Cytology and Histology

Infection of Wheat and Oats by *Pyrenophora tritici-repentis* and Initial Characterization of Resistance

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ABSTRACT

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Over a 72-hr postinoculation period, conidial germination (99%) and production of germ tubes (2.5 per conidium) by *Pyrenophora tritici-repentis* were similar on leaves of susceptible ND 495 and resistant BH 1146 spring wheats and highly resistant Lodi oats. Appressoria production was significantly greater on Lodi (2.1 per conidium) than on ND 495 (1.8 per conidium) and greater on ND 495 than on BH 1146 (1.5 per conidium), suggesting stimulation from susceptibility and from high resistance. The fungus usually entered the host epidermal cell from the appressorium with a penetration peg that subsequently developed a vesicle. Secondary hyphae formed from the vesicle and invaded the mesophyll intercellularly. Penetrations were rare 3 hr after inoculation and increasing by 6 hr, with most occurring from 12 hr on. From 24 hr on, the percentage of papilla formation around the infecting hypha was significantly greater in Lodi (29-47%) than in BH 1146 (11-13%) and greater in BH 1146 than in ND 495 (3-7%). From 12 hr on, the percentage of appressoria colonizing host cells (hyphae not covered by papillae and growing beyond initial penetration) was similar in ND 495 and BH 1146 (20-34%) and significantly lower in Lodi (4-7%). In Lodi, hyphae from successful colonizations were restricted to the intercellular area of a few epidermal and mesophyll cells, resulting in small brown leaf flecks 8 days after inoculation. Lesion size in BH 1146 was half that in ND 495, but mesophyll cells in lesions on both wheats had similar vesiculation of membranes and chloroplast degeneration. Papilla formation appeared to be an active defense against infection by *P. tritici-repentis* in Lodi and a much less active defense in BH 1146 and ND 495. Subsequent severe restriction of hyphal and surrounding lesion growth in Lodi and greater restriction in BH 1146 than in ND 495, without obvious histological or cytological structural mechanisms for restriction, suggest a molecular mechanism for resistance to fungal development, following infection and papilla formation, that is very active in Lodi and less active in BH 1146.

Additional key words: *Drechslera tritici-repentis*, *Helminthosporium tritici-repentis*, *Pyrenophora trichostoma*, tan spot, ultrastructure, yellow leaf spot.

Pyrenophora tritici-repentis (Died.) Drechs. (syn. *P. trichostoma* (Fr.) Fckl.), anamorph: *Drechslera tritici-repentis* (Died.) Shoem. (syn. *Helminthosporium tritici-repentis* Died.), the causal agent of tan spot or yellow leaf spot of wheat and leaf spot of oats and other Gramineae, has become a major pathogen of wheat in many areas of the world (4,6,11,13,14,23,26,27,32,34). The fungus has a wide host range among Gramineae (20). Lesions on wheat leaves range from small dark flecks to large tan-to-brown blotches, often with a yellow halo (11). Ascospores (26) and

conidia (11) produced on weathered wheat and other Gramineae straw and stubble are the primary inoculum, and conidia produced in lesions are the secondary inoculum (12,26). The incidence of tan spot has been related to the duration of free water on the leaves, the relative susceptibility of the cultivar, the virulence of the fungal race, and the growth stage of the host (11,22,25,26). Genotypes resistant to tan spot have been identified (5,13,22,25,26), and resistance has been shown to be polygenically controlled in some wheat cultivars (25). A single recessive gene controlling resistance in the winter wheat cultivar Carifen 12 was reported by Gough (5) and by Lee and Gough (21). Hard red spring wheat breeding line ND 495 was severely spotted by the fungus with exposure to a 6–12 hr wet period (11). Cultivar BH 1146 (PI 185831) was relatively resistant even at wet periods up to 50 hr (22,25), and the oat cultivar Lodi (CI 7561) did not show any distinct spotting even in wet periods up to 49 hr (11). Very little is known, however, about either

the infection process or the mechanisms of resistance involved in this fungus-host complex. In some other complexes, papillae have been associated with prevention of fungal penetration of host (1,2,18) and nonhost plants (3,28). Papillae have been associated with disease resistance (3,9,28) and are common in Gramineae (17,29). The objectives of this study were to investigate the infection process at the macroscopic, cellular, and subcellular levels on and in plants of differing susceptibility and to search for mechanisms of resistance to *P. tritici-repentis*.

MATERIALS AND METHODS

Inoculation procedure. Conidia of race 11 isolate PyD7 of *P. tritici-repentis*, virulent on ND 495 (22), were produced according to the technique developed by Raymond et al (26), then suspended in deionized water and filtered through four layers of cheesecloth. One drop of Tween 20 was added to each 100 ml of conidial suspension. The concentration was adjusted to 2,500 conidia per milliliter.

