Identification of Subpopulations of *Colletotrichum acutatum* and Epidemiology of Almond Anthracnose in California

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ABSTRACT

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In recent years, almond anthracnose has developed into a major problem for the California almond industry. The identification of the causal pathogen as *Colletotrichum acutatum* was confirmed using species-specific primers and restriction fragment length polymorphisms of ribosomal DNA in comparative studies with isolates of *C. acutatum* from strawberry and *C. gloeosporioides* from citrus. Two distinct clonal subpopulations among the almond isolates of *C. acutatum* were identified. These two subpopulations differed in their colony appearance (pink versus gray cultures), conidial morphology, virulence in laboratory inoculation studies, temperature relationships for growth, and molecular fingerprints using random

and simple-repeat primers in polymerase chain reactions. Both subpopulations were commonly isolated from the same orchard or even the same fruit. In other orchards, one subpopulation predominated over the other subpopulation. Using random, simple-repeat, and species-specific primers, isolates of the almond anthracnose pathogen from Israel were very similar to the California isolates that produce gray colonies. In addition to fruit, the pathogen was isolated from blighted blossoms, water-soaked or necrotic leaf lesions, symptomless peduncles, and spurs and wood from branches showing dieback symptoms, indicating that the amount of tissue that may be infected is more extensive than previously considered. Overwintering fruit mummies were identified as inoculum sources for early spring infections. Growth studies using almond kernels with different moisture contents indicated that postharvest damage of stored kernels likely originates from preharvest field infections.

Almond anthracnose was first reported in California in 1916 (5); however, only in recent years has the disease become a widespread problem in the major California almond-growing areas, causing substantial economic losses (2). A study was initiated following several outbreaks of the disease from 1992 to 1996. The causal organism was characterized using conidial morphology, temperature relationships for growth, molecular techniques, and fungicide sensitivity, and was identified as *Colletotrichum acutatum* J.H. Simmonds (2). Globally, almond anthracnose has been reported to be caused by two species of *Colletotrichum*, *C. acutatum* as identified in California and *C. gloeosporioides* (Penz.) Penz. & Sacc. in Penz. as identified in Israel (8,9,21). The disease has also been found in Italy (12), France (11,12), South Africa (6), and Tunisia (11), but unfortunately, these reports did not include a description of the causal pathogen.

Previously, symptoms of almond anthracnose have been primarily described on fruit (2,18). Early season fruit infections develop as depressed orangish lesions on the hull. Infections on mature fruit are also orangish lesions that commonly produce profuse amber-colored gum and develop through the hull into the kernel. Many of the infected fruit become mummified, and these fruit may remain on the tree throughout tree dormancy. Symptoms on blossoms, leaves, and twigs have also been associated with the disease (18). Diseased blossoms become blighted, with an appearance similar to brown rot blossom blight caused by *Monilinia laxa*. Under wet conditions, blossoms with anthracnose develop orange acervuli on the outer hypanthium. On branches bearing diseased fruit, dieback of twigs and premature defoliation may occur. Leaf symptoms range from watery spots to irregular chlorotic or necrotic lesions that occur primarily at the leaf margins. The pathogen, however, has not been isolated from woody tissues or leaves (21). More recently, anthracnose damage was also found on processed almond

kernels in storage (1). Symptoms included brown and purplish discolorations of the kernel tissue that commonly originated at the suture of the kernel. The postharvest damage was correlated with a high preharvest incidence of the disease (1).

The disease is favored by high rainfall in the spring (21). Economic losses for the California almond industry due to anthracnose were most severe in 1998, following conducive conditions during the warm and unusually wet spring and early summer weather (J. E. Adaskaveg, *unpublished data*). This increasing and widespread occurrence of almond anthracnose in California in recent years has stimulated our continued research interest in the disease. Considering the occurrence of two species of *Colletotrichum* on almond worldwide, we continued to characterize the anthracnose pathogen in California using molecular techniques. Specifically, the objectives of this research were to verify the pathogen's identity, investigate relationships between isolates of *C. acutatum* from almond and other hosts in California, investigate relationships between isolates from almond in California and in Israel, and determine the possible presence of subpopulations of the pathogen on almond. Studies were also done to investigate the distribution of *C. acutatum* populations within California and within selected orchards. Additionally, we wanted to determine the extent of colonization of almond tissues by *C. acutatum* and, subsequently, whether or not inoculum sources other than colonized fruit could be identified. This was done because, in previous studies, most isolates were obtained from infected fruit tissue, which contrasts the wide range of disease symptoms found on almond trees. Last, we wanted to determine if the occurrence of postharvest kernel infections was a result of either postharvest colonization or late season, preharvest infections of mature kernels by the fungus.

MATERIALS AND METHODS

Disease surveys, collection of diseased almond tissue, and fungal isolations. Plant tissues were collected in 37 different orchards throughout the Central Valley from 1992 to 1998 from the

