Subcuticular-Intracellular Hemibiotrophic and Intercellular Necrotrophic Development of *Colletotrichum acutatum* on Almond

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Accepted for publication 30 March 2005.

ABSTRACT

Diéguez-Urbeondo, J., Förster, H., Soto-Estrada, A., and Adaskaveg, J. E. 2005. Subcuticular-intracellular hemibiotrophic and intercellular necrotrophic development of *Colletotrichum acutatum* on almond. *Phytopathology* 95:751-758.

The early infection and colonization processes of *Colletotrichum acutatum* on leaves and petals of two almond cultivars with different susceptibility to anthracnose (i.e., cvs. Carmel and Nonpareil) were examined using digital image analysis of light micrographs and histological techniques. Inoculated tissue surfaces were evaluated at selected times after inoculation and incubation at 20°C. Depth maps and line profiles of the digital image analysis allowed rapid depth quantification of fungal colonization in numerous tissue samples. The results showed that the early development of *C. acutatum* on petals was different from that on leaf tissue. On petals, conidia germinated more rapidly, germ tubes were longer, and fewer appressoria developed than on leaves. On both tissues, penetration by the pathogen occurred from appressoria and host coloni-

zation was first subcuticular and then intracellular. On petals, colonizing hyphae were first observed 24 h after inoculation and incubation at 20°C, whereas on leaves they were seen 48 to 72 h after inoculation. Intercellular hyphae were formed before host cells became necrotic and macroscopic lesions developed on petals ≥ 48 h and on leaves ≥ 96 h after inoculation. Histological studies complemented data obtained by digital image analysis and showed that the fungus produced infection vesicles and broad hyphae below the cuticle and in epidermal cells. In both tissues, during the first 24 to 48 h after penetration fungal colonization was biotrophic based on the presence of healthy host cells adjacent to fungal hyphae. Later, during intercellular growth, the host-pathogen interaction became necrotrophic with collapsed host cells. Quantitative differences in appressorium formation and host colonization were found between the two almond cultivars studied. Thus, on the less susceptible cv. Nonpareil fewer appressoria developed and host colonization was reduced compared with that on cv. Carmel.

Species of the genus *Colletotrichum* cause economically important diseases on a wide range of crops worldwide (26). On almond (*Prunus dulcis* (Mill.) D.A. Webb), *C. acutatum* J.H. Simmonds causes anthracnose, a disease that under conducive environmental conditions is a serious concern for the almond industry in California (1). *C. acutatum* can affect leaves, branches, fruit, and harvested kernels (1,9).

Infection strategies of *Colletotrichum* spp. and plant-pathogen interactions have been extensively investigated in several systems (19,21). The initial steps of infection among species of *Colletotrichum* studied are very similar. Conidia adhere to the host surface and germinate, and germ tubes either grow to develop a mycelium that forms secondary conidia (not in acervuli) or appressoria (specialized infection structures). Appressoria form penetration pegs that directly penetrate the cuticle and host cell wall leading to colonization of the host. Two basic colonization strategies have been well described in *Colletotrichum* spp. based on colonization and host interaction: (i) intracellular hemibiotrophy, and (ii) subcuticular-intramural necrotrophy (4,19,21).

For the majority of *Colletotrichum* spp., host colonization is initially intracellular (21). The most studied system representing this type of colonization is *C. lindemuthianum* infecting *Phaseolus vulgaris* (17,18). In this pathosystem, the infection peg penetrates the epidermal cell and swells to produce an infection vesicle and broad primary hyphae that may colonize adjacent epidermal and mesophyll cells. During the early stages of this type of coloni-

zation, the interaction between the host and the pathogen is biotrophic and the host cells remain alive (17). The subsequent necrotrophic interaction coincides with the development of visible symptoms in form of water-soaked areas that turn into necrotic lesions. This interaction is also characterized by the formation of thin secondary hyphae (4,16,18,20,27) that grow intra- and intercellularly while secreting cell wall degrading enzymes and killing the host cells (4). A host-pathogen interaction like this that combines a biotrophic and a necrotrophic phase has been termed hemibiotrophy (13).

