

## Relation of Appressorium Pigmentation and Penetration of Nitrocellulose Membranes by *Colletotrichum lagenarium*

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### ABSTRACT

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Seventeen albino mutants of *Colletotrichum lagenarium* were obtained by ultraviolet irradiation or treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. These mutants formed orange colonies and colorless appressoria, whereas the parent strain formed dark-brown colonies and pigmented appressoria. More than 90% of the mutant appressoria germinated laterally and formed secondary appressoria within 48 hr, and consequently <10% penetrated nitrocellulose membranes within 72 hr. In

contrast, appressoria of the parent strain rarely germinated laterally and >70% penetrated the membranes. When conidia of the mutants were incubated in the presence of 3,4-dihydroxyphenylalanine, they behaved like the parent strain, the appressoria became pigmented, lateral germination decreased to 20%, and penetration of membranes increased to 60%. From these results, we conclude that melanin pigments in the appressorial walls are necessary for normal penetration of the host.

*Additional key words:* anthracnose, fungal melanin.

In some plant pathogenic fungi, formation of appressoria is a prerequisite for invasion of host plants (8). It is generally accepted that pigmented, thick-walled appressoria possess the capacity to endure adverse environmental conditions (8), but the physiological role of appressorial pigmentation in plant pathogenic fungi is not sufficiently understood.

Appressorium formation by *Colletotrichum lagenarium* is temperature-sensitive: at 24 C, appressoria with dark-brown pigment were formed synchronously soon after the elongation of germ tubes from spores; 32 C, the germ tubes elongated continuously without forming appressoria (11,15).

In the study reported here, mutants that produced appressoria without pigment were isolated, and their ability to penetrate nitrocellulose membranes was investigated.

### MATERIALS AND METHODS

**Spores.** Spores of *Colletotrichum lagenarium* (Pass.) Ellis and Halsted were prepared as described previously (9).

**Isolation of albino mutants.** Albino mutants that produced appressoria without pigmentation were obtained by ultraviolet light (UV) irradiation or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) treatment. Two milliliters of spore suspension ( $5 \times 10^5$  spores per milliliter) of the parent strain (104-T) in a petri dish (4.5 cm in diameter) was exposed to UV at 150 ergs/cm<sup>2</sup>/sec for 3 min

(7), or treated with NTG at 100 µg/ml for 60 min at 24 C (1). Treated spores were washed twice with ice-cold distilled water and then spread on potato-sucrose agar (PSA) medium (9). After incubation for 7 days at 24 C, albino mutants were selected as orange colonies.

**Spore germination.** Spores were suspended in sterile, deionized water to give about  $10^5$  spores per milliliter. A drop of the spore suspension was placed on a glass slide in a humid petri dish at 24 or 32 C for 24 hr. Several substances closely associated with, or known to be precursors of, melanin biosynthesis were tested for pigment induction in appressoria of the albino mutants. Spores of mutant 79215 were incubated for 48 hr at 24 C in the presence of 3,4-dihydroxyphenylalanine (DOPA), catechol, tyrosine, hydroquinone, and β-naphthol at various concentrations.

**Observation of the penetration process on nitrocellulose membranes.** A nitrocellulose membrane (2 × 2 cm), prepared from Visking cellulose tubing, with a uniform coating of spores was soaked in 2 ml of sterile, distilled water in a petri dish (4.5 cm in diameter) (15). After incubation for 72 hr at 24 C, the spores on the membrane were stained with lactophenol cotton blue or ZnCl<sub>2</sub>/KI solution. Percentages of lateral germination and penetration were based on the number of primary appressoria.

### RESULTS

Among 17 albino mutants, six mutants were obtained by UV irradiation, 10 by NTG treatment, and one appeared spontaneously. Colonies of all these mutants appeared orange on PSA medium and were distinguished easily from colonies of the parent strain, which appeared dark brown on PSA medium (Fig. 1). Growth rates, sporulation on PSA medium, and the temperature ranges for spore germination of these mutants were similar to those

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of the parent strain. All these characteristics of the mutants have remained stable for over 50 generations.

