Cycloheximide Treatments Implicate Papilla Formation in Resistance of Reed Canarygrass to Fungi

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ABSTRACT

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Reed canarygrass leaf-disks were floated on water and inoculated with spore suspensions of *Helminthosporium catenarium, H. avenae, Stemphylium botryosum, Leptosphaerulina trifolii, Botrytis cinerea,* and an *Ascochyta* sp. Only *H. catenarium* was pathogenic on reed canarygrass. With the noninfecting species, a papilla (host cell wall thickening) formed in the epidermal wall beneath each fungal appressorium from which penetration was attempted, and no penetration occurred. With *H. catenarium* most appressoria induced papilla formation and did not initiate penetration, but about 1% of appressoria initiated direct penetration through a thin-walled, poorly-formed papillar swelling.

When leaf-disks were floated on aqueous solutions of cycloheximide (10-25 μg/ml), protein synthesis was inhibited, papilla formation was prevented, and more than 80% of appressoria of all fungi gave rise to direct penetrations. Primary infection hyphae grew well and invaded adjacent epidermal cells. Transfer of leaf-disks from cycloheximide solutions to water prior to appressorium formation, restored protein synthesis, restored papilla formation, and prevented penetration. No inducible antifungal compounds could be detected by the drop-diffusate technique. Papilla formation appears to be a defense mechanism against fungal penetration that requires response-dependent protein synthesis.

Additional key words: resistance, phytoalexin, protein synthesis, lignituber, *Phalaris arundinacea.*

We have undertaken a program to discover mechanisms of resistance of reed canarygrass (*Phalaris arundinacea* L.) to fungal diseases. This effort was motivated by the general lack of definitive information on resistance mechanisms in the Gramineae, and by reports (10, 15) and personal observations that forage stands of reed canarygrass are free from serious disease problems. As reported here, we found no evidence for an inducible antifungal chemical system when we used the classical approach (6, 8) of challenging plant defenses by inoculation with fungi nonpathogenic to the species. Our concurrent observations on histological events revealed that reed canarygrass responded to nonpathogenic fungi by forming papillae in the epidermal wall beneath the fungal appressoria.

Many plant species form papillae in response to penetration activities (1, 2, 3, 5). Papillae have frequently been postulated to resist fungal invasion (2, 3, 5). This role has been questioned, because papillae also occur in compatible host-parasite combinations (1, 2), and they sometimes fail to exclude the fungus.

We report here a new approach in evaluating papilla formation as a resistance mechanism. We considered that new wall formation would be a protein-mediated process. The proteins could function as enzymes, intercalating materials or other factors in wall synthesis. We reasoned that inhibition of protein synthesis by a specific inhibitor, such as cycloheximide, might prevent papilla formation, and that if papillae were a major aspect of resistance the absence of papillae would permit penetration by nonpathogenic fungi. Effects of manipulation of cycloheximide upon papilla formation, penetration, and protein synthesis are reported in this paper.

MATERIALS AND METHODS

Reed canarygrass clone 6049 (9) was used. Nine pieces of sod, 25 cm square, were transplanted into a glasshouse bed of peat moss:vermiculite (1:1, v/v). The plants were watered and fertilized frequently to maintain vigorous growth.

We used an isolate of *Helminthosporium catenarium* Drechs. that causes a slowly expanding leafspot on reed canarygrass (K. E. Zeiders, *personal communication*). For the five other fungal species (Tables 1-2) we used isolates that were pathogenic to known hosts, but that did not cause disease on reed canarygrass.

The fungi were cultured on V-8 juice agar (11) in petri dishes at 25 C with continuous fluorescent light. Seven to 14 days after cultures were initiated, ascospores of *Leptosphaerulina trifolii* (Poll.) Graham and Luttrell or conidia of the other species were washed from the surface of cultures and suspended in water with 0.1% Tween-20 surfactant. The inoculum suspensions contained 2,500 to 10,000 spores per ml.

Leaf-disks, 8 mm in diameter, were cut with a cork

borer from unblemished, fully expanded leaves. The disks were floated on water or aqueous solutions of cycloheximide (10 μg/ml) in petri dishes. A 20-μliter drop of inoculum suspension was pipetted on the upper surface of each disk. Dishes were kept on a laboratory table under prevailing temperature (20-24 C) and light (north window and fluorescent light during working hours).

