Characterization of *Colletotrichum acutatum* Isolates Causing Anthracnose of Almond and Peach in California

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ABSTRACT

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The causal organism responsible for the recent outbreak of almond and peach anthracnose in California was identified and characterized as *Colletotrichum acutatum*. Isolates of *C. acutatum* from almond were found to be similar to California strawberry isolates and South Carolina peach and apple isolates of *C. acutatum* based on conidial morphology, temperature relationships, fungicide sensitivity, and polymerase chain reaction (PCR) methods using DNA species-specific primers. On almond, blossoms and immature or mature fruit were affected by the disease, causing direct losses of crop. On peach, the disease was observed only on mature fruit. Pathogenicity of almond and peach isolates of *C. acutatum* was demonstrated on wound- and nonwound-inoculated almond or peach fruit by fulfilling Koch's postulates. Conidial morphology of isolates was variable, depending on the medium or substrate used to culture the isolates. Isolates of *C. acutatum* from strawberry, almond, and peach were grouped together based on a similar response to temperature, with an optimal growth rate at 25°C (generally less than 10 mm/day), whereas isolates of *C. gloeosporioides*

from citrus and papaya had an optimal growth rate at 30°C (generally greater than 10 mm/day). In fungicide disk assays, isolates of *C. acutatum* from strawberry, peach, and apple, as well as almond and peach isolates from California, were less sensitive to benomyl at 300, 600, or 1,200 µg/ml. In contrast, *C. gloeosporioides* isolates from citrus and papaya were very sensitive to benomyl at all concentrations evaluated. All isolates of both species were sensitive to captan (300, 600, or 1,200 µg/ml). Oligonucleotide primers were synthesized for *C. acutatum*, *C. fragariae*, or *C. gloeosporioides* using published DNA sequences from the internal transcribed spacer 1 region of ribosomal DNA. Thirty-two *Colletotrichum* isolates from almond fruit produced DNA products with a *C. acutatum* primer (CaInt-2) that matched products and approximate molecular weight of known *C. acutatum* isolates. No PCR products were produced with primers for *C. gloeosporioides* or *C. fragariae*. Isolates from citrus and papaya produced DNA products only with primers from *C. gloeosporioides* or *C. fragariae*. Thus, worldwide, anthracnose of almonds may be caused by either *C. gloeosporioides*, as previously reported, or by *C. acutatum*, as indicated in this study.

Additional keywords: fruit and nut tree diseases, *Prunus dulcis*, *P. persica*.

The genus *Colletotrichum* Corda in Sturm. contains an extremely diverse number of fungi including both plant pathogens and saprophytes. Plant pathogenic species are important worldwide, causing pre- and postharvest losses of crops (26,30). These fungi cause diseases commonly known as anthracnose of grasses, legumes, vegetables, small fruit, and perennial tree crops (30). The disease can occur on leaves, stems, and fruit of host plants. Typical symptoms include sunken subcircular or angular lesions that produce erumpent, mucilaginous, pink spore masses under conducive environments. In California, three species have been reported on strawberry: *C. gloeosporioides* (Penz.) Penz. & Sacc. in Penz., *C. acutatum* J.H. Simmonds, and *C. fragariae* (12,14); whereas only *C. gloeosporioides* has been reported from almond (19). One of the most common species of the genus is *C. gloeosporioides* (teleomorph *Glomerella cingulata*). This species causes anthracnose on a wide range of plants including temperate and tropical fruits (8,18) and has been reported from almond (19) and citrus (22). *C. fragariae* has only been reported on strawberry in California (12,14), but *C. acutatum* occurs on a wide variety of hosts (7).

Almond and peach anthracnose have rarely been reported in California (19,27). The disease was first reported on almonds from Napa and Alameda counties in 1915 and was considered to be caused by *Gloeosporium amygdalinum* (5). The disease on almonds also occurs in Italy (5), France (13), Israel (20), and South Africa

(6). von Arx (29) considered *G. amygdalinum* a synonym of *C. gloeosporioides* and identified the pathogen of almond anthracnose as the latter fungus (20). In California, the disease was observed more recently in orchards in northern California in the mid-1980s by cooperative extension personnel and was reported to the Almond Board of California by J. E. Adaskaveg and J. M. Ogawa after several outbreaks on almond in central and northern California from 1992 to 1996 (1,2). Peach and nectarine anthracnose is commonly observed in the southeastern United States, but is uncommon in California. In 1990, the disease was reported as a postharvest pathogen of peaches and nectarines in Fresno County (19).

Species of *Colletotrichum* have been identified almost exclusively based on morphological and cultural characteristics. Characters such as conidial morphology, presence or absence of setae, presence or absence of the teleomorph, colony color, production of pigments, and growth rate have been used to differentiate species. *C. gloeosporioides* has cylindrical conidia with rounded ends, develops setae and the teleomorph state, and the dark gray colonies have an average growth rate of 13 to 14 mm/day (14,18). Isolates of *C. fragariae* are similar to *C. gloeosporioides*, but lack the teleomorph and produce abundant conidia. *C. acutatum* forms fusiform conidia, rarely produces setae, lacks a teleomorph, and the light gray colonies have an average growth rate of 8 to 9 mm/day (7, 14). Identification of these species, however, is difficult, because of the extreme variation in morphology accepted for *C. gloeosporioides* (26) and other species of the genus. Isolates of these species have overlapping ranges of conidial morphology and show wide variation in colony characteristics. Attempts to distinguish strawberry isolates of three *Colletotrichum* species based on the foregoing characteristics have been limited (26).

