeum vulgare* coleoptile cell from an appressorium of *Erysiphe graminis hordei*. The elapsed time (in hours and minutes) after the first frame is shown in the upper right corner of each frame. In this interaction, a cytoplasmic aggregate (frame 50) deposited a papilla onto the inner surface of the host wall before penetration began (frame 1 00). Within 30 min (frame 1 30) the papilla was almost fully formed, and a penetration peg (indistinctly shown) had begun to penetrate it. The peg can be more easily seen near the center of the papilla in frame 7 50 and in frame 8 00 in which peg contrast was enhanced by lactophenol-cotton blue. Legend: Agg= cytoplasmic aggregate; AW= anticlinal wall; Hk= appressorial hook; P= papilla; and PP= penetration peg. Frames 0 - 7 50,  $\times 1,500$ ; frame 8 00,  $\times 2,300$ . Scale bar calibration: 10  $\mu\text{m}$ .

secondary hooks penetrated successfully into cells which primary hooks of the same appressoria had previously failed to penetrate. Thus, if resistance alone accounts for these penetration failures, it is highly localized, even at the subcellular level. Of all suggested plant disease resistance mechanisms, only papilla formation is known to be this highly localized.

If papilla formation is a resistance mechanism, then the observed (Fig. 4) association of early (with respect to penetration pegs) papilla formation with a significant reduction in penetration efficiency certainly would be expected. A related association was reported by Bushnell and Bergquist (3). However, several lines of evidence suggest that papillae may not have adversely affected penetration. First, the absolute time of haustorium initiation was similar, regardless of when papillae were formed. Second, the interval from peg initiation to haustorium initiation was shorter when papillae preceded pegs. Third, the difference in the interval from papilla initiation to peg initiation for successful versus unsuccessful penetration attempts was not statistically significant. Fourth, because some appressoria (Fig. 1-A,B) failed to form pegs or haustoria even in the absence

of papillae, it is likely that at least some of the others [Fig. 1-(C to F)] which developed sufficiently to incite papillae failed to develop further for similar (unknown) reasons. Figure 1-E seems to support this; even though the papilla did not encase the haustorium, the latter did not develop further. Finally, because damaged haustoria are known to induce papilla formation (1), the aforementioned association of early papilla formation with penetration failures could be due to precocious secretion of papilla-inducing materials by appressorial hooks that lack the potential for further development. We are now seeking conclusive evidence showing whether or not papillae can prevent fungal ingress by comparing PE's in the presence and in the absence of experimentally controlled papilla formation.

Our present results differ markedly from those of Stanbridge et al. (9), who reported that papillae of barley leaf cells are typically formed before host walls are traversed. This difference could be due to our use of coleoptiles as host tissues, since barley leaves and coleoptiles have been shown to differ in papilla formation (3). Most of the general observations reported by Bushnell and Bergquist (3) were corroborated in the

