Papilla Formation and Hypersensitivity at Penetration Sites and Resistance to *Pseudocercosporella herpotrichoides* in Winter Wheat

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ABSTRACT

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Seedlings of six winter wheat cultivars were inoculated with conidia of *Pseudocercosporella herpotrichoides* and the infection process followed. Penetration of coleoptiles and leaf sheaths occurred sooner in susceptible than in resistant cultivars. Lignified papillae, stain halos, and a hypersensitive reaction formed in epidermal cells in response to penetration in all cultivars. More papillae were found in first leaf sheaths of resistant than of susceptible cultivars; VPM, the most resistant cultivar, had the most papillae at penetration sites (100%) and Selection 101, the most susceptible cultivar, had the fewest papillae (66%). Fewer successful penetrations occurred in resistant than in susceptible cultivars at sites where papillae were present. However, penetration always occurred when papillae did not form, regardless of host resistance. The total number of successful penetrations (sites with and without papillae) increased with increasing host susceptibility from 2.3% in VPM to 74.3% in Selection 101. The hypersensitive reaction occurred only at sites with papillae, and there were fewer successful penetrations at sites with both papillae and the hypersensitive reaction than at sites with papillae alone in all cultivars except VPM. Histochemical tests indicated lignin was present in papillae of all cultivars. These data indicate that both lignified papillae and hypersensitive reactions are involved in seedling resistance to *P. herpotrichoides*.

Additional key words: eyespot, foot rot, soilborne pathogens.

Strawbreaker foot rot (eyespot), caused by *Pseudocercosporella herpotrichoides* (Fron) Deighton, is an important disease of winter wheat (*Triticum aestivum* L.) in parts of the Pacific Northwest region of the United States. Resistance to this soilborne pathogen is available (28) and would be the most desirable control, but cultivars with high levels of resistance to *P. herpotrichoides* are not adapted to this area. Currently, selection of resistant cultivars is a slow process because plants must be grown to maturity to determine their resistance. Understanding the resistance of wheat to *P. herpotrichoides* at the seedling stage could lead to development of a quicker method of screening for resistance.

Previous work has shown that *P. herpotrichoides* colonizes tissues of resistant cultivars more slowly than tissues of susceptible cultivars (2,3,16). The same events occur during penetration and infection of both resistant and susceptible cultivars, but they occur more rapidly in susceptible cultivars (2,3,7,8). In one study, growth rate of *P. herpotrichoides* after infection was almost independent of host resistance, suggesting that cultivar differences may be expressed in the early stages of infection (25). Papillae (also called lignitubers or appositions), halos (6–10), and a hypersensitive reaction (2,3,13) occur at penetration sites in some cultivars, but their relationship to resistance has not been shown conclusively. Papillae formed more frequently and were larger in resistant than in susceptible cultivars (6), and halos were most intense on wheat, which is more susceptible to *P. herpotrichoides*, than on barley, rye, and oat (7,8). Lignification of papillae, epidermal cell walls, and pith cells in stems has also been associated with resistance to *P. herpotrichoides* in wheat (6,18,29).

In the Gramineae, papilla formation may be a general resistance response to attempted fungal penetration (27), and lignification is often associated with papilla formation (1,4,11,12,21–24,26,30,31). According to Ride (22), lignified structures may make cell walls more resistant to mechanical penetration, make cell walls or papillae resistant to attack by fungal enzymes, restrict diffusion of enzymes and toxins from the fungus, restrict diffusion of nutrients and water to the fungus, or provide low molecular weight precursors and free radicals produced during polymerization of lignin that are toxic to the fungus. Lignified halos and papillae were shown to be highly resistant to in vitro degradation by fungi and commercial preparations of macerating enzymes (21,23). Even lightly lignified tissues were highly resistant to in vitro degradation by many different fungi (24).

Most studies on the role of papilla formation and induced lignification in preventing infection have used nonpathogenic fungi (20) or fungi pathogenic to other hosts (20,21,24,27,31–33). A few studies have used fungi pathogenic to the host but only a single cultivar of the host (21,31). Less is known about the role of papillae and induced lignification in resistance of cultivars of a host to a fungal pathogen (4,26), especially to fungi that are not obligate parasites. The purpose of this study was to determine the sequence of events in the infection of wheat seedlings, to determine the time of occurrence of these events in resistant and susceptible cultivars, and to better understand the mechanism of resistance to *P. herpotrichoides* in cultivars of wheat, particularly the role of papilla formation and lignification.

MATERIALS AND METHODS

Wheat cultivars. Six wheat cultivars were selected on the basis of their resistance to *P. herpotrichoides* in the field: VPM-1124-R25-1 (VPM), highly resistant; Cappelle-Desprez (PI 262223), resistant; Viking (PI 316424) and Stephens (CI 17596), moderately resistant; and Daws (CI 17419) and Selection 101 (Sel 101) (CI 13438), susceptible (18). Seeds were sown 3 cm deep in a mix consisting of 55% peat, 35% pumice, and 10% sand (w/w) in 15-cm-diameter plastic pots with 16 seeds per pot. Experiments were conducted in a growth chamber at 13 C, with diurnal lighting (10 hr of light) of about 400 $\mu E/cm^2$ supplied by fluorescent light tubes (Sylvania GRO-VHO-WS and Westinghouse CW-SHO-EW [1:2 mixture]).

