Histochemistry of Papillae Formed in Reed Canarygrass Leaves in Response to Noninfecting Pathogenic Fungi

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ABSTRACT

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Leafspot fungi which were nonpathogenic to reed canarygrass but pathogenic to other plants, formed appressoria and penetration pegs on reed canarygrass leaves. Papillae usually formed in the outer epidermal walls beneath penetration pegs and no penetration occurred. The epidermal walls became histochemically modified in disk-shaped areas, up to 60 μm in diameter, around the sites of attempted penetration. Lateral epidermal walls beneath the disks (disk-shaped areas) became swollen and histochemically modified. Papillae in leaves inoculated with *Helminthosporium avenae* gave histochemical reactions with toluidine blue O, chlorine water-sodium sulfite, and phloroglucinol-HCl which indicated that lignified material was a major structural component of the core of the papillae and the altered lateral walls and only a minor component of the disks. Insolubility in 72% H_2SO_4 and solubility in chlorine-sulfite supported these conclusions. Tests with resorcinol blue, lacmoid, and aniline blue-fluorescence indicated that callose was present in the papillae, lateral walls, and disks. Results from tests with IKI-H_2SO_4 and toluidine blue O, and for birefringence suggested that cellulose was a significant structural component of the disks, the altered lateral walls, and the cover layer of the papillae. Tests for cutin, suberin, tannins, gums, and pectic compounds were negative.

Callose was more readily demonstrated 10-12 hours after inoculation with *H. avenae* than at later times. Lignification was detected at 10 hours, and was strong after 24-48 hours. Six clones of reed canarygrass had the same structural and histochemical reactions. Papillae and wall modifications formed at 17, 24, and 29 C in continuous light or dark had similar histochemical reactions. Positive tests for lignified material, callose, and cellulose were obtained in papillae, disks, and lateral walls associated with penetration attempts by *Botrytis cinerea, Leptosphaerulina trifolii, Stagonospora arenaria, Stemphylium botryosum, Helminthosporium catenarium,* and *Aschochyta* sp.

Additional key words: Phalaris arundinacea, Drechslera avenacea, resistance, lignituber, halo, hemicellulose, basic staining material.

Papilla formation apparently serves as a mechanism of resistance to fungal penetration in many plants (4, 5, 12). A recent study in our laboratory implicated papilla formation in resistance of leaves of reed canarygrass *(Phalaris arundinacea* L.) to many leaf-infecting fungi which are nonpathogenic to reed canarygrass (35). Papilla formation also seemed to be involved in resistance of reed canarygrass to a pathogen (*Helminthosporium catenarium* Drechs.).

Information on the structure and chemical composition of papillae is essential to understanding the physical and chemical barriers presented to fungal penetration pegs. It also provides insight into processes of new cell wall biosynthesis elicited in the papilla formation response. Considering the apparent importance of papillae in resistance, there have been few studies of the histochemistry of papillae and of altered epidermal cell walls around sites of attempted penetration.

This paper presents data on the structure and histochemistry of reed canarygrass papillae. Effects of environment, host genotype, fungal species, and papilla age also are considered.

MATERIALS AND METHODS

Preparation of plant materials.—Reed canarygrass clones 1-3, 22-2, 41-5, 263-8, 6049, and 6332 (18, 23) were grown as replicated 25 × 25 cm sod pieces in greenhouse beds of peat:vermiculite mixture (1:1, v/v) (35). The clones differed widely in alkaloid composition, palatability, and agronomic type (18, 23). Leaf segments 2-4 cm long were excised from mature, unblemished leaves and placed on wet filter paper in petri dishes. Twenty-μliter drops of spore suspension, which contained about 200 conidia of *Helminthosporium avenae* Eidam [*Drechslera avenacea* (Curtis ex. Cooke) Shoem.] and 0.1% Tween-20 surfactant, were placed on the leaf surface along either side of the midrib. Dishes

were kept on a laboratory table under prevailing light and temperature (21-24 C).

Most histochemical tests were made on fresh strips of inoculated epidermis. To prepare the strips, the inoculated surface was placed downward on the stage of a dissecting microscope, and the mesophyll, veins and opposite epidermis overlying the inoculated spot were removed by scraping with a razor. The stripped epidermal area and 2-3 mm of intact, noninoculated tissue on each side was excised and tested immediately. The intact tissue provided mechanical support. It also provided information on the staining reactions of normal cell wall constituents of reed canarygrass.