In other *Colletotrichum* spp., such as *C. capsici* infecting cotton and cow bean (21,22), host colonization is initially subcuticular and intramural. The biotrophic phase is very short or does not occur and the interaction is mostly necrotrophic (19). Following fungal penetration of the cuticle and subcuticular growth, the fungus causes swelling and dissolution of epidermal cell walls prior to entering the necrotrophic phase with the formation of inter- and intracellular hyphae. In this particular infection strategy, from the penetration peg, subcuticular hyphae develop that later give rise to intramural hyphae. No distinct primary and secondary hyphae are produced (19), in contrast to the intracellular hemibiotrophic strategy, and the fungus quickly grows both inter- and intracellularly throughout the tissue causing cell disruption and death (19,21).

Both types of infection strategies, i.e., intracellular hemibiotrophy and subcuticular-intramural necrotrophy, have been reported to occur in one single species, i.e., *C. gloeosporioides* infecting different hosts (21). Thus, with *C. gloeosporioides* infecting tangerines (5) or *Stylosanthes* spp. (25) within the same tissue the initial colonization can be either subcuticular-intramural or intracellular. Another infection strategy has been postulated for

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DOI: 10.1094/PHYTO-95-0751

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C. acutatum (previously reported as *C. gloeosporioides*) on citrus where the pathogen survives as appressoria or possibly biotrophically as quiescent infections on leaves for most times of the year while having a short necrotrophic phase on flowers (3,28). Thus, in this system the two different host interactions are found on different host tissues.

Studies on the colonization of almond by *C. acutatum* have not been done previously and information on the pathobiology of this host-pathogen system is lacking. Furthermore, there are few reports on *C. acutatum* in other host systems (7,10,28). Thus, the goal of our research was to investigate the development of *C. acutatum* on almond from conidial germination to host penetration and colonization and assess the pathogen's infection strategies using light microscopy of whole tissue mounts and histological sections. In addition, we used digital image analysis of light micrographs that allows image interpretations of samples with a large depth of field. Infection and colonization strategies were compared for leaf and flower petal tissues of two commercial almond cultivars.

MATERIALS AND METHODS

Preparation of inoculum and tissue inoculation. An isolate of the predominant "pink" phenotype of *C. acutatum* occurring in California almond orchards (9) was used in the studies. Conidial suspensions (10^6 conidia per ml) were prepared in sterile distilled water from 14-day-old potato dextrose agar cultures grown at 22 to 25°C. Potted almond plants of cvs. Carmel and Nonpareil grown in a greenhouse at 22 to 27°C were used in the experiments. For induction of flowering, plants were incubated at 4 to 5°C for 4 weeks and were then moved back to the greenhouse where they started blooming after approximately 4 weeks. During experiments, plants were kept in growth chambers under controlled environmental conditions at $20 \pm 2^\circ\text{C}$ with a 12-h photoperiod (12,000 to 15,000 lux; Sylvania Cool White F72T12/CW/VHO). Leaves or blossoms attached to the plant were inoculated with 10- μl droplets of the conidial suspension and were spaced 5 mm apart on the lower surface of mature leaves (leaves 5 to 15 from the apex of each shoot) or the upper petal surface of recently opened blossoms. To maintain a relative humidity of >95%, inoculated leaves and blossoms were covered with petri dishes that were supported by stakes and were Parafilm-sealed around the petiole or peduncle, respectively. Tissue samples (2 $\times$ 2 mm²) were taken 3, 6, 12, 24, 36, 48, 72, and 96 h after inoculation and processed as described below. Data were obtained from three independent experiments and at least 10 leaf samples were examined for each period after inoculation in each experiment.

Light microscopy and histology. For direct observations of fungal development, tissue samples (leaves and flower petals) were cleared in saturated chloral hydrate for at least 2 days, stained with 0.05% acid fuchsin for 2 min, washed in distilled water, and mounted on glass slides. Samples were evaluated for conidial germination, appressorium formation, hyphal growth and development, and host penetration by bright field microscopy (Zeiss Axioskop, Zeiss, Germany) at $\times 400$ magnification. To obtain quantitative data on conidial germination, 100 conidia were assessed from each of 10 leaf samples for each of three experiments. To obtain quantitative data on appressorium formation at each incubation period, three fields of view at $\times 400$ (0.2 mm² each field) were assessed for the presence of appressoria in each of 10 leaf samples and the experiment was done three times. The number of appressoria per square millimeter of leaf tissue was expressed as the average for each incubation period and experiment.