TABLE 1. Process of infection in wheat breeding line ND 495 (susceptible), wheat cultivar BH 1146 (resistant), and oat cultivar Lodi (highly resistant) inoculated with conidia of *Pyrenophora tritici-repentis*[a]

| | Conidia | | | | | | | | Appressoria | | | | | | | | | | | |
| | Percent germination | | | | Germ tubes per conidium | | | | Per 100 conidia | | | | Percent with papillae formed around infecting hypha | | | | Percent colonizing host cells | | | |
Hours after inoculation	495	1146	Lodi	LSD$_{0.05}$	495	1146	Lodi	LSD$_{0.05}$	495	1146	Lodi	LSD$_{0.05}$	495	1146	Lodi	LSD$_{0.05}$	495	1146	Lodi	LSD$_{0.05}$
3	78	65	74		1.5	1.4	1.5		5	4	7		...[b]	0	0		0	0	0	
6	96	95	97	1.6	2.3	2.5	2.4	0.3	62	60	58	85	1	8	17	6	6	13	1	7
12	98	99	94	1.6	2.6	2.7	3.1	0.3	90	107	118	85	3	5	8	6	23	31	5	7
24	100	99	100	1.6	2.5	2.5	2.4	0.3	188	156	221	85	3	11	29	6	25	28	4	7
48	99	100	100	1.6	2.5	2.4	2.4	0.3	255	221	307	85	5	12	44	6	20	30	7	7
72	100	100	99	1.6	2.5	2.5	2.4	0.3	286	226	345	85	7	13	47	6	32	34	8	7
6–72	99	99	98	1.5	2.5	2.5	2.5	0.1	176	154	210	13	4	9	29	5	21	28	5	7

[a] After inoculation, hosts were misted in a lighted chamber at 20 C for 48 hr, then placed on a greenhouse bench at 16–23 C. For each time after inoculation, each value is the average of 200 observations (40 conidia × five trials). For 6–72 hr after inoculation, each value is the average of 1,000 observations (40 conidia × five trials × five times). The low values at 3 hr after inoculation were not included in the SAS ANOVA computations of LSD (*P* = 0.05).
[b] Three hours after inoculation, a papilla formed beneath the one appressorium formed within 3 hr in trial five. The SAS program interpreted this papilla as the formation of papillae beneath 20% of the appresoria.
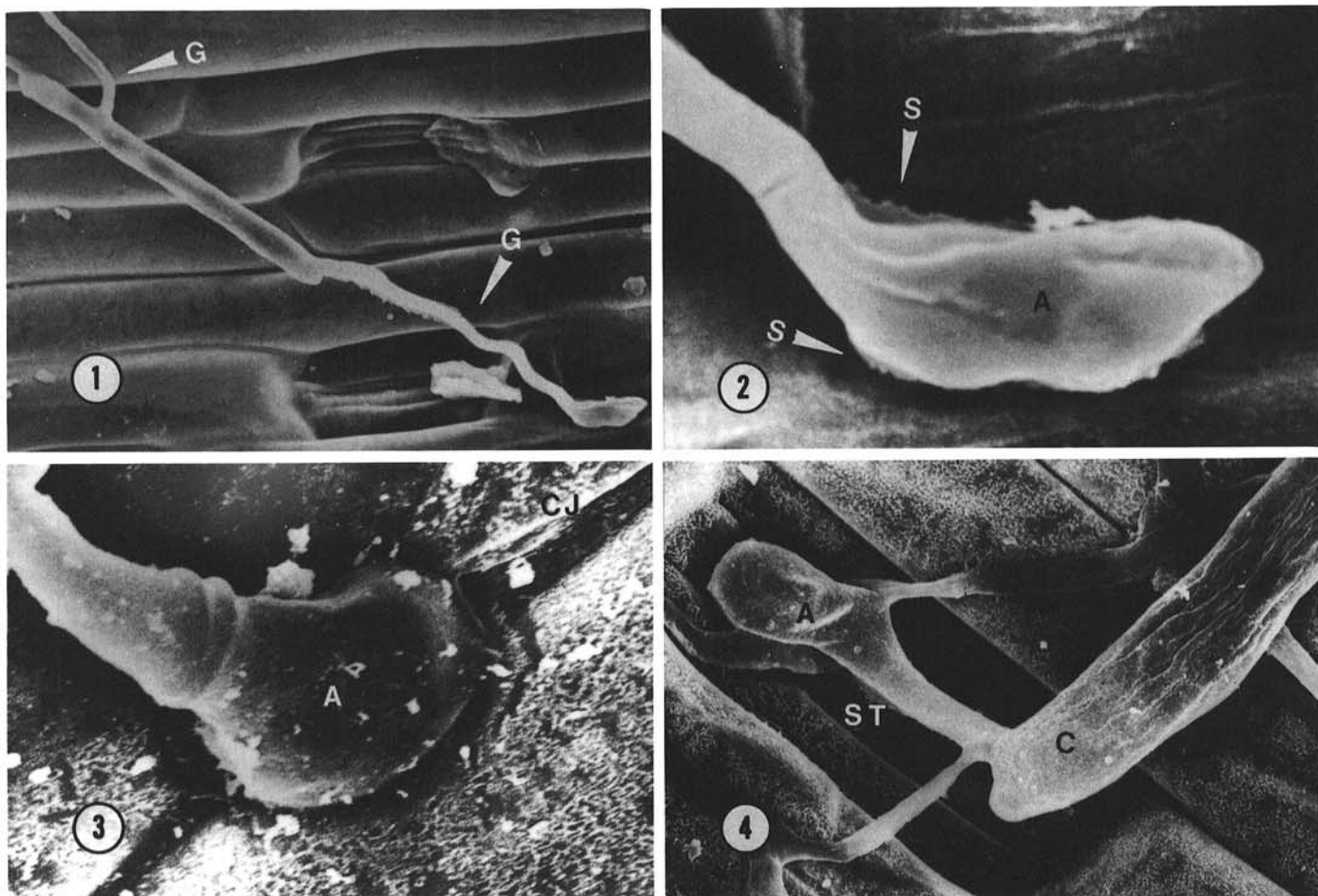
Figs. 1–4. Scanning electron micrographs of *Pyrenophora tritici-repentis* on leaf surfaces of wheat and oats. **1,** Germinated conidium showing germ tubes (G) from polar and intercalary cells. (×350) **2,** Enlargement of club-shaped appressorium (A) in Figure 1. Note extracellular sheath (S). (×2,400) **3,** Round appressorium (A) formed over cell junctures (CJ). (×2,400) **4,** Germinated conidium (C) with appressorium (A) formed over stoma (ST). Note development of secondary hyphae from germ tube. (×1,200)

