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Characterization of *Colletotrichum* Isolates Causing Anthracnose of Pepper in Korea

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A total of 33 isolates of *Colletotrichum* species obtained from pepper, apple, and strawberry in 2001 and 2002 were identified based on mycological characteristics, responses to fungicides carbendazim and the mixture of carbendazim and diethofencarb, and nucleotide sequence analysis of internal transcribed spacer (ITS) region. Most of the *Colletotrichum* isolates from pepper could be identified as *C. acutatum*. The pepper isolates produced grey white mycelia that gradually changed to dark gray. The conidia were variable in size, and almost cylindrical in shape with at least one rounded end. They could grow on PDA amended with carbendazim or with the mixture of carbendazim and diethofencarb at 10 μg/ml, to which the isolates from apple and strawberry were very sensitive. A part of the ITS regions from the *Colletotrichum* isolates was amplified with the specific primers designed for *C. acutatum* (Ca1-1) or *C. gloeosporioides* (Cg1-3). A primer pair of Ca1-1 and a universal primer (ITS4) amplified a 496-bp DNA fragment from all of the pepper isolates examined and one apple isolate. Taken together, it is conclusive that the *Colletotrichum* isolates causing the typical lesion of anthracnose on pepper fruits are *C. acutatum*.

Keywords : *Colletotrichum acutatum*, ITS region, pepper anthracnose, PCR with species-specific primer

The genus *Colletotrichum* contains a large number of fungi including both plant pathogens and saprophytes. They infect a wide range of crops, causing a disease commonly known as anthracnose in more than 197 plant species including crops, weeds, and trees (Sutton, 1992; Walter, 1992). In Korea, these fungal pathogens cause serious diseases on red pepper, strawberry, grapevine and apple (Park and Kim, 1992; Kim et al., 2007; Kim and Nam, 1999; Park et al., 1992). Especially anthracnose is one of the major limiting factors in red pepper production. The yield loss is serious since pepper fruits with anthracnose have little value for marketing.

Pepper (*Capsicum annuum*) including sweet and chili is an important crop due to its massive consumption as a seasoning vegetable in Korea and many other countries. The total annual production of pepper in Korea has been amounted to about US $1.4 billion. In Korea, the annual damage resulting from this disease has been estimated to be more than US $ 100 million, corresponding to about 10% of the total annual pepper production. Anthracnose makes lesions mainly on immature green and mature red fruits and leaves. Several *Colletotrichum* species such as *C. gloeosporioides*, *Glomerella cingulata*, *C. dematium*, *C. cocodes*, and *C. acutatum*, have been described to be involved in the anthracnose symptoms (Park and Kim, 1992). Among these, *C. gloeosporioides* was reported as the dominant pathogen as it was isolated from more than 90% of the diseased samples collected in 1992. The pathogens causing anthracnose in crops showed variable sensitivities to benzimidazole fungicides (Chung et al., 2006). Therefore, it is very important to identify the current pathogen causing anthracnose in the fields because benzimidazoles have been frequently used to control the pepper anthracnose.

The taxonomy of *Colletotrichum* spp. is in flux state. von Arx (1957) subsumed 594 species into a large one, *C. gloeosporioides*, based on mycological characteristics. To date morphological and cultural characteristics such as conidial morphology, presence or absence of teleomorph, colony color, growth rate, and sensitivity to benzimidazoles have been used to identify *Colletotrichum* species (Adaskaveg and Hartin, 1997 Freeman et al., 1998). Differential sensitivity to benomyl also has often been used in distinguishing *C. acutatum* from *C. gloeosporioides* (Adaskaveg and Hartin, 1997; Ishii et al., 1998). However, wide variations in these characteristics, together with the existence of isolates exhibiting intermediate or mixed characteristics, made their exclusive use unreliable to distinguish these two species (Marinez-Culebras et al., 2000; O'Neill et al., 1997 Sutton, 1992). This has led to unambiguous diagnosis of the

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pathogens in a number of pathosystems and implementation of control measures (Berstein et al., 1995). Nucleotide sequence of the internal transcribed spacer (ITS) 1 of ribosomal DNA gene has been used to distinguish *C. acutatum* from *C. gloeosporioides* on strawberry (Freeman and Katan, 1997). Based on the ITS1 sequence, specific polymerase chain reaction (PCR) primers for these two species have been developed (Gunnel and Gubler, 1992; Talhinhas et al., 2002). *C. acutatum* has been identified as the causal agent instead of the widely recognized *C. gloeosporioides* in the last few years (Marinez-Culebras et al., 2000; Urena-Padilla et al., 2002; Weeds et al., 2003).