For histological studies on fungal penetration and colonization, inoculated tissue samples were fixed in 2.5% glutaraldehyde in 0.05 M sodium phosphate buffer (pH 6.8) for at least 24 h at room temperature. Samples were rinsed three times for 15 min per wash in 0.05 M phosphate buffer (pH 6.8) and dehydrated in a series of

ethanol (10, 35, 50, 70, 80, and 95%) three times for 5 min per concentration. Samples were embedded in JB-4 Embedding Medium (Polysciences, Inc., Warrington, PA) using a 1:2, 1:1, 2:1, 1:0, and 1:0 dilution of resin in ethanol. Transverse, 3- μm -thick sections were obtained with a rotatory microtome (Model 820; American Optical, Buffalo, NY). Sections were stained as described previously and mounted on glass slides for light microscopy.

Digital image analysis of light micrographs. Digital image analysis was used to assess conidial germination and appressorium formation, to measure dimensions of infection structures, as well as to determine fungal penetration and the extent of colonization within host tissues. Images were captured using a Sony DKC-5000 digital camera (Sony Corp. of America, Montvale, NJ) that was mounted on the Zeiss Axioskop compound microscope. Sequential images of inoculated tissues at selected times after inoculation were captured at step-wise intervals of $\approx 1 \mu\text{m}$ using the image capture software and the program Syncroscope-Auto-montage (Microbiology International Inc., Frederick, MD). To generate a single in-focus (montage) image, the program options "blended depth" and a "patch size" between 5 and 20 pixels were used. Depth relief maps and line profile studies were done for three-dimensional analyses as described previously (8).

Data analysis. The number of appressoria formed on petals and leaves was compared using analysis of variance and least significant difference mean separation procedures of the statistical analysis system or SAS, version 6.12 (SAS Institute, Cary, NC). A regression analysis of the average number of appressoria that developed on leaf surfaces of two almond cultivars for three experiments on time after inoculation was done in a one-way fixed-effects treatment structure using SAS. Regression lines were compared following analysis of covariance and mixed model procedures (12). Hypotheses testing for the regression models were done in the following order: slopes equal to zero, slopes equal, or slopes unequal.

RESULTS

Microscopic observations on the infection process and host colonization. Light microscopic observations were made using chloral hydrate-cleared cv. Carmel almond tissues. A composite "montage" image with a large depth of field was generated from digitized sequential images with stepwise focus intervals (Fig. 1A to G). On flower petals and leaves, approximately 98% of conidia ($n = 3,000$) that germinated developed a central transverse septum before starting to germinate. Early development differed on the two tissues. On petals, germ tubes were first observed 3 h after inoculation and incubation at 20°C, whereas on leaves it was after 6 h. Germ tubes were much longer on petals (Fig. 1A) than on leaves (Fig. 1E) and 12 to 24 h after inoculation a network of mycelium could be observed on petals with some hyphae anastomosing (Fig. 1A). On both petals and leaves, appressoria from germ tubes started developing 6 h after inoculation. Appressoria were often located above anticlinal cell walls (Fig. 1B and F) and occasionally above stomata. The percentage of germ tubes differentiating into appressoria was significantly lower ($P < 0.01$) for petal than for leaf tissue, e.g., approximately 30% versus 99%, respectively. On leaves, germ tubes that did not differentiate into appressoria generally continued to elongate. Hyphal anastomosis was observed similar to petal tissues. At 72 h after inoculation, appressoria started developing from mycelium colonizing the leaf surface. On petals, appressorium formation from hyphae was already observed beginning 12 h after inoculation.

The regression models for the number of appressoria that developed on leaves between 0 and 72 h after inoculation were significant for both almond cultivars ($P < 0.001$), and the equations and R^2 values are shown in Figure 2. Slopes for each of the regression models were not equal to zero and were not equal for

the two cultivars studied. Slopes were significantly different ($P = 0.011$) and significantly more appressoria formed on cv. Carmel than on cv. Nonpareil 72 h after inoculation (420 versus 250 appressoria per mm², for cvs. Carmel and Nonpareil, respectively).