Inoculation. Four virulent isolates of *P. herpotrichoides* were

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used throughout the study; each was maintained as a mycelial culture on Difco potato-dextrose agar. Conidial inoculum was prepared either by growing the fungus on autoclaved oat kernels and incubating outdoors in the autumn on fiberglass screen or by spreading a conidial suspension on the surface of water agar (Difco, 15 g/L) in 100-mm plastic petri dishes and incubating at 5 C for 4 wk. Conidial suspensions were prepared by washing oat kernels (5) or water agar cultures and adjusting the concentration

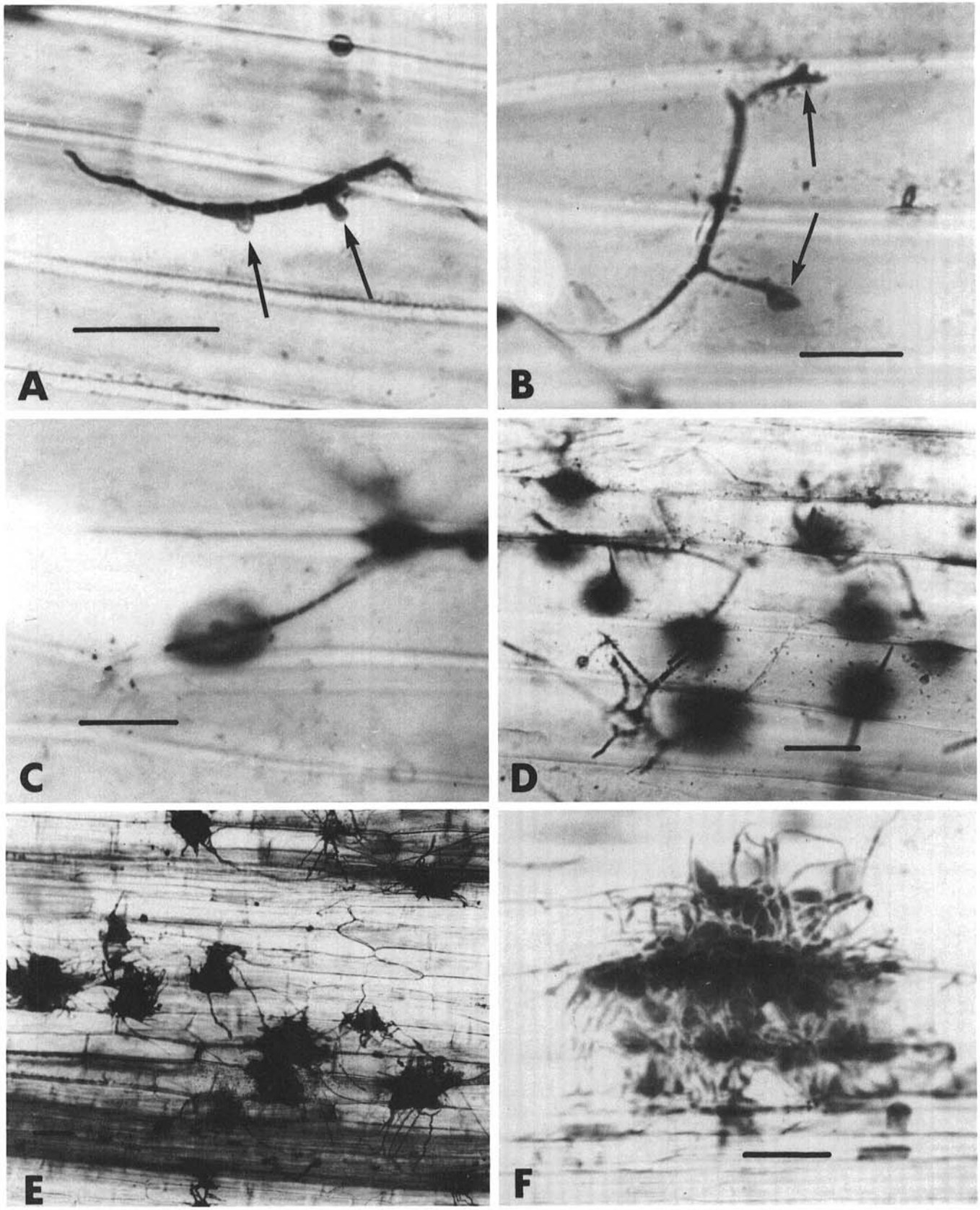

Fig. 1. Infection of winter wheat by *Pseudocercosporella herpotrichoides*: **A**–D, on coleoptiles and **E and F,** on leaf sheaths. **A,** Appressoria (arrow) forming in cells of a germinating conidium, **B,** appressoria (arrow) forming on short hyphae from a germinating conidium, **C,** halo surrounding an appressorium, **D,** halos at penetration sites, **E,** hyphal mats, and **F,** close-up of hyphal mat showing the pseudoparenchymatous nature. Scale bars: A, B, C, and F = 25 μm; D and E = 50 μm.

to 1×10^6 conidia per milliliter. Seedlings were sprayed with the suspensions at the rate of 4.4 ml per pot. Inoculated plants were incubated at 13 C in clear plastic chambers to maintain high relative humidity.

