Plant Pathol J 15(6) : 323-329 (1999)

The Plant Pathology Journal

Comparison of Pepper Anthracnose Pathogens from Korea and China

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(Received on November 19, 1999)

Anthracnose of pepper casued by *Colletotrichum* spp. has been a great problems for pepper production in Korea and China. Especially *Colletotrichum gloeosporioides* was found predominantly over cultivation areas during infection periods and caused severe rots on both unripe and ripe fruits that resulted in major yield losses. In this study, comparison of *Colletotrichum* spp. isolated from Korea and China in morphology and pathgenicity, and RAPD-PCR analysis were conducted. Based on morphological characteristics, the pathogen isolates, K1 and C1, K2 and C2, and K3 and C3 were identified as *Colletotrichum gloeosporioides* (G) type, *C. gloeosporioides* (R) type and *C. coccodes*, respectively. In pathogenicity test, K1 and C1, and K2 and C2 were found to attack mainly fruits and to be the most virulent among isolates. K3 and C3 were strongly virulent to leaves and seedling. Pathogenicity between Korean and Chinese isolates did not show any difference. Results of the RAPD-PCR analyses indicate the varying levels of molecular diversity within and between *Colletotrichum* spp. of Korea and China. The similarities between K1 and C1, K2 and C2, and K3 andC3 were 85.71%, 71.43% and 50.0%, respectively.

Keywords : anthracnose, *Capsicum annuum, Colletotrichum* spp, RAPD-PCR.

Anthracnose of pepper has been a great problem for pepper production in Korea and China (Gong et al., 1992; Hu et al., 1992; Park et al., 1992). In recent five years, the annual losses caused by the disease in the two countries amounted to 13% and 18% of total production, respectively. Five species of anthracnose, *Colletotrichum gloeosporioides, Colletotrichum coccodes, Colletotrichum dimatium, Glomella cingulata* and *Colletotrichum acutatum*, have been found on pepper in Korea and China. Anthracnose epidemics of pepper in Korea and China seem largely by *C. gloeosporioides*. This species was found predominantly over cultivation areas during major infection periods and caused severe rots on both green and red pepper fruits resulting in major yield losses (Liu et al., 1991; Hu et al., 1992; Park et al., 1992).

The objectives of this study were to compare the morphological characters of the *Colletotrichum* spp. isolates from both Korea and China, to examine their pathogenicity, and to detect the variation among *Colletotrichum* spp. isolated from Korea and China by RAPD-PCR techniques.

Materials and Methods

Collection of diseased samples. Diseased samples were collected from Young Yang Pepper Experimental Station, Korea in 1998 and those of China from pepper growing area, Hu nan province of China in 1997-1998. Collected samples were put in paper bag, labeled and kept in a cold room (4-5°C) until used. Thirteen peppei lines of Korea were supplied from Prof. Hyo Gun Park, Dept. of Horticulture, Seoul National University, and eight pepper lines of China were supplied from Chinese Academy of Horticultural Sciences.

Isolation and morphological examination. Diseased samples were cut into small pieces (3 mm^2), surface-disinfected with 70% alcohol for 2-3 sec and 0.1% mercuric chloride for 30 sec, placed on potato dextrose agar (PDA) medium, and incubated at 25-28°C for 7-10 days. Monoconidial culture was obtained from the PDA media. During the growth, colony morphology and mycelial growth rate were examined. Development of acervuli, setae and sclerotia, and the size and shape of conidia and appressoria were examined under the microscope with both the diseased samples and 7-10-day-old cultures on PDA (Parbery and Emmett 1977; Kim et al., 1986; Oh et al., 1988). Identification of the isolates was referred to morphologocal characters described by Arx (1970), Simmond (1965), Wang and Li (1987) and Wu and Zhang (1994a, b).