Host penetration could be observed microscopically on chloral hydrate-cleared samples. Fungal penetration was evident by the appearance of an internal light spot in the appressorium (Fig. 1B, E, and F). On both petals and leaves, the first internal light spots were observed 12 h after inoculation. Below the cuticle, broad hyphae that developed from the appressoria were seen 24 h after inoculation of petals (Fig. 1B) or 36 to 48 h after inoculation of leaves (Fig. 1F). Colonization of cells was observed 48 h after inoculation of petals (Fig. 1C and D) and 72 h after inoculation of leaves (Fig. 1G). At 72 h after inoculation, approximately 50% of cv. Carmel leaf samples showed extensive fungal colonization, whereas for cv. Nonpareil this was only 20%.

Digital image analysis of light micrographs. A three-dimensional interpretation of host colonization was obtained by digital image analysis where hyphae growing on or below the host surface could be differentiated and line profiles gave estimates of the depth of colonization. Thus, relief maps and line profiles of petals (Fig. 3C and D and Fig. 3E and F, respectively) and leaves (Fig. 4C and D and Fig. 4E and F, respectively) inoculated with *C. acutatum* demonstrated internal colonization including both subcuticular and intracellular growth. Hyphae h1 and h2 in Figure 3A are shown in the line profile of Figure 3E to grow on the petal surface. Hypha ih grew $\approx 12 \mu\text{m}$ deep below the petal surface. Image analyses of a representative petal infection site as shown in the micrograph of Figure 3B is presented in Figure 3D and F. Darker blue shades in the relief image (Fig. 3D) indicate greater depth. Thus, the broad hypha extending to the left from the appressorium extends to a depth of $8 \mu\text{m}$ below the petal surface as indicated in the line profile analysis (Fig. 3F). Hyphae within the host tissue ranged in diameter between 2.2 and $7 \mu\text{m}$.

During the initial host-pathogen interaction no disruption of epidermal cells was observed (Fig. 1C and Fig. 3A and B). At 48 h after inoculation the petal tissue became rapidly colonized by subcuticular and intracellular hyphae (Fig. 1C and D). At this time, intercellular hyphae 1 to $2 \mu\text{m}$ in diameter were formed and host cells became necrotic (Fig. 1D). Macroscopic lesions became visible at this time.

On leaves, colonization of tissues was subcuticular initially with formation of broad hyphae 2 to $5 \mu\text{m}$ in diameter (Fig. 4A and B). Depth relief maps (Fig. 4C and D) and line profiles (Fig. 4E and F) showed that hyphae sometimes grew under the cuticle along the contour of anticlinal epidermal cell walls (Fig. 4A and E). When line profiles were done through the internal light spot of the appressorium as in Figure 4E, a depth relief below the leaf surface was evident at the penetration site. During the initial stages

Fig. 2. Regression of number of appressoria on time after inoculation of leaf tissue of almond cvs. Nonpareil (dashed line) and Carmel (solid line). The slopes of the regression lines are significantly different ($P = 0.011$). Data are the average number of appressoria per square millimeter from 10 leaf samples per experiment. Data from three experiments are shown for each incubation period and almond cultivar.

Fig. 1. Conidial differentiation, host penetration, and colonization of petal (A to D) and leaf (E to G) tissues of almond cv. Carmel by *Colletotrichum acutatum*. Micrographs are montage images from a sequential series of partially focused images. Bars = $5 \mu\text{m}$. **A**, Early development on petal surface 12 h after inoculation. Conidia (cn) have produced long germ tubes (gt) after developing central transverse septa (arrowhead). **B**, Penetration of tissue 24 h after inoculation. An appressorium (ap) with an internal light spot (ils) developed a subcuticular hypha (sh). **C**, Intracellular colonization of epidermal cells 48 h after inoculation. Two appressoria (ap) developed from hyphae and produced a broad intracellular hypha (bh) within an epidermal cell (ec). **D**, Intercellular colonization (arrow) of epidermal cells (ec) 48 h after inoculation. **E**, Early development on leaf surface 12 h after inoculation. Conidia (cn) have formed short germ tubes (gt) and appressoria (ap) with internal light spots (ils). **F**, Penetration of tissue 48 h after inoculation. An appressorium (ap) with an internal light spot (ils) developed above anticlinal cell walls and formed a penetrating hypha (ph). **G**, Intracellular hyphae (ih) have developed from an appressorium (ap) and colonized epidermal cells 72 h after inoculation.