Growth stage at inoculation. The effect of the stage of growth of the host on infection was determined by inoculating VPM and Daws at the one-leaf, two-leaf, and one-tiller stages of growth and following the infection process. Data from the two cultivars were pooled. Experiments with plants inoculated at the one-leaf and one-tiller stages were done once and inoculation at the two-leaf stage was done twice. Seedlings were inoculated at the two-leaf stage in all subsequent experiments.

Preparation of plant samples. Five to eight plants per cultivar were randomly selected at each sampling date, and the coleoptiles and leaf sheaths were removed and fixed in glacial acetic acid and 50% ethyl alcohol (1:17, v/v) for at least 12 hr. Samples were placed in test tubes containing 0.01% trypan blue in lactophenol, heated in a water bath at 85 C for 10 min to stain and clear the specimens (2), mounted in lactophenol on glass slides, and observed with a light microscope.

Observation of events in infection process. Coleoptiles and leaf sheaths were sampled at 2-day intervals for 26 days after inoculation. The following events were recorded: germination of conidia, formation of appressoria by superficial hyphae, formation of stain halos (7), presence of papillae at penetration sites, necrosis of cells in response to attempted penetration (hypersensitive reaction) (17), successful penetration indicated by growth of hyphae in epidermal cells, formation of mycelial mats on the abaxial surfaces of coleoptiles and leaf sheaths, and appearance of macroscopic symptoms. Data were recorded as the number of coleoptiles or leaf sheaths positive for the particular event. A coleoptile or leaf sheath was rated positive for the event if more than 5% of the conidia or penetration sites exhibited the event in question. Means were calculated and plotted as the percentage of coleoptiles or leaf sheaths exhibiting the event with days after inoculation. This experiment was repeated three times with VPM and Daws and three times with all six cultivars. The data were analyzed as a randomized complete block design using experiments as blocks.

Quantifying the relationship between papillae and hypersensitive reaction and successful penetration. Coleoptiles and leaf sheaths sampled 18 days after inoculation were prepared as described, except mycelial mats on the surfaces of coleoptiles and leaf sheaths were removed by gently rubbing the surface of the tissue with a fingertip. Samples were examined for papillae and/or the hypersensitive reaction. Successful penetration was determined by observing the growth of hyphae into epidermal cells at penetration sites or through papillae. Total percentage of sites penetrated was calculated from the percentage of sites with papillae that were penetrated + the percentage of sites at which papillae did not form. (Penetration always occurred when papillae did not form at a penetration site.) This experiment was repeated three times. During the final repetition, a time-course study of the number of successful penetrations was done with samples taken 18, 20, and 25 days after inoculation.

Determining composition of papillae. The toluidine blue O test for lignin and cellulose (19), phloroglucinol-HCl test for lignin (15,26), and the IKI-sulfuric acid test for cellulose (14) were