ND 495, BH 1146, and Lodi were used as representative hosts of differing susceptibility. Six plants of each were grown in autoclaved soil in 15-cm-diameter clay pots in a greenhouse. A DeVilbiss 26 sprayer at 0.55 kg cm^{-2} pressure was used to inoculate 30-day-old plants (four- to six-leaf stage) until runoff with the conidial suspension. Plants were then placed in a mist chamber at 20 C under continuous fluorescent light. Misting with tap water for 48 hr was provided with a DeVilbiss 841 nebulizer. Plants were removed to a greenhouse bench (16–23 C) until completion of the experiment.

Light microscopy. Leaf samples from individual host plants were taken 3, 6, 12, 24, 48, and 72 hr after inoculation. Each sample consisted of three 3-cm-long leaf pieces taken from the central portion of the second leaf from the top. Samples were stored in alcoholic lactophenol (3 parts 95% ethanol, 1 part lactophenol) until examined. Samples were boiled for 3 min in the alcoholic lactophenol, placed in lactophenol-cotton blue for 2–4 hr, rinsed in deionized water, and mounted in lactophenol for observation. Percentage of spore germination, number of germ tubes, number of appressoria, number of leaf colonizations (infecting hyphae not covered by papillae and growing beyond initial penetration), and number of initially penetrating infecting hyphae around which papillae formed in epidermal cells were recorded for 40 randomly selected conidia per sample. The experiment was arranged as a split-plot with cultivars as whole plots in a randomized complete block (31) and was repeated twice, with three replication blocks in the first trial and two in the second. Analyses of variance and differences between means (Fisher's least significant difference) were determined according to the ANOVA model of the Statistical Analysis System.

Histological studies on lesions were conducted for samples of ND 495 and BH 1146 taken 72 hr and 8 days after inoculation. Individual lesions were fixed in standard formalin-aceto-alcohol, dehydrated by the tertiary-butyl alcohol method, and embedded in paraffin. Cross and longitudinal sections 10 μm thick were stained in safranine and fast green and mounted in Canada balsam (16). All material was observed with a Leitz Laborlux bright-field microscope and recorded with a Zeiss Tessovar camera assembly on Panatomic X or Kodak Technical Pan 2415 film.

Electron microscopy. Seventy-two hours after inoculation, small (1 mm or less in diameter) growing lesions were fixed for 4 hr at room temperature in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4), postfixed for 2 hr at 4 C in 1% osmium tetroxide in the buffer, and washed twice for 30 min in the same buffer.

For scanning electron microscopy, the fixed samples were dehydrated in a series of graded concentrations of ethanol, then dried in a Tousimis Autosamdri critical point drier in 100% ethanol with CO_2 as the transition fluid. Specimens were sputter-coated with gold-palladium and examined and photographed with a JEOL JSM-35 scanning electron microscope.

For transmission electron microscopy, the specimens were dehydrated in a series of graded concentrations of acetone. Samples were stained for 4 hr in 70% acetone saturated with uranyl acetate, then embedded in a plastic mixture of Araldite. Thin sections were cut with a diamond knife on a Sorvall ultramicrotome, poststained in lead citrate for 3 min, mounted on copper grids, and examined and photographed with a JEOL JEM 100S transmission electron microscope.