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Recently, Mills et al. (17) determined the nucleotide sequence of the internal transcribed spacer (ITS) 1 region of several *Colletotrichum* species. Based on this information, primers were developed and used with the conserved primer ITS 4 (31). These primers were shown to be effective for identification of *C. coccodes*, with no DNA products produced with other closely related species. The primers also were useful for identification of *C. gloeosporioides*, *C. fragariae*, and *C. kahawae*. The primer developed and tested for *C. acutatum*, however, was not effective in distinguishing this species from *C. gloeosporioides* or *C. fragariae* isolates from strawberry. Other techniques such as arbitrarily primed polymerase chain reaction (PCR) also have been used to differentiate *Colletotrichum* species (10,11,15,16).

TABLE 1. Isolates of *Colletotrichum* species, isolation hosts, and source location of collections

Isolate ^x	Host ^y	Source location ^z
EEB 99	Strawberry	California
EEB 100	Strawberry	Yolo, CA
EEB 101	Strawberry	Yolo, CA
EEB 1995	Strawberry	California
EEB 1997	Strawberry	California
JEA 92-5	Almond	Merced, CA
JEA 92-6	Almond	Merced, CA
JEA 92-8	Almond	Merced, CA
JEA 92-16	Almond	Butte, CA
JEA 92-18	Almond	Butte, CA
JEA 95-1	Almond	Butte, CA
JEA 95-2	Almond	Butte, CA
JEA 95-3	Almond	Butte, CA
JEA 95-4	Almond	Butte, CA
JEA 96-1	Almond	Merced, CA
JEA 96-2	Almond	Merced, CA
JEA 96-7	Almond	Merced, CA
JEA 96-8	Almond	Merced, CA
JEA 96-14	Almond	Merced, CA
JEA 96-15	Almond	Merced, CA
BLT 95-3	Almond	Stanislaus, CA
BLT 95-4	Almond	Stanislaus, CA
BLT 95-5	Almond	Stanislaus, CA
BLT 95-6	Almond	Butte, CA
BLT 95-7	Almond	Butte, CA
BLT 95-8	Almond	Butte, CA
BLT 95-9	Almond	Butte, CA
BLT 95-10	Almond	Butte, CA
BLT 95-11	Almond	Butte, CA
BLT 95-17	Almond	Stanislaus, CA
BLT 95-18	Almond	Stanislaus, CA
BLT 95-19	Almond	Stanislaus, CA
BTM 95-4	Peach	Yuba, CA
BTM 95-5	Peach	Yuba, CA
BTM 95-6	Peach	Yuba, CA
BTM 95-7	Peach	Yuba, CA
BTM 95-8	Peach	Yuba, CA
EIZ 719	Peach	South Carolina
EIZ 720	Peach	South Carolina
EIZ 721	Peach	South Carolina
EIZ 722	Peach	South Carolina
EIZ 723	Apple	South Carolina
JMO 94-22	Citrus	Yolo, CA
RJH 96-1	Papaya	Hawaii
RJH 96-2	Papaya	Hawaii
RJH 96-3	Papaya	Hawaii
RJH 96-4	Papaya	Hawaii
RJH 96-6	Papaya	Hawaii
RJH 96-7	Papaya	Hawaii
RJH 96-9	Papaya	Hawaii
RJH 96-10	Papaya	Hawaii

^x Identification codes refer to isolate numbers in the culture collection of the following researchers: EEB, E. E. Butler; JEA, J. E. Adaskaveg; BLT, B. L. Teviotdale; BTM, B. T. Manji; RJH, R. J. Hartin; and EIZ, E. I. Zehr.

^y Isolates from citrus and papaya are *C. gloeosporioides*, all others are *C. acutatum*.

^z Source location in the United States. For almond isolates within California, county of collection is also provided.

Because of the recent outbreak and the sporadic occurrence of almond anthracnose in the history of almond production in California and the potential to resolve *Colletotrichum* species using classical and newly developed molecular techniques, the hypothesis tested was that a species of *Colletotrichum* other than the previously reported pathogen of almond anthracnose, *C. gloeosporioides*, was responsible for the recent disease outbreaks. Thus, the objectives were to collect isolates of *Colletotrichum* species from almond and peaches in California; compare these isolates with reference isolates of *C. acutatum* and *C. gloeosporioides*; characterize cultures of *Colletotrichum* isolates with regard to pathogenicity, conidial morphology, and sensitivity to selected fungicides; and evaluate primers based on published DNA sequences of a variable region in the ITS 1 of *C. gloeosporioides*, *C. acutatum*, and *C. fragariae* for the identification of the *Colletotrichum* isolates collected.

MATERIALS AND METHODS

Collection of isolates. Thirty-two isolates of *Colletotrichum* were obtained from diseased fruit of almonds and peaches, single-spore-cultured, grown on Difco potato dextrose agar (PDA; Difco Laboratories, Detroit) at 25°C, and evaluated after 7 to 10 days for macroscopic cultural characteristics. Isolates, collection codes, original hosts, and source locations are listed in Table 1. These collections were compared with stock collections of five strawberry isolates from California and four peach isolates and one apple isolate from South Carolina designated as *C. acutatum*. Additionally, one citrus isolate from California and eight papaya isolates from Hawaii designated as *C. gloeosporioides* were also compared (Table 1). Cultures were maintained on PDA slants at 4°C.