As mentioned above, the five species of pathogens were reported as the pepper anthracnose pathogen in Korea. Although *C. gloeosporioides* sensitive to benzimidazoles was a major pathogen causing anthracnose on pepper in 1992, we found that most isolates causing pepper anthracnose were less sensitive to several benzimidazole fungicides (unpublished data). Because high degree of variation in some morphological characteristics is acceptable in *C. gloeosporioides* and other species of the genus, isolates of these species have practically overlapping ranges of conidial morphology, and show wide variation in colony characteristics. Since the traditional methods based on the morphological and cultural characteristics have not been satisfactory for identifying the species of *Colletotrichum*, molecular approaches have been used as an alternative. Especially, species-specific primers were developed and used with a conserved primer ITS4; the former were designed based on the ITS1 sequences from several *Colletotrichum* species.

It is very important to identify the major pathogen of the pepper anthracnose in the fields, because each *Colletotrichum* species shows significant variation in the response to the fungicides currently used in Korea. Four species of *Colletotrichum*, such as *C. gloeosporioides, C. acutatum, C. coccodes*, and *C. dematium*, were presented to be responsible for the occurrence of the pepper anthracnose (Park and Kim, 1992). Isolates of *C. gloeosporioides*, they insisted, composed of the field population up to 93%. Until now the outbreak of the pepper anthracnose has severely decreased the pepper production. Thus, it is very urgent to identify the major pathogen of the pepper anthracnose for early diagnosis and the disease management in the fields. The objectives of this study was to identify the *Colletotrichum* species isolated from pepper in Korean fields using ITS sequence analysis.

Materials and Methods

Collection of isolates. Thirty-three isolates of *Colletotrichum* were used in this study. During the period of 2001 and 2002, 11 isolates of *Colletotrichum* spp. were obtained from the infected pepper fruits in Korea. Four *Colletotrichum* isolates from apple and 11 isolates from strawberry were obtained from Gyeongbook National University in Daegu, Korea and Strawberry Experiment Station in Nonsan, Korea, respectively. The others were received from Korean Agricultural Culture Collection, Suwon, Korea. All the isolates were grown on potato dextrose agar (PDA; Difco, USA) at 25°C and maintained on PDA slants at 4°C.

Morphological and cultural characteristics. Fungal isolates were grown on PDA plates under a fluorescent light for 12 hrs per day at 25°C. To determine morphological characters, conidia formed on PDA for 7-day incubation were harvested with sterile distilled water and observed under a compound light microscope. One hundred conidia from each isolate were measured in length and width with 3 replicates. Colony color of each fungal isolate was also examined about 3 weeks after inoculation. For mycelial growth, petri plates containing PDA were inoculated at the center of each plate with a 5-mm-diameter mycelial disc that was taken from the margin of a 7-day old culture grown on PDA. Colony diameter of each isolate was measured daily for one week at 25 and 30°C. Three replicates of each isolate at both temperatures were evaluated.

Fungicide sensitivity test. Agar blocks were taken from the margins of the colonies grown on PDA at 25°C for 7 days, and transferred onto PDA plates containing 10 μg/ml of the fungicides. Carbendazim was dissolved in DMSO and amended into PDA at the indicated concentration just before pouring in petri plate. The untreated control was added only with DMSO. The final concentration of DMSO was below 1% in all treatments. Because diethofencarb has been used in the fields as a mixture with carbendazim, the sensitivity of isolates to diethofencarb was tested against the diethofencarb-carbendazim mixture. After incubation at 25°C for 7 days, colony diameters of each culture were evaluated on PDA with or without the fungicide.

Pathogenicity test. In laboratory, detached fruits (c.v. Nokkwang) harvested from the pepper plants in a greenhouse were used for determining the pathogenicity of each isolate. Fruits were either spray-inoculated to run-off, or wounded by a pin of the appliance (Accu-Chek® Softclix®) used for measuring the blood sugar and inoculated by dropping with 5 μl of a conidial suspension. Conidial suspension of *C. acutatum* JC24 isolated from the diseased pepper was prepared at concentration of 1×10^6 conidia/ml. Fruits were placed in plastic containers (30×20×10 cm, W ×L×H) where 3 sheets of paper towel soaked with 100 ml of distilled water were laid to maintain high humidity

(>95%, RH).