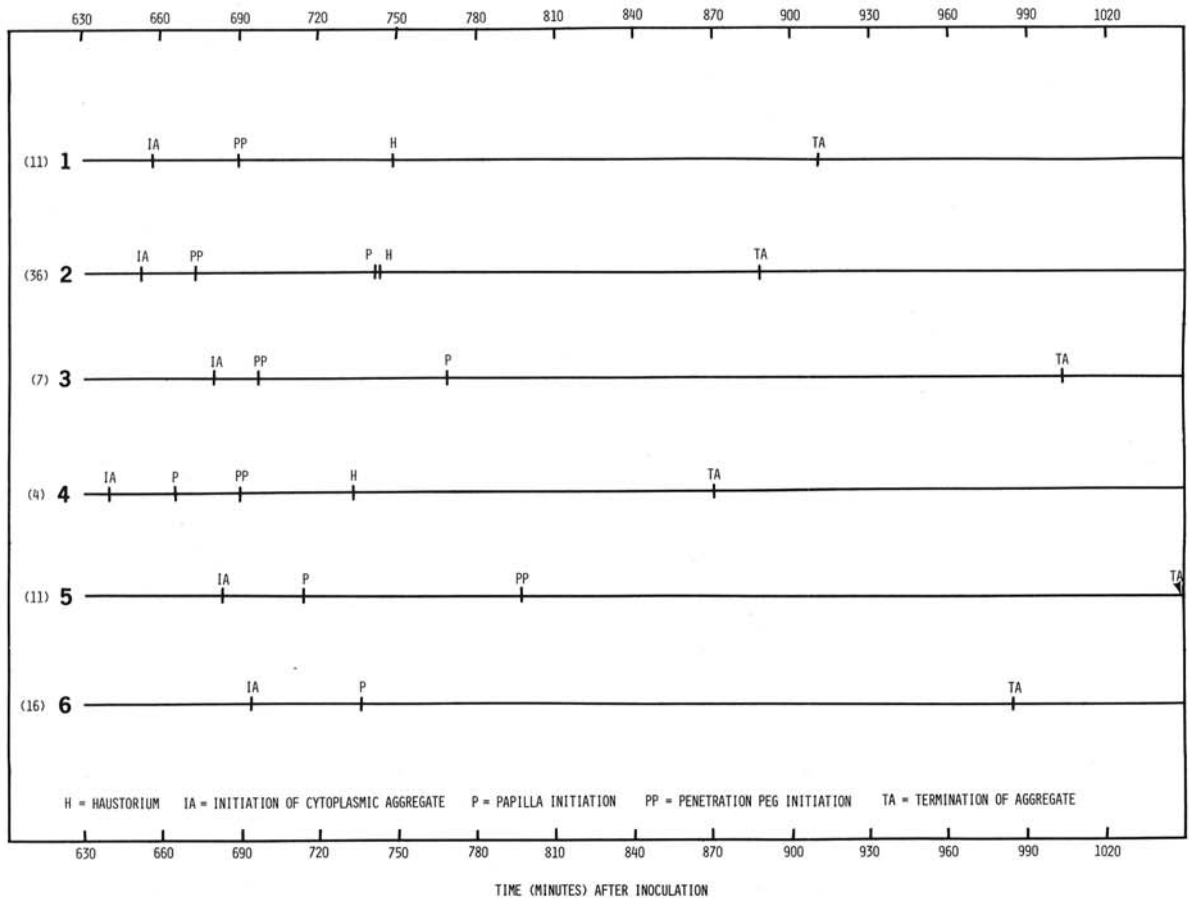


Fig. 4. Time-lines showing the courses of events during penetration of barley (*Hordeum vulgare*) coleoptile cells by *Erysiphe graminis hordei*. The 151 encounter sites studied were divided (according to relative time of penetration peg and papilla initiation and success or failure of penetration) into groups, and the groups pertinent to this study are shown. The number of sites in each group is in parentheses, and means of the absolute times of events are indicated on the lines.

present study. However, because we were able to monitor penetration peg development and because of our larger data base on a single parasite-host pairing, we were able to establish new correlations (discussed above) which bear on the function of papillae.

We found that some penetration pegs embedded in papillae grew out of the papillae and produced haustoria, whereas others did not. Furthermore, some pegs became embedded by growing into the papillae, whereas others traversed the host wall and then became enclosed by papillae. These observations point out the inadequacies of attempting to reconstruct dynamic sequences from static photomicrographs of fixed specimens; pegs embedded in papillae may or may not have been produced before the papillae, and they may or may not have produced haustoria had they not been fixed.

Because cytoplasmic aggregates and papillae were sometimes formed even when pegs failed to traverse host walls (Fig. 1-B, C), we think it is likely that both can be incited by purely chemical means, as was suggested by Bushnell and Bergquist (3). However, thin-section electron microscopy of stages preceding wall traversal should be done to substantiate this possibility.

We favor use of the single term "papilla" to denote all wall appositions [e.g., papillae, collars, and encasements (2)] which are developmentally, structurally, and chemically related, because they seem to be alternative expressions of the same basic plant cell reaction (1). Moreover, "papilla" has historical precedence (7) and has not been used for other structures with which it may be confused.

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