RESULTS

Prepenetration. The infection process was initiated with conidial
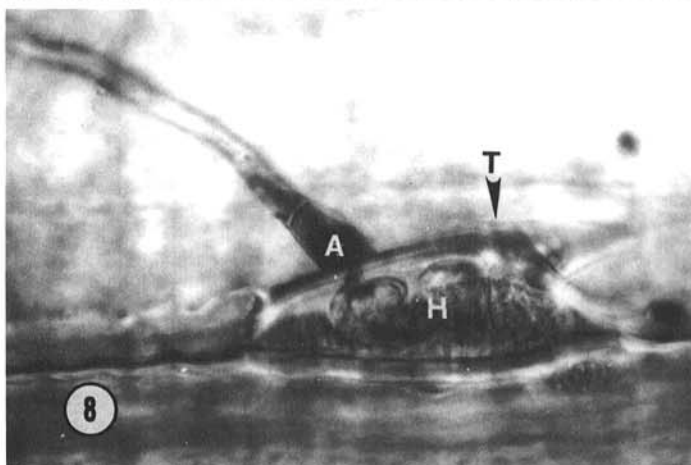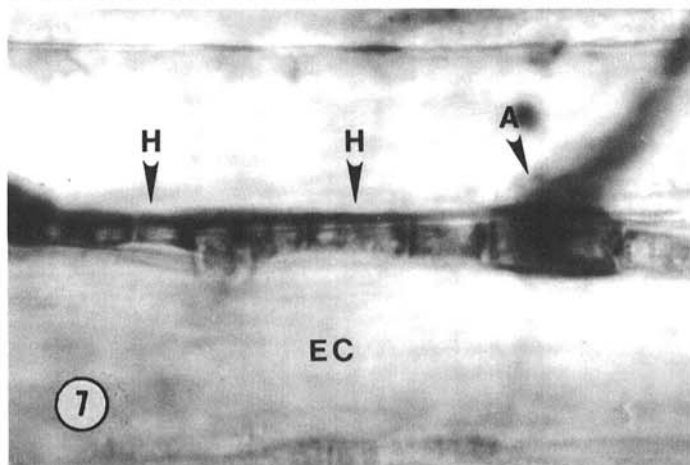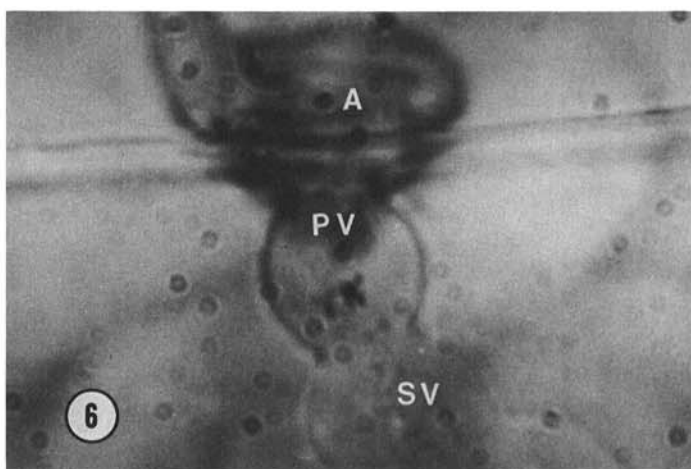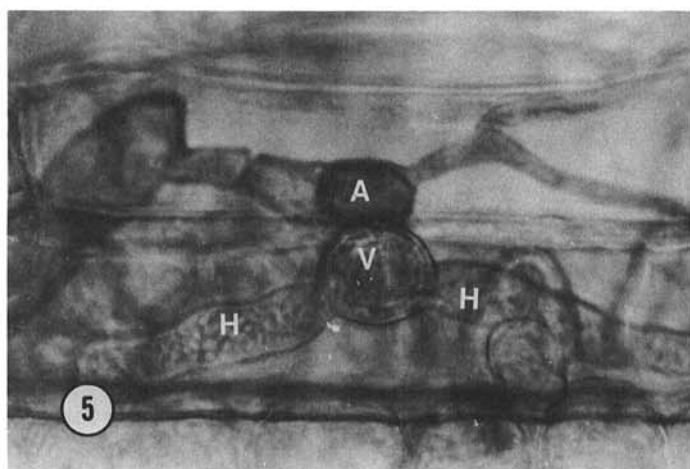
Figs. 5–8. Stages in penetration of wheat leaves by *Pyrenophora tritici-repentis*. **5,** Appressorium (A), intracellular vesicle (V), and secondary hyphae (H) in epidermal cell 24 hr after inoculation. (×1,250) **6,** Penetrated epidermal cell with development of primary (PV) and secondary (SV) fungal vesicles from appressorium (A) 24 hr after inoculation. (×3,000) **7,** Surface view of hypha (H) developed between cuticle and epidermis of cell (EC) 48 hr after inoculation. Note appressorium (A) at site of penetration. (×1,300) **8,** Appressorium (A) over penetrated trichome (T) 48 hr after inoculation. Note intracellular hyphae (H) in trichome. (×1,100)

germination, germ tube elongation, and development of appressoria. Percentage of germination and number of germ tubes per conidium did not differ significantly on the three hosts (Table 1). At least 65% of the conidia were germinated by 3 hr after inoculation, at least 95% by 6 hr, and essentially 99% from 24 hr on (Table 1). Germ tubes were produced from polar and intercalary cells of the conidium (Fig. 1). Between 6 and 72 hr after inoculation, the average number of germ tubes per conidium was 2.5 (Table 1).

Only a few appressoria developed by 3 hr after inoculation, but between 6 and 72 hr, abundant appressoria formed on all three hosts (Table 1). Because of a large standard error (LSD = 85), the higher numbers of appressoria on Lodi than on ND 495 were not significant. As time after inoculation reached 48 and 72 hr, appressoria production on Lodi became significantly greater than on BH 1146. Combining data from 6, 12, 24, 48, and 72 hr (LSD = 13) reduced the standard error and resulted in appressoria production on Lodi being significantly greater than that on ND 495 and production on ND 495 being significantly greater than that on BH 1146 (Table 1). Appressoria were club-shaped or round, formed terminally on germ tubes, and usually developed on epidermal cell junctures (Figs. 2 and 3). Appressoria sometimes developed on stomata (Fig. 4) but rarely penetrated the stomatal aperture. Penetration pegs usually entered guard cells or subsidiary cells, and hyphae subsequently grew into the stomatal chamber. Extracellular sheaths (8,35) were observed spreading out from the appressorium onto the leaf surface (Fig. 2).

Penetration and papilla development. Infection pegs grew from many appressoria and penetrated the anticlinal wall of epidermal cells. Penetrations usually resulted in papilla formation or epidermal cell colonization (Table 1). Penetrations were rare by 3 hr after inoculation. By 6 hr, penetration occurred with 7–21% of the appressoria (from papillae + colonization in Table 1) on the three hosts. From 12 to 72 hr, penetration occurred with 13–55% of the appressoria. After penetration, the fungus usually produced an intracellular vesicle that formed one or more secondary hyphae (Fig. 5). Sometimes, a secondary vesicle formed (Fig. 6) from which intracellular hyphae developed. Occasionally, no vesicle developed and the invading hypha ramified in two or more directions inside the epidermal cell. Sometimes, the fungus penetrated the cuticle, grew underneath (Fig. 7), and invaded the epidermal cell. Trichomes were also penetrated, but the fungus remained in the invaded trichome without further growth to the neighboring cells (Fig. 8).