Conidial morphology and temperature relationships. For morphological observations and measurements of conidia, representative isolates from each host were grown on PDA, pea straw agar (PSA), or almond fruit. Pea straw was sterilized using propylene oxide for 48 h (28), and several leaf and stem pieces were added to molten water agar that was poured into petri dishes (approximately 50 mg/15 ml). Plates of PDA and PSA were simultaneously inoculated using 100 µl of a conidial suspension (1×10^5 conidia/ml) of each isolate, and the suspensions were spread over the surface of the medium using a glass rod. Almond fruit were surface-disinfested, wounded, placed in plastic containers, and inoculated with 20 µl of a conidial suspension (1×10^5 conidia/ml). Inoculated plates and fruit were incubated under continuous fluorescent light for 3 to 4 days at 25°C. To determine morphology, conidia were mounted in water, observed using differential interference contrast microscopy, and photographed using a Sony DKC-5000 digital camera (Sony Corp. of America, Montvale, NJ) mounted on a Zeiss Axioskop compound microscope (Carl Zeiss, Inc., Thornwood, NY). One hundred conidia of each isolate were measured in length and width (at the middle of the spore), and the experiment was conducted twice. The factorial experiment for fungal isolate and medium or substrate was evaluated using an analysis of variance and means were separated using Duncan's multiple range test provided in the statistical algorithms of SAS version 6.04 (SAS Institute, Cary, NC).

Colony diameter of selected isolates was recorded daily for 1 week for cultures grown on PDA in the dark at 10, 15, 20, 25, 30, 35, or 40°C. Petri plates containing PDA were inoculated in the center of each plate with a 6-mm-diameter plug obtained from the margin of a 5- to 7-day-old culture also grown on PDA. Three replications of each isolate at each temperature were evaluated, and the experiment was repeated once. Growth rate was calculated as the 7-day average of mean daily growth rate (millimeters per day) for each culture and temperature.

Fungicide sensitivity assays. To determine the sensitivity of isolates to captan and benomyl, PDA petri plates were simultaneously inoculated with a conidial suspension of each isolate as described above. Filter paper disks (12 mm in diameter), previously satu-

rated with 100 μ l of 0, 300, 600, or 1,200 μ g/ml of either benomyl or captan and air-dried, were placed on the surface of inoculated PDA plates. The plates were incubated for 3 days at 25°C, the radius of the inhibition zone was recorded for each fungicide concentration evaluated, and plates were photographed using a Sony DKC-5000 digital camera (Sony Corp. of America). Three replications of each isolate for each fungicide concentration were evalu-

ated, and the experiment was repeated once. The factorial experiment was evaluated using general linear model procedures and means were separated using Tukey's studentized range test provided in the statistical algorithms of SAS version 6.04 (SAS Institute).

Pathogenicity studies. Approximately 6 to 10 weeks after bloom, almond fruit, cultivar Drake, were used in pathogenicity studies with almond isolates JEA 92-6 or JEA 92-16. In laboratory studies,

Fig. 1. Conidial morphology of *Colletotrichum* isolates from **A, B, and C**, strawberry (isolate EEB 101); **D, E, and F**, almond (isolate JEA 92-8); **G, H, and I**, citrus (isolate JMO 94-22); and **J, K, and L**, papaya (isolate RJH 96-9) grown on **A, D, G, and J**, potato dextrose agar (column one); **B, E, H, and K**, pea straw agar (column two); and **C, F, I, and L**, almond fruit (column three). Bars = 20 μ m.

detached fruit were surface-disinfested for 2 min in a 0.84% (vol/vol) solution of NaOCl (household bleach), rinsed in sterile distilled water, and then air-dried at 25°C. Fruit were either nonwounded and spray-inoculated to run-off or wounded (1 × 1 × 0.5 mm) using a sterile glass rod and inoculated with 20 µl of a conidial suspension of each fungus (30,000 conidia/ml). Conidia were obtained from 5- to 7-day-old cultures grown on PDA. Fruit were then placed on metal racks in plastic containers (30 × 20 × 10 cm) containing 100 ml of water (fruit were not in contact with the water) and incubated for 5 to 7 days at 25°C and >95% relative humidity. In field studies, nonsurface-disinfested, attached fruit on terminal branches were inoculated as previously described for both inoculation techniques. The branch was then placed in a plastic bag containing a wet paper towel, covered with a brown paper bag, and the closed bags were tied to the almond branch. Bags were removed after 18 h of wetness, and fruit were evaluated for disease incidence after 10 to 12 days. Tissue from diseased fruit was excised, surface-disinfested as described above, and placed on PDA. Sterile water was used in check treatments. For laboratory studies, four replications of five fruit were used for each inoculation treatment, and the experiment was conducted twice. For field studies, four single-tree replications of 15 to 20 fruit per replication were used for each inoculation treatment, and the experiment was done twice. Data were analyzed using analysis of variance and Duncan's multiple range test for mean separation procedures using the statistical algorithms of SAS version 6.04 (SAS Institute).

Additionally, almond fruit, cultivar Drake, or peach fruit, cultivars Ross or Elegant Lady, were wounded, placed in plastic containers, and inoculated using a conidial suspension as described previously with either of two almond isolates (JEA 92-6 and 92-8), one peach (BTM 95-4), one strawberry (EEB 99), one citrus (JMO 94-22), or one papaya isolate (RJH 96-4). Disease incidence was determined after fruit were incubated for 5 to 7 days at 25°C and >95% relative humidity. Three replications of three fruit were used, and the experiment was done twice. Data were analyzed using analysis of variance and Duncan's multiple range test for mean separation procedures using the statistical algorithms of SAS version 6.04 (SAS Institute).

