

The etiology of recent pepper anthracnose outbreaks in Florida

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ABSTRACT

Within the last 4–6 years, anthracnose has become an increasingly serious disease on un-ripe, immature (green) pepper fruit in Florida. This contrasts with earlier reports of anthracnose as strictly a ripe-rot disease of ripened (usually red) pepper fruit. The species of *Colletotrichum* associated with anthracnose on both immature and ripe pepper fruit in Florida was identified. Based on reactions with polymerase chain reaction (PCR)-specific primers, 28 of 50 isolates associated with anthracnose lesions from Florida were identified as *Colletotrichum acutatum*, including 22 of 22 recovered from immature fruit. Six of the *C. acutatum* isolates were associated with typical lesions on ripe fruit, but only in fields where lesions on immature fruit were also observed. In contrast, all 17 isolates identified by PCR as *Colletotrichum gloeosporioides* were recovered from lesions found only on ripe fruit from fields where no lesions on immature fruit were initially observed. No isolates were identified as *Colletotrichum capsici* or *Colletotrichum coccodes*. Isolates of *C. gloeosporioides* grew up to twice as fast *in vitro* as isolates of *C. acutatum*, suggesting a way to tentatively differentiate pepper isolates without PCR testing. In addition, *C. gloeosporioides* produced conidia that were longer and wider than those produced by *C. acutatum*. The name “early anthracnose” is proposed for the disease on immature fruit caused by *C. acutatum*.

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1. Introduction

Florida is second only to California in production of peppers (*Capsicum annuum* L.) in the United States. Most acreage is planted to sweet bell pepper, with the bulk of the production during the winter months (September–May) in the southeastern and southwestern areas of the state (Maynard et al., 2003). In the 2003–2004 season, 223,605,454 kg of pepper were harvested from more than 6880 ha, with a total annual value of \$175,654,000, second only to tomatoes in farm-gate value in the state (www.nass.usda.gov, December 2007).

Anthracnose has emerged as an increasingly significant disease of pepper in Florida in recent years (Roberts et al., 2001). It has been observed on both sweet bell peppers and specialty peppers, such as cubanelle, jalapeno, and scotch bonnet (*Capsicum chinense*) (McGovern and Polston, 1995). The disease is characterized by sunken, necrotic lesions on the surface of pepper fruit that usually contain an abundance of tan or salmon-colored conidia. Traditionally, the disease in Florida has been primarily associated

with ripe fruit that had already turned from green to the ripened color of the cultivar (usually red). Therefore, the causal agents were generally thought of as mostly “ripe-rot” pathogens (Roberts et al., 2001; Alexander and Pernezny, 2003). On immature fruit, typical anthracnose symptoms were generally not observed and therefore the disease was not considered a significant problem on bell peppers harvested as fully sized green fruit (by far the bulk of the harvested acreage in Florida). Within the last few years, however, the disease has been observed on immature pepper fruit grown in Florida. A similar outbreak of anthracnose on immature fruit has occurred in Ohio (Lewis-Ivey et al., 2004). The causal agent of the Ohio epidemic was identified as *Colletotrichum acutatum* (Simmonds). Although at least four species of *Colletotrichum* have been reported in the US to cause anthracnose of pepper, *Colletotrichum gloeosporioides* (Penz.), *Colletotrichum capsici*, *Colletotrichum coccodes*, and *C. acutatum* (Roy, 1996; Hadden and Black, 1988; Hadden, 1989; Alexander and Pernezny, 2003; Marvel et al., 2003; Lewis-Ivey et al., 2004), *C. acutatum* has never been identified as a pepper pathogen in Florida (McGovern and Polston, 1995; Roberts et al., 2001).

In Ohio, *C. acutatum* was reported as a more aggressive pathogen than other *Colletotrichum* species known to infect pepper, capable of causing losses in marketable yield of up to

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100% (Lewis-Ivey et al., 2004). Probably of more significance, *C. acutatum* will attack both ripe and immature fruit, unlike other species that are strictly ripe-rot pathogens. Since the epidemics of anthracnose on immature fruit in Florida have occurred at roughly the same time as those in Ohio, it is possible that *C. acutatum* is responsible for the disease in Florida as well.