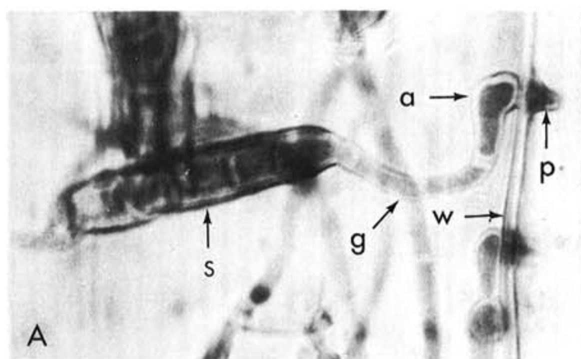
At 24 hours after inoculation, the disks were cleared and stained by simmering them for 5 minutes in cotton blue-lactophenol (1 ml lactic acid, 1 ml phenol, 1 ml glycerine, 1 ml water, 8 ml ethanol, 4 mg cotton blue). They were mounted in lactophenol for observation.

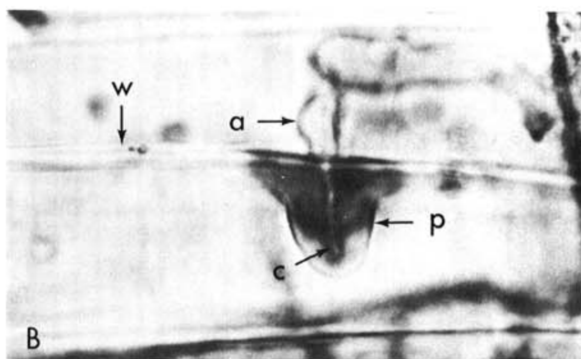
Data were taken on 50 randomly selected germinated spores per treatment per trial. We counted the number of germ tubes, appressoria, direct penetrations, and papillae associated with the 50 spores. Direct penetration was distinguished by a visible connection between the appressorium and primary intracellular hypha. Five or more disks were observed per treatment in each of two trials. Similar results were obtained in repeated experiments.

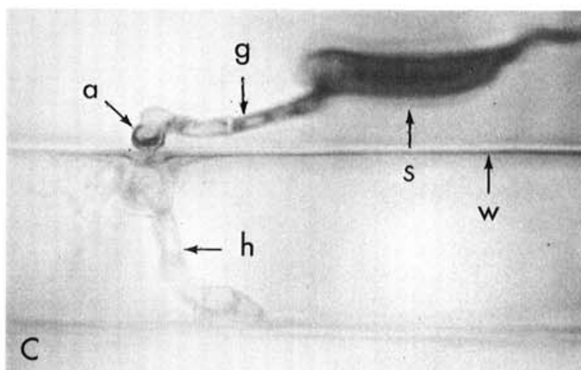
The rate of protein synthesis in water- and cycloheximide-treated disks at the time of harvesting was assayed by measuring the incorporation of ^{14}C-tyrosine into trichloroacetic acid-precipitable material (4).

The effect of cycloheximide upon mycelial growth rate of *H. avenae* Eidam, *Botrytis cinerea* Pers. ex Fr. and *Stemphylium botryosum* Wallr. was measured in vitro. Radial growth rate was measured for colonies on potato-dextrose agar and V-8 juice agar media with, and without, cycloheximide amendment (10-15 μg/ml).

The drop-diffusate method (8, 14) was used to test for inducible antifungal compounds. Excised leaf pieces, 6-10 cm long, were floated on water in 15-cm diameter petri dishes. They were inoculated with many drops of *H. avenae* spore suspension. After 36 hours of incubation on the laboratory table, the drops were collected by suction. The total diffusate from each treatment was 125 ml. The aqueous diffusate was concentrated to 12.5 ml, made acid to pH 3.0 with 1N HCl, and then extracted three times with 125-ml portions of ethyl acetate. This was designated the acid fraction. The diffusate was then made basic to pH 11.0 with 1N NaOH and extracted three times with 125 ml