Extraction of fungal DNA. Total DNA was extracted from mycelium obtained from cultures grown on PDA for 2 to 3 days at 24°C. Aerial mycelium was removed from a 2-cm² area of each culture using a sterile transfer needle and placed in a sterile 1.5-ml microcentrifuge tube containing 300 µl of extraction buffer (0.2 M Tris-HCl, 0.25 M NaCl, 25 mM EDTA, and 2% sodium dodecyl sulfate, pH 8.5). Tubes were uncapped and placed in a boiling water bath for 5 min and then cooled to 25°C; 200 µl of phenol that was equilibrated with extraction buffer (vol/vol), and 200 µl of chloroform were added. The tubes were vortexed for 4 min, and

then centrifuged at 13,844 × g (12,000 rpm) for 5 min. The supernatant was pipetted to a new sterile 1.5-ml tube and 200 µl of chloroform was added; the mixture was vortexed for 30 s and then centrifuged at 13,844 × g for 15 min. The supernatant was again transferred to a new 1.5-ml tube and 200 µl of isopropanol was added; the capped tube was inverted several times to adequately mix and precipitate DNA and then centrifuged at 13,844 × g for 15 min. The supernatant was discarded, and the nucleic acid pellet was washed in 400 µl of 70% ethanol and centrifuged at 13,844 × g for 5 min. Again the supernatant was discarded, and the nucleic acid pellet was air-dried for 10 to 15 min, resuspended in 50 µl of low-TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 8.5), and gently agitated to dissolve the DNA. DNA was treated with Ribonuclease A (R4875; Sigma Chemical Co., St. Louis) for a final concentration of 10 µg of RNase/ml for 1 h at 37°C.

PCR analysis. Species-specific target primers were synthesized for *C. gloeosporioides* (CgInt) 5'-GGCCTCCCGCCTCCGGGCGG-3', for *C. acutatum* (CaInt-1) 5'-GGCGCCGGCCCCACACGGGGA-3' and (CaInt-2) 5'-GGCGCCGGCCCCGTACGGGGG-3', and for *C. fragariae* (CfInt) 5'-GGCCTCCCGCCCCCGGCGG-3'

Fig. 2. Temperature relationships of *Colletotrichum* isolates from **A**, citrus (isolate JMO 94-22) and papaya (isolates RJH 96-4, 96-9, and 96-10); and **B**, strawberry (isolate EEB 99), almond (isolates JEA 92-8 and 92-18), and peach (isolate BTM 95-4). Growth rate was calculated over a 5-day period at each temperature. Values are the average of three replications per isolate and temperature. The experiment was conducted twice.

TABLE 2. Comparison of length-width ratios of conidia from selected isolates of *Colletotrichum* spp. originally obtained from anthracnose lesions on fruit and grown on potato dextrose agar (PDA), pea straw agar (PSA), or inoculated almond fruit^y

Isolate ^y	Original host	Length-width ratio ^z		
		PDA	PSA	Fruit
EEB 99	Strawberry	3.8 ± 0.5 a	3.6 ± 0.4 b	3.5 ± 0.5 b
JEA 92-8	Almond	2.6 ± 0.4 f	3.2 ± 0.3 c	3.1 ± 0.4 d
JEA 92-18	Almond	3.1 ± 0.4 d	3.5 ± 0.4 b	3.0 ± 0.4 d
BTM 95-4	Peach	2.9 ± 0.5 e	3.2 ± 0.3 c	3.3 ± 0.5 c
JMO 94-22	Citrus	3.28 ± 0.5 c	2.9 ± 0.3 d	3.0 ± 0.5 d
RJH 96-4	Papaya	3.54 ± 0.7 b	3.9 ± 0.3 a	4.3 ± 0.4 a

^y Cultures of each isolate were inoculated on PDA or PSA and incubated for 3 days at 25°C; on almond fruit, cultures were inoculated and incubated for 5 to 7 days at 25°C.

^z Ratios are based on the average of 100 length-width (µm) ratios of 100 conidia per isolate for each medium or substrate. Values followed by the same letter were not significantly different based on Duncan's multiple range test ($P < 0.05$).

from ITS 1 of the ribosomal DNA (rDNA) gene based on published DNA sequences (17). Target primers were used with the conserved primer ITS 4 (31). Primers were synthesized by Oligos Etc., Inc. (Wilsonville, OR).

PCR amplification was performed in a 25- μ l reaction mixture containing 1.5 μ l of DNA extract (approximately 1 to 5 ng of DNA) in low-TE buffer; 4 μ l of 200 μ M each of dATP, dCTP, dGTP, and dTTP; 2.5 μ l of 10 $\times$ *Taq* reaction buffer (500 mM KCl, 100 mM Tris-HCl [pH 9.0], and 1% Triton X-100); 0.5 μ l of 100 μ M MgCl₂; 1.0 μ l of 1 μ M target primer; 1 μ l of 1 μ M ITS 4 primer; 0.13 μ l of *Taq* DNA polymerase (0.65 units); and 14.37 μ l of sterile water. A thermocycler (model PTA-100; MJ Research Inc., Watertown, MA) was programmed for 35 cycles of denaturation

(94°C for 1 min), annealing (59°C for 2 min), and DNA extension (72°C for 2 min). Following amplification, PCR products and λ -Hind III marker were separated using electrophoresis in 1% (wt/vol) agarose gels in Tris-HCl buffer, stained in ethidium bromide (0.4 μ g/ml), viewed, and photographed using Polaroid black and white film (Polaroid Corp., Cambridge, MA) on a UV transilluminator.