Fig. 3. Digital image analysis of penetration and colonization of petal tissue of almond cv. Carmel by *Colletotrichum acutatum*. Bars = 5 μm . **A**, Montage image of hyphae h1 and h2 grow on the leaf surface and a suspected internal hypha (ih) with dotted outline develops from the appressorium. **B**, Montage image of a suspected internal hypha (ih) developing from an appressorium (ap) 24 h after inoculation. **C and D**, Three-dimensional color relief images of light micrographs **A** and **B**, respectively. The dashed lines indicate where line profile analyses were done. Depth relief is indicated by the scale bar that extends from 2 μm (white) to -8 to -14 (dark blue). The dark blue color of the internal hyphae (ih) indicates that they are below the petal surface. **C**, Hyphae h1 and h2 are light blue indicating that there is no depth relief in this area and that these hyphae are located on the petal surface. **E and F**, Line profile analysis along the above dashed lines showing the degree of penetration into the epidermis. The location of the appressorium (ap) is indicated. **E**, Line profile analysis differentiating hyphae h1 and h2 that are growing on the petal surface from an internal hypha (ih) that is growing 13 μm below the epidermis. **F**, The line profile shows the internal hypha (ih) developing 8 μm below the petal surface.

of colonization the colonized cells maintained their integrity (Fig. 4A and B). Images shown are for almond cv. Carmel. No differences in colonization strategy of leaves by *C. acutatum* were observed for cv. Nonpareil.

Histology of host penetration and colonization. Histological studies on leaf cross sections of cv. Carmel extended observations

made by digital image analysis. Thus, after penetration of the host cuticle (Fig. 5A), infection vesicles 2 to 5 μm in diameter developed subcuticularly (Fig. 5B and E) and were first observed 24 h after inoculation. Subcuticular hyphae 2 to 5 μm in diameter were present beginning 36 to 48 h after inoculation, often lifting the cuticle from the epidermal cells (Fig. 5B and C). Subsequently,

Fig. 4. Digital image analysis of penetration and colonization of almond cv. Carmel leaf tissue by *Colletotrichum acutatum*. **A**, Montage image of conidium (cn), appressorium (ap), infection vesicle (iv), and a subcuticular hypha (sh) growing along anticlinal walls (arrowheads) of epidermal cells 72 h after inoculation. Bar = 5 μm . **B**, Montage image of an advanced stage of internal colonization 72 h after inoculation. The analysis was performed on three internally colonizing hyphae (ih1, ih2, and ih3). Bar = 10 μm . **C and D**, Three-dimensional color relief images of light micrographs **A** and **B**, respectively. The dashed lines indicate where line profile analyses were done. The dotted lines indicate the location of hyphae being studied. Depth relief is indicated by the scale bar that extends from 2 μm (white) to -5 or -10 μm (dark blue). **C**, Blue color indicates that the subcuticular hyphae following the contour of the epidermal cell are below the leaf surface (white color). **D**, Hyphae ih1, ih2, and ih3 are different shades of blue, indicating that there is depth relief in these areas. **E and F**, Line profile analyses showing the degree of penetration into the epidermis. **E**, The line profile shows the subcuticular hypha (sh) 5 μm below the leaf surface. **F**, Line profile analysis indicates the depths of three internal hyphae between 7 and 10 μm below the leaf surface.

these hyphae penetrated into the epidermal cells and formed broad hyphae that grew intracellularly (Fig. 5B and C) and intercellularly (Fig. 5D). These hyphae progressively colonized new epidermal cells while becoming constricted during penetration of cell walls (Fig. 5D). Host cells still appeared to be intact at this stage (Fig. 5C). At 72 h after inoculation disruption of the epidermal cells was first evident (Fig. 5E). Subcuticular hyphae were also observed to grow intercellularly between epidermal cells and subsequently between epidermal and mesophyll cells (Fig. 5F). Narrow secondary hyphae 1 to 2 μm in diameter that were seen 96 h after inoculation grew intercellularly into the mesophyll

tissues (Fig. 5G). The chloroplasts of the mesophyll cells showed disintegration (Fig. 5G) and tissue damage became macroscopically visible as water-soaked areas 4 to 6 days after inoculation.