Various methods have been described previously to determine the species of *Colletotrichum* causing pepper anthracnose, including both molecular (Kim et al., 2002; Lewis-Ivey et al., 2004) and morphological (Hadden, 1989; Kim et al., 1999; Lewis-Ivey et al., 2004) techniques. Species-specific primers based on the rDNA internal transcribed spacer (ITS) regions of different species have been used to differentiate *C. gloeosporioides* and *C. acutatum* (Mills et al., 1992; Brown et al., 1996; Sreenivasaprasad et al., 1996; Freeman et al., 2000; Lewis-Ivey et al., 2004). Molecular methods generally have been preferred over morphological methods (Marvel et al., 2003; Lewis-Ivey et al., 2004), because the morphology between species often are quite similar and a certain degree of morphological variation is considered acceptable within a species of *Colletotrichum* (Sutton, 1992). Colony growth rate on artificial media in growth chambers has been used to differentiate between *C. acutatum* and *C. gloeosporioides* recovered from pepper (Hadden, 1989). The other species reported to occur on pepper, *C. coccodes* and *C. capsici*, are easily distinguished due to the production of abundant sclerotia on Potato Dextrose Agar (PDA) culture by *C. coccodes*, and by the distinct falcate or 'curved' shape of conidia produced by *C. capsici* (Hadden, 1989; Bailey et al., 1992; Sutton, 1992; Roy, 1996).

The purpose of this study was (i) to isolate and identify the species of *Colletotrichum* causing pepper anthracnose in Florida from both ripe and immature fruit, and (ii) identify morphological characteristics useful for differentiation of isolates identified to species by polymerase chain reaction (PCR)-DNA analysis.

2. Materials and methods

2.1. Isolates

Fifty isolates were recovered from both ripe and immature symptomatic pepper fruit from various commercial farms throughout Florida. An additional isolate from a diseased immature bell pepper in 2005 from southern Georgia was provided by D. Langston (Table 1). Forty-seven of the Florida isolates were recovered from infected pepper fruit during the 2004–2005 vegetable season, and three isolates from pepper were originally recovered in the mid-1990s by R. McGovern. Two isolates, Ca Mil-1 (*C. acutatum*) and GD (*C. gloeosporioides*) (Lewis-Ivey et al., 2004), were used as reference isolates in the PCR studies. The 50 isolates from Florida were collected from three pepper-growing areas: Indian River and St. Lucie Co. in east-central Florida, Palm Beach Co. in southeast Florida, and Collier and Hendry Co. in southwest Florida (Table 1). Fungi were isolated by rinsing symptomatic fruit with de-ionized water and placing in a closed plastic container containing a moist paper towel (ca. 100% humidity) for 24 h. Conidia from lesions on the surface of the fruit were removed with a sterile loop, which was then streaked onto the surface of 10-cm-diameter Petri plates containing water agar (15 g agar 1000 ml⁻¹ distilled water), and allowed to grow for 12–18 h. Up to four germinating, single-spores per isolation were identified under a dissecting microscope (40×), removed with a sterile needle, and transferred to a Petri dish containing 25 ml of PDA and allowed to grow at 30 °C for 7 d. One isolate per lesion was selected for further study. In some cases, two isolates were obtained from different lesions on the same fruit. Isolates were transferred to PDA plates containing small (approximately 5 mm²)

pre-cut sterilized pieces of filter paper (Whatman #4) placed directly on the surface, and incubated at 20 °C for 14 d with continuous light to induce sporulation (Bernstein et al., 1995; Freeman et al., 1998). The individual pieces of colonized filter paper were then removed from the surface of the agar using sterile forceps, allowed to dry in empty Petri dishes for 14 d, and placed in sterile vials (2.0 ml microcentrifuge tubes) for long-term storage at –4 °C. Isolates were recovered as needed by transferring filter paper units to PDA and incubating plates for a minimum of 3 d at 20 °C.