RESULTS

Cultural and conidial morphology. Among the peach and almond isolates collected from California, aerial mycelium was grayish to pink with bright orange spore masses produced outward from the center of the culture, whereas the reverse or underside

TABLE 3. Growth rate at three selected temperatures, relative sensitivity to benomyl and captan in in vitro disk assays, and polymerase chain reaction (PCR) amplification products using CaInt-2 or CgInt primers for isolates of *Colletotrichum acutatum* and *C. gloeosporioides* evaluated

Isolate ^x	Growth rate (mm/day)				Fungicide sensitivity ^y		PCR amplification products with primers ^z	
	20°C	25°C	30°C	35°C	Benomyl	Captan	CaInt-2	CgInt
EEB 99	5.9	6.7	6.0	0.2	—	+	+	—
EEB 100	4.6	5.4	3.4	...	—	+	+	—
EEB 101	4.6	5.3	3.3	...	—	+	+	—
EEB 1995	4.8	5.6	5.1	...	—	+	+	—
EEB 1997	4.6	5.5	3.9	...	—	+	+	—
JEA 92-5	7.3	8.1	7.2	...	—	+	+	—
JEA 92-6	7.0	8.5	7.3	...	—	+	+	—
JEA 92-8	6.5	7.5	7.3	0.6	—	+	+	—
JEA 92-16	7.4	8.5	8.1	...	—	+	+	—
JEA 92-18	6.7	7.6	7.0	2.1	—	+	+	—
JEA 95-1	7.9	8.1	7.8	...	—	+	+	—
JEA 95-2	7.6	8.0	7.8	...	—	+	+	—
JEA 95-3	4.3	5.3	5.1	...	—	+	+	—
JEA 95-4	7.3	7.8	7.1	...	—	+	+	—
JEA 96-1	6.0	6.4	5.6	...	—	+	+	—
JEA 96-2	7.8	8.3	7.5	...	—	+	+	—
JEA 96-7	5.7	6.2	5.6	...	—	+	+	—
JEA 96-8	7.7	8.8	8.2	...	—	+	+	—
JEA 96-14	7.5	9.9	8.7	...	—	+	+	—
JEA 96-15	4.9	6.4	6.0	...	—	+	+	—
BLT 95-3	7.7	8.8	7.3	...	—	+	+	—
BLT 95-4	7.7	8.5	7.2	...	—	+	+	—
BLT 95-5	5.8	6.9	5.7	...	—	+	+	—
BLT 95-6	5.9	6.3	5.7	...	—	+	+	—
BLT 95-7	5.9	6.5	5.3	...	—	+	+	—
BLT 95-8	7.9	8.4	7.7	...	—	+	+	—
BLT 95-9	4.4	4.9	2.9	...	—	+	+	—
BLT 95-10	7.7	8.3	7.2	...	—	+	+	—
BLT 95-11	7.9	8.9	8.3	...	—	+	+	—
BLT 95-17	5.9	6.8	5.7	...	—	+	+	—
BLT 95-18	8.1	8.8	8.9	...	—	+	+	—
BLT 95-19	9.1	9.2	7.8	...	—	+	+	—
BTM 95-4	6.6	7.8	5.9	3.1	—	+	+	—
BTM 95-5	7.4	7.9	7.4	...	—	+	+	—
BTM 95-6	7.1	8.7	8.7	...	—	+	+	—
BTM 95-7	7.7	8.4	7.2	...	—	+	+	—
BTM 95-8	7.2	8.5	7.1	...	—	+	+	—
EIZ 719	8.2	9.5	8.2	0.0	—	+	+	—
EIZ 720	8.4	9.6	5.6	...	—	+	+	—
EIZ 721	5.6	9.1	8.1	...	—	+	+	—
EIZ 722	8.6	9.4	7.7	...	—	+	+	—
EIZ 723	7.8	8.6	5.9	...	—	+	+	—
JMO 94-22	...	8.5	12.1	5.9	+	+	—	+
RJH 96-1	...	6.2	6.4	0.0	+	+	—	+
RJH 96-2	...	5.7	5.6	0.0	+	+	—	+
RJH 96-3	...	12.6	16.6	5.0	+	+	—	+
RJH 96-4	...	8.7	11.9	4.9	+	+	—	+
RJH 96-6	...	12.6	14.8	2.8	+	+	—	+
RJH 96-7	...	12.6	16.0	6.5	+	+	—	+
RJH 96-9	...	11.4	14.2	5.1	+	+	—	+
RJH 96-10	...	12.6	15.6	4.5	+	+	—	+

^x Identification codes refer to isolate numbers in the culture collection of the following researchers: EEB, E. E. Butler; JEA, J. E. Adaskaveg; BLT, B. L. Teviotdale; BTM, B. T. Manji; RJH, R. J. Hartin; and EIZ, E. I. Zehr.

^y Fungicide sensitivity of isolates based on fungicide disk assays (1,200 μ g/ml): — = no or low sensitivity, inhibition zones absent or with some colonies within inhibition zone; and + = sensitive, no colonies within inhibition zones.

^z PCR amplification with CaInt-2 or CgInt and ITS 4 ribosomal DNA primers with genomic DNA of isolates: + = DNA product present; — = DNA product absent.

was commonly orangish and occasionally reddish (pinkish) to gray. Strawberry isolates from California and peach and apple isolates from South Carolina were similar in cultural morphology. Cultures obtained from citrus and papaya had grayish aerial mycelium with bright orange spore masses. The reverse of cultures was mostly grayish-white to greenish.