Some tests were conducted on fixed and sectioned tissue. Leaf samples were fixed in formalin:acetic acid:alcohol (FAA), dehydrated through a *t*-butanol series, embedded in Tissuemat, sectioned at 12 μm, and affixed to slides (16, 17).

Histochemical tests.—Histochemical tests followed the procedures described in the references cited, except in the instances detailed below in which it was necessary to select conditions or develop modifications suitable for reed canarygrass leaves. Unless otherwise stated, leaf samples were taken 24-48 hours after inoculation. Tests (i) to (vii) were applied to two strips per treatment in all experiments. The experiments were conducted at least twice. The test procedures were:

(i) Toluidine blue O test for lignin and cellulose (28).

(ii) Chlorine water-sodium sulfite test for lignin (6, 8, 36). Tissues were soaked in a freshly prepared saturated solution of chlorine gas in water in a closed vial for 15 minutes. The tissues were rinsed with water, blotted, mounted in fresh 3% Na_2SO_3 solution, and examined within 5-10 minutes.

(iii) Phloroglucinol-HCl test for lignin (8, 17, 31). Tissues were soaked in a solution of 2% phloroglucinol in 95% ethanol for 1-2 hours. The tissue was placed in a drop of 35% HCl on a slide and heated over a low flame until the veins turned reddish-purple.

(iv) Lacmoid test for callose (16). Fresh epidermal strips were immediately placed in a 0.1% solution of lacmoid in 50% ethanol. They were stained for 12-24 hours and mounted in the same solution.

(v) Aniline blue-fluorescence test for callose (7, 10). Fresh strips and strips fixed in FAA at −75 C (10) were placed in a solution of 0.01% water-soluble aniline blue in 0.15 M potassium phosphate buffer at pH 8.4 in the dark for 0.5 to 18 hours. The tissue was observed with a Leitz fluorescence microscope equipped with a HBO 50W mercury lamp, a 2-mm thick UG-1 filter, and a K510 eyepiece filter.

(vi) IKI-H_2SO_4 test for cellulose (16).

(vii) Glycerine-ferricyanide test for reducing compounds [a new procedure based on Johansen (17, p. 186) and Dorée (8, p. 352)]. The strip was placed in 15 ml of glycerine in a small beaker and heated at 270 C for 15 minutes; then it was transferred to a slide, rinsed with water, blotted, and covered with one drop of 0.02 M $FeCl_3$ solution and one drop of 0.02 M potassium ferricyanide solution (aqueous).

(viii) Birefringence test for cellulose. Tissues were observed for birefringence using a Leitz polarizing microscope. The test was used on untreated fresh tissues, on fresh tissues from which lignin or callose was extracted (see below), and on sectioned tissue.

(ix) Basic staining material (21).

(x) Hydroxylamine-ferric chloride test for pectic compounds (16).

(xi) Sudan IV test for suberin and cutin (17). Tissues were observed after being immersed in Sudan IV solution for 2-4 hours. The solution was prepared by adding 50 ml of glycerine to 50 ml of a saturated solution of Sudan IV in 95% ethanol and filtering.

(xii) Orcinol test for gums (29).

(xiii) Resorcinol blue test for callose (10). Whole leaf tissue was fixed in FAA at −75 C, then stripped, stained, and placed in 0.2 M potassium phosphate buffer at pH 4.1 for 2 hours.

(xiv) Nitroso test for tannins (16).

Extraction methods.—To extract pectins, epidermal strips were kept in 0.5% ammonium oxalate at 98 C for 20 hours. Control tissues were kept in water at 98 C. To extract callose, tissues were kept in glycerine at 270 C for 15-60 minutes (17). To extract lignified material, tissues were soaked in fresh chlorine water for 60 minutes, rinsed in water, and soaked in 3% sodium sulfite for 5 minutes; this procedure was repeated three times.

Light and temperature study.—Leaf segments of clones 1-3 and 22-2 were inoculated with *H. avenae* and incubated in continuous darkness or continuous fluorescent-incandescent light (2,000 lux) in incubators at 17, 24, and 29 C. Samples were taken at 36 hours for tests (ii) to (vii).