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following counties: Butte, Tehama, Glenn, Sutter, Colusa, San Joaquin, Stanislaus, Merced, Fresno, Tulare, and Kern. The five primary counties surveyed were Butte, Glenn, Merced, San Joaquin, and Stanislaus. The greatest distance between orchards sampled in these counties was 580 km. Orchards surveyed in this study were selected because of concerns of growers or farm advisors of the possible occurrence of almond anthracnose. The almond cultivars sampled included Butte, Carmel, Fritz, Harvey, Mission, Monterey, Peerless, Price, Thompson, NePlus Ultra, Nonpareil, Merced, and Padre. Mummified fruit were collected in February from trees from four orchards in 1997 and from one orchard in 1998. Samples were also taken of blighted blossoms within 2 to 4 weeks after full bloom in 1996. Other samples collected during the growing seasons (from early fruit development to harvest) of 1992 to 1998 included symptomatic fruit, leaves with water-soaked or necrotic lesions, symptomless peduncles bearing symptomatic fruit, and spurs (short shoots that bear fruit) and wood from branches showing dieback symptoms. From spurs and branches, 1.5-cm segments up to 15 cm from symptomatic fruit were sampled. For fungal isolations, symptomatic fruit or leaf tissues from the margins of the lesions, or woody tissue beneath the bark, were used. Approximately 125 mm³ of fruit or woody tissue or 25 mm² of leaf tissue was surface-sterilized with 400 mg of hypochlorous acid per liter for 1 min, rinsed in autoclaved distilled water, and plated onto potato dextrose agar (PDA) amended with rifampicin (20 mg/liter) and ampicillin (130 mg/liter) to inhibit bacterial growth. Isolation plates were incubated in a growth chamber at 25°C in the dark. Fungal isolates were identified based on cultural and microscopic morphology. Cultures were maintained

on PDA (up to 1 year) at 4°C or cryogenically (23). Between 1 and 30 isolates of *Colletotrichum* spp. were obtained from each orchard. Thus, a total of over 500 isolates were available for characterization.

Morphological characterization of *Colletotrichum* spp. isolates from almond. Colony characteristics of all isolates grown on PDA were compared. To characterize conidial morphology, isolates 1286, 1288, 1732, 1776, 1782, 1793, 1796, and 1798 were grown on wheat straw agar. Wheat straw was autoclaved for 1 h, and three or four stem pieces were added to molten water agar in petri dishes. A 5-mm-diameter mycelial agar disk of each isolate was placed on the surface of the medium, and the petri dishes were incubated at 24°C for 8 to 12 days in the dark. Conidial morphology was examined using a compound microscope, photographed, and compared with isolates of *C. gloeosporioides* (isolates 8 and 1427).

Molecular characterization of *Colletotrichum* spp. from almond. Fungal DNA was isolated using a modification (15) of the Raeder and Broda (19) method using approximately 5 mm³ of mycelium. For species identification, species-specific primers targeting the rDNA region of *C. acutatum* (primers Ca1 and Ca2) and *C. gloeosporioides* (primers Cg1 and Cg2) (17) were used in polymerase chain reaction (PCR) amplification experiments as described by Adaskaveg and Hartin (2), except that 2 µl of dNTP stock (2.5 mM each dNTP) and 1 µl of 10 µM primer were used in a 25-µl reaction. In an additional test for species identification, ribosomal DNA from six almond isolates (isolates 1286, 1288, 1732, 1776, 1796, and 1813) and reference cultures of *C. acutatum* from strawberry and *C. gloeosporioides* from citrus (isolates 725 and 8, respectively) was amplified by PCR using primers NS1 and ITS2 (25)

TABLE 1. Reference cultures of *Colletotrichum* spp. used in the study

Collection no.	Species	Host	Geographic origin	Genotype designation, collectors, alternative isolate codes ^a
1286	<i>C. acutatum</i>	<i>Prunus dulcis</i>	California, Butte Co.	Genotype Ca-P
1288	<i>C. acutatum</i>	<i>P. dulcis</i>	California, Stanislaus Co.	Genotype Ca-G
1732	<i>C. acutatum</i>	<i>P. dulcis</i>	California, Glenn Co.	Genotype Ca-P
1776	<i>C. acutatum</i>	<i>P. dulcis</i>	California, Butte Co.	Genotype Ca-G
1782	<i>C. acutatum</i>	<i>P. dulcis</i>	California, Butte Co.	Genotype Ca-G
1793	<i>C. acutatum</i>	<i>P. dulcis</i>	California, Butte Co.	Genotype Ca-G
1795	<i>C. acutatum</i>	<i>P. dulcis</i>	California, Butte Co.	Genotype Ca-G
1796	<i>C. acutatum</i>	<i>P. dulcis</i>	California, Butte Co.	Genotype Ca-P
1798	<i>C. acutatum</i>	<i>P. dulcis</i>	California, Butte Co.	Genotype Ca-P
1808	<i>C. acutatum</i>	<i>P. dulcis</i>	California, Stanislaus Co.	Genotype Ca-P
1813	<i>C. acutatum</i>	<i>P. dulcis</i>	California, Stanislaus Co.	Genotype Ca-G
1814	<i>C. acutatum</i>	<i>P. dulcis</i>	California, Stanislaus Co.	Genotype Ca-P
1815	<i>C. acutatum</i>	<i>P. dulcis</i>	California, Stanislaus Co.	Genotype Ca-P
1816	<i>C. acutatum</i>	<i>P. dulcis</i>	California, Stanislaus Co.	Genotype Ca-G
1817	<i>C. acutatum</i>	<i>P. dulcis</i>	California, Stanislaus Co.	Genotype Ca-P
1819	<i>C. acutatum</i>	<i>P. dulcis</i>	California, Stanislaus Co.	Genotype Ca-G
1820	<i>C. acutatum</i>	<i>P. dulcis</i>	California, Stanislaus Co.	Genotype Ca-P
1821	<i>C. acutatum</i>	<i>P. dulcis</i>	California, Stanislaus Co.	Genotype Ca-G
719	<i>C. acutatum</i>	<i>P. persica</i>	South Carolina	E. Zehr
720	<i>C. acutatum</i>	<i>P. persica</i>	South Carolina	E. Zehr, SF-3
721	<i>C. acutatum</i>	<i>P. persica</i>	South Carolina	E. Zehr, SF23
722	<i>C. acutatum</i>	<i>P. persica</i>	South Carolina	E. Zehr, SF11
1406	<i>C. acutatum</i>	<i>P. persica</i>	California, Yuba Co.	B. T. Manji, 95-3
724	<i>C. acutatum</i>	<i>Fragaria</i> × <i>ananassa</i>	California	E. E. Butler, 99
725	<i>C. acutatum</i>	<i>Fragaria</i> × <i>ananassa</i>	California	E. E. Butler, 100
726	<i>C. acutatum</i>	<i>Fragaria</i> × <i>ananassa</i>	California	E. E. Butler, 1996
727	<i>C. acutatum</i>	<i>Fragaria</i> × <i>ananassa</i>	California	E. E. Butler, 1987
1767	<i>C. acutatum</i>	<i>Fragaria</i> × <i>ananassa</i>	California	A. O. Paulus
723	<i>C. acutatum</i>	<i>Malus sylvestris</i>	South Carolina	E. Zehr, APL-30
1415	<i>C. gloeosporioides</i>	<i>Carica papaya</i>	Hawaii	R. J. Hartin, 96-9
8	<i>C. gloeosporioides</i>	<i>Citrus sinensis</i>	California, Yolo Co.	J. M. Ogawa, 94-22 or Cg22
1387	<i>C. gloeosporioides</i>	<i>C. sinensis</i>	California	J. E. Adaskaveg
1427	<i>C. gloeosporioides</i>	<i>C. sinensis</i>	California	J. E. Adaskaveg
1484	<i>C. gloeosporioides</i>	<i>P. dulcis</i>	Israel	S. Freeman, NA1Q
1485	<i>C. gloeosporioides</i>	<i>P. dulcis</i>	Israel	S. Freeman, IKS2A
1486	<i>C. gloeosporioides</i>	<i>P. dulcis</i>	Israel	S. Freeman, BZR9A
1487	<i>C. gloeosporioides</i>	<i>P. dulcis</i>	Israel	S. Freeman, GOZ36E
1488	<i>C. gloeosporioides</i>	<i>P. dulcis</i>	Israel	S. Freeman, NBR36M