For each of the isolates evaluated, conidial morphology was compared when grown on PDA, PSA, or almond fruit (Fig. 1). On PDA, conidia ranged from short (almond isolate) or long (citrus and papaya isolates) elliptical cells with rounded ends to elliptical cells with acute ends (strawberry isolates). On PSA and almond fruit, conidia of the almond isolates had more acute ends, similar to conidia of the strawberry isolates (Fig. 1) and peach and apple isolates from South Carolina. Cell ends of conidia of the citrus and papaya isolates were rounded on PSA and almond fruit (Fig. 1). The independent variables, isolate, medium, and their interaction, had significant effects on length ($P < 0.10$), width ($P < 0.0001$), and length/width (L/W) ratio ($P < 0.0001$) of conidia of each isolate evaluated. No differences were observed between experiments ($P > 0.05$). In comparisons of L/W ratio, all isolates were significantly different when grown on PDA (Table 2). On PSA, L/W ratio of conidia from the citrus (JMO 94-22) and papaya (RJH 96-4) isolates were significantly different ($P < 0.05$) from all other isolates (Table 2). L/W ratios of the latter two isolates were also different from each other. On almond fruit, L/W ratios of conidia from almond and citrus isolates were not significantly different ($P > 0.05$), whereas those of strawberry, peach (from CA), and papaya isolates were significantly different ($P < 0.05$) from the other isolates and from each other (Table 2).

Temperature relationships. Citrus and papaya isolates had optimal growth rates at 30°C (Fig. 2A, Table 3); whereas almond, peach, and strawberry isolates had similar temperature responses with optimal growth rates of 25°C (Fig. 2B, Table 3). All isolates grew at 10 and 35°C (approximately 5 mm/day), but did not grow at 40°C after 5 days (Fig. 2A and B).

Sensitivity of *Colletotrichum* isolates to benomyl and captan. All isolates evaluated were sensitive to captan (Fig. 3A, Table 4) and showed a variable response to benomyl (Fig. 3B, Table 4). Comparing fungicides for all concentrations and isolates evaluated, inhibition zones caused by captan and benomyl were significantly different ($P < 0.001$) and averaged 3.9 and 6.1 mm, respectively. For each fungicide, the general linear model was significant

with a high level of confidence ($P < 0.001$), and the independent variables concentration, original isolation host, and their interaction had significant ($P < 0.001$) effects on the dependent variable inhibition zone. For captan, inhibition zones increased with fungicide concentration for isolates grouped by original isolation host (Table 4). Although significant differences ($P < 0.02$) in size of the inhibition zones caused by high rates of captan were observed among the grouped isolates, the inhibition zones were similar in size (Table 4). In contrast, inhibition zones caused by benomyl were significantly different ($P < 0.01$) for isolates grouped by original isolation host (Table 4). Several groups were formed depending on the fungicide concentration. In general, citrus and papaya isolates of *C. gloeosporioides* were highly sensitive to benomyl, with inhibition zones ranging from 14.7 to 19.0 mm in radius for all concentrations evaluated. Almond and peach isolates of *C. acutatum*, however, were less sensitive, with mean inhibition zones ranging from 1.4 to 8.3 mm in radius. With these isolates, numerous colonies formed within the inhibition zones (Fig. 3). The strawberry

TABLE 4. Toxicity of benomyl or captan (300, 600, or 1,200 µg/ml) in agar disk assays to selected isolates of *Colletotrichum* spp. identified by their original host^y

Fungicide	Original host	Isolates	Mean radius of inhibition zone (mm) per fungicide concentration (µg/ml) ^z		
			300	600	1,200
Captan	Citrus	JMO 94-22	4.7 a	4.8 ab	6.6 ab
		RJH 96-4	3.4 a	5.9 a	8.6 a
		RJH 96-7			
	Almond	JEA 92-8	4.5 a	5.0 ab	6.7 ab
		JEA 92-18			
		JEA 95-4			
Benomyl	Peach	BTM 95-1	3.4 a	6.0 a	6.7 ab
		EEB 99	3.0 b	3.8 b	5.1 b
	Strawberry				
	Citrus	JMO 94-22	18.5 a	18.5 a	19.0 a
		RJH 96-4	14.7 b	14.7 a	15.7 a
		RJH 96-7			
	Almond	JEA 92-8	2.5 c	3.0 bc	3.3 c
		JEA 92-18			
		JEA 95-4			
	Peach	BTM 95-1	1.4 c	4.1 b	8.3 b
		EEB 99	0.0 c	0.0 c	0.2 c

^y Potato dextrose agar plates were simultaneously inoculated with each isolate using 100 µl of a conidial suspension (30,000 conidia/ml), filter disks saturated in 0, 300, 600, or 1,200 µg/ml were placed on the agar surface, and the plates were incubated for 3 days at 25°C. No inhibition zone occurred at the 0 µg/ml concentration of each fungicide evaluated.

^z Mean radius of inhibition zones of six replications from two experiments (three replications/experiment) for the isolates evaluated for each host and fungicide concentration. For each fungicide and concentration, values followed by the same letter were not significantly different using Tukey's studentized range test ($P < 0.01$).

TABLE 5. Effect of inoculation method on disease incidence of almond fruit inoculated with an isolate of *Colletotrichum acutatum* from almond under laboratory and field conditions

Inoculation method ^y	Incidence (%) of fruit infection ^z	
	Laboratory	Field
Check	0 c	0 c
Nonwounded, spray	40.0 b	7.8 b
Wounded, drop	100.0 a	67.3 a

^y Fruit were inoculated with JEA 92-6 or 92-16 (30,000 conidia/ml). For both field experiments, daily average temperature and relative humidity ranged from 57 to 75°C and 37 to 66%, respectively.

^z Evaluations were made after 10 days. For laboratory studies, values are the average of eight (five fruit/replication) replications from two experiments. For field studies, values are the average of eight replications (15 to 20 fruit/replication) from two experiments. Values within each column followed by the same letter were not significantly different based on Duncan's multiple range test for mean separation ($P < 0.05$).

