Effect of Tricyclazole on Appressorial Pigmentation and Penetration from Appressoria of *Colletotrichum lagenarium*

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ABSTRACT

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Tricyclazole (5-methyl-1,2,4-triazole (3,4-b) benzothiazole) inhibited pigmentation of appressoria at concentrations higher than 1 μM in *Colletotrichum lagenarium*, and the color of appressoria varied from dark brown to light brown depending on tricyclazole concentrations. In the presence of 100 μM tricyclazole, appressorial pigmentation was completely inhibited. The colorless appressoria germinated laterally and consequently could not penetrate nitrocellulose membranes. Furthermore, tricyclazole treatment after appressorial pigmentation did not affect penetration into the membranes. These characteristics of appressoria formed in the presence of tricyclazole were the same as those of an albino mutant. On host leaves, colorless appressoria formed in the presence of tricyclazole and those of the albino mutant did not penetrate the host plant cell wall. From these findings, it is concluded that pigmentation of appressoria is essential for host penetration by *C. lagenarium*.

Additional key words: anthracnose of cucumber, fungal melanin, fungicide.

In some plant pathogenic fungi, formation of appressoria is a prerequisite for invasion of host plants (1). Some plant pathogenic fungi form pigmented appressoria, but the physiological role of appressorial pigmentation in the penetration process is not sufficiently understood.

In *Colletotrichum lagenarium* Ellis and Halsted, pigmentation of appressoria is important for penetration of nitrocellulose membranes (4). In the present study, tricyclazole, which is reported to inhibit melanization of several fungi (7–10), was used to establish the morphological and physiological similarity between appressoria formed in the presence of tricyclazole and those of an albino mutant (4). The significance of appressorial pigmentation to the ability to penetrate the cell wall of the host plant is also discussed.

MATERIALS AND METHODS

Spores. The parent strain 104-T of *C. lagenarium* and its albino mutant (4) were cultured on potato sucrose agar medium at 24 C for 7 days. Spores on the mycelial mat were collected with a brush and washed three times by centrifugation with ice-cold distilled water (3).

Spore germination. Spores were suspended in sterile, deionized water to give about 10^5 spores per milliliter. Drops of the spore suspension containing tricyclazole (5-methyl-1,2,4-triazole (3,4-b) benzothiazole) (Eli Lilly Research Laboratories, Greenfield, IN, USA) were placed on a glass slide in a humid petri dish at 24 C.

Observation of the penetration process on nitrocellulose membranes. A nitrocellulose membrane (2 × 2 cm) with a uniform coating of spores was soaked in 2 ml of sterile, deionized water containing tricyclazole in a petri dish (4.5 cm in diameter) (5). To observe the effect of tricyclazole on the formation and elongation of penetration hyphae, spores were incubated on nitrocellulose membranes and the water was exchanged for 2 ml of tricyclazole solution after appressorial pigmentation. After incubation for 72 hr at 24 C, the spores on the membranes were stained with lactophenol cotton blue or $ZnCl_2$/KI solution. The percentage of lateral germination and formation of penetration hyphae was based on the number of primary appressoria (4). Approximately 300 appressoria were observed in each sample.

Hyphal growth. Spores were incubated in the presence of Czapek's liquid medium containing 100 μM tricyclazole at 24 C for 24 hr. Under this condition, germ tubes from spores developed hyphae without forming appressoria. The length of hyphae was measured with a micrometer.

Pathogenicity tests. Cucumber plants (*Cucumis sativus* L. 'Suyo') were grown in vermiculite in a greenhouse at 20–25 C. Thirty microliters of spore suspension (10^5 spores per milliliter) was spotted on the surface of excised leaves. After inoculation, the leaves were placed in humid petri dishes at 24 C, maintained in a dark chamber for 24 hr, transferred into a chamber, and exposed to 1,200 lux of fluorescent light and maintained at 24 C for an additional 4 days. Tricyclazole was applied at the time of inoculation by spotting a spore suspension containing 100 μM tricyclazole on leaves. For microscopic observation, 30 μl of spore suspension was spotted on the lower epidermis of cucumber cotyledons. After 5 days, the lower epidermis was stained with lactophenol cotton blue and observed by light microscopy. Each experiment was replicated three times.