Formation of a papilla around the infecting hypha beneath an appressorium was common in all three hosts. The beginnings of papillae were first observed during onset of penetration 3 hr after inoculation. A papilla began as an aggregation of dense cytoplasm (Fig. 9) and when completely developed, appeared mostly hemispheric from a side view (Fig. 10) (1). In a few cases, the infecting hypha extended beyond the papilla (Figs. 11 and 12). At 6 and 12 hr after inoculation, papillae were evident around 1–17% of the infecting hyphae (Table 1). From 24 hr on, papillae developed under significantly higher percentages of appressoria on Lodi than on BH 1146 and ND 495 (Table 1); the percentage on BH 1146 was twice that on ND 495, but the difference was not significant and did not appear to affect percentage of colonization (Table 1). In all three hosts, a clear inverse relationship existed between the percentage of appressoria with papillae beneath them and the percentage of appressoria colonizing epidermal cells (Table 1). Lodi had the highest percentages of papillae developed beneath appressoria and the lowest percentages of colonization, whereas
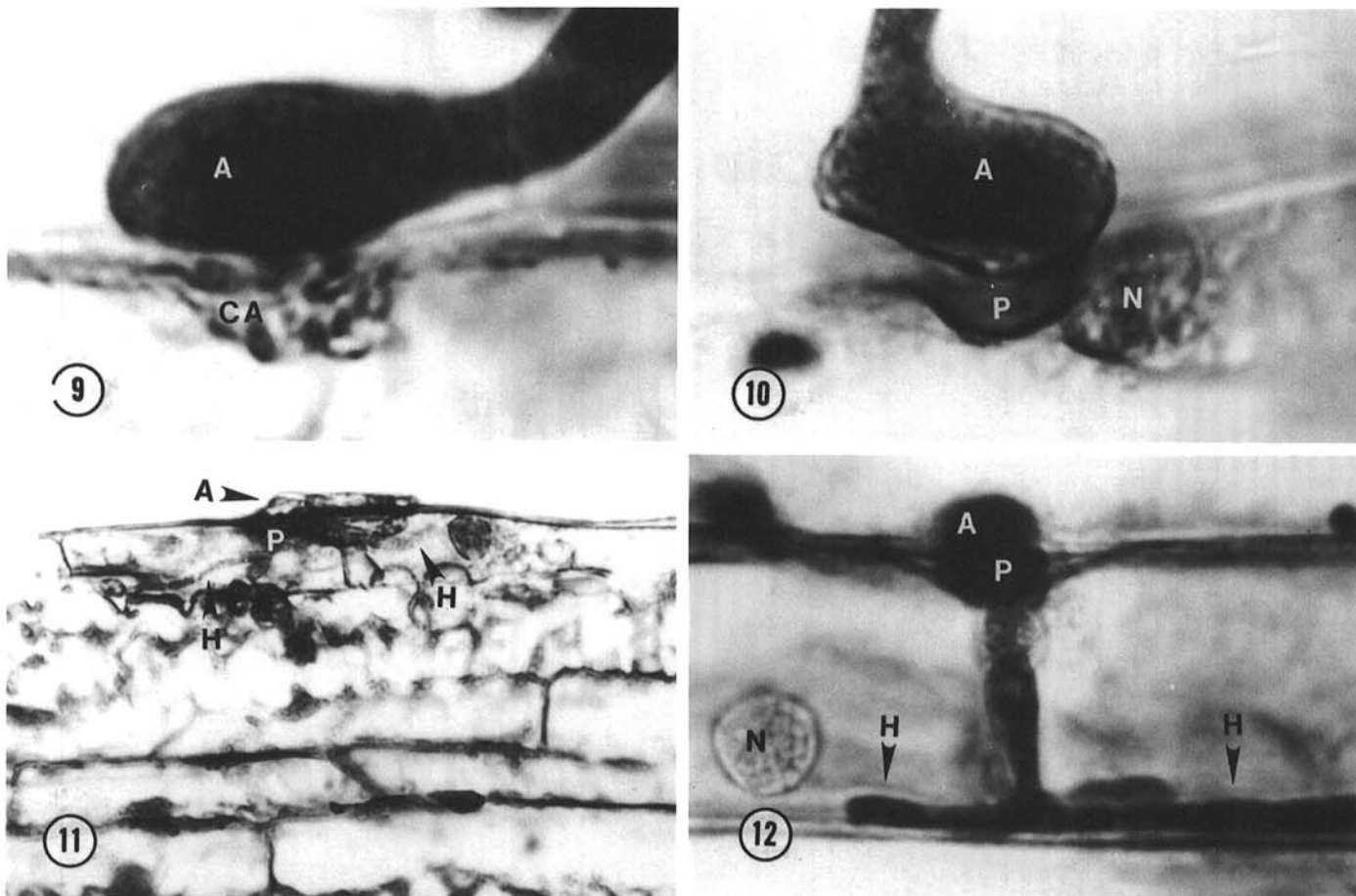

Figs. 9–12. Penetration by *Pyrenophora tritici-repentis* and development of papillae in wheat leaves. **9,** Cytoplasmic aggregate (CA) beneath appressorium (A) 3 hr after inoculation. (×4,200) **10,** Completely developed papilla (P) beneath appressorium (A) 12 hr after inoculation. Note host nucleus (N) close to papilla. (×3,200) **11,** Longitudinal section of papilla (P) 72 hr after inoculation. Note appressorium (A) at site of penetration and intracellular hyphae (H). (×750) **12,** Appressorium (A) on papilla (P) with intracellular hyphae (H) extending beyond it 48 hr after inoculation. Note host nucleus (N). (×1,800)

BH 1146 and ND 495 had the lowest percentages of papillae and the highest percentages of colonization.