Fig. 3. Fungicide disk assay showing inhibition zones of three *Colletotrichum* isolates (petri dishes; left to right): strawberry (isolate EEB 101), citrus (isolate JMO 94-22), and almond (isolate JEA 92-8) for A, captan; or B, benomyl. Disk concentrations in each plate were 0, 300 (top disks; left to right), 600, and 1,200 µg/ml (bottom disks, left to right) of either captan or benomyl. Note the numerous colonies within inhibition zones of the almond isolate to benomyl. Bars = 21 mm.

isolate of *C. acutatum* was least sensitive, with a mean inhibition zone of 0.2 mm at only the 1,200 µg/ml of concentration of benomyl. No differences were observed between experiments ($P > 0.10$).

Pathogenicity of *Colletotrichum* isolates on almond and peach. In both laboratory and field studies using almond fruit, wounded and nonwounded inoculations using two almond isolates resulted in fruit infections (Table 5). Fruit symptoms from both inoculations were similar to those observed naturally in the field, and reisolations yielded the *Colletotrichum* isolates. Significant differences ($P < 0.05$) were observed between inoculation methods (Table 5). In both laboratory and field studies, wounded drop inoculations produced a higher incidence of disease than did non-wounded spray inoculations. In laboratory wound-inoculation studies, all isolates evaluated were pathogenic to almond and peach fruit. No difference ($P > 0.05$) in disease incidence caused by the isolates evaluated was observed.

Molecular characterization of *Colletotrichum* isolates. Four species-specific target primers from ITS 1 and primer ITS 4 regions were used in PCR to amplify rDNA from genomic DNA of the isolates used in this study (Table 1). Both CaInt primers specific to *C. acutatum* produced PCR products of approximately 0.5 kb

(as indicated by the λ -Hind III marker) with strawberry, almond, apple, and peach isolates (Figs. 4A and C and 5A). No PCR products were produced with either CaInt (-1 or -2) primer for the citrus and papaya isolates of *C. gloeosporioides* (Table 3). PCR products were produced with the CgInt primer for the citrus and papaya isolates of *C. gloeosporioides* (Figs. 4B and 5B), however, the CfInt primer only produced products with the papaya isolates (Fig. 4D). Neither CgInt nor CfInt primers produced PCR products with *C. acutatum* isolates from strawberry, almond, and peach (Figs. 4B and D and 5B). No PCR products were produced with water checks in any amplifications. A summary of DNA amplification products for primers CaInt-1 or CgInt and ITS 4 for all isolates used in this study is shown in Table 3.

DISCUSSION

Almond and peach isolates of *C. acutatum* were demonstrated to be pathogenic on wounded and nonwounded fruit and were reisolated, fulfilling Koch's postulates. An anthracnose disease outbreak of fruit on almond and isolated occurrences on peach in California were caused by these isolates. Almond and peach isolates

Fig. 4. Amplification products and markers in agarose gels stained with ethidium bromide for ITS ribosomal DNA primers **A**, CaInt-2; **B**, CgInt; **C**, CaInt-1; and **D**, CfInt and photographed using transmitted UV light. Isolates of *Colletotrichum* species are indicated across the top, and original isolation hosts across the bottom. JMO and RJH isolates are *C. gloeosporioides* and all others are *C. acutatum*. Marker used was λ -Hind III, and molecular weights are shown on the right for each gel. LW = loading well.

from California were compared with strawberry, peach, and apple isolates of *C. acutatum* and citrus and papaya isolates of *C. gloeosporioides* using classical criteria such as conidial morphology, temperature relationships, and fungicide sensitivity, as well as by PCR amplification techniques based on published rDNA sequence information of selected *Colletotrichum* species. Based on these comparative studies, isolates of *Colletotrichum* from almond and peach in California were characterized as most similar to *C. acutatum*, as in a preliminary study (2), rather than the previously reported *C. gloeosporioides* (19,20). In our study, almond and peach isolates from California produced a PCR amplification product of approximately the same molecular weight as that from known *C. acutatum* isolates from strawberry, apple, and peach (from SC). Temperature relationships and benomyl sensitivity were less definitive, but also indicated similarity of the almond and peach isolates to strawberry, apple, and peach (from SC) isolates of *C. acutatum*. Using these criteria, all of the almond and peach isolates from California were distinct from the *C. gloeosporioides* isolates from papaya and citrus. Freeman et al. (9), using arbitrarily primed PCR, also indicated that almond isolates from California were distinct from almond isolates from Israel. On peach and other fruit tree crops, Bernstein et al. (4) indicated that two species of *Colletotrichum*, *C. gloeosporioides* and *C. acutatum*, occur in the southeast United States. Thus, this is the first report of *C. acutatum* on almond and the second report of this species on peach. Both reports are new for California.

In a previous study of isolates of *Colletotrichum* (17), the CgInt and ITS 4 primers produced PCR products with DNA from *C. gloeosporioides*, *C. fragariae*, and *C. kahawae*, but not with DNA from *C. acutatum* or several other species of *Colletotrichum*. In our study, the CgInt and ITS 4 primers only produced PCR products with citrus and papaya isolates representing *C. gloeosporioides*. Interestingly, the CfInt and ITS primers only produced PCR products with papaya isolates and not with our citrus isolate. The CfInt primer differed from the CgInt primer in three base pairs

(bp) and, thus, differences among isolates of *C. gloeosporioides* could be detected with the use of these primers. Sreenivasaprasad et al. (23,24), using restriction fragment length polymorphism analyses of ribosomal and mitochondrial DNA and sequence analyses, however, suggested that, because of the molecular similarity of *C. gloeosporioides*, *C. fragariae*, and *C. kahawae*, they should not be considered separate species.