Appressorium formation of the parent strain was temperature sensitive; pigmented appressoria were formed when spores were incubated below 24 C, but were not formed at 32 C (11). These mutants also showed the same temperature sensitivity relative to appressorium formation, but colorless appressoria were formed when spores were incubated for 12 hr at 24 C (Fig. 2A and B). After further incubation at 24 C, about 90% of the appressoria germinated laterally and the tips of germ tubes from colorless appressoria became swollen and formed secondary appressoria. By contrast, lateral germination from pigmented appressoria of the parent strain was rarely observed (Fig. 2A and C). Pigmentation of appressoria of mutant 79215 was induced in the presence of DOPA, whereas pigmentation of appressoria was not induced by other precursors examined (Table 1 and Fig. 2D). Appressorial pigmentation of the other 16 mutants was also induced by the addition of DOPA. Pigmented appressoria induced by DOPA were similar morphologically to those of the parent strain (Fig. 2A and D). Furthermore, in the presence of 1 mM DOPA, lateral germination decreased to 20%, whereas lateral germination from colorless appressoria in the absence of DOPA was about 90% (Table 2). At this concentration, appressorial pigmentation was delayed relative to the parent strain and 10–20% of appressoria had already germinated laterally before appressoria began to darken.

About 70% of appressoria of the parent strain penetrated nitrocellulose membranes after incubation for 72 hr. The membranes around the penetration sites did not stain with ZnCl<sub>2</sub>/KI solution, forming clear haloes (15), which seemed to be caused by chemical dissolution of membranes. Penetration hyphae growing in the membranes were readily observed (Fig. 3A). In albino mutants, however, most appressoria germinated laterally on the surface of the membranes and the percentage of penetration into membranes from appressoria was below 10% (Figs. 3B and 4). The penetration was mostly from the primary appressorium, and lateral germination and secondary appressorium formation were

not observed from primary appressoria that had penetrated membranes. On the other hand, when spores of mutants were incubated in the presence of 1mM DOPA for 72 hr at 24 C, appressoria with pigment were formed and lateral germination was suppressed. From about 60% of these pigmented appressoria, penetration into the membranes, formation of haloes, and elongation of penetration hyphae were observed (Figs. 3C and 4).

## DISCUSSION

In this experiment, 17 albino mutants that formed orange colonies on PSA medium were obtained by UV irradiation, by NTG treatment, or spontaneously. These mutants formed colorless appressoria, whereas the parent strain formed pigmented appressoria. This result suggests a close relationship between colony color and pigmentation of appressoria in *C. lagenarium*.

In all albino mutants, lateral germination from colorless appressoria occurred very frequently in contrast to the parent

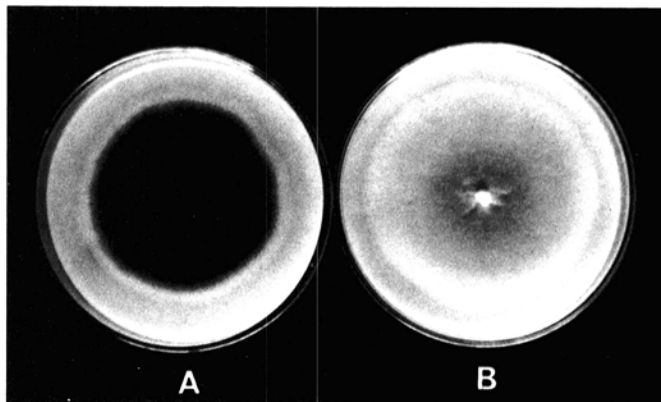


Fig. 1. Colonies of parent strain 104-T (A) and mutant 79215 (B) of *Colletotrichum lagenarium* on PSA medium.