^a Genotype designations: Ca-G = *Colletotrichum acutatum* genotype G, forming gray colonies on potato dextrose agar; and Ca-P = *C. acutatum* genotype P, forming pink colonies on potato dextrose agar. Collectors: E. Zehr, Clemson University, SC; B. T. Manji, E. E. Butler, and the late J. M. Ogawa, University of California, Davis; A. O. Paulus and R. J. Hartin, University of California, Riverside; and S. Freeman, Volcani Center, Israel.

under the recommended amplification conditions. The amplification products were subsequently digested with the restriction endonucleases *Hae*III, *Hha*I, *Rsa*I, *Taq*I, and *Msp*I and separated by agarose gel electrophoresis. The population substructure was investigated using the simple repeat primers (CAG)₅ and (GACA)₄ (8) in PCR amplifications and the random primers OPX-12 and OPY-11 (Operon Technologies, Inc., Alameda, CA) in random amplified polymorphic DNA (RAPD) PCR amplification experiments. Amplification reactions were carried out in 25- μ l volumes containing 1 μ l of DNA extract, 14.3 μ l of sterile distilled water, 2.5 μ l of 10 $\times$ reaction buffer (500 mM KCl; 100 mM Tris-HCl, pH 8.3; and 1% Triton X-100), 2 μ l of dNTP stock (2.5 mM each dNTP), 4 μ l of 10 mM MgCl₂, 1 μ l of primer (10 μ M), and 1 unit (primers [CAG]₅ and [GACA]₄) or 1.5 units (primers OPX-12 and OPY-11) of *Taq* DNA polymerase (Promega Corp., Madison, WI). Amplifications were performed in a PTC-100 thermal cycler (MJ Research Inc., Watertown, MA) programmed for the following cycling conditions: initial denaturation at 94°C for 30 s; 35 amplification cycles consisting of 30 s at 94°C, 30 s of annealing, and 1 min of extension at 72°C; and final extension at 72°C for 7 min. The annealing temperature was 60°C for primer (CAG)₅, 48°C for primer (GACA)₄, and 35°C for primers OPX-12 and OPY-11. Reaction products were separated by electrophoresis in 1.5% agarose using 0.5 $\times$ Tris-borate-EDTA buffer at 10 to 15 V/cm. Using this method, 255 of the over 500 isolates of *Colletotrichum* spp. collected from almond were characterized. Reference cultures of *Colletotrichum* spp. that were used for comparisons in these studies are listed in Table 1.

Population structure of the almond anthracnose pathogen within selected orchards in three counties. In the summer of 1998, the frequency of occurrence of the two subpopulations of the anthracnose pathogen (as identified below) was investigated within four orchards in Butte County, two orchards in Stanislaus County, and one orchard in Merced County. Fifty to ninety isolates were obtained from 100 mature, symptomatic fruit collected from 38 to 46 trees per orchard (one to four fruit per tree). Fungi were iso-

lated as described previously. Subpopulations as determined by cultural pigmentation (pink versus gray) were verified for approximately 20% of the isolates by RAPD analysis. The distribution of both subpopulations was then mapped for each orchard.

