Timing and Significance of Papilla Formation During Host Penetration by Olpidium brassicae

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ABSTRACT

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Olpidium brassicae has been reported to incite host cells to form papillae at penetration sites before penetration, and it has been suggested that the papillae stopped the advancing pathogen. By means of interference contrast microscopy, we investigated timing of papilla formation in relation to growth of *O. brassicae* penetration tubes, from zoospore cysts, through walls of living kohlrabi (*Brassica oleracea*) root hairs. Most papillae were initiated after penetration tubes appeared, were conical, and were attached to the tubes (tube papillae). Those initiated before tube development were disk- or dome-shaped and were attached to the host walls (wall papillae). Tube and wall papillae both were present at a few encounter sites. Although wall and tube papillae were initiated at about the same absolute time after inoculation, cysts which induced wall papillae were significantly later than other cysts in producing tubes. Therefore, production of tubes was already tardy when wall papillae were initiated. Furthermore, failure of some cysts to penetrate was clearly unrelated to papilla formation. On the other hand, penetration efficiency of cysts that induced wall papillae was merely half that of cysts which induced only tube papillae. Further experiments will be required to determine whether penetration failures are caused by the papillae or by an inherent inability of certain cysts to complete penetration.

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

Many plant cells react to fungal attack with a localized aggregation of cytoplasm and deposition of materials in the paramural space at the point of attack (encounter site) (1). These depositions, termed papillae (10) [= callosities, calli, lignitubers (3)], have been assigned a role in disease resistance by some workers, primarily because the depositions often coincide with unsuccessful penetration attempts (1). Evidence presented thus far in the literature is inadequate to conclude that papillae are the cause of penetration failures, because a number of alternative causes have not been ruled out (1).

Kusano (7) and Karling (6) presented correlative evidence that hosts of *Olpidium* spp. may prevent penetration by forming papillae, a view apparently accepted by Temmink and Campbell (13). Work on the penetration of kohlrabi (*Brassica oleracea*) root epidermis by *Olpidium brassicae* showed this to be a highly compatible parasite-host system (9); later work (8) implied that the parasite must penetrate a papilla before establishing contact with host cytoplasm. Thus, papillae could cause a low percentage of penetration attempts to fail in this system. Furthermore, because of certain similarities between that system and the one studied by Aist and Williams (2), in which the time course of penetration from zoospore cysts of *Plasmodiophora brassicae* into living root hairs of cabbage was observable in great detail, we judged the *Olpidium–Brassica* system to be optically well-suited for similar studies.

The main purpose of this study was to determine whether papillae are produced before, or after, penetration is completed and whether or not there is a correlation between early papilla formation and the apparent effectiveness of papillae in resisting intrusion. A secondary purpose was to establish the overall time courses of visible events associated with penetration.

MATERIALS AND METHODS

A culture of *Olpidium brassicae* (Wor.) Dang. (8), obtained from Prof. W. H. Fuchs, Göttingen, was maintained in roots of a highly compatible host, *Brassica oleracea* L. sp. *gongyloides* Ducks. 'White Vienna', grown in autoclaved sand with added nutrients in an environmental control chamber. The chamber was

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programmed for a 16-hour photoperiod (19,000 lux of mixed incandescent and fluorescent lights), a diurnal temperature of 22 C day and 18 C night, and 65% relative humidity.