TABLE 1. Effect of several precursors of melanin biosynthesis on spore germination, appressorial formation, and pigmentation of mutant 79215 of *Colletotrichum lagenarium*<sup>a</sup>

Precursor	Concentration (mM)	Germination (%)	Appressorial formation (%)	Appressorial pigmentation
DOPA	10	87.8	93.8	+
	1	95.0	98.0	+
Catechol	10	0	0	—
	1	70.2	96.5	—
Tyrosine	1	98.7	100.0	—
	0.1	13.7	21.4	—
β-naphthol	0.01	100.0	96.7	—
	10	0	0	—
Hydroquinone	1	77.0	90.9	—

<sup>a</sup>Spores were incubated at 24 C for 48 hr on a glass slide.

TABLE 2. Effect of DOPA on lateral germination from primary appressoria of mutants of *Colletotrichum lagenarium*<sup>a</sup>

Isolate	DOPA (1 mM)	Percentage lateral germination
104-T	—	2.0
	+	6.0
79215	—	98.3
	+	20.3
8054	—	89.4
	+	21.3
8119	—	97.8
	+	22.1

<sup>a</sup>Spores were incubated at 24 C for 48 hr on a glass slide in the presence (+) or absence (—) of DOPA.

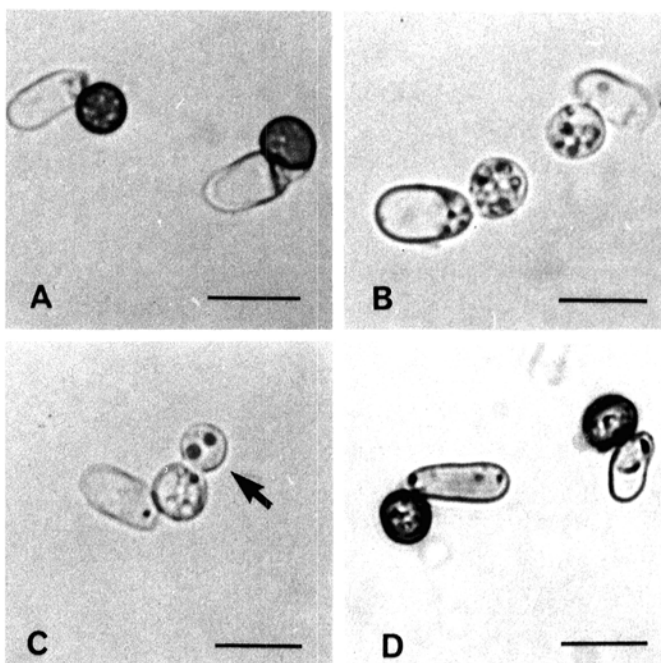


Fig. 2. Appressoria of the parent strain and mutant 79215 of *Colletotrichum lagenarium*. Spores were incubated at 24 C on glass slides: (A) pigmented appressoria of the parent strain at 24 hr; (B) colorless appressoria of mutant 79215 at 24 hr; (C) secondary appressorium (←) of mutant 79215 at 48 hr; (D) pigmented appressoria of mutant 79215 at 48 hr in the presence of 1 mM DOPA. Bar markers represent 10 μm.

strain, and the percentage of penetration into nitrocellulose membranes from these appressoria was markedly lower than that of the parent strain. Moreover, these contrasting characteristics of the mutants were partially reversed when pigmentation of appressoria was induced by DOPA. These results suggest that both the increase in lateral germination from appressoria and the decrease in penetration into nitrocellulose membranes in albino mutants are due to the lack of pigmentation of appressoria. It is generally accepted that the dark-brown pigment occurring in fungi possesses characteristics typical of melanin (2,4,5). Since pigmentation of appressoria of albino mutants was induced by DOPA, a melanin precursor, it is probable that the 17 albino mutants isolated in this experiment are defective in melanin biosynthesis.

