Conidial Germination and Appressorium Formation of *Colletotrichum coccodes* on Tomato Foliage

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ABSTRACT

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Conidial germination and infection processes of *Colletotrichum coccodes* were quantified on foliage of processing tomatoes (*Lycopersicon esculentum* Mill.). The abaxial surface of two opposing terminal leaflets removed from a fully expanded leaf at the 4th to 5th node was inoculated with 10- μ l droplets of *C. coccodes* conidial suspension. Leaflets were incubated for 2 to 24 h in 2-h intervals at 25°C under high relative humidity. Explants with the conidial droplet were fixed, cleared, and preserved for microscopic observation. The percentage of germinated conidia and those with unmelanized and melanized appressoria was determined for each leaf disk. Conidial germination increased linearly with time ($R^2 = 0.73$) ($P = 0.001$), maximizing (68.3%) 24 h after inoculation. The percentage of germinated conidia with unmelanized appressoria peaked 6 h after inoculation (38.3%). Melanized appressoria formation followed a linear trend ($R^2 = 0.74$) ($P = 0.001$), maximizing (62.0%) 24 h after inoculation. Infection vesicles were produced in 2.7% of conidia by 22 h, indicating successful infection.

Additional keywords: anthracnose, foliar, fruit, fungal disease, histological, latent infections, sporulation

Anthracnose (*Colletotrichum coccodes* (Wallr.) S. J. Hughes) is a devastating fungal disease affecting processing tomato fruit grown in the eastern and midwestern United States. Fruit grown for processing are harvested when fully ripe and are especially vulnerable to infection by *C. coccodes* (7,5). Fruit symptoms begin as small, dark, sunken lesions that have a water-soaked appearance, increase in diameter, and coalesce, leaving a large sunken soft area (17). Under favorable temperatures, lesions on ripe fruit become visible within 5 to 6 days after infection (3). Black acervuli are produced just beneath the skin of the infected fruit. As lesions mature, setose sclerotia develop from the stromata of acervuli within the infected fruit tissue (17).

C. coccodes can overwinter as sclerotia in soil and infested tomato debris for at least 1 year (4). Acervuli form on infested debris in soil, producing conidia that serve as the primary source of inoculum of tomato fruit (8,15). Moisture is necessary for

conidial release from the gelatinous matrix in the acervulus. Conidia are disseminated by splashing rain, and severe disease incidence most often is associated with high rainfall during the growing season (3).

Colletotrichum coccodes does not cause foliar blight on tomato, although it can infect foliage, producing small necrotic spots with a chlorotic halo where sporulation may occur (18). Inoculation of tomato seedlings with *C. coccodes* results in necrotic lesions and premature senescence of cotyledons (10). Hausbeck and Linderman (6) determined that at 15°C, a minimum of 16 h of leaf wetness is necessary for infection, whereas only 12 h are necessary at 20 and 25°C. Illman (8) conducted histological studies of foliar infection by *C. coccodes* and determined that appressoria formed by 19 h after inoculation, and a germ tube was present and had infected a neighboring epidermal cell by 42 h after inoculation. However, this study was conducted under unspecified environmental conditions and included observations at only two periods.

The significance of foliar inoculum in fruit infections was investigated by Kendrick and Walker (10) by inoculating plants with an albino, morphologically recognizable strain of *C. coccodes*. After failing to recover the albino strain from infected fruit, the researchers concluded that sporulation of *C. coccodes* in foliar lesions was not a significant source of secondary inoculum for fruit infection (10). However,

these results were likely influenced by the reduced ability of albino isolates to penetrate tissue (12). In contrast, Pantidou and Schroeder (15) concluded from studies using morphologically recognizable *C. coccodes* strains that *C. coccodes* can infect foliage and subsequently sporulate, providing significant inoculum for fruit infection.

The objective of this study was to establish the time necessary for *C. coccodes* conidial germination and infection processes on tomato foliage under controlled environmental conditions, thereby providing the necessary framework for additional studies regarding the role of foliar sporulation in fruit infection.

