Centrifugation Studies Help to Clarify the Role of Papilla Formation in Compatible Barley Powdery Mildew Interactions

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ABSTRACT

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In compatible interactions between barley and *Erysiphe graminis hordei*, failures of fungal penetration are commonly associated with host papillae. To determine if papillae are responsible for these failures, and if enhanced papilla formation increases the percentage of failures, papilla deposition was experimentally altered in host cells. During attempted penetrations of coleoptile cells from fungal appressoria, the living coleoptiles were centrifuged sufficiently to produce two distinct zones [cytoplasm-rich (CR) and cytoplasm-poor (CP)] within individual host cells. Papilla deposition occurred at interaction sites in CR zones but not in CP zones. Using interference contrast microscopy, we observed the outcomes of penetration attempts from appressoria at pairs of interaction sites, one member of each pair located over each zone. Comparisons among the zones and noncentrifuged coleoptiles were made of two parameters: (i) the percentage of successful penetrations (penetration efficiency, PE); and (ii) the percentage of appressoria with pegs that failed to produce haustoria. In CP zones (where papillae did not occur) and noncentrifuged coleoptiles these parameters did not differ significantly. Therefore, we inferred that papillae do not cause the penetration failures that occur normally in this host-pathogen combination. Further data are needed to substantiate this inference. In CR zones, PE was only one third that in either the CP zones or in the noncentrifuged controls. Further, it appeared that in CR zones the only penetration attempts that were affected by the resistance response were by those appressoria that induced papillae. Thus, it appears that a centrifugally enhanced papilla (or some mechanism linked to papilla formation) has the potential to prevent fungal ingress.

Additional key words: primary penetration, resistance, wall appositions, cytology, host-pathogen interactions.

Individual plant cells respond to wounding by depositing materials between the cell wall and the plasmalemma at the wound site. Wall appositions (7) at sites of fungal penetration attempts are called papillae (15); appositions caused otherwise are called wound plugs (1).

Papillae were observed more than a century ago (10) and have been suggested to prevent or impede fungal ingress (1). However, direct evidence for such a role is incomplete (1) since the problem has only recently been approached experimentally. Vance and Sherwood (17) treated leaf disks of reed canarygrass with cycloheximide to reduce the amount of papilla deposition and found that several nonpathogenic fungi were able to penetrate more often into treated leaves than into nontreated controls. Papilla deposition was thought to be responsible for the penetration failures observed in the nontreated leaves, but the results did not eliminate possible cycloheximide effects on other resistance mechanisms.

Aist and Israel (5) used heat shock to prevent and delay papilla deposition at sites of attempted penetrations (encounter sites) between *Olpidium brassicae* and *Erysiphe graminis hordei*, and their respective compatible hosts, kohlrabi and barley. Most penetration attempts by these fungi on their hosts were successful, but each fungus had a subpopulation whose penetration attempts failed in the presence of papillae (3, 4). Papillae were not responsible for the failures of some penetration attempts in the *E. graminis*-barley system, but the results were inconclusive and could not explain all penetration failures because heat shock indirectly affected penetration peg production.

Low-speed centrifugation of inoculated barley coleoptiles provides an alternate method for studying papilla deposition as an impediment to fungal penetration (18). With this technique, the fungus is able to develop penetration and infection structures, 95% of the host cells remain alive, and the observable centrifugation effects on the host are readily reversible. Centrifugal localization of host cytoplasm to one end of each cell creates a cytoplasm-rich (CR) and a cytoplasm-poor (CP) zone. The restriction of papilla deposition to CR zones permits simultaneous studies of penetration attempts both in the presence and absence of papilla deposition.

The main purposes of this study were: (i) to determine whether or not papilla deposition is responsible for the

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failure of certain penetration attempts by *E. graminis hordei* on noncentrifuged barley coleoptiles, (ii) to evaluate the effects of centrifugal enhancement of host cytoplasmic aggregates and papilla deposition on parasite penetration efficiency, and (iii) to determine what effects low-speed centrifugation might have on parasite development during primary penetration.

MATERIALS AND METHODS

General.—Methods for growth, maintenance (4), preparation, and centrifugation of plant and fungal materials, and inoculation were described previously (18) and are only briefly outlined here.