In general, pigmented, thick-walled appressoria possess the capacity to endure adverse conditions (8), and cell walls with melanin possess more structural rigidity than do those without

melanin, due to the physical properties of melanin (3,6,12). Campbell et al (5) reported that structural changes due to lack of melanin in cell walls of *Alternaria brassicicola* weakened the walls and that their strength was restored by addition of some precursors of melanin biosynthesis, such as catechol. Also, in *C. lagenarium* appressoria, dark pigment was thought to occur in the walls of appressoria (10). Furthermore, in anthracnose fungi such as *Colletotrichum* (10,13) or *Gloeosporium* (14), germ pores were observed at the site of contact of appressoria with the host plant. In our preliminary experiment, germ pores were observed by scanning electron microscopy to form at the point of contact between the membrane surface and the appressoria of the albino mutants

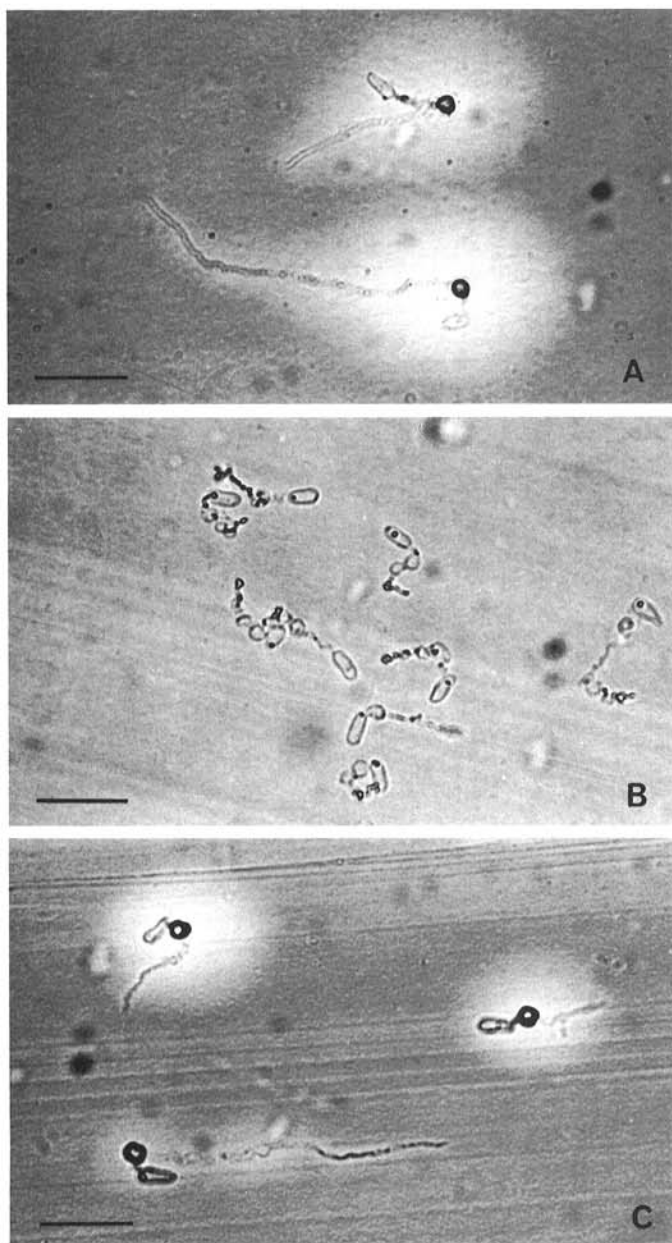


Fig. 3. Penetration into nitrocellulose membranes from appressoria of parent strain 104-T (A) and mutant 79215 (B and C) of *Colletotrichum lagenarium*. Spores were incubated on nitrocellulose membranes at 24 C for 72 hr; (C) Spores were incubated in the presence of 1 mM DOPA. Bar markers represent 30 µm. PH penetration hyphae; SA secondary appressorium.