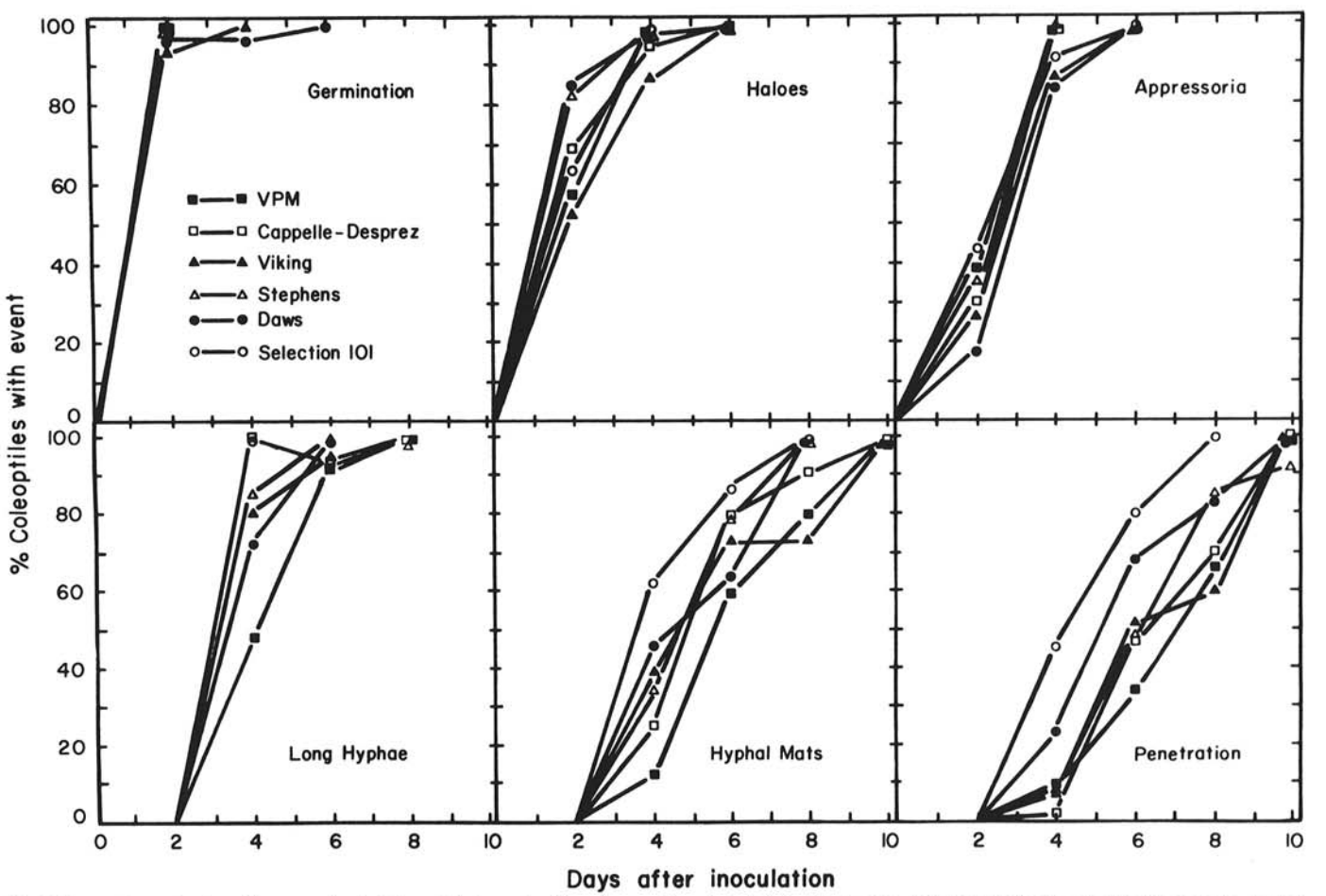

Fig. 2. Percentage of coleoptiles examined with conidial germination, trypan blue stain halos, appressoria, long hyphae from germinating conidia, hyphal mats, or penetration after inoculation of resistant and susceptible wheat cultivars with *Pseudocercosporella herpotrichoides*. VPM = highly resistant, Cappelle-Desprez = resistant, Viking and Stephens = moderately resistant, and Daws and Selection 101 = susceptible. Data points are the mean of six replicates for VPM and Daws and three replicates for all others.

conducted to determine if lignin and/or cellulose were present in papillae and surrounding cell walls. All tests were performed on inoculated coleoptiles and leaf sheaths.

RESULTS

Infection of coleoptiles. Germination of conidia began within 24 hr of inoculation, and by 2 days, most conidia had germinated (Figs. 1A and 2). There were no differences in rate of germination of conidia on these cultivars. Halos surrounding penetration sites began to appear on coleoptiles within 24 hr of inoculation (Figs. 1B–D and 2); most coleoptiles showed halos within 4 days of inoculation. Halos were small, distinct, and encircled individual appressoria (Fig. 1C,D). Differences were not observed in shape of halos or in the time of their appearance among these cultivars.

Appressoria were detected within 2 days of inoculation and occurred on most of the coleoptiles examined by 4 days after inoculation (Figs. 1A,B and 2). Appressoria formed from one or more of the cells of a germinating conidium (Fig. 1A) or on a short hypha from a germinating conidium (Fig. 1B). Long hyphae on the surfaces of coleoptiles were apparent after 4 days (Figs. 1D and 2). Hyphal mats were also apparent after 4 days but occurred less frequently on resistant than on susceptible cultivars (Figs. 1E and 2). Hyphal mats were composed of compacted hyphae and appeared pseudoparenchymatous (Fig. 1F).

Penetration (indicated by the presence of hyphae in epidermal cells) was evident 4 days after inoculation and increased in frequency up to 10 days after inoculation (Fig. 2). Time required for penetration of 50% of the coleoptiles was shortest for Sel 101 and Daws (4.4 and 5.2 days, respectively) and longest for VPM and Cappelle-Desprez (7.4 and 6.6 days, respectively) (Fig. 2). Penetration of host cell walls was initiated from either appressoria, individual hyphae, or hyphal mats. In general, penetration from appressoria occurred most rapidly, followed by penetration of individual hyphae, and then from hyphal mats. Penetration of the coleoptile was most frequent from hyphal mats.

Infection of first leaf sheaths. Penetration of first leaf sheaths was more frequent from hyphal mats (Fig. 1E,F) than from hyphae. Hyphal mats formed on most leaf sheaths within 8 days (Fig. 3), and halos occurred within 10 days of inoculation. Halos were larger and more diffuse than on coleoptiles and encircled many papillae (Fig. 4B). Papillae formed on most leaf sheaths within 10 days (Figs. 3 and 4B–E) and formed slightly sooner on the resistant VPM and Cappelle-Desprez than on the susceptible Daws and Sel 101.