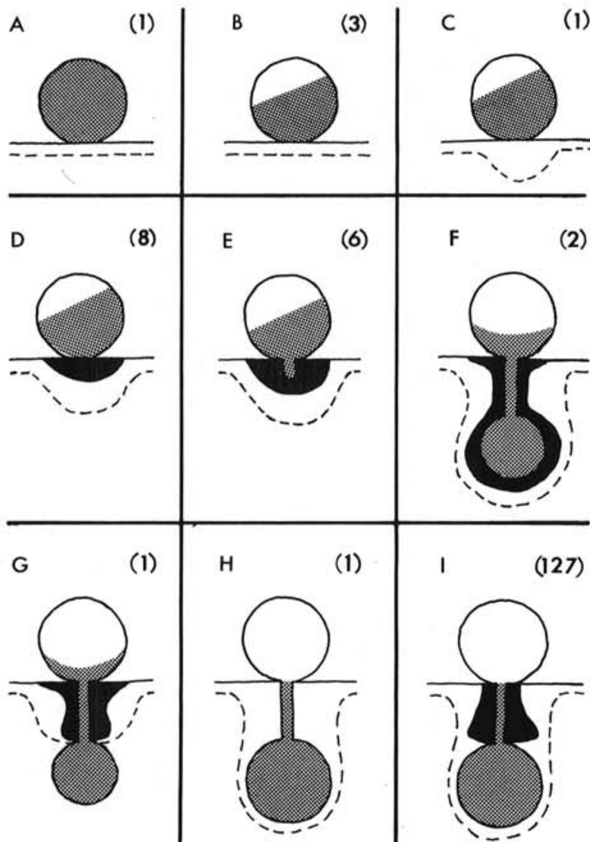


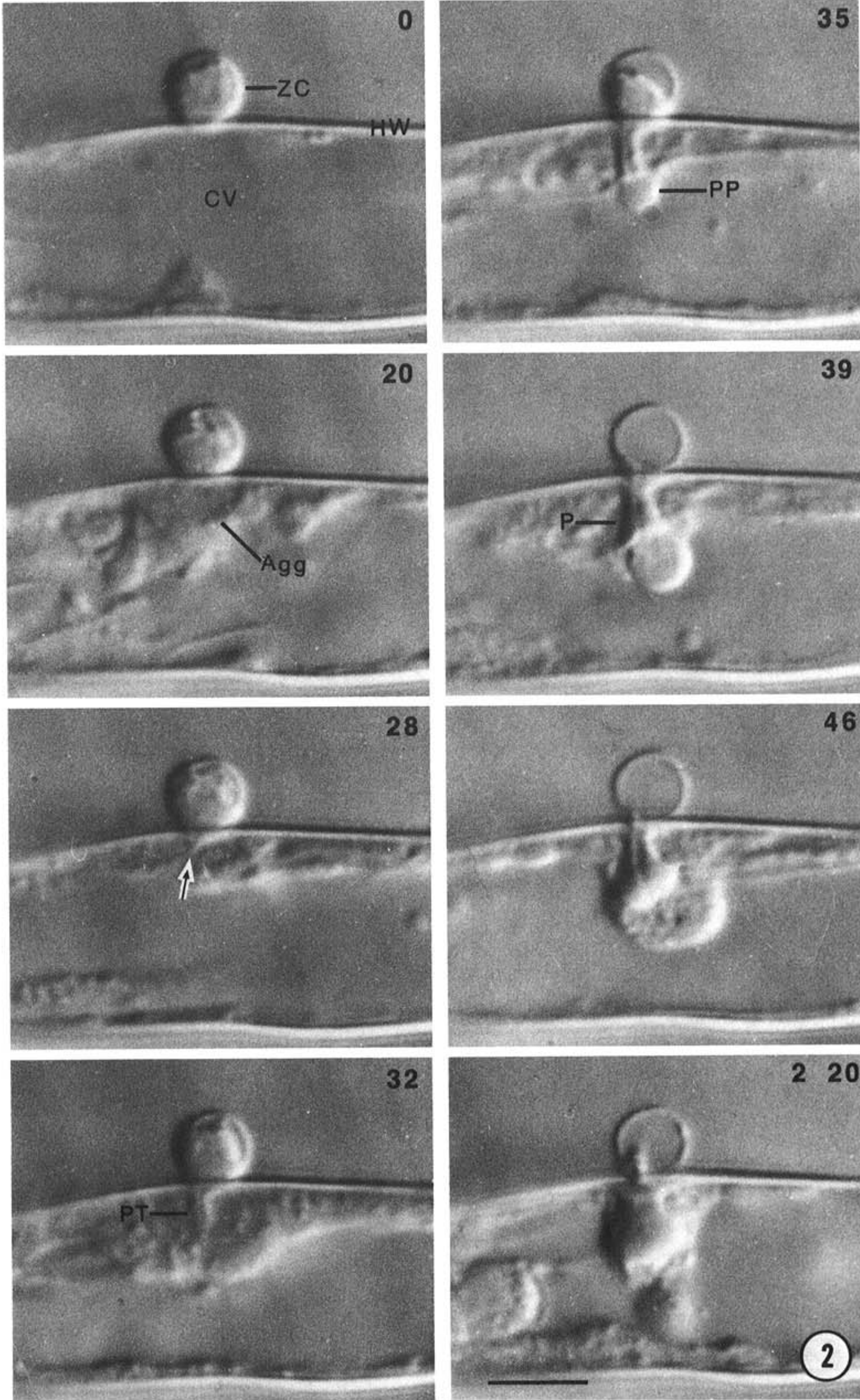
Fig. 1. Diagrammatic representation of types of interactions between kohlrabi (*Brassica oleracea*) root hairs and *Olpidium brassicae* zoospore cysts. These are based on the same data as the time courses in Fig. 5. The number of sites in each type is given in parentheses. The host tonoplast is represented by dashed lines, papillae by solid black areas and parasite cytoplasm by stippled areas. Some cysts did not germinate or induce papillae (A-C); some did not germinate, but, nevertheless, induced papillae (D); some germinated and induced papillae, but failed to complete penetration (E-G); one penetrated without inducing a papilla (H); and most, about 85%, penetrated successfully and induced papillae (I).