Pathogenicity assays. To compare the pathogenicity of the almond isolates (isolates 1286, 1288, 1732, and 1776) with those of *C. acutatum* isolates from strawberry (isolates 724 and 1767) or peach (isolate 1406), almond fruit were collected from orchards in Kern and Fresno Counties with no history of almond anthracnose. Detached almond fruit (cv. Butte) with no symptoms of disease were wounded using a stainless steel rod (6 mm in diameter) with a sharp wounding edge (1 $\times$ 2 $\times$ 2 mm) and then inoculated using 20 μ l of an aqueous suspension of conidia (10⁵ conidia per ml). The control fruit were wounded, and 20 μ l of autoclaved distilled water was placed on the wound. Fruit were then incubated in a plastic container at 20°C and >95% relative humidity. After 14 days, outside lesion diameters of each fruit were recorded at each inoculation site. Additionally, fruit were cut perpendicularly to the longitudinal axis of the fruit through the center of the lesion and the widest lesion diameter was measured. For each fungal isolate, there were three replications of six to eight fruit per replication and the experiment was done twice (once in each of 2 years). Lesion diameters of inoculated fruit caused by the fungal isolates evaluated were statistically analyzed using analysis of variance. Data for the two experiments were treated as blocks, and means of the combined data from both experiments were compared using the least significant difference (LSD) test of SAS (version 6.12; SAS Institute, Cary, NC).

Temperature relationships for mycelial growth of the two subpopulations of *C. acutatum* from almond. To determine the growth rate at selected temperatures, agar plugs (5 mm in diameter) obtained from the margin of 5- to 7-day-old cultures (gray isolates: 1782, 1793, 1795, 1813, 1816, 1819, and 1821; and pink isolates: 1796, 1798, 1808, 1814, 1815, 1817, and 1820) were placed on the surface of PDA in 10-cm-diameter petri dishes. There were three replicate petri dishes for each isolate and temperature. Cultures were incubated at 3, 10, 15, 20, 25, 32, or 36°C. Growth was recorded daily for 5 days, and radial growth rates were calculated as the 5-day mean daily growth (mm per day) for each culture and temperature. This experiment was carried out twice. Growth rate data for both subpopulations of *C. acutatum* for each temperature were statistically analyzed using analysis of variance. Data from the two experiments were treated as blocks, and means for combined data from both experiments were compared using the LSD test of SAS. Average daily growth rates for each subpopulation from both experiments combined were also analyzed using general linear model and regression analysis procedures of SAS.

Effect of kernel moisture content on growth of *C. acutatum*. To define environmental conditions that lead to postharvest kernel damage, almond kernels that were inoculated in the laboratory either

Fig. 1. Distribution map of almond anthracnose in California. Symbols indicate counties where the disease was verified with isolation data from 1991 to 1998. The two sites of the initial disease outbreaks were in Butte County (northern California) and Merced County (central California).

Fig. 2. Conidial morphology of *Colletotrichum* spp. isolated from almond and citrus in California and grown on wheat straw agar ($\times$ 550). **A.** *C. acutatum* Ca-P (pink colonies, isolate 1286) from almond. **B.** *C. acutatum* Ca-G (gray colonies, isolate 1288) from almond. **C.** *C. gloeosporioides* from citrus (isolate 8).

with conidial suspensions or agar disks of the pink cultural type were incubated for up to 3 months at selected levels of relative humidity using salt or sugar solutions. Almond kernels (cv. Carmel) were split in two halves along the sutures and were sterilized by autoclaving for 20 min. Kernels were then placed on sterile wire racks in clear plastic boxes (30 × 23 × 10 cm) containing 200 ml of one of the following solutions: saturated KNO₃, saturated K₂HPO₄, saturated KCl, saturated NaCl, or 6 molal of sucrose. These solutions served to initially obtain and subsequently maintain different levels of humidity within the boxes (13,20) and, therefore, different kernel moisture contents. The boxes were sealed with silicon sealant or plastic tape, and the kernels were allowed to equilibrate for 7 days at 20 to 24°C. The kernels were then inoculated with either 10 µl of a spore suspension (10⁶ spores per ml) or with mycelial agar plugs (5 mm in diameter, 2 mm thick) of isolate 1288 of *C. acutatum*. Three boxes were prepared for each solution, and each box contained three replications of 10 kernel halves for each inoculation method and three replications of 20 kernel halves for measuring kernel moisture contents. The boxes were incubated at 20 to 24°C for up to 3 months. Kernels were checked weekly for fungal growth. When fungal growth was observed, aliquots of the noninoculated kernels were ground using a food processor and passed through a 12-mesh (1.7 mm) sieve. The moisture content of the ground almond kernels was determined using a moisture balance (Mettler LP16; Mettler Instrument Corp., Hightstown, NJ). Kernels of treatments that did not show any fungal growth after 3 months of incubation were processed and the moisture contents were determined. To test the viability of the fungus in these latter treatments, the dried-up agar plugs were removed from the kernels and plated on PDA. If no growth occurred after 5 days, the fungus was considered nonviable. This experiment was done four times for each salt or sugar solution. Kernel moisture contents using the same salt or sugar solutions were not exactly the same among experiments. Based on kernel colonization, kernel moisture contents were grouped as either 4.3 to 10.1% or >13.1%. Data for percent colonization of kernels and percent viability of the fungus were transformed using the arcsine-square root percentage transformation and were analyzed for the two kernel moisture content groups using general linear model procedures of SAS. Experiments were treated as blocks, and means for combined data sets were compared using the LSD test of SAS.

Disease surveys, collection of diseased almond tissue, and fungal isolations.