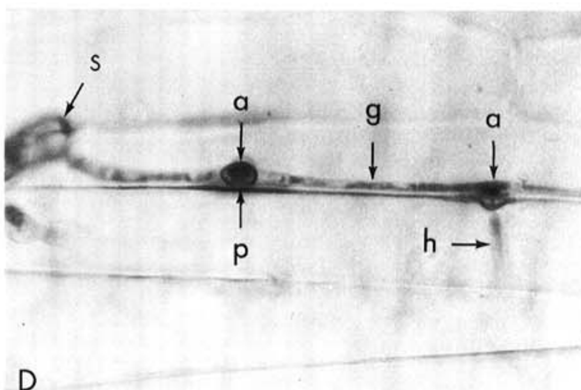


Fig. 1-(A to D). Papilla formation and penetration in reed canarygrass leaf epidermis inoculated with spores of *Helminthosporium avenae*. The whole mounts were stained with cotton blue-lactophenol. **A)** Area of a leaf disk floated on water and examined 18 hours after inoculation. The spore (s) formed a germ tube (g) and appressorium (a). A papilla (p) formed in the outer epidermal wall (w) beneath the appressorium. (× 757). **B)** Appressorium and papilla 48 hours after inoculation. The central unstained channel (c) is thought to be an extension of the penetration peg. (× 1,691). **C)** Area of a leaf-disk floated on a solution of cycloheximide (10 μg/ml) and sampled 24 hours after inoculation. The fungus penetrated the outer epidermal wall via a thin penetration peg from the appressorium and formed an intracellular hypha (h). (× 472). **D)** Area of a leaf-disk transferred from water to cycloheximide solution 8 hours after inoculation and sampled at 24 hours. The germ tube grew from left to right. The first-formed appressorium incited papilla formation and the second-formed appressorium initiated penetration of the plant cell. The papilla and intracellular hypha were clearly visible in a lower focal plane. (× 472).

portions of chloroform. This was designated the basic fraction. The remaining diffusate was designated the aqueous neutral fraction. The ethyl acetate, chloroform, and aqueous neutral fractions were then evaporated to dryness under reduced pressure at 30 C. All treatments were then redissolved in 2 ml of methanol. These solutions were then used for chromatography and spore germination bioassays.

Thin-layer chromatography (TLC) was done with silica gel and cellulose plates using isopropanol-ethyl acetate-ammonia (20:5:1, v/v/v), toluene-ethyl formate-formic acid (5:4:1, v/v/v), and benzene-acetic acid-water (10:7:3, v/v/v) solvent systems.

Bioassay of TLC plates was performed by spraying air-dried plates with a spore suspension of *Cladosporium fulvum* and then overspraying with warm liquid potato-dextrose agar. The plates were then incubated in a plexiglass moist chamber placed in an incubator at 24 C. The plates were observed after 48 hours for the formation of inhibition zones.

Spore germination studies were done with *H. avenae* and *S. botryosum*. A 0.05-ml aliquot of the original unextracted drops and the acid, basic, and neutral fractions from extracted-inoculated and control drops were added to a sterile 5-ml vial. The methanol was allowed to evaporate in a transfer chamber. To this was added 0.50 ml of a spore suspension (1×10^4 spores/ml) of each fungus tested. The percentage germination was calculated from a count of 100 spores at random 18-20 hours later. Data were the average of three experiments with three replicates for each treatment.

RESULTS

Papilla formation.—The spores germinated by forming germ tubes (Fig. 1-A; Tables 1-2). One or more lateral cells of the germ tubes, or the terminal cell, differentiated into a unicellular, saccate appressorium. The appressoria of *H. avenae* and *H. catenarium* formed within 6-10 hours after inoculation. Those of other fungi formed later. Within 2 hours after appressorial formation, a small refractive spot appeared in the outer surface of the epidermal wall beneath the appressorium, and the host cell wall beneath the appressorium showed a slight thickening. The refractive spot was believed to be the penetration peg. The thickening

TABLE 1. Number of germ tubes, appressoria, direct penetrations, and papillae formed by four fungi on leaf-disks of reed canarygrass floated on water or solutions of cycloheximide (10 μg/ml)

Fungus[b]	Treatment[c]	Number of structures per 50 spores[a]			
		Germ tubes	Appressoria	Direct penetrations	Papillae
Ascochyta sp.	Control	50	50	0	43
	Cycloheximide	50	50	45	0
Stemphylium botryosum	Control	191	212	0	175
	Cycloheximide	205	112	89	0
Leptosphaerulina trifolii	Control	350	137	0	108
	Cycloheximide	363	119	95	0
Botrytis cinerea	Control	83	117	0	97
	Cycloheximide	72	88	70	0

[a]Each value is the mean of two experiments with at least five disks counted for each experiment.
[b]All fungi were nonpathogenic on reed canarygrass.
[c]Control disks were retained on water for the entire 24 hr from inoculation to sampling. Cycloheximide-treated disks were retained on 10 μg/ml cycloheximide for the entire 24 hours.