Penetration of first leaf sheaths of susceptible or moderately

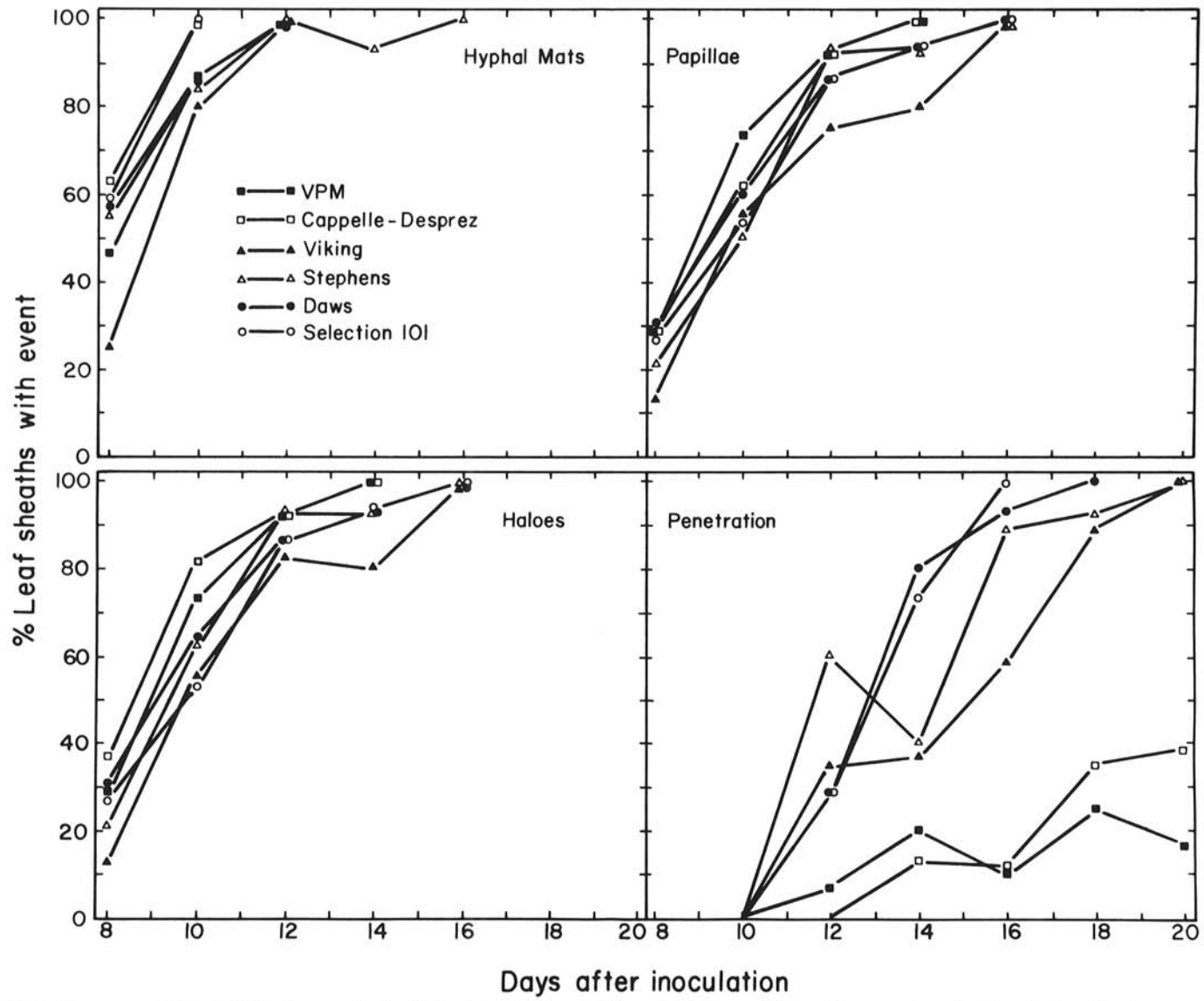

Fig. 3. Percentage of first leaf sheaths examined with hyphal mats, trypan blue stain halos, papillae, and penetration after inoculation of resistant and susceptible wheat cultivars with *Pseudocercosporella herpotrichoides*. VPM = highly resistant, Cappelle-Desprez = resistant, Viking and Stephens = moderately resistant, and Daws and Selection 101 = susceptible. Data points are the mean of six replicates for VPM and Daws and of three replicates for all others.

resistant cultivars occurred within 12 days, with most leaf sheaths penetrated by 15 days (Figs. 3 and 4A). In contrast, most leaf sheaths of the resistant cultivars were not penetrated even after 20 days.

Macroscopic symptoms appeared within 16–18 days and consisted of elliptical, necrotic lesions near the soil surface. Lesions were smaller and appeared lighter brown on resistant than on susceptible cultivars.