Two-week-old plants were inoculated by pouring a suspension of zoospores into the sand in which the plants were growing and were used 8-14 days later as a source of inoculum for laboratory experiments. Portions of infected roots were placed in a small beaker containing deionized, distilled water and were agitated gently with forceps to remove sand. The root pieces were then moved to a petri dish and washed again. To avoid loss of zoospores, the above steps were completed in less than 2 min. The washed roots were then placed into a beaker containing a dilute salts solution (14) for 5 min and gently stirred intermittently. The zoospore concentration thus obtained was checked microscopically and adjusted, if necessary, to that which would result in about 5-10 zoospore cysts per root hair after a 5-min pulse inoculation (14). The suspension of zoospores was then poured through a single layer of cheesecloth into glass shell vials.

Kohlrabi seeds were germinated on moist filter paper in petri dishes and grown for 3 days at 18 C with cool-white fluorescent illumination. The seedlings were then transferred, one each, to shell vials containing dilute salts solution. For inoculation, the seedlings were transferred for 5 min to the vials containing zoospores and then returned to their original vials.

After a 1.25-hr incubation at 18 C under cool-white fluorescent illumination, each inoculated seedling was placed on a 0.8-1.0 mm-thick microscope slide in solution from its own vial and a 22 × 40 mm #1 coverslip was applied to the root. Roots mounted in this way apparently were not compressed by the coverslips and would grow under them for several days. Because root hairs selected for study were located near the coverslips, they each were bent slightly near the base; even so, cyclosis and root hair growth seemed unaffected. After the corners of the coverslips were secured with molten paraffin, the preparations were observed with interference contrast optics at a magnification of ×2,000 or ×2,500. A heat-absorbing filter was used to reduce the likelihood of adverse effects from illumination. Cysts for study were selected on the bases of (i) their lateral position (with respect to the optical axis) on a root hair, which greatly enhanced the likelihood that all parasite structures would be in one focal plane during penetration (ii) their overall apparent vitality, and (ii) their distance from other cysts. The observations, which were conducted in a room maintained at 18 ± 1 C, began 1.75 hr after inoculation

Fig. 2. A time-lapse series of interference contrast micrographs showing progressive events at a single encounter site during penetration of a living kohlrabi (*Brassica oleracea*) root hair by a zoospore cyst of *Olpidium brassicae*. The elapsed time (in minutes and hours) 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 20). The penetration tube then emerged from the inner surface of the host wall (arrow, frame 28) and extended fully into the aggregate (frame 32) before papilla deposition began. Migration of the parasite protoplast into the host cell began at about the time papilla deposition was initiated (frame 35). The parasite protoplast lingered momentarily at the end of the penetration tube as papilla deposition continued (frame 39) and then was swept away from the encounter site by host cyclosis (frame 46). Finally, the cytoplasmic aggregate terminated (frame 2 20), leaving the cyst, the papilla, and a scant amount of host cytoplasm at the site. Note that the papilla was initiated after the cell wall was breached, and that it was attached to the penetration tube (tube papilla). Legend: Agg = cytoplasmic aggregate; CV = central vacuole; HW = host wall; P = papilla; PP = parasite protoplast; PT = penetration tube; and ZC = zoospore cyst. ×3,000. Scale bar calibration: 5 μm.


0
ZC
HW
CV
35
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20
Agg
39
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28
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2

(prior to any visible interaction) and were made every 5 min on each encounter site over a 2-hr period. That all potential penetrations occurred during this period was corroborated in separate experiments by observing appropriate sites again, after an additional 20-hr incubation. The following were recorded: (i) relative amount of cytoplasm in the cyst, (ii) initiation and termination of a host cytoplasmic aggregate (5, 7) at the encounter site, (iii) initiation and enlargement of a papilla beneath the cyst, (iv) initiation and growth of a penetration tube, and (v) intracellular occurrence and eventual removal of the parasite protoplast from the encounter site by host cyclosis. Five cysts on each root were studied, until, over a period of several months, sufficient data were accumulated. Light micrographs were taken at ×640 on Kodak Tri-X film in a Zeiss Photomicroscope II equipped with an electronic flash attachment.