Pathogenicity tests. Pathogenicity of the *Collectotrichum* isolates K1 and C1 was examined on leaves, seedling, and unripe and ripe fruits of pepper lines were collected from Korea and China. Conidial suspension of the isolates at concentration of 1×10^6 spore/ml prepared from 7-10-day-old culture were used as an inoculum. Detached unripe (after flowering for 35-day-old) and ripe fruits (over 90% red-coloring) were washed with tap water, and point-inoculated by dropping 10 ml conidial suspension on each sample using micro-syringe (Park et al., 1992). After inoculation, the fruits were kept at 28°C, and near 100% relative humidity for 48 hr, incubated for an additional 5-8 days, and

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examined for lesion development. For seedling inoculation, the same conidial suspension was sprayed to 60-day-old plants (Liu et al., 1991; Gong et al., 1992; Hu et al., 1992). The disease symptoms were investigated 7-10 days after the inoculation.

Genomic DNA analysis of *Colletotrichum* spp. The six isolates and control isolate (*C. acutatum*) were grown on PDA medium for 7-10 days at about 25-28°C. The mycelium was harvested, ground under liquid nitrogen in a motar and pestle. Genomic DNA was extracted by a modified benzyl chloride extraction protocol (Zhu et al., 1994; Li et al., 1998). Final concentration of the DNA solution (in distilled, deionized water) was adjusted to 100 ng/μl.

Each reaction volume, determined empirically at 50 μl, was composed of 0.1 mM dNTP 4 μl, 2.0 μM primer 2 μl, 100 ng of template 2 μl, 2 unit of *Taq* polymerase 1 μl (Promega), *Taq* buffer (10) 4 μl, 2.5 mM $MgCl_2$ 4 μl and water to 50 μl. The PCR mixture was subjected to 40 cycles of 45 sec at 94°C, 1 min at 36°C and 1.5 min at 72°C, preceded by 4 min denaturation at 94°C and followed by a 10 min extention at 72°C

PCR products (20 μl) were loaded into the wells of a 1.2% w/v agarose gel containing ethidium bromide (0.4 μg/ml), and electrophoresed at 50 V for 1-2 hr along with a molecular size marker (*Hin*dIII-digested λDNA), and photographed on a transilluminator.

Each isolate was amplified by using 10-base random primers (Shi et al., 1996). The primers were designed and synthesized in Zhejiang Univ. DNA fragments of the same size were assumed to represent the same genomic locus and scored as either present or absent. The cluster analysis of the data was done based on a similarity matrix derived from the formula: $[(2\ N_{ab}) / N_a + N_b] \times 100\%$, where N_{ab} is the number of shared fragments between two samples, a and b, N_a and N_b are total numbers of fragment in each of the samples (Adaskaver et al., 1989; Guthrie et al., 1992; Sreenivasaprasad et al., 1992; Stanley et al., 1996).

Results

Identification of the fungal pathogens. Six isolates of anthracnose fungi from Korea and China were grouped into two *Colletotrichum* species, *C. gloeosporioides* and *C. coccodes*. In *C. gloeosporioides*, type G and type R were recognized based on recent taxonomic criteria. The morphological characteristics of Korean isolates were compared with those of the Chinese isolates (Table 1, 2 and Fig. 1).

The isolates K1 and C1, identified as *C.gloeosporioides* (G) type, were frequently isolated from lesions of both unripe and ripe fruits. The type G was found to develop initially sunken lesions on fruits that enlarged to form water-soaked, dark brown round lesions, where bright orange-reddish conidial mass was formed on the surface. Conidia were cylindric, monocell, dyed color, 15.5-19.5 × 4.5-5.7

Table 1. Comparison of conidial and appressorial size and shape of *Colletotrichum* isolates from Korea and China associated with pepper anthracnose

Isolates[Y]	Conidial shape	Conidial size (μm)[Z]	Appressorial shape	Appressorial size (μm)
K1	Cylindric	16-19 × 4.5-4.7	Near round	2-8.5 × 3.5-5.5
C1	Cylindric	5-19.5 × 4.5-5.0	Near round	5-8.5 × 4-5 5
K2	Cylindric	5-17.5 × 3.5-4.5	Near round - round	5.7-6.7 × 4-4 5
C2	Cylindric	13-18.5 × 3.5-5.0	Near round - round	5.5-7 × 4.5
K3	Cylindric, long	5-19 5 × 3.4	Round	5-8.5 × 7-8.5
C3	Cylindric, long	5-20 × 3.5-4.5	Round	7-9 × 7.5-9.5

[Y] K1, K2 and K3 were collected from Korea, and C1, C2 and C3 were collected from China.
[Z] Conidia and appressoria size were measured under microscope (15 × 40). These values are averages from 100 spores.