2.2. PCR amplification

PCR amplification was used to putatively identify isolates as species of *C. acutatum* or *C. gloeosporioides* using species-specific primers as previously described (Mills et al., 1992; Sreenivasaprasad et al., 1996; Lewis-Ivey et al., 2004). The species-specific primers for *C. gloeosporioides* (CgInt; 5'-GGC-CTCCCGCTCCGGGCGG-3') (Mills et al., 1992) and for *C. acutatum* (CaInt2; 5'-GGGGAAGCTCTCGCGG-3') (Sreenivasaprasad et al., 1996) from the ITS 1 region of the rDNA were used in combination with the conserved primer ITS 4. Prior to conducting PCR, the DNA of each isolate was extracted according to the protocol previously described (Lee and Taylor, 1990) and modified by Lewis-Ivey et al. (2004). Each 25 µl reaction mixture contained: 2.5 µl of extracted DNA (50 ng µl⁻¹), 0.125 µl of each 10 µM primer, 0.08 µl 10 mM dNTP, 0.5 µl Taq Polymerase (5 U µl⁻¹), 1.5 µl of 25 mM MgCl₂, 2.5 µl 10× polymerase buffer, and 16.9 µl sterile de-ionized water. The PCR was performed with a MJR PTC-100 thermocycler (MJ Research Inc., Waltham, MA) using the following temperature-cycle program: 5 min at 94 °C, 30 cycles of 1.5 min at 94 °C, 2 min at 55 °C, and 3 min at 72 °C, followed by a 10 min final extension at 72 °C. The PCR products (7 µl) were mixed with 3 µl of loading dye (5 mg bromophenol blue, 5 ml 5× TBE, 2 g sucrose) and separated by horizontal gel electrophoresis in 1.5% agarose in 0.5× TBE buffer at 110 V for 150 min. Correct product size was confirmed using a 1 kb DNA ladder marker as previously described (Lewis-Ivey et al., 2004). Gels were then stained in dilute ethidium bromide (2 µg ml⁻¹), visualized under UV light, and the image was captured using the Kodak Electrophoresis Documentation and Analysis System (EDAS) 290 (Eastman Kodak Company, New Haven, CT). The PCR procedure was conducted three times for each isolate.

2.3. Growth rate in vitro

Radial growth rate (mm) was determined for each isolate. Isolates were grown on PDA for 3–5 d and were transferred to each of three replicate PDA Petri dishes using plugs made with a #3 cork-borer. Plates were placed into a growth chamber (Enviro chamber, Detroit, MI) at 30 °C in continuous darkness, and arranged within the growth chamber in a completely randomized design. At 5 d, the radius of each colony was measured and recorded. Mean growth rates of the three replicates were calculated for all isolates and were compared statistically using ANOVA ($P < 0.05$). The experiment was repeated once.

2.4. Conidial measurements

Isolates VB07, VB09, MF05, MF08, MG01, HB01, HJ01, HC02, and GA01 were grown on PDA for 5 d under continuous fluorescent light at 25 °C to promote sporulation. Conidia were suspended in sterile water using a sterile loop and mounted on a microscope slide. Length and width (µm) were measured for 25 conidia per isolate using an ocular scale at 700× magnification (10× ocular,

Table 1
Isolates of *Colletotrichum* spp. recovered from pepper fields throughout Florida (sample no. 1–7) or Georgia (sample no. 8)^a

Sample no.	Isolate designation	Location (Co.)	No. of isolates	Host sample description	Species recovered
1	MF01–MF09	St. Lucie	9	Red bell 'Olympus'	<i>C. gloeosporioides</i>
2	VB01–VB10	Indian River	10	Red jalapeno 'Milta'	<i>C. gloeosporioides</i>
3	PB01–PB06	Palm Beach	6	Green bell 'Brigadier'	<i>C. acutatum</i>
4	HB01–HB08	Hendry	8	Green/Red bell 'Aristotle'	<i>C. acutatum</i>
5	HC01–HC09	Hendry	9	Green Cubanelle 'Aruba'	<i>C. acutatum</i>
6	HJ01–HJ05	Hendry	5	Green/Red jalapeno 'Tormenta'	<i>C. acutatum</i>
7	MG01–MG03	Collier	3	Red bell, Scotch bonnet, Thai	<i>C. gloeosporioides</i>
8	GA01	Tift (GA)	1	Green bell	<i>C. acutatum</i>

^a All isolates were recovered in 2004 and 2005, except for those collected from Collier Co. (MG01–MG03), collected in the mid-1990s by R. McGovern, University of Florida.