In addition to the hemispheric papillae, Lodi had larger papillae or encasing sheaths (Figs. 13 and 14). In a few cases, encasing sheaths did not completely cover intracellular hyphae (Fig. 15). In samples observed 8 days after inoculation, some papillae occupied the whole diameter of the cell (Fig. 16). In some instances when epidermal cells were penetrated, intracellular hyphae were highly

branched, with numerous septa, and formed chlamydospore-like cells (Figs. 17 and 18). These hyphae did not grow into the intercellular spaces of the mesophyll. Occasionally, papillae developed on the internal walls of the cells in contact with but not penetrated by the hyphae (Fig. 19). The fungus usually remained restricted to the invaded cell but occasionally branched into a very limited area of the intercellular spaces of the mesophyll. Only a few mesophyll cells around the point of penetration were affected, and
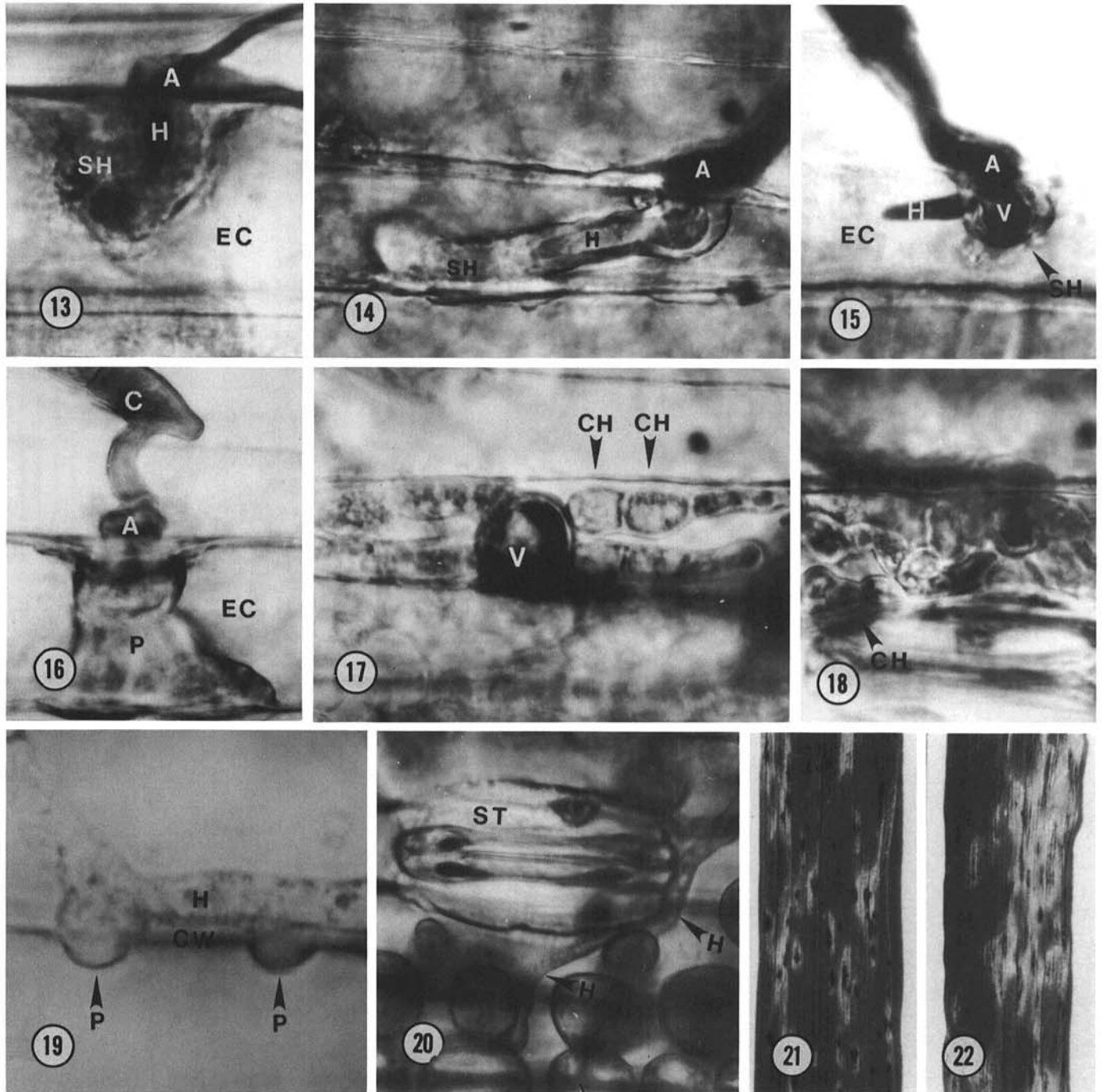