MATERIALS AND METHODS

Plant culture. Tomato seedlings (cv. Ohio 7814) were grown in a research greenhouse in 168-cm³ cell packs containing Baccto soilless medium (Michigan Peat Company, Houston) for 4 to 5 weeks, then transplanted to individual 15.2-cm clay pots. Plants were watered with drip tubes to prevent leaf wetness and fertilized with 6 g of 15-10-12 slow-release fertilizer per pot (Sierra Chemical Company, Milpitas, CA) at transplanting.

Inoculation. An isolate of *C. coccodes* was isolated from an infected tomato fruit and grown on medium containing V8 juice (30%) (Campbell Soup Co., Camden, NJ) and agar (2%) (Difco Laboratories, Detroit) in 100 by 15 mm petri dishes at 20°C under continuous cool-white fluorescent light. Before inoculation, 10 plates of 14-day-old cultures were flooded with 10 ml of sterile distilled water and gently scraped with a glass rod. The conidial suspension was adjusted to 5×10^5 conidia/ml using a hemacytometer.

Plants with 7 to 9 nodes were chosen. Two opposing terminal leaflets were removed from a fully expanded leaf at the 4th or 5th node and assigned unique incubation treatments. Each leaflet was placed abaxial surface up on filter paper moistened with 2 ml of sterile distilled water in an inverted 100 by 15 mm petri dish. Using a 100- μ l Hamilton syringe (Hamilton Company, Reno, NV), 6 to 8 10- μ l droplets of conidial suspension were placed on the abaxial surface of each leaflet, avoiding areas with pronounced venation. Petri dishes were closed to maintain high relative humidity and prevent evaporation of the inoculum droplets.

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Incubation. Inoculated leaflets were incubated for up to 24 h in a dew chamber (Percival Manufacturing Co., Boone, IA) in continual darkness at 25°C. Samples were removed at 2-h intervals starting 2 h after incubation began. Using a 1-cm-diameter cork borer, 4 disks, each with a conidial suspension droplet, were excised from each incubated leaflet. Leaf disks were fixed with an FAA solution (1:18:1 vol/vol/vol) for 2 h and cleared in a saturated solution of chloral hydrate (250 g/100 ml) for 5 days. Disks were preserved in 1-dram vials of lactophenol solution (20 g phenol, 20 ml lactic acid, 40 g glycerin, 20 ml water) until microscopic observations were made.

Observations. Cleared leaf disks were stained with cotton blue in lactophenol solution (100 ml lactophenol, 1 ml 1% aqueous cotton blue, 20 ml glacial acetic acid) and mounted in glycerol. All conidia within four arbitrarily chosen microscopic fields ($\times 400$) were counted on each of the 8 leaf disks comprising each incubation treatment. The percentage of germinated conidia and those with unmelanized and melanized appressoria was determined for each disk. The percentage of infection vesicles produced by conidia with melanized appressoria was determined on disks incubated for 22 and 24 h. Fields including

pronounced venation where conidia were pooled in large concentrations were avoided.

A conidium was considered germinated if the germ tube was at least as long as the width of the conidium (13) (Fig. 1A). Appressoria were recognized by their globose, sometimes slightly lobed structures formed terminally on a germ tube (Fig. 1B). Melanized appressoria were distinguished by their darkened appearance (Fig. 1C). Infection vesicles were hyaline, globose structures without lobes, adjacent to the melanized appressoria (Fig. 1D). Infection vesicles ranged from one-half to two-thirds the size of the adjacent appressorium.

Replications consisted of the 8 leaf disks taken from the incubated leaflets in each treatment. Each microscopic field was considered one subsample of the leaf disk. The experiment was repeated once. Data were subjected to an arcsine square root transformation in order to normalize the variance (14). Because variance for both experiments was similar using Bartlett's test at χ^2 of 0.95, data were combined before analysis. Analysis of variance (ANOVA), analysis of trend effect, and regression analysis were done using the ANOVA, GLM, and REG procedures of the Statistical Analysis System (SAS Institute, Cary, NC).