For the time-course analysis, the encounter sites were divided into groups, four of which were defined by whether papillae were initiated at least 5 min before or after penetration tubes and whether penetration was successful or not. Differences in penetration efficiency between groups were analyzed by the χ^2 test (11). To detect significant time differences between events within a group, the paired *t*-test (12) was used. Differences between groups in the time of occurrence of the same event and in intervals between events were evaluated by the nonpaired *t*-test (12). Although all possible comparisions among events in the four groups in Fig. 5 were made, only those which seemed relevant to papilla function were analyzed statistically.

Successful penetration was equated with migration of the parasite protoplast into the host cell. Penetration efficiency (PE) was defined as the number of empty cysts observed, divided by the total number of cysts observed, the quantity multiplied by 100%.

RESULTS

In this in vivo system, the penetration tubes were readily detected during or just after their passage through the host walls. The tubes were easily seen in papillae also, but tended to lose contrast after a time (Fig. 2-4). In preliminary comparisons of the time courses and PE's of cysts on roots observed in wet mounts versus cysts on roots incubated simultaneously in vials, no differences were noted. The pulse inoculation method created a high degree of synchrony in early parasite development; nearly all penetrations occurred between 135 and 165 min after inoculation (Fig. 5).

Interaction types.—The parasite-host interactions took various courses during the period of observation and are diagrammed in Fig. 1 as interaction types which are grouped according to the final outcome at each encounter site. A few of the zoospore cysts failed to penetrate or induce detectable papillae [Fig. 1-(A to C)]. Others induced papillae, but did not produce detectable penetration tubes (Fig. 1-D). Still others induced papillae and formed penetration tubes, but did not develop further (Fig. 1-E, F). In one case, penetration was not consummated although the papilla was breached by the penetration tube (Fig. 1-G). One parasite protoplast entered the host cell without inciting papilla formation (Fig. 1-H); however, the physical proximity of the host nucleus at that encounter site denied the host cytoplasm access to the site. About 85% of the zoospore cysts penetrated successfully and were associated with papillae in the form of inverted cones which encased the penetration tubes (Fig. 1-I). Successful penetrations and penetration failures occurred simultaneously on the same root hairs, usually within 40 μm of each other.

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. In some cases, the penetration tube grew to its full length before papilla deposition began (Fig. 2). The papilla was deposited along the wall of the penetration tube and was initiated at about the time the parasite protoplast began to enter the root hair. When completely inside the host cell, the protoplast lingered momentarily and then was swept away from the encounter site by host cyclosis. Those that lingered longer often became partially enclosed, for a moment, by papillae which enlarged primarily at the distal ends of the penetration tubes. Papilla deposition ceased when the cytoplasmic aggregate terminated (Fig. 2). Sometimes cytoplasmic aggregation and deposition of a thin, broad papilla began almost simultaneously (Fig. 3). At about the time the penetration tube began to penetrate the papilla, the cytoplasmic aggregate terminated, but it formed again when the penetration tube had grown through the papilla. Subsequent events were similar to those described for Fig. 2, so that two distinct forms of papillae were deposited at the encounter site. One form (wall papilla) was deposited onto the host wall before penetration began; the other (tube papilla) onto the penetration tube after host wall traversal was completed (Fig. 3, frame 44).

The failure of a penetration tube to breach a papilla is illustrated in Fig. 4. A papilla was deposited before the tube appeared. Growth of the penetration tube into the papilla was coincident with renewed host cytoplasmic activity (Fig. 4, frame 1 58) and the deposition of a large, hemispherical papilla beneath the cyst. Five hours after inoculation the cyst was only half empty, and the cytoplasmic aggregate had terminated (Fig. 4, frame 3 05.).

To obtain evidence as to whether or not papillae, like that illustrated in Fig. 4, function in disease resistance, we divided the 150 encounter sites examined into groups as described in Materials and Methods. Relatively few (~10%) penetration tubes encountered papillae which were formed 5 min or more before the tubes were initiated (Fig. 5, lines 2, 3). When papillae preceded successful penetratin tubes (Fig. 5, line 2), the absolute times to penetration tube initiation and cyst evacuation were significantly (P = .10 and .01, respectively) greater than when successful tubes preceded papillae (Fig. 5, line 1). Additionally, the interval from tube initiation to empty cysts was significantly (P = .01) greater (Fig. 5, lines 1,2). PE varied significantly (P = .01) according to whether tubes or papillae were initiated first: 99% PE (114/115) when tubes preceded papillae (Fig. 5, line 1) and 50% PE (7/14) when papillae preceded tubes (Fig. 5, lines 2, 3).