Figs. 13–22. 13–19. Formation of papillae and encasing sheaths and restriction of intracellular hyphal growth of *Pyrenophora tritici-repentis* in epidermal leaf cells of oat cultivar Lodi. 13, Encasing sheath (SH) in epidermal cell (EC) 8 days after inoculation. Note hypha (H) in encasing sheath and appressorium (A) over site of penetration. (×1,200) 14, Encasing sheath (SH) with internal hypha (H). Note appressorium (A) on site of penetration. (×1,200) 15, Fungal vesicle (V) in encasing sheath (SH) and secondary hypha (H) formed from vesicle and growing into epidermal cell (EC). Note appressorium (A) at site of penetration. Sample taken 72 hr after inoculation. (×1,200) 16, Appressorium (A) formed from conidium (C) induced formation of large papilla (P) occupying whole diameter of epidermal cell (EC). Sample taken 8 days after inoculation. (×1,200) 17, Epidermal cell containing profuse hyphal growth 48 hr after inoculation. Fungal cells have thick cell walls and resemble chlamydospores (CH). Fungal vesicle (V) also has thick cell wall. (×900) 18, Fungal chlamydospore-like cells (CH) in epidermal cell. (×1,000) 19, Intracellular hypha (H) incited formation of papillae (P) on internal cell wall (CW) of neighboring epidermal cell 48 hr after inoculation. (×3,100) 20, Hypha of *P. tritici-repentis* (H) in intercellular spaces of mesophyll of wheat leaf close to stoma (ST) 48 hr after inoculation. (×1,100) Symptoms of tan spot on 21, wheat cultivar BH 1146 (resistant) and 22, wheat breeding line ND 495 (susceptible) 8 days after inoculation. Plants were in mist chamber for 48 hr at 20 C after inoculation.

8 days after inoculation, these areas appeared macroscopically as minute necrotic flecks.

Fungal and lesion development in the host tissue was similar in ND 495 and BH 1146. By 24 hr after inoculation, secondary hyphae produced from intracellular vesicles grew into the neighboring epidermal cells or directly into the intercellular spaces of the mesophyll (Fig. 20). Mesophyll cells did not appear to be penetrated. Lateral growth of the fungus continued until hyphae contacted the larger vascular bundles, where further growth was often blocked. Small bundles were not a barrier to lateral spread, and the hyphae were seen in the surrounding parenchyma. Eight days after inoculation, no evidence was found of xylem or phloem invasion, although hyphae were occasionally seen in the cells of the bundle sheath. At this time, lesions on BH 1146 were typically dark
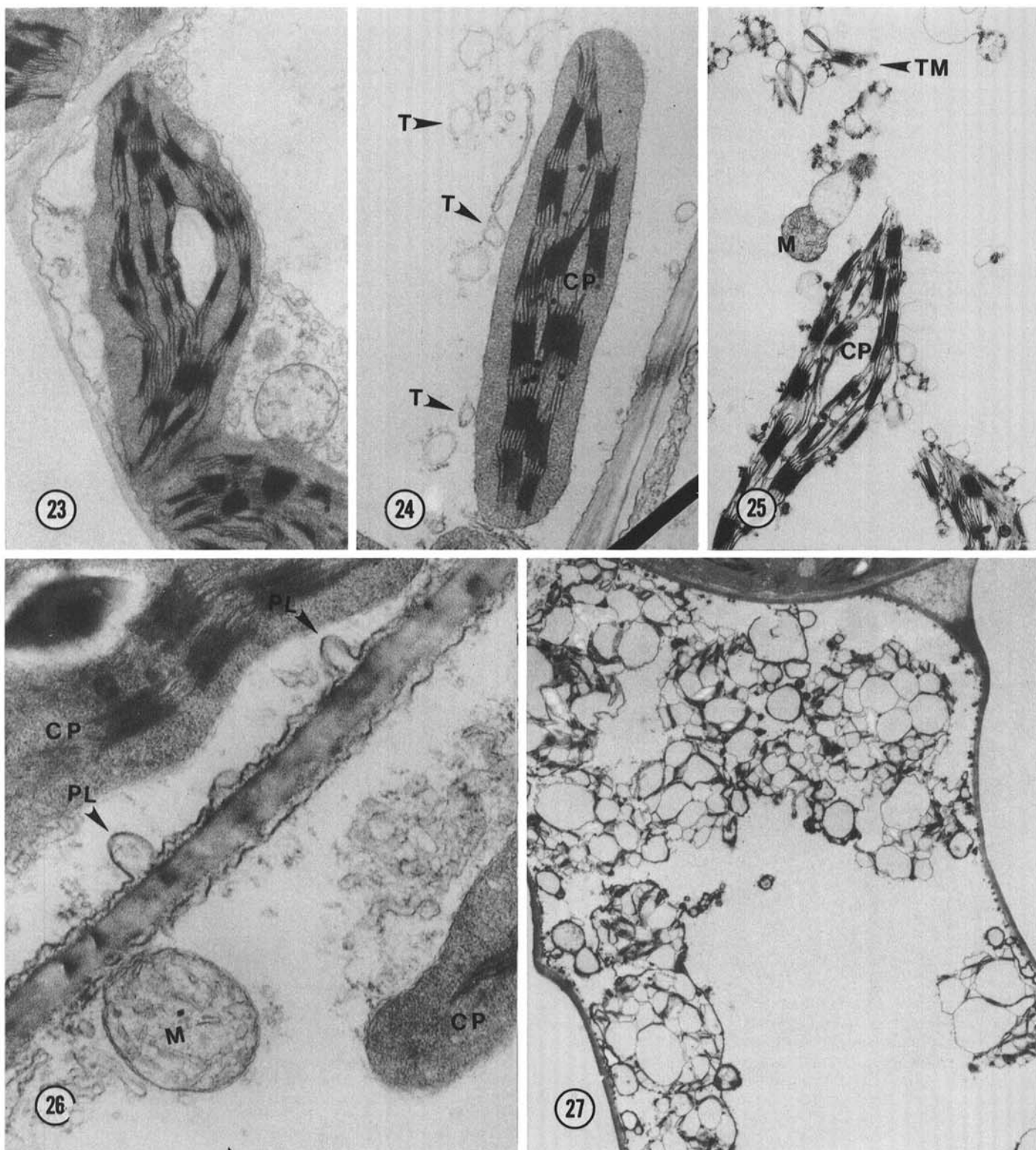
Figs. 23–27. Transmission electron micrographs showing effect of *Pyrenophora tritici-repentis* infection on mesophyll cells in tiny growing lesions on wheat leaves 72 hr after inoculation. Location of cells undetermined but beyond growing hyphae. 23, Uninoculated control. (×14,600) 24, Affected cell showing pieces of vesiculated tonoplast (T). Chloroplast (CP) appears unaffected. (×15,900) 25, Affected cell with damaged chloroplast (CP) and mitochondrion (M). Note pieces of thylakoid membrane (TM). (×12,000) 26, Affected cell showing partial vesiculation of plasmalemma (PL). Mitochondrion (M) and chloroplasts (CP) appear unaffected. (×32,700) 27, Damaged cell with no evidence of normal membranes or organelles. (×4,000)