TABLE 2. The effect of time of application of cycloheximide (10 μg/ml) on number of germ tubes, appressoria, direct penetrations and papillae formed per 50 spores by *Helminthosporium avenae* on leaf disks of reed canarygrass over 24 hours

Time after inoculation when disks were placed on cycloheximide[b] (hours)	Number of structures per 50 spores[a]			
	Germ tubes	Appressoria	Direct penetrations	Papillae
24 (water control)	99	116	0	102
0	94	107	91	0
4	106	122	101	2
8	113	117	52	55
12	75	79	27	51
16	102	116	3	105
20	110	120	0	101
24	92	98	0	82

[a]Each value is the mean of two experiments with at least five disks counted for each experiment.
[b]Control disks were retained on water for the entire 24 hours from inoculation to sampling.

expanded into a dome-shaped or elongate-conical papilla extending into the epidermal cell lumen (Fig. 1-A, B). The papillae induced by *H. avenae* and *H. catenarium* were typically 5-9 μm in diameter by 5-12 μm long at 48 hours. Papillae were smaller with most other fungi. They stained well with cotton blue, but had a nonstaining channel, 0.3-1.0 μm in diameter, extending through the center of the papilla to within 1-2 μm of the tip. The channel may have been an extension of the penetration peg. Elongation of papillae usually ceased by 48 hours.

Papillae formed beneath 85 to 90% of the appressoria of the five noninfective fungi (Tables 1 and 2). Papillae did not form in association with any other fungal structure. Penetration never occurred through any of these papillae, nor through any of thousands of additional papillae that were studied. No penetration attempts were seen from appressoria which were not associated with a papilla.

When attached leaves of whole plants were inoculated with *H. avenae,* papillae formed beneath appressoria, and no penetration occurred.

Papillae formed beneath 78% of the appressoria on leaf disks inoculated with the pathogen, *H. catenarium*. Penetration and hyphal invasion were effected from 1.1% of the appressoria. Penetration occurred directly through unswollen walls and through thin-walled, poorly stainable, and poorly organized papillae. No penetration occurred through well developed papillae.

Effect of cycloheximide on papillae and penetration.—When the disks were floated on cycloheximide solutions (10 μg/ml), germ tubes and appressoria were formed at a frequency (Tables 1-2) and rate comparable to those formed on disks on water. Papillae never were formed. Appressoria led to penetration by all five fungi. The percentage of appressoria leading to penetration (80%) was similar to the percentage which induced papillae (88%) in water controls. Penetration began 8 hours after inoculation and was abundant by 14 hours. This rate of penetration was similar to or only slightly slower than that for papilla appearance in water controls. The intracellular hyphae (Fig. 1-C) grew well, branched, and often entered adjacent epidermal cells. They resembled primary hyphae in normal penetrations by *H. catenarium*.

To determine whether the resistance mechanism inhibited by cycloheximide could be restored, we floated disks on cycloheximide solutions for 24 hours, then blotted them and transferred them to water for 12-14 hours before inoculating them with *H. avenae*. Papillae formed in the treated disks as they did in water controls. In a sample of 100 spores there were 191 germ tubes, 299 appressoria, 267 papillae and two penetrations (without papillae).

Disks were transferred from water to cycloheximide solutions at various times after inoculation with *H. avenae*. No papillae formed in disks that were immediately placed on cycloheximide solution (Table 2). With increasing time until transfer to cycloheximide, there were increasing numbers of papillae and decreasing numbers of penetrations. Only papillae formed in disks transferred at 20 hours. In disks transferred at 8 hours certain germ tubes gave rise to both a papilla without penetration and a penetration without a papilla (Fig. 1-D). The appressorium leading to the papilla was nearer to the spore than was the appressorium leading to penetration, and presumably the papillar appressorium formed earlier than the other appressorium. In a low percentage of penetrations a poorly staining, thickened collar of wall material encircled the penetration peg. The fungus never penetrated well developed papillae in tissue that was inhibited with cycloheximide.

Protein synthesis.—The rate of protein synthesis in cycloheximide-treated disks was 6% (3,797 dpm/g fresh wt.) as compared to that in water controls (55,172 dpm/g fresh wt.). Protein synthesis was restored to 90% (48,947 dpm/g fresh wt.) of the level of controls within 8 hours after cycloheximide-treated disks were transferred to water.

Effect of cycloheximide on mycelial growth.—The radial growth rate of *S. botryosum* on agar media with cycloheximide at 10-15 μg/ml was 33% of that on unamended media. The growth rates for *H. avenae* and *B. cinerea* were, respectively, 50 and 66% of that on controls.