Extraction of fungal genomic DNA. Total DNA was extracted from mycelia obtained from PDA culture grown on at 25°C for 7 days. Aerial mycelia were harvested from the culture plates 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). Uncapped tubes were placed in a boiling water bath for 5 min, and then cooled to 25°C. Two hundred µl of phenol that was equilibrated with extraction buffer (v/v) and 200 µl of chloroform was added. The tubes were vortexed for 4 min, and then centrifuged at 13,000 *g* for 5 min. The supernatant was transferred to a new sterile 1.5-ml tube, and 200 µl of chloroform was added; the mixture was vortexed for 30 sec, and then centrifuged at 13,000 *g* for 15 min. The supernatant was extracted with 200 µl of isopropanol, and centrifuged at 13,000 *g* for 15 min. The nucleic acid pellet, after washing with 70% ethanol, followed by air-dried for 15 min, was resuspended in 50 µl of TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 8.5). DNA was finally treated with Ribonuclease A.

Sequencing of ITS regions. Primers ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') were used for amplifying ITS region. Amplification was conducted in a total reaction volume of 25 µl using the PCR kit (Bioneer Inc., Korea). The parameters used were as follows: A hold of 2 min at 95°C, 40 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C, and a final extension for 5 min at 72°C. The amplicons were column-purified with PCR purification kit (Atmanbio Inc., Korea), and cycle-sequenced by Eugentech Inc (Korea). The sequences were analyzed using both EditSeq and MegAlign programs (DNA Star Inc, USA).

PCR for the identification of *Colletotrichum* spp. Oligonucleotide primers Cg1-3 (5'-TAG GGT CTC CGC GAC CCT CCC GG-3) and Ca1-1 (5'-CAG GGG AAG CCT CTC GCG GGC CT-3') were designed based on the sequence similarity of ITS1 regions within/between species of both *C. acutatum* and *C. gloeosporioides*. These primers were used to specifically amplify the ITS1 regions of each species. Either of Cg1-3 (specific to *C. gloeosporioides*) or Ca1-1 (specific to *C. acutatum*) was used along with the backward primer ITS4 that was conserved in both *C. acutatum* and *C. gloeosporioides*. PCR reaction (30 µl) contained 50 ng of DNA, 1 M of each primer, and 15 ml of PCR Master mix (Promega, USA). Amplifications were conducted in a program as follows: 1 cycle of 4 min at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 65°C, and 1 min at 72°C, ending with 7 min at 72°C. PCR products were separated on a 0.7% agarose gels. PCR amplification was repeated at least 3 times.

Results

Mycological characteristics. Both cultural and morphological characteristics of the *Colletotrichum* isolates were examined. The colony color of the isolates from pepper was grey white at the beginning and gradually changed to dark

Table 1. Cultural characteristics of the *Colletotrichum* spp. isolated from pepper, apple and strawberry anthracnose

Isolate	Host	Growth rate (mm/day)[a]		Colony color[b]
		25°C	30°C	
KACC40700	pepper	8.1	5.9	gray
KS21	pepper	7.2	4.1	gray
JC24	pepper	8.1	6.1	gray
CJ38	pepper	7.3	4.4	gray
YS29	pepper	7.0	4.2	gray
02GG01	pepper	7.2	3.7	gray
02YY02	pepper	6.7	4.6	gray
02OG04	pepper	7.0	5.4	gray
02OG07	pepper	7.1	4.0	gray
02CO01	pepper	7.0	4.3	gray
2001-44	pepper	10.3	9.7	gray
KACC40690	pepper	10.2	10.0	white
B88	apple	7.8	6.0	orange
B165	apple	11.7	11.8	gray
B13	apple	13.0	10.6	white
B138	apple	11.0	11.6	white
B161	apple	12.3	12.5	gray
B176	apple	11.7	11.2	gray
B90	apple	11.8	10.6	gray
B92	apple	13.8	12.8	gray
B143	apple	11.6	12.7	gray
B147	apple	12.5	11.8	gray
CGF225	strawberry	14.4	16.0	gray
CGF50	strawberry	12.9	12.0	gray
CGF56	strawberry	12.7	11.8	gray
CGF214	strawberry	11.7	10.5	gray
CGF210	strawberry	12.1	12.0	gray
CGF212	strawberry	11.3	11.6	gray
CGF215	strawberry	10.8	11.1	gray
CGF250	strawberry	11.8	12.4	gray
CGF253	strawberry	10.1	12.2	gray
CGF254	strawberry	11.0	12.8	gray