70× objective) using bright field microscopy (Leitz, Germany). The length and width were compared statistically using a one-sided *t*-test between the two species.

3. Results

3.1. PCR amplification with species-specific primers

Twenty-eight of the 50 pepper isolates from Florida were identified as *C. acutatum*, using the species-specific primer Calnt2 in conjunction with the ITS4 primer for *C. acutatum*. Seventeen isolates were identified as *C. gloeosporioides*, using the CgInt and ITS4 primers specific for *C. gloeosporioides*. The *C. gloeosporioides* isolates were recovered exclusively from ripe pepper fruit in St. Lucie and Indian River Co. in 2004–2005, and in the mid-1990s in Collier Co. The *C. acutatum* isolates were recovered in 2004–2005 from anthracnose lesions on immature fruit, or adjacent ripe fruit. PCR reactions of several representative isolates are shown (Fig. 1) and compared with the previously published reference isolates, Ca Mil-1 (*C. acutatum*) and GD (*C. gloeosporioides*) (Lewis-Ivey et al., 2004). Five pepper isolates (VB01, VB03, VB04, VB05, and VB06) did not produce a PCR product with either primer mixture, likely due to an unsuccessful DNA extraction or contamination of the sample. The isolate from the immature fruit in southern Georgia (GA01) also was identified as *C. acutatum* (Fig. 1).

3.2. Colony growth rate

In two separate tests, isolates identified by PCR as *C. gloeosporioides* grew significantly faster ($P < 0.0001$) than those identified as *C. acutatum* (Fig. 2). The 17 isolates of *C. gloeosporioides* grew at an average of 5.91 and 5.93 mm d⁻¹ in Test 1 and Test 2, respectively, while the 28 isolates of *C. acutatum* grew at an average of 2.96 and 3.54 mm d⁻¹ in Test 1 and Test 2, respectively. The five isolates that did not produce an identifiable PCR product (VB01, VB03, VB04, VB05, and VB06) grew at a mean rate of 5.85 mm d⁻¹ (data not shown), consistent with that of the growth rate for isolates of *C. gloeosporioides*. The isolate identified by PCR as *C. acutatum* from the immature pepper fruit in Georgia (GA01) grew at a mean rate of 3.37 mm d⁻¹ (data not shown), consistent with the growth rate of isolates of *C. acutatum*.

3.3. Conidial measurements

Conidia length and width (μm) were measured for five isolates previously identified by PCR as *C. gloeosporioides*, and four isolates of *C. acutatum*. The isolates designated as *C. gloeosporioides* had an average conidial size of 17.96 × 6.37 μm (standard error = 0.146 × 0.041), whereas isolates designated as *C. acutatum* had

an average conidial size of 16.79 × 4.49 μm (standard error = 0.172 × 0.049). The length and width were statistically analyzed using a one-way ANOVA *t*-test, and both were significantly larger for *C. gloeosporioides* ($P < 0.0004$ and $P < 0.0001$, respectively).

4. Discussion

C. acutatum has been identified as the causal agent of the recent epidemics of anthracnose on immature pepper fruit in Florida. This result is based primarily on reaction of DNA from all isolates from lesions on green fruit with PCR-specific primers for *C. acutatum*. All isolates identified by PCR as *C. gloeosporioides* were recovered from ripe fruit, never from immature fruit. A few *C. acutatum* isolates were from ripe fruit, indicating that *C. acutatum* can attack pepper fruit during all stages of maturity. *C. gloeosporioides*, on the other hand, seems to be strictly a ripe-rot pathogen on pepper. These results parallel those reported recently in Ohio (Lewis-Ivey et al., 2004). Pepper can now be added to the list of hosts for *C. acutatum* in Florida (Brown et al., 1996; Legard, 2000; Timmer and Brown, 2000; Lahey et al., 2004; Peres et al., 2005) and other locations (Bailey et al., 1992; Freeman et al., 1998, 2001; Adaskaveg and Förster, 2000; Correll et al., 2000; Freeman, 2000; Peres et al., 2002). Although only one isolate from Georgia was included in our study, it seems likely that anthracnose on immature peppers in Georgia is also caused by *C. acutatum*. However, more isolates from Georgia need to be recovered and identified to confirm this contention. Although this study concentrates on the occurrence of *C. acutatum* on pepper in Florida, it is likely that this species of *Colletotrichum* could emerge and cause epidemics in other pepper-growing regions throughout the United States.