Coleoptiles from 3-day-old seedlings of barley *(Hordeum vulgare* L. 'Proctor') were split lengthwise to expose the inner epidermis and were attached to the vertical glass tube supports of an apparatus designed specifically for centrifugation of fungus-inoculated plant tissues. The exposed inner surfaces of the coleoptiles were inoculated with 12- to 24-hr-old conidia of *Erysiphe graminis* DC. f. sp. *hordei* Em. Marchal (race Ao). Each apparatus with two mounted, inoculated coleoptiles was inserted into a translucent, plastic centrifuge tube. Sufficient $Ca(NO_3)_2$ at 0.01 M was added to immerse only the cut proximal ends of the coleoptiles, and the tubes were capped to maintain high relative humidity within. After incubation at 18 C under cool-white fluorescent lights for 8 hr, during which time conidia germinated and immature appressoria were formed, the tubes and their contents were centrifuged at 4,750 *g* and 20 ± 2 C for 12-14 hr. Noncentrifuged coleoptiles were mounted, inoculated, and incubated as above, but for 22 hr.

Immediately after centrifugation, or after the appropriate incubation period for noncentrifuged coleoptiles, the coleoptiles were excised from the apparatus, fixed for 1-2 hr at 0-4 C in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7), and then washed in the buffer. Centrifuged coleoptiles were mounted in the buffer on glass slides, whereas noncentrifuged coleoptiles were mounted in aniline blue-lactophenol containing Tween-20 (polyoxyethylene sorbitan monolaurate). The preparations were covered with coverslips, sealed with paraffin or Permount (Fisher Scientific Co., Fair Lawn, NJ 07410), and refrigerated.

Within 2 days the preparations were observed at ×500 and ×1,250 with Nomarski interference contrast or bright-field optics on a Zeiss Photomicroscope II. Light micrographs of the selected encounter sites on centrifuged preparations were taken on Polaroid Type-107 film. Other light micrographs were taken at ×400 on Kodak Panatomic × film.

Selection of pairs of encounter sites.—Encounter sites on centrifuged preparations were selected for study only when: (i) the host epidermal cell in question had well-compacted cytoplasm and a sharp interface between CR and CP zones, (ii) the appressorium was attached to the conidium and was lobed, and (iii) this lobe was located on the exposed periclinal wall of a suitable epidermal cell. Observations were made on pairs of encounter sites, each pair having one site over a CR zone and the other site being the nearest encounter site possible, but over a CP zone. The average distance between sites was 145 μm and ranged from 26 to 342 μm. In only one case were both sites on the same cell. Since the CR zones typically occupied only 50 μm (about 20%) of the cell length, the consequent infrequency of mature appressoria over CR zones was a major limiting factor in sample size. A total of forty-five encounter site pairs, distributed among 20 centrifuged coleoptiles, was observed during 10 trials.

Pairs of encounter sites on noncentrifuged coleoptiles were selected to correspond to those on centrifuged coleoptiles. These pairs consisted of one encounter site located within 50 μm of the basal (centrifugal counterpart) endwall and the nearest encounter site at a distance greater than 50 μm from the basal endwall of its respective host cell.

Observations.—Encounter sites were described in terms of selected parameters: presence of penetration pegs and/or haustoria, and presence and diameter of papillae. Penetration efficiency (PE) was defined as the number of mature haustorial central bodies observed, divided by the total number of appressoria observed, ×100 (4). A mature haustorial central body was equated with successful penetration (4).

The effect of a centrifugal force of 4,750 *g* on the ability of appressoria to produce penetration pegs was determined by comparing the proportion of penetration pegs in centrifuged and noncentrifuged preparations.

Appressoria were oriented at various angles with respect to the direction of centrifugal force. Because this orientation could affect the outcomes of penetration attempts, the centrifuged appressoria, their arms, and their lobes were classified according to (i) orientation with respect to the direction of centrifugal force, and (ii) location over CR or CP zones. These data were obtained from the Polaroid micrographs of encounter sites. The total number of penetration attempts and the number that resulted in success or failure were recorded for both locations and orientations.

Because all penetration attempts recorded for CR zones occurred within 50 μm of the basal endwalls of the host cells, comparisons in noncentrifuged coleoptiles were made between encounter sites that occurred less than 50 μm and those that occurred more than 50 μm from basal endwalls. Selected parameters were scored as in centrifuged preparations to detect normally occurring differences in these regions that might affect PE.