[a]Values are means of 3 replicates on 5 days after incubation.
[b]Colony color of *Colletotrichum* spp. on PDA at 25°C on 7 days after inoculation.

Table 2. Comparison of conidial size of the *Colletotrichum* spp. isolated from pepper, apple and strawberry anthracnose

Isolate	Host	Conidium size[a] (μm)
JC24	pepper	4.2×14.7 (3-6×10.5-25.5)
YS29	pepper	4.0×14.8 (3-6×9-24)
02GG01	pepper	4.7×14.9 (3-6×9-21)
02CO01	pepper	4.1×16.0 (3-6×7.5-25.5)
2001-44	pepper	5.7×16.5 (3-7.5×9-22.5)
B88	apple	3.2×10.5 (3-4.5×6-22.5)
B165	apple	4.9×16.9 (3-6×9-24)
B161	apple	5.1×15.5 (3-7.5×7.5-27)
B176	apple	4.7×16.5 (3-6×13.5-21)
B90	apple	4.6×15.1 (3-6×10.5-24)
CGF56	strawberry	4.7×16.7 (3-7.5×10.5-30)
CGF212	strawberry	4.6×16.2 (3-6×10.5-25.5)
CGF215	strawberry	5.1×17.1 (3-7.5×12-22.5)
CGF253	strawberry	5.8×15.2 (3-7.5×9-2.5)
CGF254	strawberry	3.6×11.4 (3-6×7.5-18)

[a]Mean of 100 conidia.

gray; the bottom of PDA cultures for the isolates was grey (Table 1). Because of wide variation in conidial size, it was not appropriate to identify the isolates only based on the conidial size (Table 2). Conidial shapes of the *Colletotrichum* isolates tested were almost cylindrical, having both round ends (Fig. 1).

Fungicide sensitivity. As shown in Fig. 2, most of the pepper isolates could grow on PDA amended with carbendazim or with the mixture of carbendazim and diethofencarb at 10 μg/ml. However, two isolates, 2001-44 and KACC40690 did not grow at that concentration. The response of the

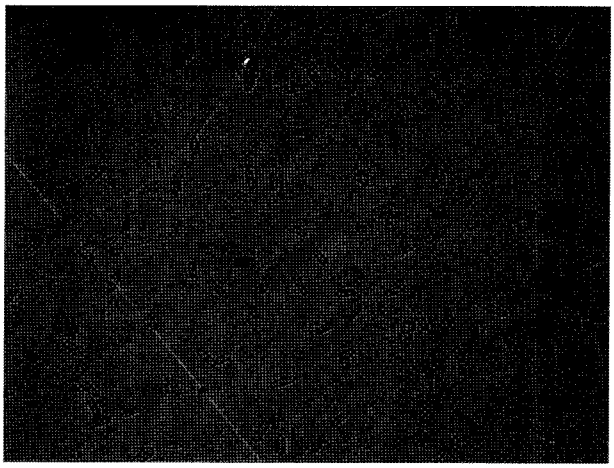

Fig. 1. Conidia of *Colletotrichum acutatum* JC24 grown on PDA at 25°C for 7 days.

isolates from apple and strawberry to carbendazim was diverse, ranging from sensitive to resistant, but all of them were very sensitive to the mixture of carbendazim and diethofencarb (Fig. 2). The two pepper-isolates showed the same response to the tested fungicides as did the apple- and the strawberry-isolates.

Pathogenicity of *C. acutatum* JC24 on pepper fruits. Through the pathogenicity test on the pepper fruits, it was confirmed that the *Colletotrichum* isolate JC24 from pepper was able to cause typical anthracnose lesions on the pepper fruits by both the wound or the non-wound inoculation (Fig. 3). The other isolates obtained from infected red peppers also showed a strong pathogenicity.