Emergence of penetration tubes from cysts which did not completely penetrate papillae (Fig. 5, line 3) was also

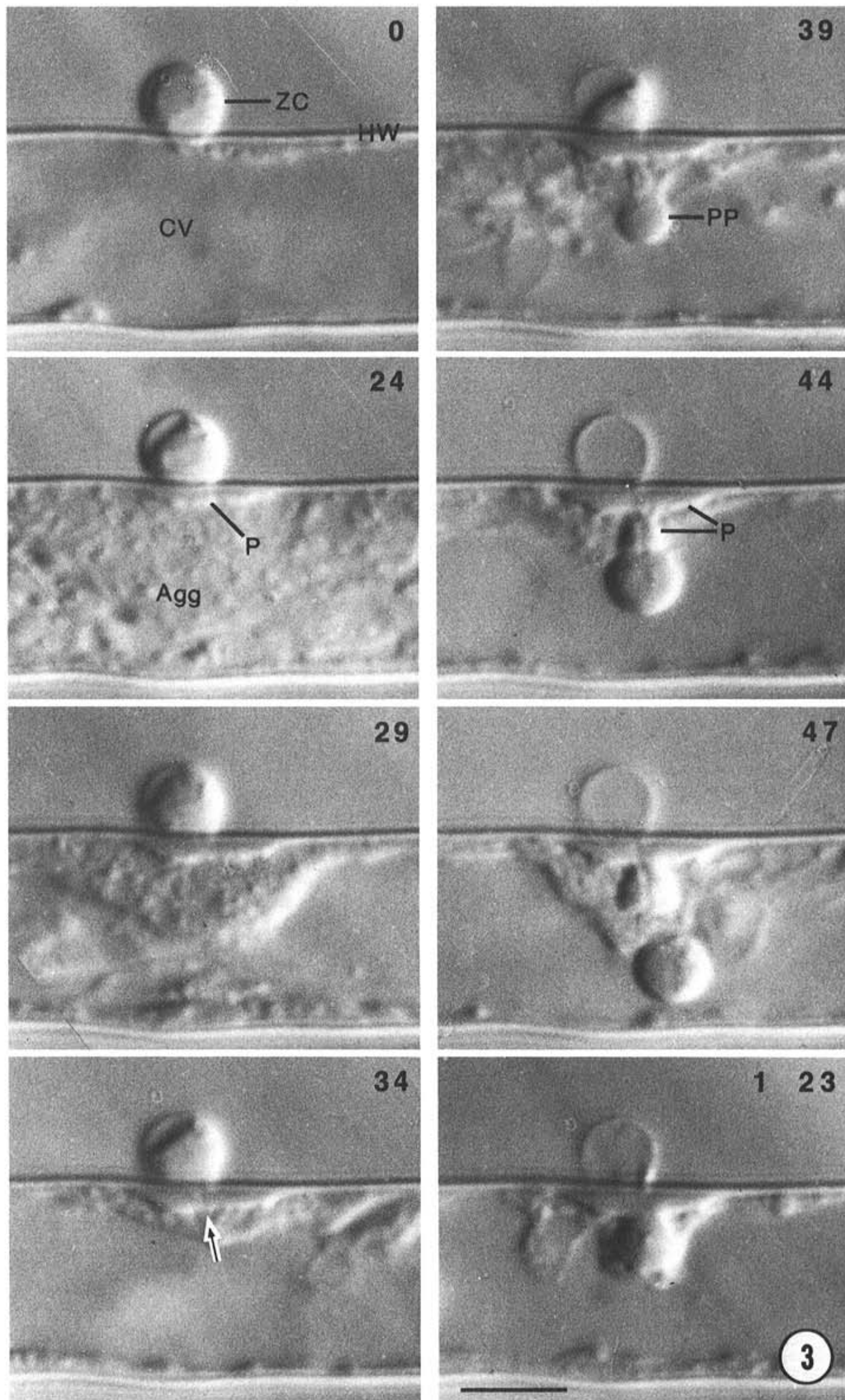

Fig. 3. A time-lapse series of interference contrast micrographs showing progressive events at a single encounter site during penetration of a living kohlrabi (*Brassica oleracea*) root hair by a zoospore cyst of *Olpidium brassicae*. The elapsed time (in minutes and hours) after the first frame is shown in the upper right corner of each frame. At this encounter site, a massive cytoplasmic aggregate formed and deposited a broad, thin papilla onto the host wall before the penetration tube appeared (frame 24). Ten min later (frame 34) the aggregate had almost completely terminated although a penetration tube (arrow) was growing through the papilla. Once the tube emerged from the papilla (frame 39), the events were similar to those described for Fig. 2, frames 35 to 2 20. Here, two forms of papillae (frame 44) were produced; the first type formed on the host wall (wall papilla) before the wall was penetrated, whereas the second type formed on the penetration tube (tube papilla) after host wall penetration. Legend: Agg = cytoplasmic aggregate; CV = central vacuole; HW = host wall; P = papilla; PP = parasite protoplast; and ZC = zoospore cyst. ×3,000. Scale bar calibration: 5 μm.