Since the cytoplasm in centrifuged cells is slightly stratified (18), possible gross effects of stratification on papilla and haustorium production were evaluated. The relative location of each of the 43 photographed encounter sites located in CR zones was diagrammed on a single graph to reveal any obvious patterns in spatial distributions of papillae and haustoria.

The application of centrifugal force to an enclosed, fluid-filled body (the coleoptile epidermal cell) causes a pressure gradient within the body, with the greatest pressure at the centrifugal pole. Because some leaves and fruits of plant cultivars resistant to powdery mildews have turgor pressures 4-5 bars greater than susceptible cultivars (9, 11, 19), we estimated whether the increased turgor in CR zones (centrifugal poles) of epidermal cells was sufficiently large to affect PE. To do this, we calculated the change in pressure established within centrifuged epidermal cells by the formula:

$$\Delta P = \rho \cdot g \cdot h \cdot CF$$

where ΔP = change in pressure (bars), ρ= density of a nucleus (g/cm^3), g = acceleration due to gravity (cm/sec^2), h = distance between midpoints of CR and CP zones of an average sized, 3-day-old, epidermal cell, and CF = centrifugal force used (cm/sec^2). Nuclei generally were positioned at about the midpoint of CR zones. The value used for the density of a nucleus was 1.24 g/cm^3 based on the density of sucrose used to separate nuclei from other cell components (6). The value for h was 1.25×10^{-2} cm, since the average length of a 3-day-old epidermal cell equaled 2h and was approximately 2.5×10^{-2} cm.

To determine whether centrifugation would cause most CR zones to have a turgor pressure greater than that in noncentrifuged cells, we estimated the range of turgor pressures among cells within noncentrifuged coleoptiles by plasmolysis on sucrose solutions, noting concentrations at which plasmolysis was first observed, and minimum concentrations required to plasmolyze all cells. Five 7-day-old coleoptiles were mounted in plastic wafers as described previously (4), and were incubated on 0.29 M sucrose for 1 hr. At 30-min intervals thereafter they were transferred to increasingly more concentrated sucrose solutions (0.02 M increments) until a concentration was reached at which all cells within a coleoptile were plasmolyzed.

RESULTS

Table 1 shows percentages of penetration pegs, haustoria, and papillae seen in CR and CP zones and in noncentrifuged coleoptiles, and thus provides a general comparison of events at these three locations. The percentage of penetration pegs was similar at all three locations. Although haustoria were produced at encounter sites at each location, CR zones had only one-third the percentage of haustoria produced in CP zones and noncentrifuged coleoptiles. Papillae were observed at similar percentages in CR zones and noncentrifuged coleoptiles, but were not seen at all in CP zones. Thus, the only effects of centrifugation revealed in this broad comparison were: (i) prevention of papilla formation in CP zones, and (ii) a marked reduction in percent haustoria (PE) in CR zones.

The percentages of various interaction types (combinations of penetration pegs, haustoria, and papillae) at encounter sites in CR and CP zones and in noncentrifuged coleoptiles are shown in Table 2. Encounter sites at each location were typified by different interaction types. In noncentrifuged coleoptiles, 61% of the encounters resulted in haustorium formation and papilla deposition. In CP zones, 64% of the interactions were typified by haustorium formation in the absence of a papilla (illustrated in Fig. 1). In CR zones, 64% of the encounter sites had no haustorium and a papilla was present (illustrated in Fig. 2).

In CP zones and noncentrifuged coleoptiles, a similar percentage [9 and 13, respectively (Table 2, 3rd and 4th columns from left)] of appressoria produced pegs that failed to develop into haustoria. Further, most haustoria in both noncentrifuged coleoptiles and CR zones were associated with papillae, but a similar small percentage [8 and 11%, respectively (Table 2)] at both locations was not. The latter result suggests that the only portion of the appressorial population whose PE was affected by the resistance response of the CR zone was the portion that induced papillae. Because of limitations imposed by the small sample size, these two comparisons could not be tested adequately for significance. They may, nevertheless, have heuristic value.

Although the incidence of papillae in CR zones and in noncentrifuged coleoptiles was not significantly different, mean papilla diameter in CR zones was significantly ($\rho = 0.01$) larger than in noncentrifuged coleoptiles (Table 3). Also, papillae associated with penetration failures were significantly ($\rho = 0.01$) larger than those associated with successful penetrations in both locations (Table 3).