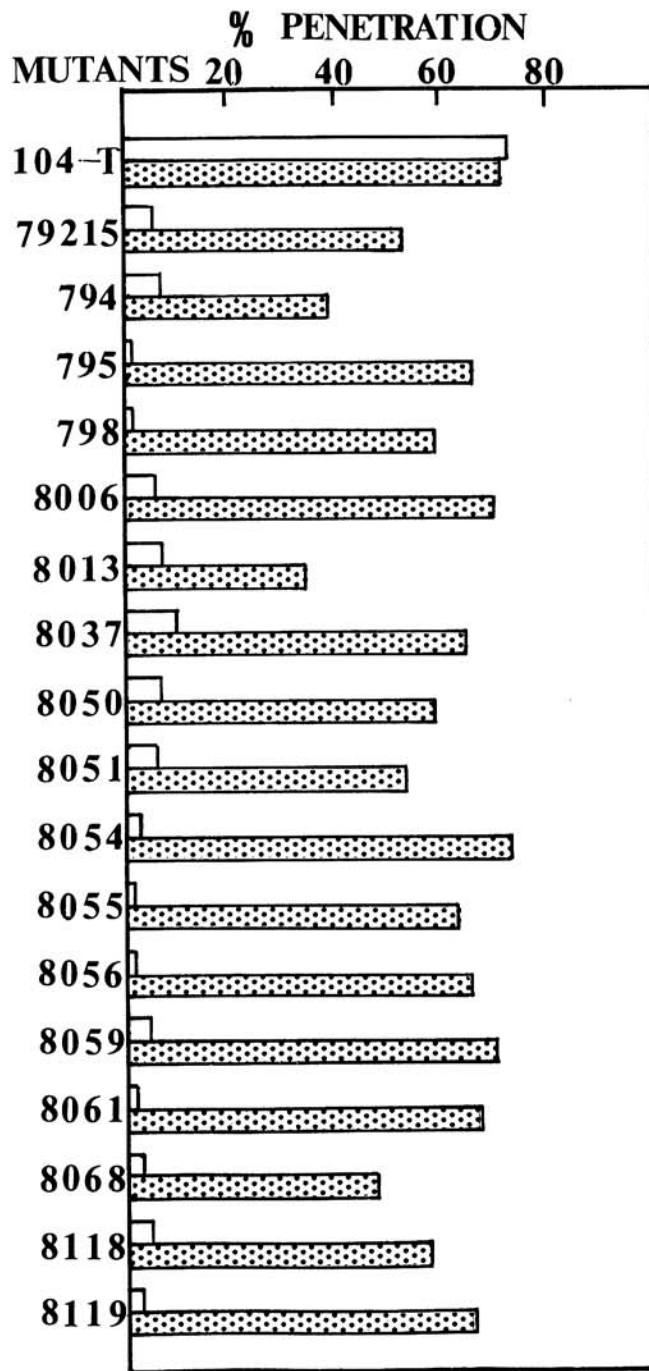


Fig. 4. Restoration of penetration into nitrocellulose membranes from appressoria of 17 albino mutants of *Colletotrichum lagenarium*. Spores of the parent strain 104-T and 17 albino mutants were incubated on nitrocellulose membranes in the absence (□) or in the presence (▨) of 1mM DOPA at 24 C. After incubation for 72 hr, appressoria with penetration hyphae were scored.

(*unpublished*). These facts and the results in this study suggest that appressorial pigmentation suppresses lateral germination and increases penetration from the germ pores of appressoria into nitrocellulose membranes. Perhaps pigmentation of appressoria plays an important role, not only in survival, but also in the ability to penetrate host plants. Inoculation of host plants is the obvious next step.

Over 90% of colorless appressoria of mutants germinated laterally and formed secondary appressoria, as shown in Fig. 2C. Like formation of primary appressoria, formation of secondary appressoria was also temperature sensitive (*unpublished*). Sometimes third and fourth appressoria were formed after further incubation. Thus, appressoria as well as spores of *C. lagenarium* can form appressoria.

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