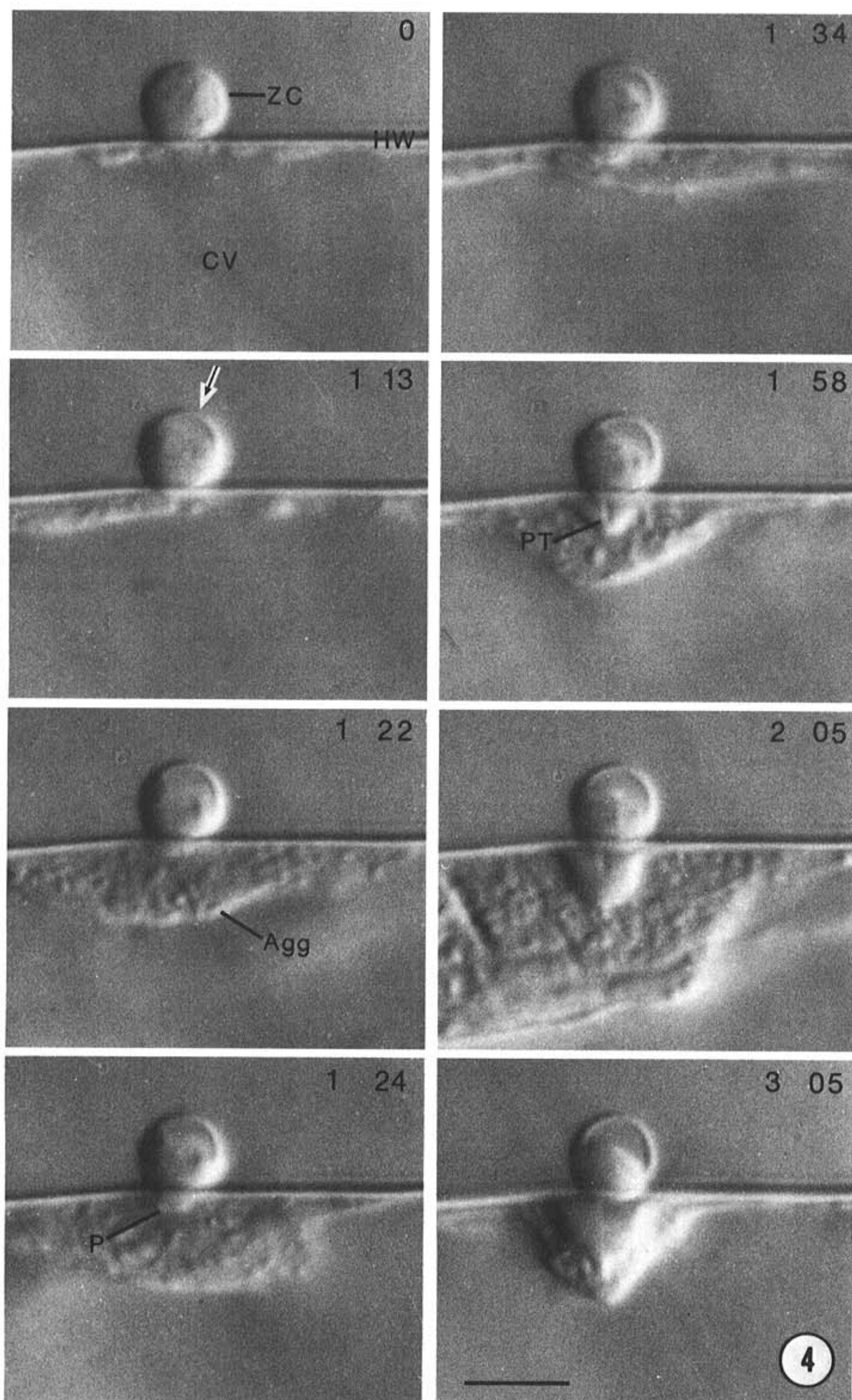

Fig. 4. A time-lapse series of interference contrast micrographs showing progressive events at a single encounter site during penetration of a living kohlrabi (*Brassica oleracea*) root hair by a zoospore cyst of *Olpidium brassicae*. The elapsed time (in minutes and hours) after the first frame is shown in the upper right corner of each frame. The enlarging cyst vacuole (arrow, frame 1 13) was apparent before the initial visible host response. The host produced a papilla (frame 1 24), and the aggregate terminated momentarily (frame 1 34), before the host wall was traversed. When the penetration tube appeared (frame 1 58), a new cytoplasmic aggregate was formed, and it deposited a large, hemispherical papilla in which the tube was embedded (frame 1 58 and 2 05). Although the site was observed for an additional 11 -hr period after frame 3 05, penetration was not completed. Legend: Agg = cytoplasmic aggregate; CV = central vacuole; HW = host wall; P = papilla; PT = penetration tube; and ZC = zoospore cyst. ×3,000. Scale bar calibration: 5 μm.

significantly ($P = .05$) later than emergence from those which did (Fig. 5, line 2). There was also a significant difference ($P = .05$) between these two groups in the interval from papilla initiation to penetration tube appearance.