brown, 1.0–2.5 mm^2, and surrounded by a narrow tan area (Fig. 21). Lesions on ND 495 were brown, 2.5–4.0 mm^2, and surrounded by a wide tan area (Fig. 22).

The effects of the fungus at the subcellular level were similar in ND 495 and BH 1146. In small (1 mm or less in diameter) growing lesions observed 72 hr after inoculation, the fungus affected host cells beyond the hyphae with varied intensity. The most evident features of affected host cells, compared with cells of uninoculated controls (Fig. 23), were vesiculation and collapse of the membranes. In some host cells, the tonoplast was broken and tended to form small vesicles free in the cytoplasm, around the cell organelles (Fig. 24). Breakdown of chloroplasts was sometimes evident, with vesiculation of the external membrane, accompanied by disruption of the chloroplast matrix; thylakoid membranes could be seen in the surrounding cytoplasm (Fig. 25). In other cells in the same lesion, mitochondria and chloroplasts appeared structurally unaffected (Figs. 24 and 26). The plasmalemma tended to separate from the cell wall and become vesiculated (Fig. 26). In severely affected cells, the protoplast appeared completely disrupted, with collapsed chloroplasts and no evidence of normal mitochondria or normal cell membranes (Fig. 27).

DISCUSSION

Percentage of germination and number of germ tubes per germinating conidium (Table 1) were unrelated to host resistance and followed the same general pattern observed in several other *Helminthosporium* spp. (8,10,15,18,19,24). Hau and Rush (8) reported that *H. orizae* formed fewer appressoria and longer germ tubes on resistant than on susceptible cultivars and attributed this phenomenon to resistance expressed before infection. According to this line of reasoning, our data for 6–72 hr after inoculation (Table 1) suggest that compared to susceptible ND 495 wheat there is a decrease in appressoria production in response to moderate resistance in BH 1146 wheat and a greater increase in response to high resistance in Lodi oats.

Initial tissue colonization by *P. tritici-repentis* and fungal development in the intercellular spaces of the mesophyll were similar to those reported with other *Helminthosporium* spp. (10,15,30,33). Also, changes at the cellular level followed, in general, the same pattern observed with other species of this fungus genus (18,24,30,33).

Concerning the instances in Lodi in which hyphae remained restricted to the invaded epidermal cell and developed many short chlamydospore-like cells, Hargreaves (7) reported modifications in the cell walls of intracellular hyphae of *P. teres* in oats. He indicated that the invaded cell died as a response to fungal invasion but did not indicate if the fungus remained alive. The chlamydospore-like appearance of some intracellular hyphae observed in Lodi suggests that the fungus might be able to form dormant cells that survive the unfavorable conditions created by the uncongenial host. Krupinsky (20) recovered *P. tritici-repentis* from small lesions that developed on oats 9 days after inoculation; whether the recovered fungus remained as normal mycelium in oats or formed resistant structures is an intriguing area for exploration.

Failures in penetrations by *P. tritici-repentis* on wheat and oats were associated with papilla formation. Similar results on barley were reported by Aist and Israel (2) for *Erysiphe graminis* and by Keon and Hargreaves (18) for *Pyrenophora teres*. Because Lodi had much higher percentages of papilla formation and much lower percentages of colonization than BH 1146 and ND 495 (Table 1), papillae appeared to be a major source of resistance in Lodi. Hargreaves (7) proposed that papilla formation was the most common means of preventing penetrations in oats by *P. teres*. The marginally significant higher percentage of papilla formation in BH 1146 than in ND 495 and the marginally higher colonization in BH 1146 (Table 1) suggest that papilla formation in BH 1146 was not a major source of resistance.

In Lodi, in addition to producing papillae and encasing sheaths, some mechanism in or related to the host cells restricted the growth of *P. tritici-repentis* hyphae to only a few epidermal and/or

mesophyll cells. In wheat, some mechanism in the epidermal and mesophyll cells of BH 1146 kept lesion size to about one-half that in ND 495. This cellular resistance, with no observed association with organelles in wheat, appeared to be a second major resistance in Lodi (following papilla formation) and the major resistance in BH 1146. The absence of any obvious histological or cytological structural mechanisms for this resistance suggests a molecular mechanism for resistance.

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