A disease survey was carried out in the major almond-growing regions within California from 1991 to 1998. Anthracnose was reported and the pathogen was verified by our isolations from Butte County of the northern Sacramento Valley and from Merced County of the northern San Joaquin Valley since 1991 (Fig. 1). From 1994 to 1998, Colusa, Glenn, San Joaquin, Stanislaus, Sutter, and Tehama Counties had confirmed reports. Since 1997, other counties where the disease was confirmed included Fresno and Madera. The pathogen was confirmed only in two samples from Kern and Tulare Counties of the southern San Joaquin Valley during our surveys. Most of the 35 isolates from almond that were collected from 1992 to 1995 were also part of a previous study (2). From March to September 1996, 240 isolates of *C. acutatum* were obtained from 30 almond orchards in the five primary counties. In February of 1997, *Colletotrichum* spp. were isolated from 25 mummified fruit from hull, shell, and endosperm tissue. The recovery rate in two orchards was 100% based on the number of mummies investigated. Due to unusually dry spring weather conditions, disease levels remained very low in 1997 and only 16 isolates originating from five orchards were obtained from collected fruit (14 isolates) and wood (2 isolates) samples. In the winter of 1998, however, the fungus still was isolated from mummified fruit. During a major disease outbreak in 1998, isolates were obtained from 28 orchards in six counties. The disease also occurred in orchards that were symptomless in 1997. Overall, isolates were obtained from blighted blossoms, sunken orangish fruit lesions, irregular necrotic or watery lesions of leaves, peduncles of diseased fruit, and other woody tissues of spurs and branches with dieback symptoms. Generally, isolations from fruit showing sunken, orangish lesions were highly successful, with recovery rates of 95.6%. High recovery rates (81%) were also obtained with peduncles, whereas isolations from leaf and woody tissue were significantly ($P < 0.05$) less successful (13.6, 11.6, and 6.3% recovery rates for leaves, spurs, and wood, respectively), regardless of extensive branch dieback and foliar chlorosis. All successful isolations of *C. acutatum* from spurs and wood were from tissue samples that were taken within 9 cm of where an infected fruit was attached. Isolations from blossoms were only obtained in 1996 from the almond cvs.

Fig. 3. Amplification products of DNA extracted from *Colletotrichum acutatum* subpopulations Ca-P (pink colonies) and Ca-G (gray colonies) from almond in California, from isolates of *C. gloeosporioides* from almond in Israel (isolates 1415, 1387, and 8). **A**, Different band intensities for subpopulations P and G using the *C. acutatum*-specific primers Ca1 or Ca2 were observed. **B**, Using the *C. gloeosporioides*-specific primer Cg2, amplification products were only generated with the three isolates from citrus, but not with almond isolates from California or Israel. Lambda *Hind*III-*Eco*RI was used as a DNA size standard (St).

Merced and NePlus Ultra. The disease was not observed on blossoms in 1997 or 1998. Other organisms isolated from fruit, leaves, and wood included *Monilinia laxa*, *Botrytis cinerea*, *Alternaria* spp., *Cladosporium* spp., *Aureobasidium* spp., *Trichothecium* spp., *Epicoccum* spp., *Aspergillus niger*, *Aspergillus* spp., *Penicillium* spp., *Phomopsis* sp., and unidentified yeasts. *C. gloeosporioides* was not isolated from any of the almond tissues sampled throughout this study.

Morphological characterization of *Colletotrichum* spp. isolates from almond. Two types of *Colletotrichum* spp. colonies were recognized during the 1996, 1997, and 1998 isolations. The majority of cultures (62%) had a pink pigmentation when freshly isolated, with the reverse of the culture also being pink. The remaining cultures were gray in appearance, with the reverse of the culture being cream to gray; these cultures did not sporulate as profusely on PDA. When grown on wheat straw agar, the conidia from the cultures with pink pigmentation were clearly pointed at each end, whereas those of the gray cultures were less pointed, but distinctly different from conidia of *C. gloeosporioides*, which have broadly rounded ends (Fig. 2). There was no difference in the disease symptoms on almond fruit that yielded either colony type. Both types were commonly isolated from the same orchard or even the same fruit.

Molecular characterization of *Colletotrichum* spp. isolates from almond. When PCR amplifications were conducted using species-specific primers for *C. acutatum* and *C. gloeosporioides*,

DNA products were obtained only using the *C. acutatum*-specific primers Ca1 and Ca2 (Fig. 3). Interestingly, the two cultural types of the almond isolates reacted differently with the two *C. acutatum* primers. Whereas with primer Ca1 amplification products were more abundant using DNA from the pink colonies, with primer Ca2 band intensities for the gray colony type were either the same or more intense compared with the pink colony type (Fig. 3A). The species-specific primers were also used to characterize three *Colletotrichum* spp. isolates obtained from almonds in Israel. All

Fig. 4. Restriction fragment length polymorphism analysis of ribosomal DNA from *Colletotrichum acutatum* isolates from strawberry (isolate 725, first lane each), *C. acutatum* subpopulations Ca-G (gray colonies, isolate 1288, second lane each) and Ca-P (pink colonies, isolate 1286, third lane each) from almond, and *C. gloeosporioides* from citrus (isolate 8, fourth lane each). Ribosomal DNA was obtained in polymerase chain reaction amplifications using the universal primers NS1 and ITS2 and was then digested with either *Hae*III, *Hha*I, *Rsa*I, *Taq*I, or *Msp*I. Lambda *Hind*III-*Eco*RI was used as a DNA size standard (St).

Fig. 5. Random amplified polymorphic DNA analysis of California *Colletotrichum acutatum* isolates from almond using primer OPX-12, showing subpopulations Ca-P (pink colonies) and Ca-G (gray colonies). Lambda *Hind*III-*Eco*RI was used as a DNA size standard (St).