Papillae formed almost immediately upon initiation of persistent cytoplasmic aggregates when papillae preceded tubes (Fig. 5, lines 2, 3) but were significantly ($P < .01$) later than aggregates when tubes preceded papillae (Fig. 5, line 1). A difference between the times of aggregate and papilla initiation also occurred when papillae, but not penetration tubes, were formed (Fig. 5, line 4). The mean time for papilla initiation preceded that for aggregates in one group (Fig. 5, line 3) because early aggregates did not persist at several of the encounter sites; initiations of only persistent aggregates were included in the means. The only significant difference in the duration of cytoplasmic aggregates occurred between the group (Fig. 5, line 4) in which papillae, but not tubes, were formed and the group (Fig. 5, line 1) in which tubes preceded papillae and penetration succeeded ($P = .05$). The mean times of papilla initiation for all groups, and especially those with penetration tubes, did not differ significantly ($P = 0.2$-0.5).

Encasement of parasite protoplasts (Fig. 1) occurred in two different ways. Sometimes part of the protoplast was injected into a papilla, while other times a papilla was deposited precipitously about a partially injected protoplast. The relative frequency of these two modes of encasement was not determined because of the small number of encased parasites observed.

DISCUSSION

Our results agree in general with those of earlier workers (6, 7, 8, 13) regarding the events of penetration by *Olpidium* spp. However, contrary to reports based solely on electron microscopy (8, 13), papillae usually were not formed until long after the host wall was breached. This discrepancy could be due, in one case (13), to differential reactivity of lettuce and kohlrabi to penetration, or to differential papilla induction by the different *O. brassicae* isolates. In view of the close timing between penetration tube and papilla initiation which we found by in vivo analysis, it appears that electron microscopy alone is inadequate to determine the relative sequence of events; electron micrographs of encounter sites with papillae could depict penetration failures, like the one shown in Fig. 4.

Some of the interactions (Fig. 1-C, D) support the hypothesis that cytoplasmic aggregates and papillae can