Table 2. Comparison of morphological characteristics of *Colletotrichum* isolates from Korea and China associated with pepper anthracnose

Species[X]	Setae	Acervuli	Sclerotia	Colony color on PDA	Linear growth (mm/day)[Z]
K1	-[Y]	+	-	White-Light grey	8.7
C1	-	+	-	White-Light grey	8.8
K2	+/-	+/-	+/-	Dark grey	10.6
C2	+/-	+/-	+/-	Dark grey	10.8
K3	++	+	++	Drak grey	11.4
C3	++	+	++	Dark grey	11.1

[X] K1, K2 and K3 were collected from Korea, C1, C2 and C3 were collected from China.
[Y] – absent, + present, +/– present or absent, ++ abundant.
Observation was based on both the diseased samples and the cultures on PDA medium.
[Z] Mycelial growth was measured on PDA.

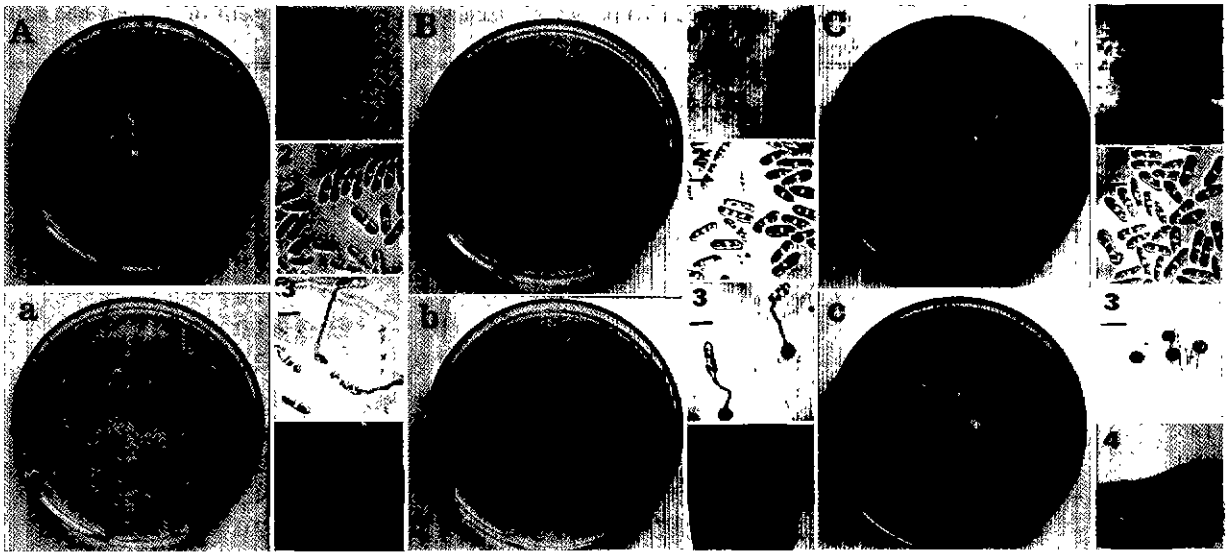

Fig. 1. Morphological characteristics and the disease symptoms of pepper anthracnose pathogens from both Korea and China (A and (a): *Colletotrichum gloeosporioides* G type; B and (b): *C. gloeosporioides* R type; C and (c): *C. coccodes*)
A: K1, B: K2, and C: K3, origin is Korea. (a): C1, (b): C2, and (c): C3, origin is China.
1: Production of sclerotia on PDA. K1 and C1 did not produced sclerotia. 2: Conidia of each isolates. Bar=10 μm. 3: Appressoria of each isolates. Bar=10 μm. 4: Disease symptom on pepper fruits.