RESULTS

Effect of tricyclazole on appressorial pigmentation. When spores of *C. lagenarium* were incubated at 24 C, dark-brown appressoria were formed within 12 hr (Fig. 1A). Inhibition of appressorial pigmentation by tricyclazole was observed at concentrations >1 μM, and the color of appressoria varied from dark to light brown depending on the concentration of tricyclazole. In the presence of 100 μM of tricyclazole, pigmentation of appressoria was completely inhibited, and colorless appressoria (Fig. 1B) resembling those of albino mutant 79215 (Fig. 1C) were formed. After further incubation at 24 C, over 80% of the colorless appressoria formed in the presence of tricyclazole germinated laterally and formed secondary appressoria as in mutant 79215 (Fig. 1D). Lateral germination from pigmented appressoria was rarely observed in the absence of tricyclazole (4).

Behavior of tricyclazole-treated spores on nitrocellulose membranes. Over 80% of the appressoria in the parent strain penetrated nitrocellulose membranes during incubation for 72 hr at 24 C (Fig. 2). The membrane around the penetration sites did not

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stain with $ZnCl_2$/KI solution; thus, clear haloes were formed, which seemed to reflect chemical dissolution of the membranes (5,6).

In contrast, in the presence of 100 μM tricyclazole, >90% of appressoria germinated laterally on the surface of the membranes, and the percentage of formation of penetration hyphae and haloes was less than 5% (Fig. 2). This behavior was the same as that of albino mutant 79215. The frequency of lateral germination and formation of penetration hyphae depended on the concentration of tricyclazole. The percentage of lateral germination was about 25% at 1 μM tricyclazole and 90% at 100 μM, while the percentage of formation of penetration hyphae decreased conversely from 65% at 1 μM to 3% at 100 μM (Fig. 2). The penetration from appressoria formed in the presence of tricyclazole occurred mostly from primary appressoria that did not germinate laterally.

Tricyclazole at 100 μM did not inhibit hyphal growth in Czapek's liquid medium. Moreover, when 100 μM tricyclazole was applied after appressorial pigmentation, neither the elongation of penetration hyphae nor halo formation was affected.

Pathogenicity. When cucumber leaves were inoculated with a spore suspension of the parent strain, lesions were observed within 5 days. Pigmented appressoria and elongation of penetration hyphae were observed by light microscopy. In contrast, when the leaves were inoculated with spores of mutant 79215 or spores of the parent strain treated with 100 μM tricyclazole, lesions were rarely observed (Fig. 3). Light microscopic observations indicated that the behavior of mutant 79215 and the tricyclazole-treated parent strain was the same as on the nitrocellulose membranes; appressoria were nonpigmented and germinated laterally on the surface of the leaves, and elongation of penetration hyphae was rarely observed.

DISCUSSION

Although tricyclazole is a new systemic agent which controls rice blast disease caused by the fungus *Pyricularia oryzae* (2) and was reported to interfere with melanin biosynthesis in this fungus (7,10), a direct relationship between the inhibition of melanization and blast control has not been established. In the present experiment, we indicated that tricyclazole inhibited appressorial pigmentation of *C. lagenarium* and that the colorless appressoria had little ability to penetrate cucumber leaves.

The color of appressoria varied from dark to light brown depending on the concentration of tricyclazole, and at 100 μM colorless appressoria were formed. The frequency of lateral germination and penetration into the nitrocellulose membranes also depended on tricyclazole concentrations. These results support our previous finding that appressorial pigmentation is necessary for penetration from appressoria (4). The behavior of colorless appressoria formed in the presence of 100 μM tricyclazole was similar to that of appressoria of albino mutant 79215. Neither the development of penetration hyphae in the membranes nor the formation of haloes was affected at this concentration. These results indicate that tricyclazole inhibits specifically appressorial pigmentation during morphogenesis from spores and that other factors involved in the penetration process such as chemical dissolution (5,6) were not affected.

On host leaves, the behavior of spores treated with tricyclazole and those of mutant 79215 was the same as on nitrocellulose membranes. Colorless appressoria germinated laterally on the