Our use of a 23- and a 22-bp sequence from a variable region of ITS 1 was specific to *C. acutatum* isolates in North America. In contrast, Mills et al. (17) used a 17-bp ITS 1 primer that produced DNA products with isolates of *C. acutatum*, but also with one isolate each of *C. gloeosporioides* and *C. fragariae*. Recently, Sreenivasaprasad et al. (25) also developed a *C. acutatum*-specific, 17-bp primer from the variable ITS 1 region of *C. acutatum* that also amplified DNA extracted from infested strawberry tissue. These specific primers probably can be used for rapid and accurate identification of almond tissues infected by *C. acutatum* in California.

Growth rates of almond and peach isolates from California and isolates of *C. acutatum* from strawberry, apple, and peach (from SC) were generally slower at all temperatures compared with isolates of *C. gloeosporioides*. Maximum growth rates for *C. acutatum* isolates were between 5 and 10 mm/day after 5 days at 25°C, whereas isolates of *C. gloeosporioides* had maximum growth rates between 10 and 15 mm/day after 5 days at 30°C. These results are consistent with other studies that used temperature relationships to distinguish *C. acutatum* from *C. gloeosporioides* on other hosts (4,14,21,26).

The fungicide sensitivity assays using benomyl indicated differences between *C. acutatum* and *C. gloeosporioides*. Strawberry isolates showed no inhibition zones at the 300 and 600 µg/ml of concentrations of benomyl evaluated. Almond and peach isolates showed some sensitivity, with an average inhibition zone of 1.4 to 8.3 mm to the concentrations of benomyl (300 to 1,200 µg/ml) evaluated. Numerous small colonies, however, also developed within the inhibition zone, suggesting a possible selection of benomyl-resistant subpopulations among conidial suspensions of each isolate. In contrast, all concentrations of benomyl produced large distinct inhibition zones to citrus and papaya isolates. Bernstein and Miller (3) and Bernstein et al. (4) also indicated the sensitivity of *C. gloeosporioides* and the insensitivity of *C. acutatum* to benomyl. Benomyl has been used commonly in both strawberry fields and almond/peach orchards in California for management of gray mold (*Botrytis cinerea*) and brown rot (*Monilinia* spp.), respectively. We can speculate that the recent outbreak of anthracnose in almonds and peaches is from endemic populations of *C. acutatum* that were previously exposed to benomyl or that the species is inherently resistant to benomyl and was introduced into California. More importantly, these results indicate that benomyl may not provide disease control in the field, whereas captan should provide adequate management against *Colletotrichum* isolates from almonds and peach in California.

The limited number of morphological and cultural characters of *Colletotrichum* species, the lack of standardization of cultural conditions among different researchers around the world, and the inherent plasticity of individual isolates of these fungi have led to confusion and uncertainty in the characterization of many species of *Colletotrichum* (26). Additionally, many species of *Colletotrichum* have been recorded on a large number of hosts and, thus, species concepts within the genus are broad and have been based on morphology, presence of a teleomorph, and host symptoms. Identification based only on these criteria often lacked consistency and precision (26). We confirmed these inherent taxonomic problems with the species of *Colletotrichum* found on almonds and peach in California using classical morphological criteria. The range of cultural characteristics overlapped somewhat. Conidial morphology was variable depending on the medium or substrate used. Similarly, Gunnell and Gubler (14) found that a strawberry

Fig. 5. Amplification products and markers in agarose gels stained with ethidium bromide for ITS ribosomal DNA primers **A**, Calnt-2; and **B**, CgInt and photographed using transmitted UV light. Isolates of *Colletotrichum* species are indicated across the top, and original isolation hosts across the bottom. JMO and RJH isolates are *C. gloeosporioides* and all others are *C. acutatum*. Marker used was λ -Hind III, and molecular weights are shown on the right for each gel. LW = loading well.

leaf medium allowed for isolates of *C. acutatum*, *C. gloeosporioides*, and *C. fragariae* to be distinguished morphologically. In our study, L/W ratios were not as useful for distinguishing the *Colletotrichum* species evaluated. Lack of standardization of cultural conditions to obtain conidial morphology data for species identification has lead to greater taxonomic confusion (26).

Freeman et al. (9), using PCR analyses, identified 57 almond isolates from nine different geographical regions within Israel as *C. gloeosporioides*. Using four distinct primers, almond isolates from California were different from those from Israel, but they were not identified to species (9). Our collection of California isolates from almond are similar to and can be characterized as *C. acutatum*, although slight differences in conidial morphology and benomyl sensitivity were observed. Additional molecular comparisons are needed to determine if almond and peach isolates from California are distinct populations of *C. acutatum*.

In the current study, all of the *Colletotrichum* isolates evaluated were able to infect wounded almond fruit regardless of the original host. Both avocado and almond isolates of *C. gloeosporioides* from Israel were able to infect nonwounded almond fruit, with relatively little difference in severity; however, almond isolates caused only small infections on avocado (9). Bernstein et al. (4) also indicated that *C. acutatum* and *C. gloeosporioides* occur on apple, peach, and pecan. Because these species have a broad host range and they occur on strawberry (14), other species of *Colletotrichum* may occur on almond in California. Larger samples of populations of *Colletotrichum* from almond and peach in California are needed to determine if *C. gloeosporioides* or other species occur on *Prunus* spp. in addition to *C. acutatum*. Currently, on a worldwide basis, almond anthracnose may be caused by populations of either *C. gloeosporioides* (e.g., Israel), as previously reported, or by *C. acutatum* (e.g., California), as indicated in this study.

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