μm in size. Setae and sclerotia were not found on both the diseased samples and the PDA culture. On PDA, this type initially formed whitish colony, produced light orange conidial mass from the center, and developed concentric zones of acervuli, later its colony surface turned to whitish grey. The mycelial growth rate was slow with 8.7-8.8 mm/day. Appressoria were near round, 7.25-8.5 × 3.5-5.5 μm in size.

The isolate K2 and C2 isolated from lesions of only ripe fruits were identified as *C. gloeosporioides* (R) type. The type was found to develop initially sunken lesions on fruits that enlarged to form water-soaked, dark black spot, later numerous small black acervuli were developed on the lesions surface. Conidia were cylindric, monocell, dyed color, 13.0-18.5 × 3.5-5.5 μm in size, sometimes setae and sclerotia were found on both diseased samples and PDA culture. On PDA medium, this type was initially whitish grey, later turned to dark grey, occasionally produced orange conidial mass on the center. The mycelial growth rate was 10.6-10.8 mm/day, faster than G type. Appressoria were near round or round, 5.5-7.0 × 4.0-5.0 μm in size. Appressoria of China R type was bits of rounder than that of Korea.

The isolates K3 and C3 isolated from fruits and leaves, particularly ripe pepper and mature leaves were identified as *C. coccodes,* were Conidia were cylindric, monocell, dyed color, 16.5-20.0 × 3.0-4.5 μm in size, but long and narrow comparing to *C. gloeosporioides.* This species produced abundant setae and sclerotia. The mycelial growth rate was 11.1-11.4 mm/day. Appressoria shape were round, 7.0-9.0 × 7.0-9.5 μm in size, On PDA medium, the fungus formed initially whitish grey colony, later turned to dark grey. This species was found to produce irregular black sunken lesions, and numerous small black spot on the lesion surface.

No difference of morphological characteristics was found between Korean and Chinese isolates within each species or types, but, the difference among *C. gloeosporioides* (G) type, *C. gloeosporioides* (R) type, and *C. coccodes,* isolates were distinguished.

Pathogenicity tests. Pathogenicity of Korea and China isolates was examined on 21 lines of pepper from Korea and China. K1 and C1 (=*C. gloeosporioides* G type) isolates were most virulent to both green and red fruits, but not pathogenic on leaves and seedlings. These isolates developed brown sunken lesions on fruits.

K2 and C2 (=*C. gloeosporioides* R type) isolates produced severe necrotic lesions on ripe fruits, and ambiguous lesion or lightly-rotted lesions, if wounded, on unripe fruits. These isolates were also not pathogenic on leaves and seedlings.

K3 and C3 (=*C. coccodes*) isolates were found strongly virulent to both seedlings and mature leaves. They were produced lesions on unripe fruits, but their virulence on unripe fruits were weak. These isolates were not pathogenic on red fruits. Most of seedling leaves were defoliated 7-10 days after spray inoculation, and on seedling stems oval shape- or irregular brown sunken lesions were developed. These isolates produced brown, water-soaked, small lesions

Table 3. Pathogenicity of *Colletotrichum* isolates associated with pepper anthracnose on various plant parts of pepper when artificially inoculated in the laboratory and greenhouse