Fig. 6. Random amplified polymorphic DNA analysis using primer OPY-11 for California isolates of *Colletotrichum acutatum* from almond (subpopulations Ca-P = pink and Ca-G = gray colonies) and strawberry (from left to right: isolates 724, 725, 726, 727, and 724), and South Carolina isolates of *C. acutatum* from peach (from left to right: isolates 719, 720, 721, and 722) and apple (isolate 723). Lambda *Hind*III-*Eco*RI was used as a DNA size standard (St).

Fig. 7. Random amplified polymorphic DNA analysis using primers OPX-12 and OPY-11 for almond anthracnose isolates of *Colletotrichum acutatum* from California (subpopulations Ca-P = pink and Ca-G = gray colonies) and from Israel. Banding patterns of the isolates from Israel were very similar to those of California Ca-G. Lambda *Hind*III-*Eco*RI was used as a DNA size standard (St).

three isolates produced a band using primers Ca1 and Ca2, whereas no band was present after amplification with the *C. gloeosporioides*-specific primers (Fig. 3A and B).

For additional species verification, ribosomal DNA was amplified using the universal primers NS1 and ITS2 and subjected to restriction enzyme analysis. With three of the restriction enzymes (*Hae*III, *Hha*I, and *Msp*I), representatives of the pink and gray almond isolates had identical fragment patterns, which were also identical to the one of *C. acutatum* from strawberry (Fig. 4). With two additional restriction enzymes (*Rsa*I and *Taq*I), polymorphisms between the two cultural types from almond were evident. Using *Rsa*I, unique fragment patterns were generated for *C. acutatum*

from almond, *C. acutatum* from strawberry, and *C. gloeosporioides* from citrus. Using *Taq*I, the DNA banding pattern for the pink almond type was identical to the one for *C. acutatum* from strawberry, whereas the pattern for the gray almond type was identical to the one for *C. gloeosporioides* from citrus.

Molecular differences between the pink and gray almond isolates were much more pronounced using short random primers or simple repeat primers during PCR amplifications. Following electrophoretic separation of the amplification products, very distinct banding patterns were evident for the two colony types (Fig. 5). All representatives of each colony type (160 isolates with pink colonies and 95 isolates with gray colonies) were analyzed in

Fig. 8. Frequency of occurrence of *Colletotrichum acutatum* subpopulations Ca-P (pink colonies) and Ca-G (gray colonies) within four orchards in Butte County, two orchards in Stanislaus County, and one orchard in Merced County in the summer of 1998. Isolations were carried out from infected fruit (one to four fruit per tree, 100 fruit per orchard). Each symbol represents a fruit from which *C. acutatum* was isolated. ● = Ca-P was isolated from fruit, □ = Ca-G was isolated from fruit, ■ = both subpopulations were isolated from the same fruit, and × = no successful isolations. Blanks next to tree numbers represent trees without diseased fruit. M = missing tree.

Fig. 9. Effect of temperature on in vitro mycelial growth of isolates of *Colletotrichum acutatum* from almond (subpopulations Ca-P = pink and Ca-G = gray colonies). **A**, Growth rate of Ca-P and Ca-G subpopulations at 3 to 36°C. Each data point represents the mean daily growth rate (mm/day) on potato dextrose agar of seven isolates of each of the two subpopulations for both experiments. **B and C**, Regressions of growth on time of incubation for each subpopulation at 10 and 32°C, respectively.

these PCR amplification studies) had identical patterns with all four primers.

RAPD banding patterns of the almond isolates were distinct from those of isolates of *C. acutatum* from strawberry in California (six isolates) and from apple (one isolate) and peach (four isolates) in South Carolina (Fig. 6). Two distinct banding patterns were recognized among the California strawberry isolates. All four California peach isolates were identical, and their banding patterns were the same as those for the California almond subpopulation producing pink colonies (data not shown). RAPD banding patterns of the California isolates from almond were also compared with those of three isolates from almond in Israel that had been identified as *C. gloeosporioides*. The three isolates from Israel had identical banding patterns, very similar to those of the gray isolates of *C. acutatum* from almond in California (Fig. 7).

Population structure of the almond anthracnose pathogen within selected orchards in three counties. Fifty to ninety isolates of *Colletotrichum* spp. were obtained from each of four orchards in Butte County, two orchards in Stanislaus County, and one orchard in Merced County. The proportion of isolates with the pink pigmentation within the population was 97.6, 83, 60.7, and 15% in the Butte County orchards, and 100, 94.3, and 83.3% in the two Stanislaus County orchards and the Merced County orchard, respectively. Thus, both subpopulations were found in all orchards except in one orchard in Stanislaus County, and the relative abundance of the two types varied among orchards (Fig. 8). For approximately 20% of the isolates, the subpopulation was also determined by RAPD analysis. In these studies, all isolates with gray colonies had banding patterns specific for this gray type, whereas all isolates with pink colonies had banding patterns specific for the pink type.

Pathogenicity assays. When almond fruit were wound-inoculated in the laboratory with representatives of the two California almond anthracnose pathogens and of California isolates of *C. acutatum* from strawberry and peach, the outside and inside fruit lesion sizes were very similar after an incubation period of 2 weeks. The analysis of variance table indicated that significant differences ($P < 0.01$) were found for fungal isolates and experiments, but not for the interaction of fungal isolate and experiments ($P > 0.05$). Thus, for the combined data set, significantly ($P < 0.05$) larger average lesion sizes were caused by isolates of the pink almond sub-

Fig. 10. Colonization of almond kernels by *Colletotrichum acutatum* after inoculation with agar plugs or a spore suspension and viability of agar plug inoculum at different levels of kernel moisture. Almond kernel halves (cv. Carmel) were incubated for up to 3 months in plastic boxes over selected salt and sugar solutions to obtain and maintain different kernel moisture contents. Data were the averages of all experiments.

population and the California peach isolate (14.9 and 12.2 mm outer lesion diameter, respectively) compared with the strawberry isolates (10.5 mm) or the gray isolates from almond (8.8 mm). Thus, in wound-inoculation assays with detached fruit, the two California subpopulations of the almond anthracnose pathogen differed significantly in their virulence. However, the disease symptoms were identical, with both subpopulations producing circular, orangish to tan lesions.