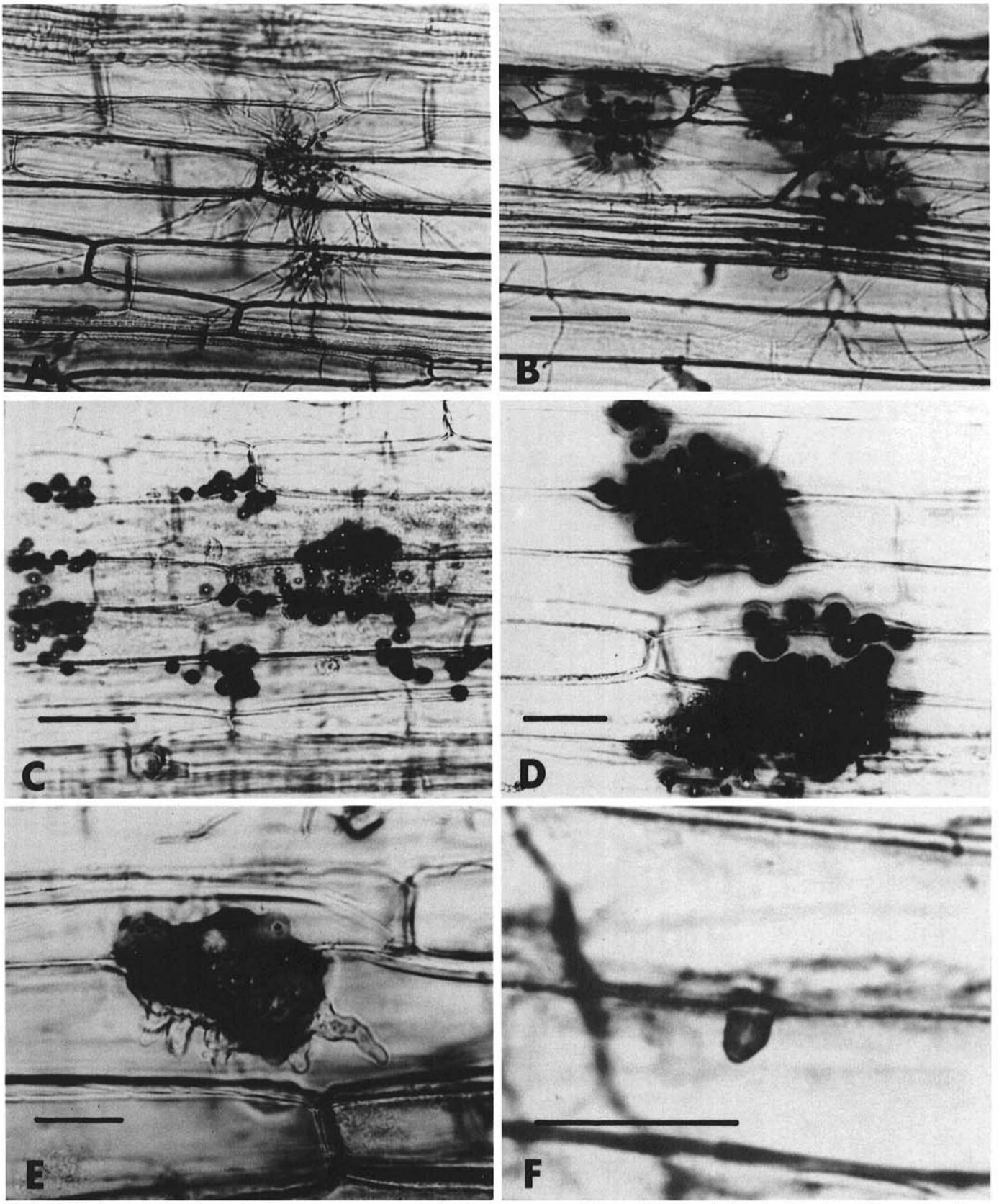

Fig. 4. Papilla formation in winter wheat in response to penetration by *Pseudocercosporella herpotrichoides*: **A**–E, on leaf sheaths and **F,** on coleoptile. **A,** Successful penetration indicated by hyphae in epidermis cells where papillae did not form, **B,** halos surrounding papillae and successful penetration through papillae, **C and D,** unsuccessful penetration through papillae, **E,** lignified papillae showing fingerlike protruberances ensheathing penetration hyphae, and **F,** structure of papilla in coleoptile (note translucent center). Scale bars: A, D, E, and F = 25 μm; B and C = 50 μm.

Relationship between stage of host development and time required for infection. The time required for penetration of 50% of the coleoptiles decreased from 12.5 to 4.5 days when inoculation was delayed from the one-leaf to the one-tiller stage (Fig. 5). Likewise, the time required for penetration of 50% of leaf sheaths decreased from 21 to 11 days when inoculation was delayed from the one-leaf to the one-tiller stage. Plants inoculated at the two-leaf stage were intermediate in time required for penetration of 50% of either coleoptiles or first leaf sheaths.

On coleoptiles, formation of long hyphae and hyphal mats on the surface of the host occurred sooner when seedlings were inoculated at the one-tiller stage than at either the one- or two-leaf stage. Stage of plant development at inoculation had no effect on the rate of conidial germination or frequency of appressoria or halos. On leaf sheaths, formation of hyphal mats, halos, and papillae all occurred sooner when inoculation was delayed until the one-tiller stage.

TABLE 1. Percentage of penetration sites on first leaf sheaths with papillae, penetrated papillae, and total penetrations 18 days after inoculation with *Pseudocercosporella herpotrichoides*

Cultivar	Relative resistance[a]	No.	Percent penetration sites with[b] Papillae	Penetrated papillae[c]	Total penetrations[d]
VPM	HR	1,467	100.0	2.3	2.3
Cappelle-Desprez	R	1,364	99.2	7.8	8.6
Stephens	MR	940	88.7	32.8	42.7
Viking	MR	761	88.7	31.4	44.1
Daws	S	1,442	81.1	41.9	60.8
Sel 101	S	1,196	66.0	40.3	74.3
LSD (5%)[e]			7.8	13.9	17.8

[a] Field reactions to *P. herpotrichoides*: HR = highly resistant, MR = moderately resistant, R = resistant, and S = susceptible.
[b] Figures represent the mean of three experiments.
[c] Penetration through papillae was indicated by the presence of hyphae in epidermal cells. Penetration always occurred when papillae did not form.
[d] Total penetrations are calculated from the percentage of sites with penetrated papillae and sites at which papillae did not form = % sites with penetrated papillae + (100 − % sites with papillae).
[e] Fisher's protected least significant difference.