Fungal species.—Conidial and ascosporic inoculum of six fungal species was produced on V8 juice agar (35). Inoculated leaf segments were kept on a laboratory table. For tests (i) to (vii), samples were taken 3.5-5.0 days after inoculation.

Time-course study.—Leaf segments of clones 1-3 and 41-5 were inoculated with *H. avenae* and incubated in the dark at 24 C. Samples were taken 6, 10, 12, 18, 24, and 48 hours after inoculation. Histochemical tests (i) to (vii) were run.

RESULTS

Structural and histochemical responses to attempted penetration were visible in three areas; these were designated by us as the papilla, the disk, and the altered lateral wall (Fig. 1-C). The six clones gave the same structural developments and the same results in histochemical tests (i) to (vii) (Table 1). Tests (viii) to (xiii) were run on only one or two clones. Results of all

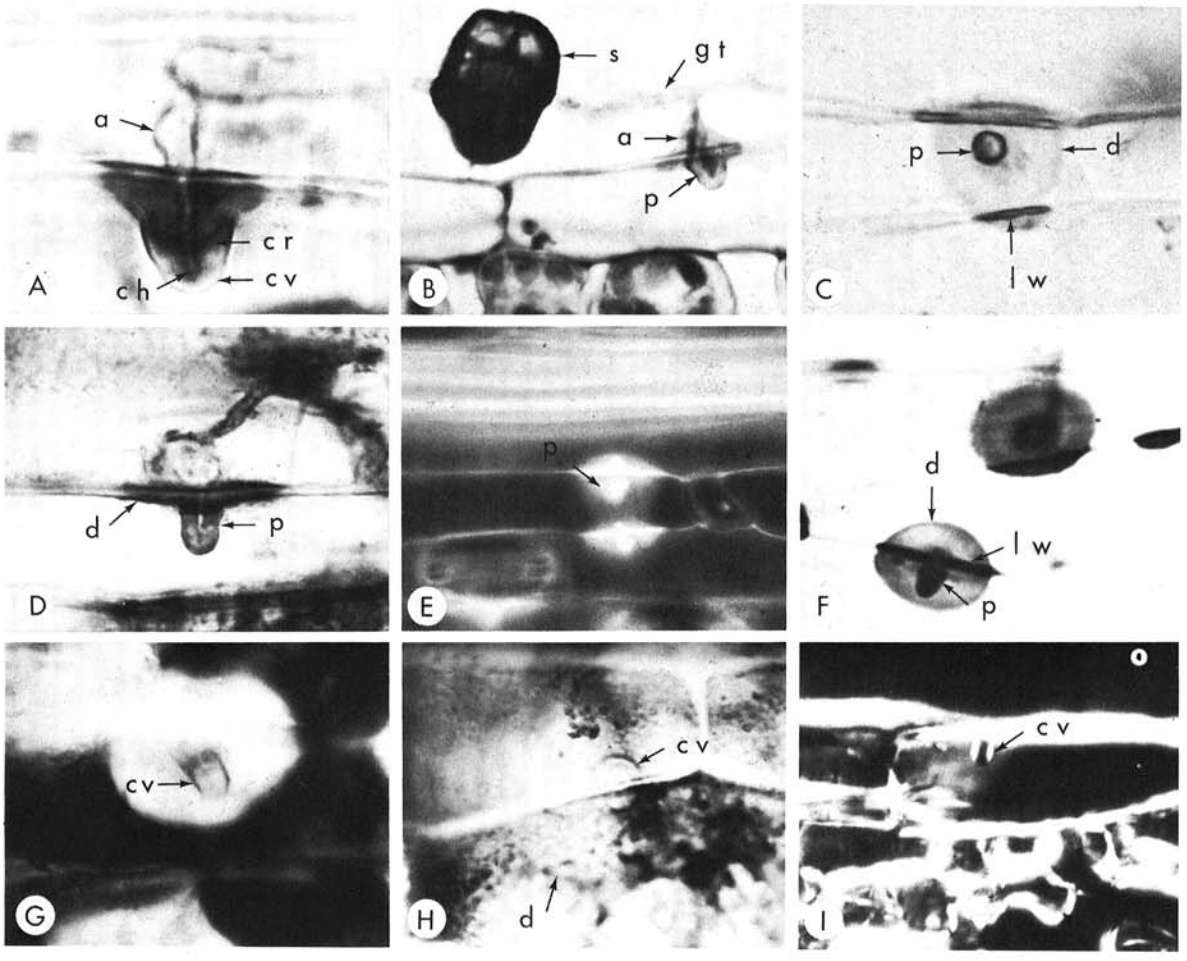

Fig. 1-(A to I). Histochemical reactions of papillae and associated epidermal walls in reed canarygrass leaves inoculated with *Helminthosporium avenae* (A, and C through I) or *Stemphylium botryosum* (B). Fresh epidermal strips were used in A and C through H; fixed, paraffin-embedded longitudinal sections were used in B and I. Colors are described in Table 1 and the text. **A)** Cotton blue-lactophenol stain showing the cover (cv), core (cr) and channel (ch) of a papilla, and the appressorium (a) of *H. avenae* (× 1,500). **B)** Safranin and fast green stain showing a conidium(s), germ tube (gt), appressorium, and papilla (p) (× 1,000). **C)** Phloroglucinol-HCl test showing a vertical view of a papilla(e), disk (d), and altered lateral wall (lw) (× 700). **D)** Lacmoid test showing staining of the disk and papilla in longitudinal view (× 800). **E)** Aniline blue-fluorescence test showing fluorescence of two disks, papillae, and altered lateral walls in a vertical view (× 500). **F)** Glycerine-ferricyanide test in a vertical view (× 700). **G)** IKI-H_2SO_4 test at an early stage showing differential color of papilla cover (× 800). **H)** IKI-H_2SO_4 test at a later stage (× 800). **I)** Longitudinal section viewed through a polarizing microscope showing birefringence of the basal part of the papilla cover nearest the outer epidermal wall (× 800).

histochemical tests, except the aniline blue-fluorescence test, were consistent among papillae of similar size within a sample and among different samples and trials.