Sequence analysis. A phylogenetic relationship among the

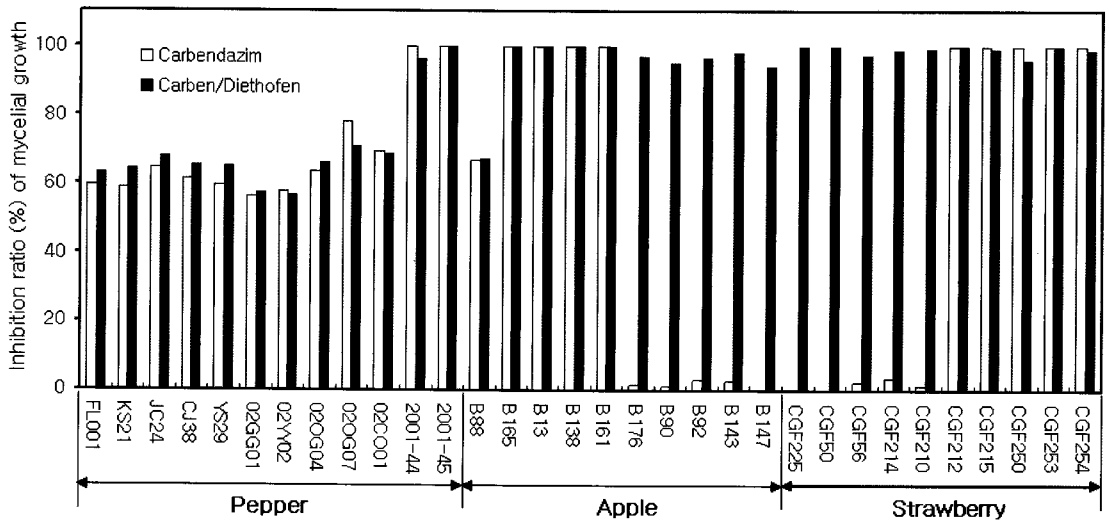

Fig. 2. Inhibitory activities of carbendazim and the mixture of carbendazim and diethofencarb against the mycelial growth of *Colletotrichum* spp. on PDA amended with 10 μg/ml of the fungicide.

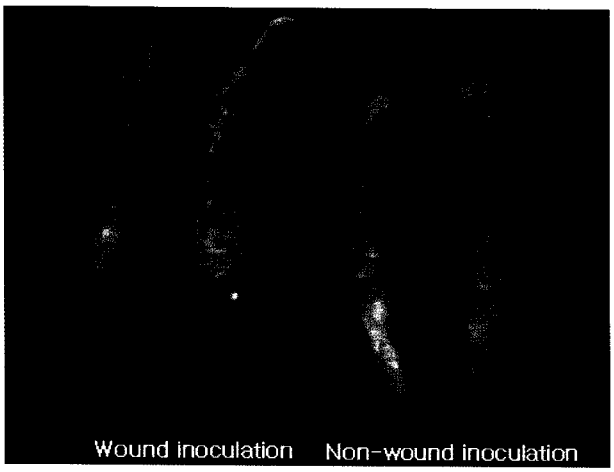

Fig. 3. Pathogenicity of *Colletotrichum acutatum* JC24 on the pepper fruits. Fungal conidia were harvested from PDA culture grown at 25°C for 7 days and adjusted to 1×10^6 conidia/ml for inoculation.