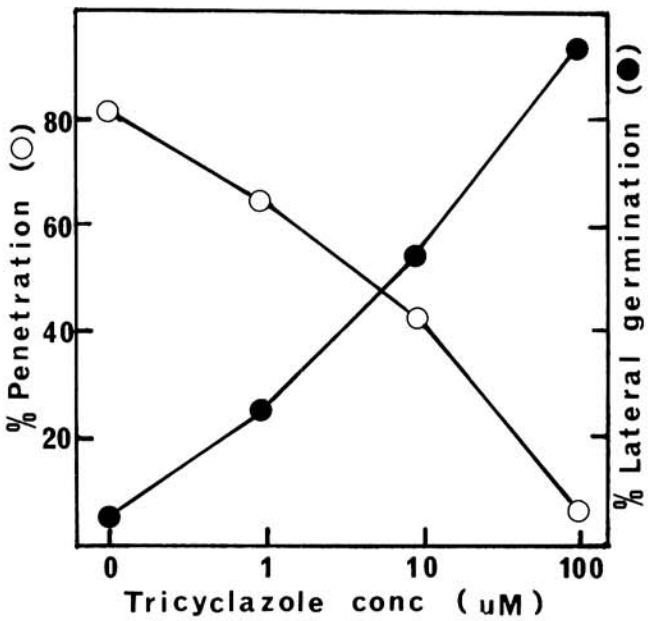

Fig. 2. Effect of tricyclazole on lateral germination (●) and formation of penetration hyphae (○) by primary appressoria of *Colletotrichum lagenarium*. Spores of the parent strain were incubated on nitrocellulose membranes for 72 hr in the presence of various concentrations of tricyclazole. Percentage was based on the number of primary appressoria.

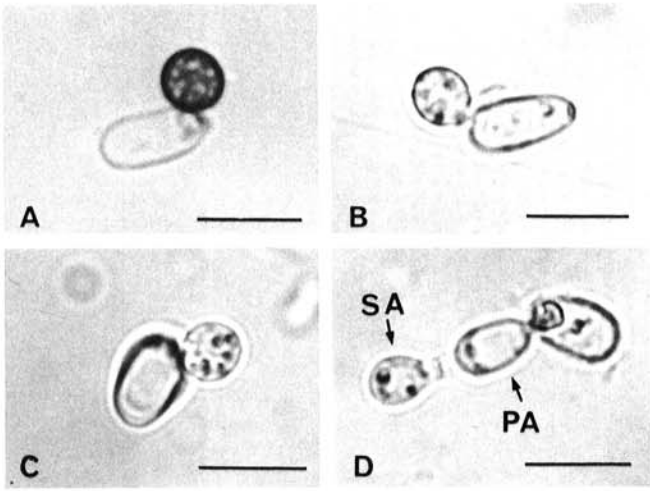

Fig. 1. Effect of tricyclazole on appressorial pigmentation in *Colletotrichum lagenarium*. Spores of the parent strain (104-T) were incubated: in water for 12 hr, **A;** in the presence of 100 μM tricyclazole for 12 hr, **B;** or 24 hr, **D.** Spores of the albino mutant 79215 were incubated for 12 hr, **C.** Bar markers represent 10 μm. PA, primary appressorium; SA, secondary appressorium.

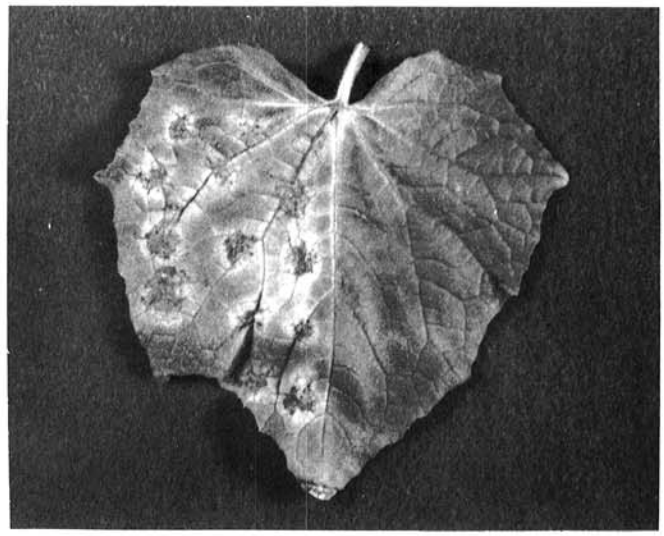

Fig. 3. Pathogenicity of albino mutant 79215 of *Colletotrichum lagenarium*. Drops of spore suspension of the parent strain (left) and mutant 79215 (right) were spotted on the cucumber leaves and incubated for 5 days at 24 C.

surface of the leaves and no lesions were observed. From these results, we conclude that pigmentation of appressoria is essential for host penetration by *C. lagenarium*. Colorless appressoria cannot penetrate, but instead will germinate and form another appressoria just as if they were spores. In contrast, pigmented appressoria germinate vertically and readily penetrate cell walls of host plants. These findings suggest a new approach to the prevention of penetration by appressoria. We consider that pigmentation of appressoria is one of the useful targets for the screening of new fungicides which do not adversely affect the metabolism of host plants.

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