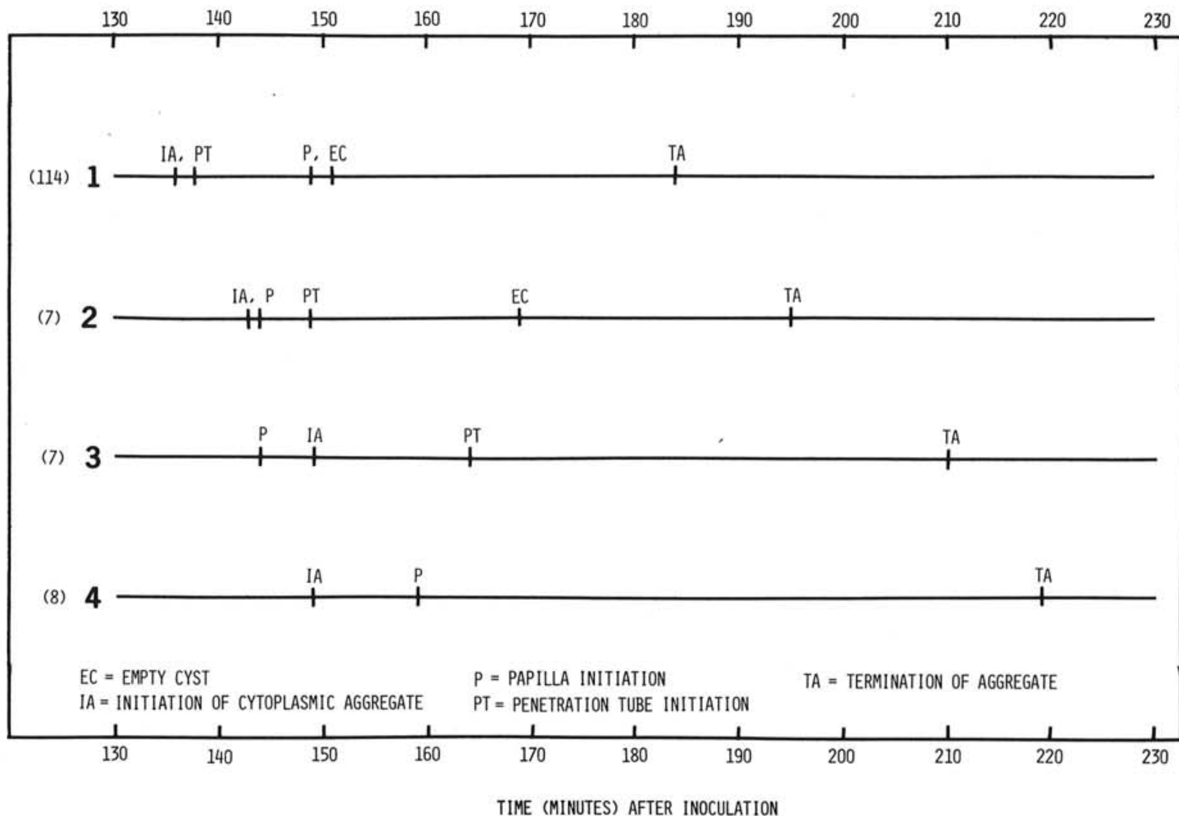

Fig. 5. Time lines representing the courses of events during penetration of kohlrabi (*Brassica oleracea*) root hairs by *Olpidium brassicae*. The 150 selected encounter sites were divided (accodring to relative time of penetration tube and papilla formation and success or failure of penetration) into groups, four of which are pertinent to this study. The number of sites in each group is in parentheses, and mean times of events are indicated on the lines.

be incited solely by fungal secretions (1). However, we have not ruled out the possibility that some degree of cell wall penetration occurred in those interactions, because we could not always identify penetration tubes within the host walls. Fine-structure analysis would resolve the question, but as yet we do not have a practical method to thin-section specific encounter sites selected in vivo.

Papillae and encasements (4) are similar, if not identical, in origin and structure (1). In this study, parasites became encased either by partial injection of a parasite protoplast into a papilla or by continued growth of a papilla to enclose a parasite. Thus, papillae and encasements resulted from the same deposition process. We feel that use of the term encasement for a papilla that encloses part of a parasite implies more dissimilarity than is known to exist between these depositions and should therefore be abandoned.

The morphology and position of papillae varied according to whether or not they preceded penetration tubes. Those which preceded tubes were hemispherical or discoidal and attached to the host walls, whereas those which followed tubes were conical and attached to the tubes. Papilla morphology apparently is influenced by the shapes of the structures onto which papillae are deposited. However, it is more difficult to explain why papillae that are initiated after tubes develop do not form on the host walls in addition to the tubes, and why they grow faster at the ends of the tubes than elsewhere. One possible explanation is that the source of the irritant which induces papillae formation is at the tip of the incipient or growing penetration tube; a chemical inducer of papillae could be secreted along with wall materials by exocytosis. In fact, cytoplasmic vesicles which could be involved in the process are known to gather at the site of the incipient penetration tube before penetration begins (8, 13).

Since penetration failures commonly occurred simultaneously within 40 μm of successful penetrations of the same host cells, resistance mechanisms which would act over large areas of a given cell (i. e., virtually all suggested resistance mechanisms other than papilla formation) were probably not involved. The observed correlations (Fig. 5) of papillae formed before penetration tubes with relatively slower development of penetration tubes and empty cysts and with lower PE are consistent with the concept of papillae as active agents of resistance. Accordingly, the interval between papilla initiation and penetration tube appearance was greater for unsuccessful than for successful cysts. However, a strong case can also be made for the view that the unsuccessful cysts failed because of inherent (developmental deficiencies. First, since some cysts [Fig. 1-(A to C)] failed to penetrate even in the absence of papillae, one might expect that at least some of those [Fig. 1-(D to G)] which did incite papillae failed to penetrate for a similar (unknown) reason. The cyst in Fig. 1-G seems to represent a relatively clear-cut example of this, because the papilla was successfully breached. Second, our analysis showed that papillae were formed at about the same absolute time whether or not they were preceded by a penetration tube; i.e., production of tubes which were preceded by papillae was already tardy when the papillae were initiated. Third, the above correlation between time of papilla formation and rate or extent of fungal development would occur if the cycsts were developmentally deficient and unaffected by papillae, because the developmental deficiency would ensure that cyst development would be partial and would terminate with the tubes embedded in papillae. An experimental approach whereby papillae are prevented from forming is now being used to determine whether or not the cysts which fail to penetrate in the presence of papillae will also fail in their absence.

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