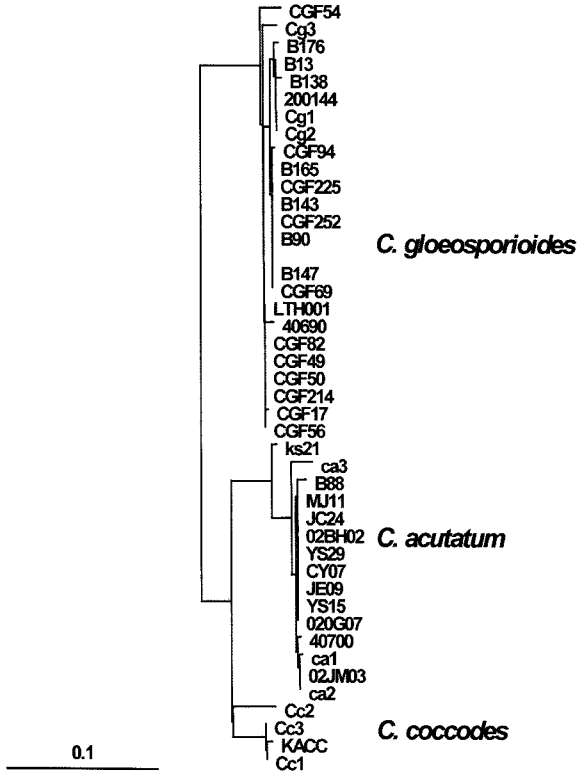

Fig. 4. A ITS-based phylogenetic tree of the *Colletotrichum* isolates. The tree was constructed using the neighbor-joining algorithm. The orders of branching were similar in all tree construction approaches used. Scale bar indicates estimated 10% sequence divergence.

Colletotrichum isolates was inferred from neighbor-joining analysis of the nucleotide sequences of amplified ITS regions (Fig. 4). Phylogenetic analysis revealed that the *Colletotrichum* isolates examined fitted well into three

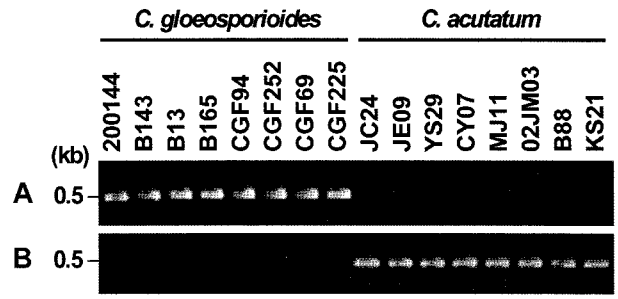

Fig. 5. Amplification of a species-specific fragment from fungal DNA using the primer pairs Cg 1-1 and ITS4 (A) or Ca 1-3 and ITS4 (B).

distinct groups of *C. acutatum*, *C. gloeosporioides* and *C. coccodes*; this grouping is consistent with that based on both mycological characteristics and fungicide sensitivity. All of the pepper isolates, except for 2001-44 and KACC40690, had the ITS sequence of *C. acutatum*. However, all of the 11 strawberry isolates were belong to the group of *C. gloeosporioides*. Among the apple isolates, six were identified as *C. gloeosporioides*, and the other, B88, was *C. acutatum*. KACC40700 was identified as *C. coccodes* based on the sequence similarity to that species.

Species-specific identification. A part of ITS regions was amplified from the 31 *Colletotrichum* isolates from pepper, apple, and strawberry using a specific primer set designed for *C. acutatum* (Ca1-1) or *C. gloeosporioides* (Cg1-3). The primer sequences were derived from the regions showing species-specific variation among the ITS regions. With a primer set of the *C. acutatum*-specific primer Ca1-1 and a universial primer ITS4, a 496-bp DNA fragment was amplified from all the pepper isolates (JC24, JE09, YS29, CY07, MJ11, 02JM03, and KS21) and one apple isolate (B88) (Fig. 5). Although 2001-44 was isolated from the infected pepper, a specific ITS region (495 bp) could be amplified with the *C. gloeosporioides*-specific primer pair, but not with the *C. acutatum*-specific primer pair. Using Cg1-3 and ITS4, the 495-bp DNA fragment was amplified from 3 isolates from apple (B13, B143, and B165) and 4 strawberry isolates (CGF69, CGF94, CGF225, and CGF252). Based on these results, it was confirmed that all of the 11 strawberry isolates and 7 out of 8 apple isolates were *C. gloeosporioides*.

Discussion

It has been known that the pepper anthracnose is caused by several different species of *Colletotrichum*. Each of these species was reported to differently respond to the fungicides used in the Korean pepper fields. For example, a wild-type isolate of *C. gloeosporioides*is is sensitive to benzi-