Temperature relationships for growth of the two *Colletotrichum* spp. subpopulations from almond. For all temperatures evaluated, no significant difference ($P > 0.05$) was observed for experiments or for the interaction of experiments and growth rate of the subpopulations. Optimal growth rates for both subpopulations were observed at 25°C, with a radial growth of 3.5 mm $\pm$ 0.1 mm/day for the gray colony type and 3.6 mm $\pm$ 0.2 mm/day for the pink colony type (Fig. 9A). At 20°C, both types grew at a rate of 3.0 mm $\pm$ 0.1 mm/day. No significant differences were found in growth rate of the two subpopulations at these two temperatures (20 and 25°C). At 10, 15, and 32°C, significant differences ($P < 0.05$) in mean growth were evident between the two subpopulations (Fig. 9A). The gray cultures grew faster at 10°C (Fig. 9B) and 15°C as compared with the pink cultures using the combined data from both experiments. At 32°C, however, growth of the gray cultures almost completely stopped, whereas the pink cultures still grew (Fig. 9C). Linear regressions of growth of each subpopulation on time of incubation were significant ($P < 0.01$) with significant differences ($P < 0.01$) in both slopes and midpoints of each line at 10 and 32°C (Fig. 9B and C). At 36°C, neither the pink nor the gray isolates showed any growth during the 5-day experimental period.

Effect of kernel moisture content on growth of *C. acutatum*. Almond kernel moisture contents using the same salt or sugar solutions were not exactly the same among experiments. Kernel moisture contents ranged from 4.3 to 5.9, 6.6 to 9.6, 8.3 to 8.6, 7.8 to 10.1, and 13.1 to 21.2% for K₂HPO₄, sucrose, KCl, NaCl, and KNO₃, respectively. Significant differences ($P < 0.01$) were observed in kernel colonization between the two kernel moisture content categories (4.3 to 10.1 and $>13.1\%$), whereas no significant difference was observed between experiments or the interaction of experiments and kernel colonization. At kernel moisture levels $>13.1\%$, kernel colonization by the fungus occurred after 7 to 20 days and incidence of colonization at each of these kernel moistures was nearly 100% using either inoculation method (Fig. 10). No growth of the fungus was observed after 3 months at or below kernel moisture contents of 10.1%. The fungal mycelium, however, was still viable, with nearly 100% recovery at all kernel moisture contents evaluated after the same time period and no significant difference between the two kernel moisture categories. The moisture content of 4.3% was the lowest value obtained with any of the treatments in the experiments. No data were obtained for kernel moisture contents between 10.1 and 13.1%.

DISCUSSION

Worldwide, two species of *Colletotrichum* have been identified to cause almond anthracnose: a clonal population of *C. gloeosporioides* as described from Israel (8,21) and *C. acutatum* as identified in California (2). The only other more recent reports of almond anthracnose are from France (11,12), but no description of the pathogen is presented and the author of these reports assumes the pathogen is *C. gloeosporioides*. *C. gloeosporioides* has been isolated from other crops in California, such as citrus. To date, however, the fungus has not been reported from stone fruit. The species identity of the almond pathogen in California was substantiated in the current study, and this confirmed previous results (2). Using restriction enzyme analysis of ribosomal DNA, the almond isolates were more similar to a reference culture of *C. acutatum* from strawberry than to *C. gloeosporioides* from citrus. Consequently, together with the

traditional characters that are used to separate *C. acutatum* from *C. gloeosporioides*, such as conidial shape, slower growth rate, lower optimum temperature (2), and insensitivity to the fungicide benomyl (2,22,24), the almond anthracnose pathogen fits the species description of *C. acutatum*. Interestingly, three isolates from almonds with anthracnose from Israel that had been identified as *C. gloeosporioides* (8) had almost identical DNA banding patterns after RAPD analysis when compared with almond *C. acutatum* isolates with gray colonies from California. These three isolates also produced DNA bands when *C. acutatum*-specific primers were used in DNA amplifications. Additionally, the isolates from Israel and the isolates with gray colonies from California were very similar in colony appearance and in their conidial morphology. Therefore, the identification of the causal organism of almond anthracnose in Israel needs to be reevaluated.