Structure of the papilla.—The papilla was a domelike or conical growth extending from the epidermal wall beneath the appressorium into the cell lumen (Fig. 1-A, B, D). Papillae often formed at or near the junction of two cells (Fig. 1-C, E, F). Forty-eight hours after inoculation with *H. avenae,* papillae were about 5-12 μm long and 5-9 μm in diameter at the base.

The papillae contained two principle areas: the cover and the core. The cover was a thin (less than 1 μm), continuous layer between the host cell lumen and the core (Fig. 1-A, D). The cover appeared to be continuous with the inner cellulose layer of the epidermis; however, this relation could not be unequivocally established by light microscopy. The core was the main body of the papilla, and it enlarged as the papilla expanded. A thin, single, or sometimes forked, channel passed from the penetration peg through the center of the core to within 1-2 μm of the growing point of the papilla (Fig. 1-A). The channel was refractive and ranged from 0.3 to 1.0 μm in diameter. It probably represented an extension of the penetration peg. In many papillae, the basal portion of the core appeared to be distinctly wider and stained more darkly than the distal portion (Fig. 1-A, D). Careful focusing suggested that the dark, enlarged base was partly an illusion created by curvature of the stained, thickened outer epidermal wall.

TABLE 1. Histochemical color reactions of papillae, modified epidermal walls, and normal cell walls in reed canarygrass leaves inoculated with *Helminthosporium avenae* and tested by six histochemical procedures[a]

Area tested	Toluidine blue O	Chlorine water-sodium sulfite	Phloroglucinol-HCl	Lacmoid	Aniline blue-fluorescence	IKI-H_2SO_4
Reaction area						
Papilla cover	Reddish-purple	Orange to red	Medium red	Reddish-blue	Quench	Y - O - B[d]
Papilla core	Blue-green	Orange to red	Medium red	Medium blue	Yellow or quench	Y - O - B
Disk[b]	Clear or faint pink	Unstained	Faint pink	Faint blue	Yellow or quench	Y - O - B
Altered lateral wall[c]	Blue-green	Yellow to orange	Faint red	Medium blue	Yellow or quench	Y - O - B
Normal cell walls						
Epidermal long and short cells	Reddish-purple	Light yellow	Faint yellow	Rust	Faint yellow-green	Blue
Guard cells	Blue-green	Faint pink	Red	Unstained or rust	None	Blue
Prickle-hairs	Blue-green	Faint pink to orange	Faint red	Faint blue	Yellow	Blue
Epiveinal fibers	Blue-green	Orange	Medium red	Faint red-blue	. . .	Green to blue
Tracheary elements	Blue-green	Red	Rust	Rust	. . .	Green to blue
Expected color						
Lignified walls	Green or blue-green	Orange to red	Red to purple	Reddish	Faint greenish	Yellow to orange
Callose	Unstained	Unstained	Unstained	Blue	Yellow	. . .
Cellulose	Red or reddish-purple	Unstained	Unstained	Reddish	Faint greenish	Blue