midazoles, whereas *C. acutatum* isolate is not sensitive to the same fungicide. Therefore, it is necessary to exactly identify the pathogen(s) for selecting cost-effective fungicides for the disease control. Morphological identification that had distinguished each pepper anthracnose pathogen as *C. gloeosporioides*, *C. acutatum*, *C. coccodes*, and *C. dematium*, respectively is now not reliable for pathogen identification, because of overlapping morphological characteristics between two species of *Colletotrichum*. In this respect, one of the most serious challenges in this study is that two pepper fruit-infecting pathogens, *C. gloeosporioides* and *C. acutatum*, could not be easily differentiated based on morphological and cultural characteristics. Adaskaveg and Hartin (1997) presented morphological characteristics of *C. acutatum* isolates causing anthracnose of almond and peach in California, but the isolates were highly variable, and affected by the both media and substrates used for culture. Physiological characteristics such as growth rate, optimal growth temperature, and fungicide sensitivity were also used to distinguish *C. acutatum* from *C. gloeosporioides*. Several researchers reported that the growth rate of *C. acutatum* was much lower than that of *C. gloeosporioides* (Agostini et al., 1992; Berstein et al., 1995; Liyanage et al., 1992). Based on the mycelial growth response to temperature, *C. acutatum* from strawberry, almond, and peach was considered to grow at 25°C as the optimum temperature, while the *C. gloeosporioides* isolates from citrus and papaya grew well at 30°C (Adaskaveg and Hartin, 1997). In the fungicide assay, the isolates of *C. acutatum* from several crops showed the less sensitivity to benomyl, whereas the *C. gloeosporioides* isolates from citrus and papaya were very sensitive to the fungicide at all applied concentration such as 300, 600 and 1,200 μg/ml. Based on the responses to benomyl and diethofencarb, Ishii et al. (1998) divided *Colletotrichum* spp. into three groups as 1) benomyl-sensitive and diethofencarb-resistant, 2) benomyl-resistant and diethofencarb-sensitive, and 3) insensitive to both benomyl and diethofencarb. They identified the last group as *C. acutatum*.

Sequence of the ribosomal DNA region containing the ITS1, ITS2, and the 5.8S rRNA gene has been analyzed for phylogenetics and systematics of *Colletotrichum* spp. (Afanador-Kafuri et al., 2003). Therefore, this approach may be applied to verify the identification of *Colletotrichum* spp. from pepper, apple and strawberry in Korea. Based on the sequence data of the ribosomal DNA region, the isolates of *Colletotrichum* spp. tested were divided into three species, *C. gloeosporioides, C. acutatum*, and *C. coccodes*. Except for KACC40700 identified as *C. coccodes*, 32 isolates were verified as *C. gloeosporioides* or *C. acutatum*. This result was consistent with PCR amplification using species-specific primers. The PCRs with *C. gloeosporioides*- or *C. acutatum*-specific primer have been used in identification of *Colletotrichum* population infecting various host plants. For example, it was confirmed that *C. acutatum* was the causal organism responsible for the recent outbreak of the almond and peach anthracnose in California, and that all lupin *Colletotrichum* isolates tested were positive with *C. acutatum*-specific PCR but not with *C. gloeosporioides*-specific PCR. In this study, all of the isolates from pepper, except the isolate 2001-44, showed positive response to *C. acutatum*-specific primer, too. Based on these results, the major pathogen of anthracnose in Korean pepper fields may be *C. acutatum* rather than *C. gloeosporioides*.

In the case of strawberry, *C. gloeosporioides* and *Glomerella cingulata* were reported as the major pathogens of anthracnose in Korea (Kim and Nam, 1999). Also, all strawberry isolates of *Colletotrichum* spp. not only were included into *C. gloeosporioides* based on the sequence data but also showed positive response with *C. gloeosporioides*-specific primer. Among the apple isolates, B88 was not positive with the *C. gloeosporioides*-specific primer, whereas all remaining isolates were positive the same primer. Although the number of *C. acutatum* tested in this study was relatively low, it is important that Ca1-1 is able to detect the isolate (B88) from apple. Therefore, Ca1-1, a species-specific primer for *C. acutatum*, could be applied to identify *C. acutatum* isolates infecting other host plants. Recently, it was known that the population of *C. acutatum* causing the apple anthracnose increased continuously in fields (unpublished data). In Korea, the *C. acutatum* population in several crops might increase because of several factors, such as resistant responses to benzimidazoles and a strong field fitness of a specific pathogen.

In this report, it was verified that the anthracnose pathogens isolated from infected peppers were *C. acutatum* by using molecular techniques. Furthermore, *C. acutatum* was re-identified as a major pathogen in many crops. For the control of pepper anthracnose, it was of importance to identify an anthracnose pathogen of pepper, and to continuously monitor the population shift of a major pathogen in the fields.

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