Neither the orientation of the whole appressoria nor that of their arms (the long portion connected to the germ

TABLE 1. Percentage of encounter sites with selected parameters in cytoplasm-rich and -poor zones and in noncentrifuged coleoptiles of *Hordeum vulgare* inoculated with conidia of *Erysiphe graminis hordei*

Encounter site location	N	Encounter sites with: Penetration pegs	Haustoria (PE)	Papillae
Cytoplasm-rich	45	87	22[a]	76
Cytoplasm-poor	45	73	64	0[a]
Noncentrifuged	90	82	69	71

[a]Value differs significantly from others in the same column ($\rho = 0.05$ by χ^2 analysis of raw data).

TABLE 2. Percentage of encounter sites with indicated interaction type in cytoplasm-rich and -poor zones and in noncentrifuged coleoptiles of *Hordeum vulgare* inoculated with conidia of *Erysiphe graminis hordei*

Encounter site location	N	Encounter sites with: Appressorium only (%)	Peg only (%)	Peg and papilla only (%)	Peg and haustorium only (%)	Peg, haustorium, and papilla (%)
Cytoplasm-rich	45	13	0	64[a]	11	11
Cytoplasm-poor	45	27	9	0	64[a]	0
Noncentrifuged	90	18	3	10	8	61[a]

[a]Value differs significantly from others in the same column ($P = 0.05$ by χ^2 analysis of raw data).