[a]Papillae and modified walls were tested in fresh epidermal strips and normal cells were tested in adjacent unstripped tissue. Duplicate tests of six clones inoculated for 48 hours. See text for procedures.

[b]Outer epidermal wall within 30 μm of the penetration peg.

[c]Lateral wall beneath the disk.

[d]Y - O - B = bright yellow turning to bright orange or amber within a few minutes, followed by development of scattered blue areas.

Chemical extractions also suggested layering of papillae. Extraction for 18 hours in a permanganate solution which dissolved lignin but not cellulose resulted in a hollowing out of the core, leaving the cover. Extraction of lignin by repeated cycling between chlorine water and sodium sulfite gave a progressive loss of structure. By the end of the third cycle, the only structure remaining was a 2-3 μm area immediately around the original bore hole and, sometimes, the cover.

Histochemistry of the papilla. —The channel did not stain with any histochemical reagents. It did not contain visible structure or organelles. The wall of the channel stained faintly with toluidine blue O and fast green in a manner suggestive of cellulose (Fig. 1-B).

Lignin.—Toluidine blue O gave a positive test for lignin in the core and a test for cellulose in the cover of fresh and fixed papillae (Table 1). Safranin and fast green also differentiated the core (safranin) and cover (fast green) (Fig. 1-B). Phloroglucinol-HCl (Fig. 1-C) gave a faint to moderate test for lignin, and chlorine water-sodium sulfite gave a moderate test for lignin, throughout the papillae. Failure to obtain localization between core and cover using the latter two tests may have resulted from diffusion of the colored products. Girder sclerenchyma, tracheary elements, guard cells, and prickle-hairs (26) showed staining colors and intensities similar to that in the core of the papillae. The results indicated that the core contains lignified material which is similar to that in normally lignified cell walls of reed canarygrass.

Callose.—The lacmoid test indicated that callose was present in the papillae and was particularly concentrated in the core (Table 1, Fig. 1-D). The prickle-hairs and certain guard cells also appeared to have callose, as expected from the report by Currier (7).

The resorcinol blue test further indicated that papillae contained callose. When strips that were stained in resorcinol blue in tap water for 10 minutes were placed in buffer at pH 4.1, the papillae remained blue, but all other cell walls became reddish.

The aniline blue-fluorescence test for callose gave variable results. Some papillae fluoresced greenish-yellow (Fig. 1-E). Other papillae in the same area showed no increase in fluorescence compared with the faint yellowish-green background fluorescence characteristic of all tissue and which was present in control strips incubated in buffer alone. Still other papillae appeared black under ultraviolet (UV) light.

Extraction with hot glycerine caused little or no reduction in mass of the papillae. This indicated that callose was not necessary for structural integrity of papillae.

Cellulose.—In the IKI-H_2SO_4 test (Fig. 1-G), normal epidermal walls rapidly became intense blue to deep indigo, indicative of their cellulosic nature (16). The papillae were at first yellowish (Fig. 1-G). This soon turned to intense orange or amber as expected for lignin (16). After several minutes, there was an admixture of blue with the amber which was most intense in the cover and in an area immediately around the bore hole. The blue color was more prominent with small, young papillae (Fig. 1-F). The subsidiary cells were the only leaf cells to give the sequence of yellow to amber to blue seen with papillae. The girder fibers and tracheary elements became greenish, then blue, indicating a close relation of lignin and cellulose (16). The prickle-hair base soon became blue.

The cover stained reddish-purple with toluidine blue O, and greenish with safranin and fast green. The basal portion of some covers showed birefringence in paraffin longitudinal sections mounted in Permount (Fig. 1-I). Other papillae in the same slide lacked birefringence. The results suggested that there may be cellulose in the cover of the papillae.