DISCUSSION

This work constitutes the first study, to our knowledge, on the infection and colonization process of almond tissues by *C. acutatum*. We used novel digital image analysis methods that complemented results obtained on the infection process on flower petals and leaves using traditional microscopic and histological tech-

Fig. 5. Montage images of almond leaf cross sections showing the penetration and colonization by *Colletotrichum acutatum*. Bars for **A to F** = 5 μm . Bar for **G** = 10 μm . **A**, Lower side of healthy leaf with epidermal cells (ec), cell wall (cw), and cuticle (c). **B**, An appressorium (ap) forming an infection vesicle (iv) subcuticularly 36 h after inoculation. The infection vesicle develops broad subcuticular hyphae (sh). Intracellular hyphae (ih) grow in epidermal cells. **C**, A subcuticular hypha (sh) and an intracellular hypha (ih) in an epidermal cell 48 h after inoculation. The protoplasts of the epidermal cells do not appear to be disrupted. **D**, Colonization of an adjacent host cell in the vascular tissue by an intercellular hypha (ih). The hypha becomes constricted during growth through the cell wall (arrow). **E**, Advanced stage of epidermal cell colonization with numerous intracellular hyphae 72 h after inoculation. Below the appressorium (ap) is the infection vesicle (iv). The epidermal cell wall and the cell contents show signs of disintegration (arrowhead). **F**, Subcuticular (sh) and intercellular (arrowheads) colonization 72 h after inoculation; ec = epidermal cell. **G**, Intercellular hypha (arrow) between mesophyll cells 96 h after inoculation. Some host cells are disintegrating (arrowheads).

niques. Digital image analysis software allowed us to generate a single, completely focused montage image with a continuous in-focus depth of field from a series of sequential, partially focused digital micrographs. One of the key advantages of using image analysis on cleared whole tissue mounts was that many samples could be evaluated, resulting in a more representative study of the infection process than can be done when using more laborious histological methods. Digital image analysis previously helped to demonstrate that the internal light spots of the appressoria of *C. acutatum* corresponded to the penetration pores and the developing infection pegs (8). The present study expanded this finding to the next level by focusing on subsequent stages in the infection process. Hyphae on the host surface could be easily differentiated from fungal structures within the host tissue at different depths. Subcuticular and intracellular growth, however, could not be differentiated by digital image analysis because in infected tissue the cuticle sometimes became separated from the epidermis in this host-pathogen system. Thus, depth relief of the colonizing hypha could not always be assigned to subcuticular or intracellular space. In addition, measurement of depth of hyphal growth within whole tissue samples was restricted to the outer cell layer because the resolution of depth in light microscopy is limited by background information from nontarget structures. Thus, the interpretation of digital images has to be carefully considered.

Studies were conducted on attached almond flower petals and leaves. These tissues, in addition to fruits and woody tissues, are infected by the pathogen during disease outbreaks, although infections of leaves are generally of less economic importance. Almond fruits were not used in the current studies for several reasons. Fruits are difficult to obtain on greenhouse-grown plants, they are available in the field for only a short time of the year, and microscopic observations on fruits are difficult due to their dense layer of trichomes. Still, field observations on susceptible almond cultivars suggested a similar susceptibility of leaves and fruits to anthracnose (2).