Growth rates in culture were clearly different for *C. acutatum* and *C. gloeosporioides*. Isolates of *C. gloeosporioides* grew 50–200% faster than those of *C. acutatum*. These observations serve as the basis of a suggestion that colony growth rate under very specific conditions (30 °C in complete darkness on PDA plates) can be used to tentatively separate these two species. Other researchers (Kim et al., 1986; Hadden, 1989; Sutton, 1992; Brown et al., 1996; Marvel et al., 2003) have also suggested that colony growth rate of isolates can be of taxonomic significance. Tolerance to benomyl also tends to vary between these two species (Bernstein et al., 1995; Adaskaveg and Hartin, 1997; Freeman et al., 1998; Peres et al., 2004). The difference in conidial size between the two species was less distinct than growth rate differences in our studies. However, conidia of *C. acutatum* were significantly smaller than conidia of *C. gloeosporioides* in both length and width. Differentiation between these two species on conidial size alone could prove difficult, due to size variation within an isolate and the similarity of spore shape between the two species. However, for laboratories

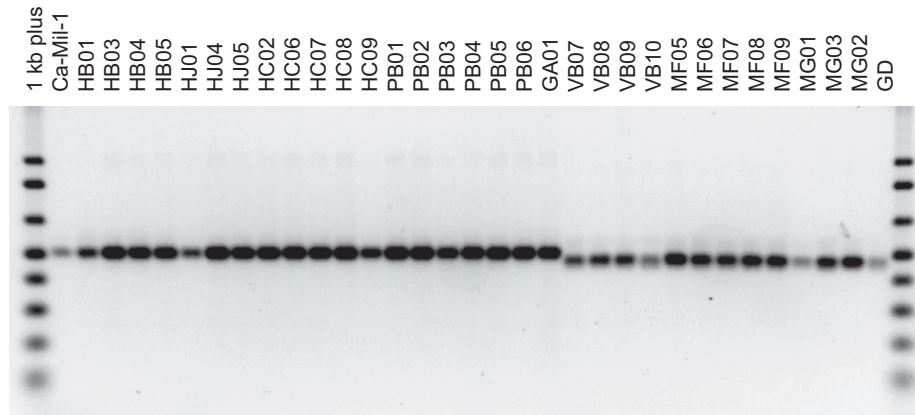


Fig. 1. An agarose PCR gel of isolates of *Colletotrichum* spp. collected from Florida and Georgia that have produced amplified DNA fragments with either Calnt2 (20 isolates from left) or CgInt (13 isolates from right) species-specific primer. Ca Mil-1 and GD are reference isolates of *C. acutatum* and *C. gloeosporioides*, respectively (15).