Papillae did not lose their structure when strips were kept in 72% H_2SO_4 to hydrolyze cellulose. Papilla in fresh strips did not show birefringence, although normal cell walls of the strips showed strong birefringence. We concluded that cellulose is not the major component of reed canarygrass papillae.

Structure of the disk.—The disk was a circular or elliptical area of altered epidermal wall extending outward from the point of attempted penetration (Fig. 1-C, F). The disk was 22-60 μm in diameter. The margin was sharply delimited and smooth. Most disks were histochemically quite homogeneous. They were best revealed by the glycerine-ferricyanide test (Fig. 1-F). When papillae formed near the junction of two cells, often the part of the disk over one cell had a different radius than the part over the other cell. The glycerine-ferricyanide test indicated that every papilla was accompanied by a disk; however, some disks did not have a papilla.

Histochemistry of the disk.—The disk gave a faint pink or red color with hot phloroglucinol-HCl (Fig. 1-C). It was not structurally degraded by 72% H_2SO_4. It was not stained by chlorine-sulfite or toluidine blue. Thus, we concluded that the disk may contain a small amount of lignified material, but not as much as the papilla or altered lateral wall.

The disk gave a faint to medium blue color with lacmoid or resorcinol blue. It often gave a faint yellow fluorescence with aniline blue (Fig. 1-E). Apparently callose was present.

The IKI-H_2SO_4 test gave a conspicuous amber reaction (Fig. 1-G). This was followed by development of blue fibrillar streaks or punctations (Fig. 1-H). Extraction of lignin left the disk with a blue reaction to IKI-H_2SO_4 and normal birefringence. Thus, cellulose appeared to be a significant component of the framework of the disk.

The border of the disk was histochemically distinct. It often appeared black under fluorescent light. It infrequently stained dark with cotton blue and with nitroso reagent.

Structure of the altered lateral wall.—Any part of a lateral wall which passed beneath a disk was slightly thickened and histochemically altered (Fig. 1-C, E, F). The alteration occurred whether the lateral wall was medially or tangentially located with respect to the disk or the papilla. Sometimes disks with papillae formed over the central face of a cell and did not have an altered lateral wall. Deposition of lignin in a wall can cause swelling (36).

Histochemistry of the altered lateral wall.—The lateral wall stained faint orange with chlorine-sulfite and faint red with phloroglucinol-HCl. The lacmoid and aniline blue-fluorescence tests for callose gave the same reaction and intensity as observed in papilla. The IKI-H_2SO_4 test

was also like that of papilla. Birefringence was readily seen in fresh and fixed material without extraction.

We concluded that the altered lateral wall contained cellulose, lignified material, and some callose. It seemed to contain more lignified material than the disk, but less than the papilla.

Basic staining material.—The test (21) for 'basic staining material' (BSM) was applied to fixed material sampled 28 hours after inoculation. The papillae and altered lateral walls stained red (positive), and the disks stained light red. Walls of a few guard and subsidiary cells were stained. No other plant cell gave a positive test.

Other compounds.—Older papillae and altered lateral walls were naturally yellowish brown. The color was not removed by soaking in dilute acid or base, ethanol, acetone, ethyl acetate, or chloroform. The nature of this naturally occurring pigment was not known.

Negative results were obtained in the following tests on fresh and fixed material: (i) Sudan IV test for cutin and suberin; (ii) 50% chromic acid solubility test for cutin and suberin (17); (iii) orcinol test for gums; (iv) nitroso reaction for tannins; and (v) $FeCl_3$ test for tannins. The hydroxylamine-ferric chloride test for pectins gave positive results only in reed canarygrass veins before extraction with ammonium oxalate. Use of nonstained controls and careful focusing was essential to avoid being misled by color aberrations in the cover and associated wall.

Light and temperature study.—Papillae, disks, and altered lateral walls which formed in dark or light at 17, 25, and 29 C gave the color reactions described above in tests (ii) to (vii). Test (i) was not run. The leaves at 29 C had few and small papillae. Color intensity was related to papilla size. There were no major differences among treatments when papillae of equivalent size were compared.