RESULTS

Conidial germination increased linearly with time following inoculation ($P = 0.0001$, $R^2 = 0.73$). Two hours after inoculation, 17.9% of conidia had germinated, with a maximum germination (68.3%) 24 h after inoculation (Fig. 2). Conidial germination rarely was associated with trichomes or stomatal openings in this study. Penetration was not associated with anticlinal cell walls as reported by Pantidou and Schroeder (15); instead, there was direct penetration through periclinal cell walls. In addition, germination occurred and appressoria formed on veins in the leaf tissue, conidia were often pooled in high concentrations along the veins. Data were not taken for these sites to insure consistency of epidermal cell type.

Development of melanized appressoria increased linearly with time following inoculation ($P = 0.0001$, $R^2 = 0.74$). Appressoria melanization began 4 h after inoculation (0.69%) and increased to 27.0% 6 h after inoculation (Fig. 3). The percentage of germinated conidia with melanized appressoria maximized 24 h after inoculation (62.0%).

Infection vesicles were produced in 2.7% and 1.8% of conidia with melanized appressoria 22 and 24 h after inoculation, respectively, indicating successful infection by *C. coccodes*.

DISCUSSION

This study provides a chronological guide of the events involved in infection of tomato foliage by *C. coccodes* under controlled environmental conditions. Earlier histological studies of foliar infection by *C. coccodes* were conducted under unspecified environmental conditions and did not include observations before 19 h after incubation (8,15). Results of this study show that much of the infection process is completed by that time. At 25°C, conidia germinated within 2 h of inoculation, with formation of unmelanized appressoria peaking 6 h after inoculation. In this study, melanization of appressoria began 4 h after inoculation. Tomato foliage was successfully infected by 22 h after inoculation, documented by the presence of infection vesicles. The documentation of successful infection of tomato foliage by *C. coccodes* indicates that the role of foliar sporulation in fruit infection should be reexamined using controlled environmental conditions. Previous research concluding that foliar sporulation is an insignificant source of inoculum for fruit infections may not have included environmental conditions conducive to foliar infection. Furthermore, albino isolates used by Kendrick and Walker (10) may have been unable to penetrate intact leaf tissue because of their lack of melanization. Appressorial melanization is necessary for successful penetration of intact tissue by many *Colletotrichum* species (16). Melanin strengthens the appres-

Fig. 1. *Colletotrichum coccodes* conidia germinated at 25°C. (A) Conidia producing germ tubes 2 h after inoculation. (B) Globose, unmelanized appressorium produced 6 h after inoculation. (C) Melanized appressoria produced 18 h after inoculation. (D) Hyaline infection vesicle produced by a melanized appressorium, 24 h after inoculation. Scale bars = 10 μ m.

Fig. 2. *Colletotrichum coccodes* conidia germinated at 25°C. Error bars represent standard deviation of the mean.

Fig. 3. Percentage of germinated *Colletotrichum coccodes* conidia with unmelanized and melanized appressoria at 25°C. Error bars represent standard deviation of the mean.

sorial wall, allowing a buildup of the internal hydrostatic pressure necessary for cuticle penetration (11).

Although intracellular primary hyphae grow from infection vesicles into adjacent cells (1), there were no intracellular primary hyphae during the 24-h observation period in this study. Illman et al. (9) determined that although infection by *C. coccodes* occurs readily in fruit, roots, and stems, development of *C. coccodes* ceases after a brief initial period of rapid growth until the surrounding host tissues become senescent. Hornby (7) observed that while the rate at which *C. coccodes* colonizes healthy tomato roots is initially slow, it increases with the onset of flowering. Cerkaskas (2) detected latent colonization of *Colletotrichum* spp. on tomato stems following surface disinfestation and treatment with paraquat. Using this treatment to simulate the effects of senescence, acervuli developed on previously symptomless stems. Latent infections are common in green fruits where lesion development is delayed until fruit ripening (5). Latency of infection in green fruit can be overcome by a period of low temperature storage prior to ripening at room temperature (9). Further time course studies are needed to determine the length of the latency period and factors involved in releasing latent infections of *C. coccodes* in tomato foliage.

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