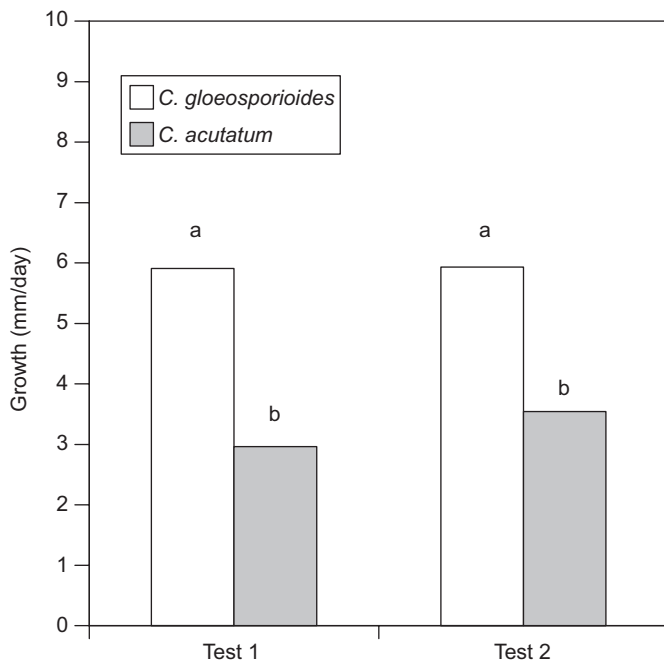


Fig. 2. Average radial growth per day of 45 isolates representing the two species of pepper anthracnose isolates recovered from Florida as determined by PCR. Colony radius (mm) was measured for three colonies per isolate after mycelial plugs were allowed to grow on artificial media (PDA) at 30 °C for 5 d in two separate tests (Test 1 and 2). The species of each isolate was determined previously by PCR using species-specific primers for *Colletotrichum gloeosporioides* (CgInt/ITS4) and *C. acutatum* (CaInt2/ITS4).

without access to many modern molecular techniques, colony growth rates, conidial size, and other phenotypic characteristics may be very important for initial identification of fungal isolates.

On a particular host, *Colletotrichum* species may exist as a hemi-biotroph or necrotroph using the terminology of Bailey et al. (1992) and O'Connell et al. (2000), and more recently Diéguez-Urbeondo et al. (2005). When *C. gloeosporioides* attacks pepper fruit, its lifestyle is probably that of a hemi-biotroph. Most likely, it initially colonizes the space directly below the cuticle (Prusky and Plumbley, 1992). Only as the fruit ripens does it produce enzymes that kill tissue and allow for development of the typical sunken lesions characteristic of anthracnose. When immature green bell pepper fruit were inoculated with *C. gloeosporioides* in field plots, lesions did not form until 45 d later when the pepper

fruit ripened and turned red (Harp, unpublished). Previous work has been reported that evaluates the genetic and physiological mechanisms that occur in pepper fruit in relation to susceptibility of *Colletotrichum* infection (Kim et al., 2001).

C. acutatum, in contrast, seems to establish as a necrotroph on immature fruit, producing symptoms in 7–10 d, long before fruit turn color (Lewis-Ivey et al., 2004). When immature green bell pepper fruit were inoculated with *C. acutatum* in field plots, lesions formed within 5 to 7 d (Harp, unpublished). However, one cannot easily predict how a particular species will react on any given crop. For example, *C. acutatum* acts as a hemi-biotroph, not a necrotroph, on apple, blueberry, and peach (Bernstein et al., 1995; Milholland, 1995; Jones et al., 1996; Zaitlin et al., 2000; Peres et al., 2005), and probably other crops (Prusky and Plumbley, 1992; Timmer et al., 1998).

Because most bell pepper in Florida is harvested as fully-sized green fruit, until recently, anthracnose had been a problem only when crops were extended to the ripened stage. Indeed, in the past, some growers and extension personnel referred to pepper anthracnose as “ripe-rot” to reflect its impact on ripe fruit only. However, the recent emergence of *C. acutatum* as a pathogen of immature pepper fruit raises the status of anthracnose to a potentially major disease problem throughout the industry. This likely means that anthracnose control measures must be initiated earlier and followed diligently throughout the crop cycle. This “new” anthracnose disease is sufficiently different from the traditional ripe-rot anthracnose to merit, in our opinion, a distinctive name. We propose the name “early anthracnose” for the disease of immature fruit caused by *C. acutatum*.

The presence of early anthracnose on pepper in Florida could have dire consequences for pepper growers throughout the state. Florida's humid and wet environment is most likely conducive to anthracnose diseases and could be cause for potentially dramatic yield losses. In addition, early anthracnose could emerge as a significant threat to other pepper-producing states in the future. More research is needed on this pepper disease, such as pathogen host range and efficacy of fungicides.

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