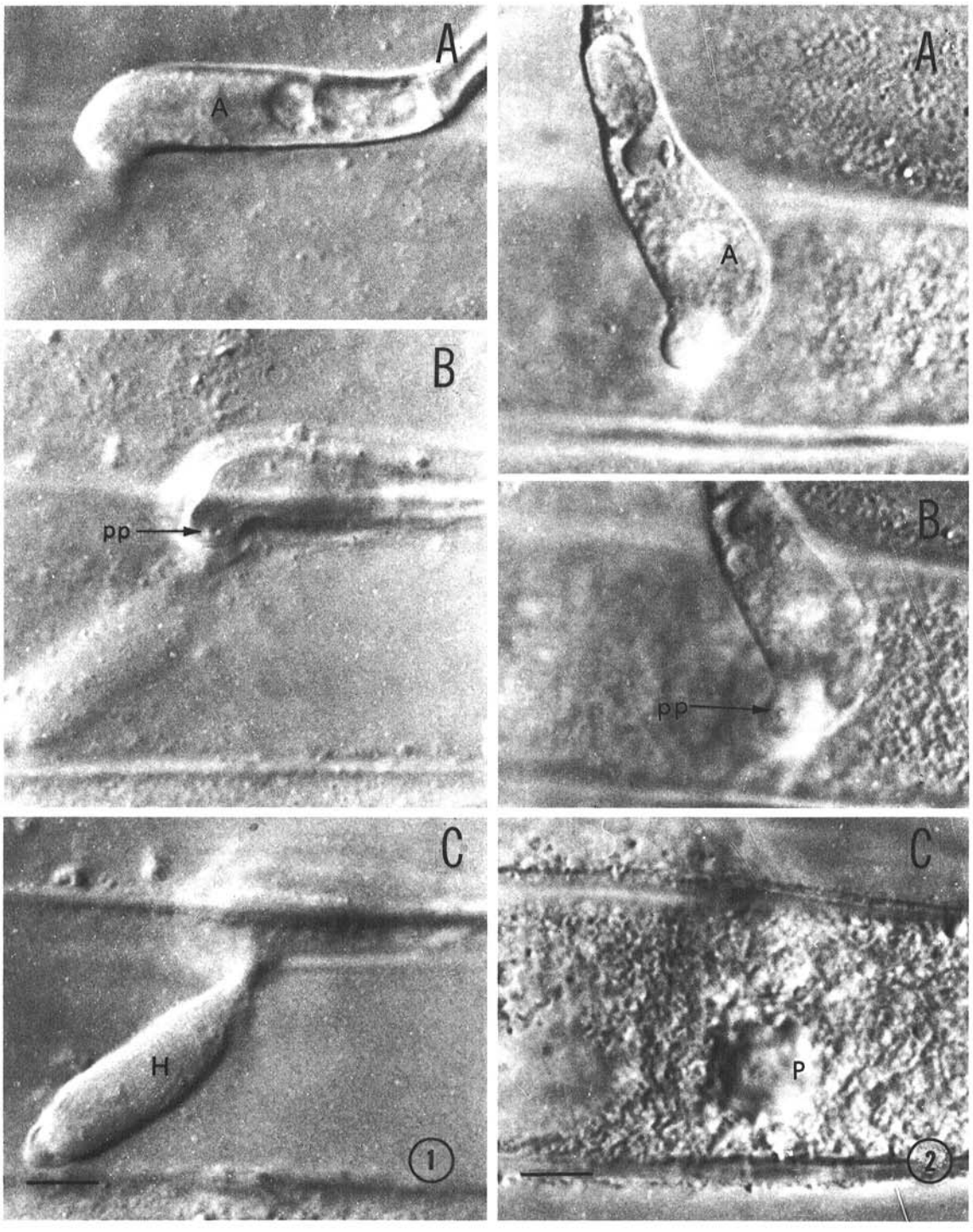

Fig. 1, 2. Three successive focal planes in each of two typical encounter sites observed in centrifuged cells of *Hordeum vulgare* inoculated with conidia of *Erysiphe graminis hordei*. **1)** Interaction in cytoplasm-poor zones. **A)** The lobed appressorium (A) on the surface of the host cell, **B)** produced a penetration peg (pp) which passed through the host cell wall, and **C)** developed into a haustorium (H) without inducing a detectable papilla. **2)** Interaction in cytoplasm-rich zones. **A)** The lobed appressorium (A) on the surface of the host cell, **B)** produced a penetration peg (pp) which passed through the host cell wall and **C)** aborted in a host papilla (P). Calibration bars = 5 μm.

tube) and lobes with respect to centrifugal force affected PE in CR or CP zones (differences not significant at $\rho = 0.05$ according to χ^2 analysis; data not shown). Also, the numbers of appressoria with various orientations at either location were not significantly different.

The frequencies of interaction types for encounter sites less than and more than 50 μm from the basal endwall of epidermal cells of noncentrifuged coleoptiles did not differ significantly (Table 4).

We detected no obvious patterns in the spatial distribution of interaction types occurring in CR zones that could be related to the slightly stratified host cytoplasm.

The pressure difference between CR and CP zones, due to centrifugation, was calculated to be 0.08 bars. Turgor pressures detected among cells within a given coleoptile ranged from a lower limit of 8.9 - 9.7 bars (calculated from the sucrose concentration at which plasmolysis was first detected) to an upper limit of 9.7 - 11.3 bars (calculated from the minimum sucrose concentration required to plasmolyze all cells). The mean difference between the least and the greatest turgor pressures was calculated to be 1.2 bars.

DISCUSSION

The results of this study suggested that papilla deposition was not responsible for papilla-associated penetration failures observed in a population of *E. graminis hordei* appressoria on noncentrifuged, compatible barley coleoptiles. In addition, they indicated that a centrifugally enhanced host response, such as papilla deposition, can confer resistance on CR zones of a normally compatible host tissue.

Our comparisons of the proportions of interaction types that occur in CP zones and in noncentrifuged coleoptiles revealed that a similar proportion of appressoria produced pegs that failed to develop into haustoria. Further, the penetration efficiencies of the appressorial populations were not significantly different, even though papillae were not detected in CP zones. Therefore, that portion of the normal, noncentrifuged appressorium population whose penetration attempts failed in the presence of papillae apparently did not fail because of papilla deposition, but for some other, as yet unknown, reason. This inference could be tested by repeating the experiment with a larger sample size. The present data corroborate the previous observation that in heat shocked, *Erysiphe*-inoculated barley some papilla-associated penetration failures occurred for reasons other than papilla deposition (5). A corresponding result was reported for *Olpidium brassicae* on compatible kohlrabi (3). None of these studies have supported Held's (13) hypothesis that papillae enhance penetration, since PE was not reduced in the absence of papillae.

By the centrifugal displacement of host cytoplasm, host responses were enhanced and PE was significantly lowered in CR zones; i.e., normally susceptible host cells became locally resistant to penetration. Papillae were significantly larger and may have been deposited faster and earlier in CP zones since the cytoplasm required for deposition (18) was present earlier and in larger quantity than in noncentrifuged coleoptiles (4, 8). If, as our results suggested, the only portion of the appressorium population whose PE was affected by the resistance response of the CR zone was that portion which induced papillae, the induced resistance would have been linked to papilla formation.