Search for induced antifungal compounds.—TLC chromatography and subsequent bioassay of the plates indicated that there were no induced antifungal compounds in drop diffusates of reed canarygrass. Inhibition zones were observed on plates, but no differences were observed between inoculated and control drop diffusates.

Spore germination bioassays indicated that no compounds were induced in either control or inoculated drop diffusates which could inhibit spore germination or germ tube growth of *H. avenae* and *S. botryosum*. The percentage of germination was 87-95% with the acid, basic, and neutral fractions of both inoculated and control drop diffusates. Spore germination bioassays with the original unextracted drop diffusate showed no induction of new antifungal compounds. The percentage of germination varied from 89-96% with both inoculated and control diffusates.

DISCUSSION

These results support the hypothesis that papilla formation functions as a resistance mechanism in reed canarygrass. Penetration never occurred through any of thousands of well formed papillae which were observed. When papilla formation was prevented by cycloheximide treatment, there was no remaining chemical or physical barrier to penetration; successful penetrations occurred and mycelium ramified through the tissue. When cycloheximide was withdrawn, protein synthesis and papilla formation were restored and no penetration occurred. This showed that treatment with cycloheximide did not irreparably damage or kill the tissue, but on the contrary the tissues were capable of renewed resistance to penetration when the effects of the inhibitor were removed. The timing of papilla formation was consistent with the hypothesis. Swelling was observed at the earliest stages of penetration peg formation. Papilla formation in untreated disks occurred at or before the time when penetration occurred in treated disks.

When disks were transferred to cycloheximide 8 hours after inoculation, individual appressoria gave rise to both papillae without penetration and penetrations without papillae. When both a papilla and a penetration occurred from a single germ tube, the papilla was nearer the spore

and therefore was thought to have been initiated before cycloheximide inhibited the resistance mechanism. The fact that penetration occurred from the same germ tube after initial formation of a papillae suggests that the fungus was not killed or inhibited from further attempts at penetration when the first penetration attempt was stopped by a resistance reaction. In disks transferred to cycloheximide solution, penetrations never occurred through papillae that had already been formed.

In other studies we found that papillae of leaf disks inoculated with these fungi gave histochemical reactions indicative of lignification (13). Lignified wall material is believed to be more resistant to fungal degradation and penetration than nonlignified wall (7, 12).

The possibility that constitutive or inducible antifungal compounds are involved in resistance of reed canarygrass must also be considered. The results seem to rule against stable, preformed antifungal compounds as significant factors in preventing penetration, because stable, preformed compounds would be expected to remain effective in cycloheximide-treated tissues.

We were unable to detect induced antifungal compounds in the infection drops which could inhibit fungal growth or spore germination. These results suggest that the typical phytoalexin response (6, 8) may not be an important aspect of the resistance of reed canarygrass to penetration. Our data do not, however, eliminate the possibility of an internal localized defense response which may involve induction of fungitoxic compounds.

When cycloheximide was applied after inoculation, the resistant (papilla) and susceptible (penetration) responses often occurred in adjacent cells. They were not seen within the same cell. Resistance was localized. This is not consistent with a mechanism based upon a diffusable, low molecular weight, antifungal compound (6, 8).

It may be contended that resistance to penetration by these fungi was due to induced antifungal processes acting locally at the interface between the penetration peg and host wall, and that the papillae served to seal the wound at the site of attempted penetration. Within this concept, cycloheximide would inhibit not only protein synthesis required for new wall formation, but also protein synthesis required for the postulated antifungal process.

It is interesting that papillae formed nearly as frequently in response to *H. catenarium* as in response to nonpathogens, and that only a few appressoria of the pathogen initiated successful penetrations. The same mechanism that conferred resistance to penetration by nonpathogenic fungi may also limit the number of successful penetrations by a pathogen. The penetrations occurred through small, thin-walled, poorly stainable papillae. This observation suggests that the pathogen may have a mechanism for inhibiting normal papilla formation.

We view papilla formation as a dynamic response mechanism of the plant for containing the fungus at the penetration site. Papilla formation occurred only at the interface with the penetration apparatus, thus it is conservative of energy and resources. The act of wounding by the advancing penetration peg may alone be sufficient to elicit the papilla formation response. This does not negate the possibility that these dynamically expanding wall appositions may be a sufficient barrier to penetration by virtue of their biochemical-biophysical makeup. Indeed, the results strongly indicate that the process is an effective resistance mechanism in reed canarygrass leaves.

We do not suggest that papilla formation is the only resistance mechanism in reed canarygrass. Other resistance mechanisms undoubtedly occur which restrict the lesion size of resistant plants penetrated by pathogens.

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