Two clonal subpopulations of the California almond anthracnose pathogen were identified in the current study, in contrast to the presence of a single homogeneous population in Israel (8,9). These two California subpopulations differ in colony appearance (pink versus gray cultures), conidial morphology, virulence in laboratory inoculation studies, and temperature-growth relationships, and they have distinct molecular fingerprints. Using four different short primers during DNA amplifications, all representatives of each type had identical patterns with each primer suggesting the presence of two clonal subpopulations or genotypes. Therefore, we refer to *C. acutatum* isolates with pink colonies as genotype Ca-P and *C. acutatum* isolates with gray colonies as genotype Ca-G. In previous studies, cultural pigmentation has been used to differentiate *Colletotrichum* spp. For example, in a study with isolates from peach, apple, pecan, and other hosts, *C. acutatum* isolates produced pink colonies, whereas *C. gloeosporioides* had gray colonies (4). Likewise, Sutton (24) characterizes *C. acutatum* as having pigmented colonies in reverse. This latter author, however, clearly states that colony pigmentation is a poor character for species differentiation in *Colletotrichum* spp. taxonomy that should not be over-emphasized. Recently, Johnston and Jones (14) and Lardner et al. (16) recognized five morphological and genetically distinct groups within *C. acutatum* sensu lato in addition to two genetically distinct groups within *C. acutatum* f. sp. *pineum*. Cultural pigmentation varied widely among the groups of *C. acutatum*, including a group of isolates from fruit rots from New Zealand producing gray mycelium. Thus, the evidence we are providing in our study, including the use of molecular methods, supports the species designation of almond isolates from California as *C. acutatum* and, furthermore, supports the designation of genetic and morphological subpopulations of *C. acutatum* from almond producing either pink (Ca-P) or gray (Ca-G) colonies.

Isolations from naturally infected tissues demonstrated that both subpopulations are able to cause severe damage in the field; however, in laboratory studies, subpopulation Ca-G was less virulent. At their optimal temperature of 25°C, both subpopulations grew at the same rate. This growth rate was lower than that reported for *C. gloeosporioides* (2). Growth of Ca-G at 32°C was much reduced compared with Ca-P. In contrast, at 10°C, the growth rate of Ca-G was much higher compared with Ca-P. Consequently, Ca-G may be better adapted to cooler environments in the field. This may explain why Ca-G was more abundant within four orchards in Butte County compared with three orchards in the more southern and warmer Stanislaus and Merced Counties. Additional population studies, however, are needed to substantiate this association. The relative abundance of the two subpopulations was very different between the orchards surveyed.

Both subpopulations were identified during the 1996, 1997, and 1998 disease surveys. Ca-G was not detected among isolates from earlier surveys. This could be explained by the limited number of isolates that was available from samples taken from 1992 to 1995. It is also conceivable that subpopulation Ca-G may have been over-

looked in earlier isolations. Alternatively, a more recent introduction of Ca-G cannot be ruled out.

Isolations of the pathogen were most successful from fruit tissues. The high recovery rate from overwintering fruit mummies strongly suggests that fruit mummies are inoculum sources for early spring infections caused by rain splash dispersal of conidia. Removal of these mummies, therefore, is likely to reduce the initial disease level. Future studies are required to determine if peduncles and spurs from which the fungus has been isolated may also serve as overwintering inoculum sources. Isolations from tissues other than fruit indicate that the amount of tissue infected by the pathogen is more extensive than previously considered. Isolations of the pathogen from leaf and woody tissues were much less successful, even with the occurrence of extensive leaf yellowing and branch dieback. These low recovery rates could mean that the pathogen often does not invade these types of tissues. Isolations from branches, however, may have been obscured by extensive growth of secondary organisms. Considering leaf tissues, isolations generally were not obscured by secondary organisms, except by the omnipresent species of *Aureobasidium*. Leaf isolations were more successful early (spring) and late (fall) in the season; we speculate that this is because, at these times, the fungus is able to infect leaf tissue due to favorable weather conditions. During the summer, however, host responses to secondary metabolites could be responsible for yellowing leaves and branch dieback. Phytotoxic compounds have been described from other species of *Colletotrichum*, and their involvement in symptom development has been suggested by a number of authors (3,7,10).

Based on preharvest losses in 1996 and 1998 (J. E. Adaskaveg, unpublished data) and the postharvest damage to the crop, anthracnose potentially represents one of the most serious threats to the almond industry in California. Because of an unusually dry spring, the disease was not a problem in 1997; however, the fungus persisted and caused problems in orchards with high-angle sprinkler irrigation. In 1998, the disease was also found in orchards that were symptomless in 1997. Anthracnose was observed throughout the major almond-growing areas of California. Continued yearly outbreaks of almond anthracnose, however, only occurred in the northern and central growing regions, where rainfall is more abundant and the rainy season is longer.

As our data for growth of the fungus on almond kernels with different moisture levels indicate, postharvest damage to almond kernels appears to be the result of preharvest epidemics that were not managed. Following inoculation, no growth of the fungus occurred below a kernel moisture content of 10.1%; whereas nearly 100% growth occurred at each kernel moisture content evaluated at or above 13.1%. Because the moisture content of almond kernels in storage is kept between 5 and 10%, new infections are unlikely to occur in storage. We cannot rule out, however, that fungal growth may occur from preexisting infections over long periods (up to 1 year) in storage.

The origin of the pathogen and the cause of its sudden spread through the California almond-growing regions remain to be investigated. Almond anthracnose was first reported in the early 1900s (5). We do not know, however, if the same species and strains of *Colletotrichum* were responsible for this initial outbreak. In a search for alternative hosts of the pathogen that could have served as the original hosts, we conducted molecular comparisons with a limited number of *C. acutatum* cultures from different crops in California. Peach isolates were found to be identical to genotype Ca-P. Anthracnose on peach is not a significant problem in California; therefore, its initial role as an inoculum source is unlikely. Two distinct fragment patterns were found among California strawberry isolates in a RAPD analysis. These patterns were very different from those of the two genotypes of *C. acutatum* from almond. Thus, from these comparisons, it is unlikely that the almond isolates originated from strawberry, where the disease occurred prior to the almond anthracnose epidemic of the 1990s. More isolates from

different hosts will have to be collected in the future to possibly gain insight into the origin and the population dynamics of almond anthracnose.

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