Relationship between papilla formation and successful penetrations. Papillae formed in response to attempted penetration of both coleoptiles (Fig. 4F) and leaf sheaths (Fig. 4B–E), but they were more frequent in the latter. The percentage of penetration sites with papillae in leaf sheaths ranged from 100% in VPM to 66% in Sel 101 (Table 1). VPM and Cappelle-Desprez, the most resistant cultivars, had only 2.3 and 7.8% successful penetrations, respectively, at sites with papillae, whereas Sel 101, the most susceptible cultivar, had 40.3% successful penetrations at sites with papillae (Table 1). Penetration always occurred at sites without papillae (Fig. 4A) regardless of host resistance. There were fewer total successful penetrations in resistant than in susceptible cultivars, ranging from 2.3 and 8.6% in VPM and Cappelle-Desprez, respectively, to 74.3% in Sel 101 (Table 1). The percentages of penetration sites with papillae, penetrated papillae, and total penetrations did not differ significantly when leaf sheaths were examined 18, 20, or 25 days after inoculation. Papillae in coleoptiles and leaf sheaths varied in shape from fingerlike structures that ensheathed penetration hyphae (Fig. 4E) to small appositions on cell walls (Fig. 4F).

Relationship between the hypersensitive reaction and successful penetration. In VPM, 45.5% of the penetration sites in first leaf sheaths exhibited the hypersensitive reaction, but in Sel 101, only 11.2% of the penetration sites exhibited the hypersensitive reaction (Table 2). The hypersensitive reaction was characterized by necrosis of epidermal cells near the point of penetration and was always associated with papillae. Fewer penetration pegs invaded the epidermal cells of leaf sheaths, and fewer hyphae outgrew hypersensitive cells of resistant cultivars than of the susceptible cultivars. All cultivars except VPM had fewer successful penetrations at sites with both papillae and the hypersensitive reaction than sites with papillae alone (Table 2).

Composition of papillae and halos. The toluidine blue O and phloroglucinol-HCl tests gave positive reactions for the presence of lignin in papillae and altered epidermal cell walls (halos). The toluidine blue O test stained papillae and halos blue-green and unaltered epidermal cell walls red-purple. The phloroglucinol-HCl test stained papillae and halos pink. The IKI-H_2SO_4 test stained papillae and halos yellow-orange and unaltered epidermal cell walls blue. These results confirmed the presence of lignin in papillae and halos and of cellulose in unaltered epidermal cell walls (26).

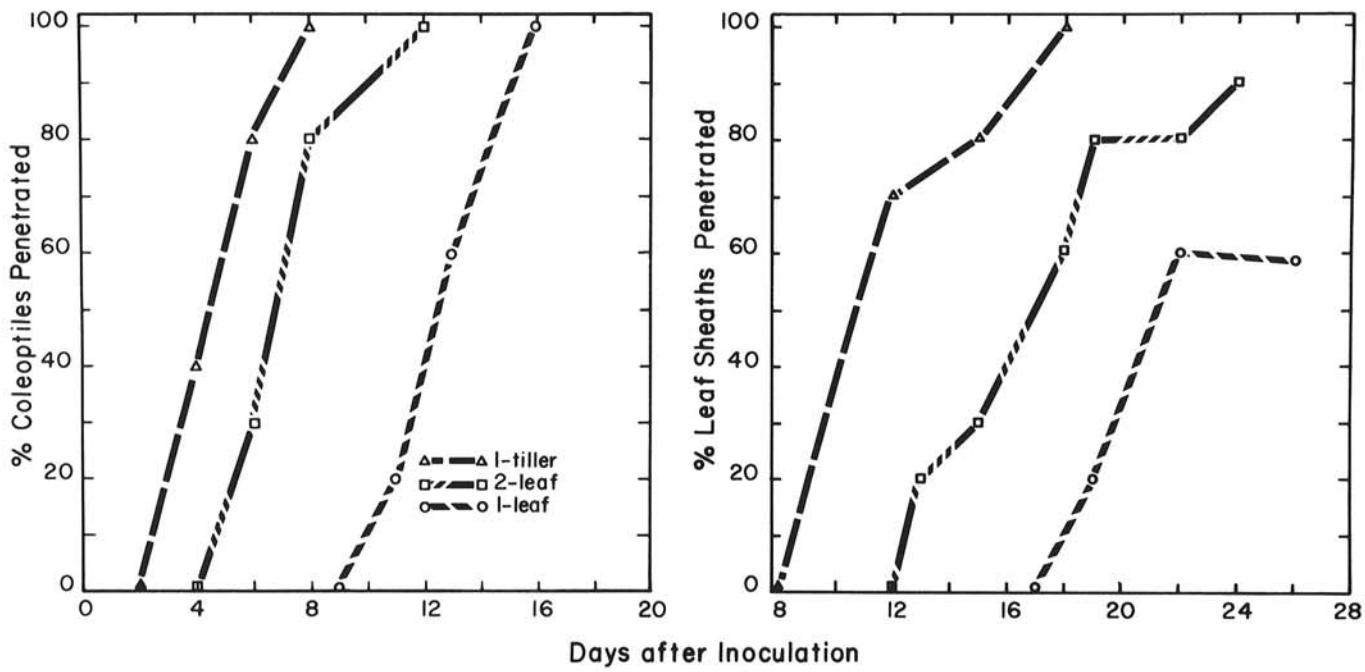

Fig. 5. Percentage of successful penetrations of coleoptiles and leaf sheaths of wheat seedlings inoculated at the one-leaf, two-leaf, or one-tiller stage of growth with *Pseudocercosporella herpotrichoides*. Data represent the average of VPM and Daws; one-leaf and one-tiller inoculations were done once and two-leaf inoculations were done twice.