Early germ tube development was different on petals than on leaves. As on citrus tissues that were inoculated with *C. acutatum* (28), germ tubes on leaves were much shorter than on flower petals where they grew profusely and started anastomosing 12 h after inoculation. The percentage of germ tubes differentiating into appressoria was lower on almond petal than on leaf tissue (30% versus 99%, respectively). Still, all appressoria caused infections on petal tissue with the presence of internal light spots, but some infections may possibly have resulted directly from germ tubes because of the low occurrence of appressoria on germ tubes. This is in contrast to citrus petals where appressoria were very rare and host penetration was concluded to be direct (28). On almond leaves, the fungus occasionally gained access into the host by means of undifferentiated germ tubes that penetrated stomatal openings as observed in several other *Colletotrichum*-host interactions (11,23) including *C. acutatum* on strawberry stolons and petioles (7). Internal light spots that developed in most of the appressoria beginning 12 h after inoculation were taken as indicators for successful penetration (8). On both almond tissues, *C. acutatum* formed appressoria preferentially on the anticlinal walls of epidermal cells as described for strawberry (7,10) and other *Colletotrichum*-host systems (14,15,24). Colonization of almond petals was more rapid than that of leaves, presumably due to the more delicate structure of the petals, but there may also be critical differences in chemical composition. No infection vesicles were observed on petals and hyphae developed directly from the penetration sites of the appressoria.

Tissue colonization strategies of *C. acutatum* on almond were different from the majority of other *Colletotrichum* spp. interactions described previously, including *C. acutatum* on strawberry, the only other *C. acutatum*-host system where the host-pathogen interaction has been studied in detail (7). In this latter system the pathogen began the subcuticular invasion of the host

tissue with a brief biotrophic phase before entering an extended necrotrophic phase. Because the biotrophic phase lasted less than 12 h, Curry et al. (7) considered this colonization strategy a modification of necrotrophy rather than biotrophy or hemibiotrophy. Thus, this relationship could be described as subcuticular-intramural necrotrophy where cells are killed prior to penetration. On both almond tissues, petals and leaves, our observations indicated that *C. acutatum* combined the two known types of infection strategies, i.e., intracellular hemibiotrophy and subcuticular-intramural necrotrophy. Intramural growth, however, could not be verified in our light microscopic studies. Because hyphal diameters often exceeded those of almond cell walls, we assume that intramural growth may not occur in our host-pathogen system. In our studies, we commonly observed subcuticular infection vesicles, subcuticular, intracellular, and intercellular hyphae of different types (including broad primary and narrow secondary hyphae), and an extended biotrophic phase of approximately 3 days in leaves. With these characteristics, this interaction is most similar to intracellular hemibiotrophy with the exception of subcuticular growth which is common in subcuticular-intramural necrotrophy. Thus, almond tissue colonization by *C. acutatum* could be described as subcuticular-intracellular hemibiotrophy and intercellular necrotrophy.

Among the few other *Colletotrichum* species that combine the two known infection strategies is *C. gloeosporioides* on tangerine (5), avocado (6), and *Stylosanthes* spp. (25) and *C. magna* on watermelon (19). Interactions on tangerine and species of *Stylosanthes*, however, were described as only biotrophic, possibly because observations were made up to 72 h after inoculation. In our studies with almond leaves, necrotrophy was not observed until after 72 h after inoculation. A combination of the two infection strategies has also been described for *C. acutatum* on citrus (3,28). The two strategies, however, were restricted to specific host tissues, i.e., blossoms and leaves. The necrotic phase on citrus blossoms was very similar to that on almond blossoms in our study. On almond, however, the biotrophic phase on the leaves developed into a necrotrophic interaction, in contrast to the situation on citrus leaves where the fungus survives as appressoria on the leaf surface and possibly in quiescent infections.

The relative importance of the different stages of subcuticular-intracellular hemibiotrophy and intercellular necrotrophy during colonization of the almond host depended on the tissue infected. Thus, on leaves subcuticular growth usually extended for longer distances than on petals before penetration of epidermal cells occurred. This, in addition to the delayed appressorium formation and penetration of leaves, could explain the later appearance of visible macroscopic symptoms on leaves compared with that on petals. No differences in infection strategies were found when we compared fungal growth on the more susceptible almond cv. Carmel with cv. Nonpareil, which is considered the least susceptible among the cultivars commercially grown in California. Quantitative differences in appressoria formation and host colonization, however, were observed on leaves. Thus, on cv. Nonpareil fewer appressoria developed and host colonization was reduced compared with that on cv. Carmel. Appressoria are responsible for host penetration and consequently a reduction in the number of appressoria on any tissue may be related to lower levels of disease incidence and severity.

ACKNOWLEDGMENTS

We thank the Almond Board of California and the J. M. Ogawa Endowment for financially supporting this project.

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