Fungal species.—Six fungal species induced papillae, disks, and lateral walls which gave histochemical reactions identical with those of *H. avenae* in tests (i) to (vii). Papillae grew moderately rapidly under appressoria of *Helminthosporium catenarium, Ascochyta* sp., *Stagonospora arenaria* Sacc., and *Stemphylium botryosum* Wallr., and leaves inoculated with those fungi were tested 3.5 days after inoculation. Papillae were small under appressoria of *Botrytis cinerea* Pers. and *Leptosphaerulina trifolii* (Rostrup) Petrak, and leaves were tested at 5 days. Papillae of comparable size gave comparable results. In this study, we did not examine the poorly developed papillar areas through which *H. catenarium* penetrated (33).

Time-course study.—Four hours after inoculation, appressoria were small. No disks or papillae were found.

At 6 hours there were many bore holes with surrounding disks in the cuticle surface. Not all disks had visible bore holes. There were a few small papillae.

At 6 hours, some of the papillae, associated lateral walls, and disks gave positive tests for callose. The lacmoid test gave a mdium intensity stain of papillae from 10 through 48 hours. The aniline blue-fluorescence test was stronger at 10 and 12 hours than at later times.

The IKI-H_2SO_4 tests for cellulose showed the progression from yellow to orange to partial blue in disks, lateral walls, and papillae at all times from 6 to 48 hours. At 6 and 10 hours, the blue color developed rapidly and was more pronounced in disks and papillae than at later times.

The toluidine blue O reaction for lignin was observed at 10 hours and thereafter. The phloroglucinol-HCl reaction was faint or absent at 10, 12, and 18 hours. It was stronger thereafter. The chlorine-sulfite reaction for lignin was faint at 12 hours.

DISCUSSION

Structure.—The structure of reed canarygrass papillae agreed with limited reports from other crops. Ito [reported by Akai (1)] illustrated a thin covering layer, a "middle layer" (the core) divided into lightly stained distal and darkly stained basal areas, and a channel in papillae of sweet potato. Some electron micrographs of papillae of barley (9, 24, 33), pea (12), and wheat (4) show two principle layers and irregular membraneous material within each layer.

Many authors have described and illustrated a platelike, homogeneous, histochemically altered area of epidermal wall surrounding the penetration site. These areas have been referred to as disks (25, 37), disk-shaped areas (25), areas (25, 33), rings (37), and halos (3, 19, 22, 25). Since the illustrations show them to be, like those of reed canarygrass, platelike and uniform, the term disk seems most appropriate. Chemical treatment was usually necessary to reveal disks. However, Young (37) observed that some disks were auto-stained yellowish or grey.

The origin and structure of the disk is not understood. The disks of barley are about 30 μm in diameter (25, 33). This corresponds to the area of barley epidermal wall underlain by an aggregation of highly active host cytoplasm during papilla formation (5). McKeen et al. (25) and Kunoh and Akai (19) believed their tests suggested that the normal cell wall constituents of the barley disks are degraded by an enzyme(s) produced by the fungus. We consider it unlikely that fungal enzymes for penetration operate over an area this wide. We suggest that host enzyme activity associated with papilla synthesis plays a role in the chemistry of the disk area. The present results show that the reed canarygrass disk contains wall materials. The relation of cytoplasmic aggregation to modification of the wall merits further study.

There are few previous descriptions of altered lateral walls. Young (37) depicted a prominent middle lamella in vertical walls in the disk area in wheat and soybean. McKeen et al. (25) noted that when an *Erysiphe* infection peg was within 15 μm of a lateral wall, the wall stained readily. Figures in Akai et al. (3) and Kunoh and Akai (19) show strong staining of lateral wall in the disk area.

Histochemistry.—Lignin, callose, and cellulose were detected in each main part of the reaction area; e.g., papilla, disk, and lateral wall. Lignification was more readily detected in papillae and lateral walls than in the disks. Cellulose was not readily found in papillae. Lignin normally occurs in close association with other wall polysaccharides. We believe that the papilla core probably contains a polysaccharide matrix within which lignified material is deposited, but our tests did not reveal it.

The composition of papillae and associated walls was relatively constant over a range of genetic and environmental conditions. Papilla formation in reed

canarygrass appeared to be a biochemically stable mechanism, controlled by the host and operating as a general response to a variety of incitants. Because of variation in normal cell wall composition between plant species, it is likely that papilla composition varies between species.