TABLE 3. Diameters of papillae induced in cytoplasm-rich zones and in noncentrifuged coleoptiles of *Hordeum vulgare* inoculated with conidia of *Erysiphe graminis hordei*[a]

Encounter site location	Mean papilla diameter in μm ± SD (sample size)		
		Papillae at encounter sites	
	All papillae	Without haustoria	With haustoria
Cytoplasm-rich	7.3 ± 2.2 (n = 34)■	7.8 ± 2.0 (n = 29)▲	4.6 ± 0.36 (n = 5)▲
Uncentrifuged	6.1 ± 2.2 (n = 64)■	8.1 ± 2.0 (n = 9)●	3.8 ± 4.1 (n = 55)●

[a]Values followed by common symbols indicate comparisons tested by a weighted, unpaired "*t*" test (16). In all three comparisons, differences were significant at $P = 0.01$.

TABLE 4. Incidence of interaction types at encounter sites from two locations on noncentrifuged coleoptiles of *Hordeum vulgare* inoculated with conidia of *Erysiphe graminis hordei*[a]

Distance from basal endwall	Total sites (no.)	Encounter sites with:				
		Appressorium only (no.)	Peg only (no.)	Peg and papilla only (no.)	Peg and haustorium only (no.)	Peg, haustorium, and papilla (no.)
< 50 μm	45	9	1	4	2	29
> 50 μm	45	7	2	5	5	26

[a]No significant differences were detected within colums by χ^2 analysis ($P = 0.05$).

The present experiments do not exclude the possibility that some mechanism linked to papilla deposition, but different from it, could be responsible for the resistance observed in cytoplasm-rich zones. For example, noncentrifuged cytoplasm could contain sublethal concentrations of a substance toxic to appressoria; such a substance could reach a more effective concentration in CR zones. A small subpopulation of appressoria might still be insensitive to the toxin, even at the higher concentration, due to the usual spread of toxin sensitivities in a fungal population. If only toxin-damaged appressoria could induce papillae, then only the insensitive units would have interactions without papillae in both CR zones and noncentrifuged coleoptiles. This speculation is supported by the finding that papillae have been induced by fungal metabolities (12), and by the report that phytoalexins may be involved in resistance of barley to *E. graminis hordei* (14). As early as 1939, Kusano (see ref. 1 for translation) recognized the possibility that papilla-associated penetration failures could be due to some toxic constituent of the host cytoplasm rather than to papillae.

A simpler explanation of centrifugally-induced resistance would be that the papilla, at some stage, acted as a structural and/or chemical barrier to penetration. In a related study, Aist (2) recently demonstrated a potential role of wall apposition formation in induced resistance of kohlrabi to penetration attempts by *O. brassicae*. Although papillae associated with penetration failures were significantly larger than those associated with successful penetration attempts in noncentrifuged coleoptiles, other results of this study suggested that papillae do not cause penetration failures in noncentrifuged coleoptiles; the relationship between papilla diameter and the outcomes of penetration attempts on noncentrifuged coleoptiles apparently was fortuitous. Therefore, resistance in CR zones, if caused by papillae, is probably not a function of papilla diameter alone, although increased diameter may have an additive effect in combination with some other aspect of papilla deposition (e.g., time of deposition).

The possibility that the low PE observed in CR zones could be due to something other than a host response was considered. Our data show that toxicity from moribund host cells, the hypothetical occurrence of some natural factor in the basal 50 μm of epidermal cells, or strata of centrifuged cytoplasm did not influence the PE. The pressure gradient established within cells during centrifugation probably did not affect PE since the calculated increase in pressure in CR zones would not put most of these zones outside the normal range of turgor pressures for barley epidermal cells. Any direct effect of centrifugation on fungal development (and consequently on PE) should have been, but was not, observed equally in all populations of centrifuged appressoria; penetration efficiency in CP zones was the same as in noncentrifuged coleoptiles, but was much lower in CR zones.

Thus, there are apparently two general possibilities to explain the artificially induced resistance to penetration in CR zones: papillae, or some mechanism linked to papilla formation, prevented many appressoria from forming haustoria.

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