TABLE 2. Percentage of penetration sites with the hypersensitive reaction (HSR) and penetrated papillae with (HSR+) or without the hypersensitive reaction (HSR−) on first leaf sheaths 18 days after inoculation with *Pseudocercosporella herpotrichoides*

Cultivar	Relative resistance[a]	Percent penetration sites with HSR[b]	Percent penetration sites with penetrated papillae	
			HSR+[c]	HSR−
VPM	HR	45.5	1.5	0.8
Cappelle-Desprez	R	29.8	2.3	5.5
Stephens	MR	16.0	2.4	29.2
Viking	MR	15.3	3.0	29.8
Daws	S	14.7	6.1	35.9
Sel 101	S	11.2	6.2	34.0
LSD (5%)[d]		18.9	3.1	13.7

[a] Field reactions to *P. herpotrichoides*: HR = highly resistant, MR = moderate resistance, R = resistant, and S = susceptible.
[b] Figures represent the mean of three experiments.
[c] HSR+ = penetration sites with necrosis of epidermal cells, HSR− = penetration sites without necrosis of epidermal cells.
[d] Fisher's protected least significant difference.

DISCUSSION

The percentages of penetration sites with papillae, penetrated papillae, and total successful penetrations in winter wheat seedlings were positively correlated with field resistance of mature plants to *P. herpotrichoides*. More papillae formed in first leaf sheaths of resistant than of susceptible cultivars (Table 1), and more formed in first leaf sheaths than in coleoptiles in all cultivars. Furthermore, fewer successful penetrations were associated with the papillae in resistant than in susceptible cultivars (Table 1) (Fig. 4B–D). The total number of successful penetrations, calculated from the percentage of penetration sites with penetrated papillae+ penetration sites where papillae did not form, was also positively correlated with field resistance to *P. herpotrichoides*; fewer successful penetrations occurred in the most resistant cultivar, VPM (2.3%), than in the most susceptible cultivar, Sel 101 (74.3%).

Other workers have shown that penetration occurred when papillae were small or did not form or when their formation was prevented by cycloheximide (27,31). Papilla formation was not artificially prevented in this study; therefore, other possible resistance mechanisms could not have been inhibited. Although these data do not preclude other possible mechanisms, they implicate papillae in seedling resistance to *P. herpotrichoides*.

Histochemical tests indicated that lignin was present in papillae and halos surrounding penetration sites in all cultivars. Although the amount of lignin in papillae of different cultivars was not determined, increased lignin content of tissues after inoculation has been reported (32). Murray and Bruehl (18) showed that lignified tissues were damaged less by the pathogen and considered early lignification of stems to be an important component of resistance.

In previous reports of seedling resistance to *P. herpotrichoides*, hypersensitivity was not related to cultivar resistance (2,3,13). In this study, however, the hypersensitive reaction formed more frequently at penetration sites of resistant than of susceptible cultivars and was always associated with papillae (Table 2). Successful penetrations at sites with papillae alone decreased from an average of 22.5 to 3.6% when the hypersensitive response occurred in conjunction with papilla. Only VPM had fewer successful penetrations with papillae alone. The percentage of penetration sites with papillae, penetrated papillae, and total penetrations differentiated resistant and susceptible cultivars more clearly and were better indicators of host resistance than the hypersensitive reaction. The hypersensitive reaction differentiated the highly resistant from the susceptible cultivars but could not differentiate the resistant from the moderately resistant or susceptible cultivars (Table 2).

The fact that cultivars did not differ in the types of events in the infection process but that penetration occurred sooner on susceptible than on resistant cultivars is consistent with other work (2,3,10,16,25). Penetration occurred sooner on both coleoptiles and leaf sheaths of susceptible than of resistant cultivars, but differences among cultivars were most apparent on leaf sheaths (Figs. 2 and 3).

Early senescence of coleoptiles and increasing host susceptibility resulted in most rapid penetration, whereas delayed senescence and increased host resistance resulted in slower penetration. Coleoptile senescence occurred nearer to the time of inoculation when plants were inoculated in the one-tiller stage than in the two-or one-leaf stages. Likewise, penetration occurred sooner when plants were inoculated at the one-tiller stage than at the two- or one-leaf stages (Fig. 5). These data support the hypothesis that *P. herpotrichoides* causes a disease of senescent tissue (3) and that infection of coleoptiles is an important stage in establishing differences among cultivars (2,3,7,8). Differences in growth stage at inoculation may also account for differences with other studies in the timing of the various events in the infection process (2,3,7,8). For example, in one study, penetration of leaf sheaths was not observed until 35 days (2), but in another study, penetration was observed within 2 days of inoculation (7). Differences in temperature among studies may also influence timing of events and the results obtained (23).

Evaluation of resistance of wheat cultivars to *P. herpotrichoides*, using the number of successful penetrations of first leaf sheaths, is correlated with field resistance. Determining total penetrations, frequency of papillae and the hypersensitive reaction, and subsequent growth of hyphae into host tissues is more reliable on leaf sheaths than on coleoptiles and is easily observed within 3 wk of inoculation. These characteristics may provide the basis for a seedling test for resistance in which leaf sheaths of individual seedlings could be evaluated for resistance to *P. herpotrichoides*.

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