In previous studies, phloroglucinol-HCl positive material was observed in papillae of wheat (11, 37), morning glory (2), and hop (34). Papillae of tomato gave negative phloroglucinol-HCl tests, but positive Maule tests for lignin (13, 14). Tests for fluorescence provided evidence for callose in barley papillae (33). Other reports (4, 9) of callose in fungus-induced wall appositions referred to other structures or were unsubstantiated. There was little evidence for cellulose in papillae (4, 13, 14). In the most elaborate previous study, Fellows (11) concluded that papillae of wheat roots were composed chiefly of lignin with a trace of suberin; callose, cellulose, and hemicellulose were not detected. Earlier histochemical tests of disks suggested lignified material in wheat (37), and callose in barley (33). It was shown that cellulose is chemically modified in disks in several host species (3, 19, 25). The modified lateral wall was not studied.

As is true of all histochemical tests, the specificity of the tests for lignin (6, 8, 28), callose (10), and cellulose (16) has been questioned. However, no better histochemical tests are available (31).

The red color developed with chlorine-sulfite indicated the presence of syringyl units in lignin (36). This was expected, because grass lignin is a 'guaiacyl-syringyl' lignin (30). The phloroglucinol-HCl test indicated cinnamaldehyde end groupings (36), and was less sensitive (fainter) than the chlorine-sulfite test as has been reported for certain other grass tissues (32). In the phloroglucinol-HCl test it usually was necessary to heat the tissue to obtain color in lignified cells and papillae of reed canarygrass. The conditions give a cherry-red color with xylan skeletal hemicellulose (17). Thus, we cannot exclude the possibility that papillae contain hemicellulose.

There was a discrepancy between the erratic appearance of aniline blue-fluorescence and the consistent positive lacmoid test for callose. Callose deposits are believed to be homogeneous and present for only short periods (15). This is more in keeping with the fluorescence results than the lacmoid results. Deposition of lignin may block reactive groups in other wall compounds (36). Thus, the presence of lignin or other materials in older papillae may alter the fluorescence properties of callose; or lacmoid may react with other groups than those detected by fluorescence.

The results of chemical extractions suggested that the structural integrity of the core is primarily dependent upon lignified material, and that the cover is of a different composition. In contrast, Griffiths and coworkers (13, 14) reported that the core of papillae in tomato was removed by a cellulose-dissolving reagent and suggested that the layer immediately around the penetrating hypha was cellulosic and that the outer layers became lignified.

Role of new cell wall formation in resistance.—The time-course study showed that callose was synthesized early in attempted penetration. Callose is deposited rapidly in response to chemical and mechanical injury (7, 10, 15, 27). Not only does callose seal or plug wounds rapidly, but experiments with ^{14}C-thymidine (15) suggested that callose may restrict the passage of small molecules between tissues. The nutritional and toxicological consequences of restricted interchange of metabolites would have a direct bearing upon the establishment of pathogenic relations.

Lignification began as early as 10 hours after inoculation and apparently continued in close proximity to the penetration peg as long as the peg continued to elongate. Except for certain wood-rotting or soil-inhabiting fungi, most fungi lack the capacity to degrade lignin effectively (20). Lignification increases the resistance of cell walls to compressive forces (36). Thus, lignified wall appositional material may present a formidable biochemical and mechanical barrier to the penetration pegs of leafspot fungi.

Basic staining material (BSM) is associated with resistance to *Erysiphe* in barley (21). We found that the distribution of BSM in reed canarygrass papillae and guard cells corresponded to the distribution of a yellow pigment in these cells. The relation of BSM to the yellow pigment and to resistance should be explored.

Lignin, cellulose, and callose biosynthesis are protein-mediated processes. Earlier studies showed that when leaf disks were floated on cycloheximide solutions, protein synthesis was inhibited, papillae did not form, and the fungi initiated successful penetration from the appressoria (35). The inhibition of papilla formation may have resulted from inhibition of protein synthesis necessary for the synthesis and deposition of these specific cell wall materials.

Our earlier studies (35) did not provide evidence for induced, diffusible (phytoalexin-like), antifungal compounds. We did not rule out the possibility of localized, induced antifungal substances acting at the penetration site. We believe that continuing formation of lignified material and callose as a response to elongation of the penetration tube may be a sufficient mechanism for resistance to penetration.

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