Isolates[W]	K1 and C1				K2 and C2				K3 and C3				HR[X]
	Leaf	Seedling	Unripe fruit	Ripe fruit	Leaf	Seed-ling	Unripe fruit	Ripe fruit	Leaf	Seed-ling	Unripe fruit	Ripe fruit	
C1081	–	–	2.9[Y]	2.8	–	–		2 9	2.9	3.5	0.2	–	I
C1108	–	–	3.2	3.2	–	–		3.4	3.2	4.0	0 2	–	S
C1188	–	–	2.2	2.3	–	–		2 4	2 3	3.0	0.1	–	I
C1194	–	–	1 0	0.9	–	–		0.9	0.9	1.0	–	–	R
C1201	–	–	0 9	0.9	–	–		0.9	0.9	1.0	–	–	R
C1204	–	–	1 8	1.9	–	–		1.9	1.7	2.5	0.1	–	I
C1232	–	–	3.2	3.3	–	–	0.1	3.4	3.3	4.5	0.2	–	S
C1285	–	–	0 7	0.7	–	–		0.8	0.8	1.0	–	–	R
K10	–	–	2.2	2.2	–	–		2.3	2.2	3.0	0.1	–	I
K23B	–	–	3.7	3.6	0.1	–	0.1	3.6	3.7	4.5	0.1	–	S
K29	–	–	1.4	1.5	–	–		1.5	1.5	2.0	0.1	–	I
K33	–	–	2.4	2.3	–	–		2.5	2.4	3.0	0 1	–	I
K46	–	–	2.6	2.7	–	–		2.7	2.7	3.5	0.1	–	I
K47	–	–	3.1	3.2	–	–		3.3	3.2	4.0	0 1	–	S
K142	–	–	4.2	4.3	0.2	–	0.2	4.4	4.3	5.0	0.1	–	S
K149	–	–	0.8	0 9	–	–		0.9	0.8	1.5	–	–	R
K191	–	–	0.7	0.7	–	–		0.7	0.7	1.0	–	–	R
K207	–	–	1.5	1.6	–	–		1.7	1.6	2.5	0.1	–	I
K209	–	–	0.9	0.9	–	–		0.9	0.9	1.0	-	–	R
K210	–	–	3.8	3.9	–	–	0.1	3.8	3.9	4.5	0.3	–	S
K212	–	–	3.4	3.5	–	–	0.1	3.6	3.6	4.5	0.2	–	S

[W] C1, C2 and C3 isolates, and 'C' lines were collected from China.
K1, K2 and K3 isolates, and 'K' lines were collected from Korea.
[X] HR (=Host Response), R=Resistant, S=Susceptible, I=Intermediate.
[Y] Disease index: 1, No symptom, 2; Lesion diameter < 1 mm, on 1-2 leaves disease incidence, 3; Lesion diameter > 1 mm, on 2 true leaves disease incidence, 4; Over 2 true leaves disease incidence, 5; Plant just before die or already dead. Numbers are means of the average of observed on 40-50 pepper seedlings per lines

on leaves.

The results showed that the pathogenicity between Korea isolates and China isolates did not show difference (Table 3).

RAPD-PCR analysis of *Colletotrichum spp* isolates from Korea and China. For RAPD-PCR analysis, 140 random primer have been used. The banding patterns obtained by using primers A_5 (5' AGGGGTCTTG 3'), M_{20} (AGGTCTTGGG), O_2 (ACCTTAGCGTC), (Fig. 2, respectively) P_{12} (AAGGGCGAGT) and O_4 (AAGTCCGCTC), O_{20} (ACACACGCTG) (data not shown).

Amplification products ranged from approximately 1.5 to 6 kb. Polymorphism of RAPD-amplified fragments among *Colletotrichum* isolates from two countries and their mutual similarities were compared. The results showed that the similarities varied within 15.4%-85.7% among the tested isolates. The similarities between K1 and C1, K2 and C2, and K3 and C3 were 85.71%, 71.43%, and 50.0%, respectively. The similarities between *C. gloeosporioides* (G) type and *C. gloeosporioides* (R) type, *C. gloeosporioides* and *C. coccodes* were 28.57% and 15.38%, respectively (Table 4 and Fig. 3).

Discussion

The genus *Colletotrichum* is an important taxon of plant pathogenic fungi, commonly causes anthracnose on a variety of plants. Disease symptoms caused by *Colletotrichum* species develop at all growth stages of some plants. The fungal species also cause preharvest and postharvest losses in some kinds of fruits and seeds. The taxonomy of *Colletotrichum* species is mainly based on morphological characteristics of conidia, setae, sclerotia, and appressoria and supplementarily cultural characterisitics and host specificity.

In this study, six isolates of anthracnose fungi of Korea and China were isolated and identified. Higgins (1926) reported several pathogenic fungal species causing anthra-

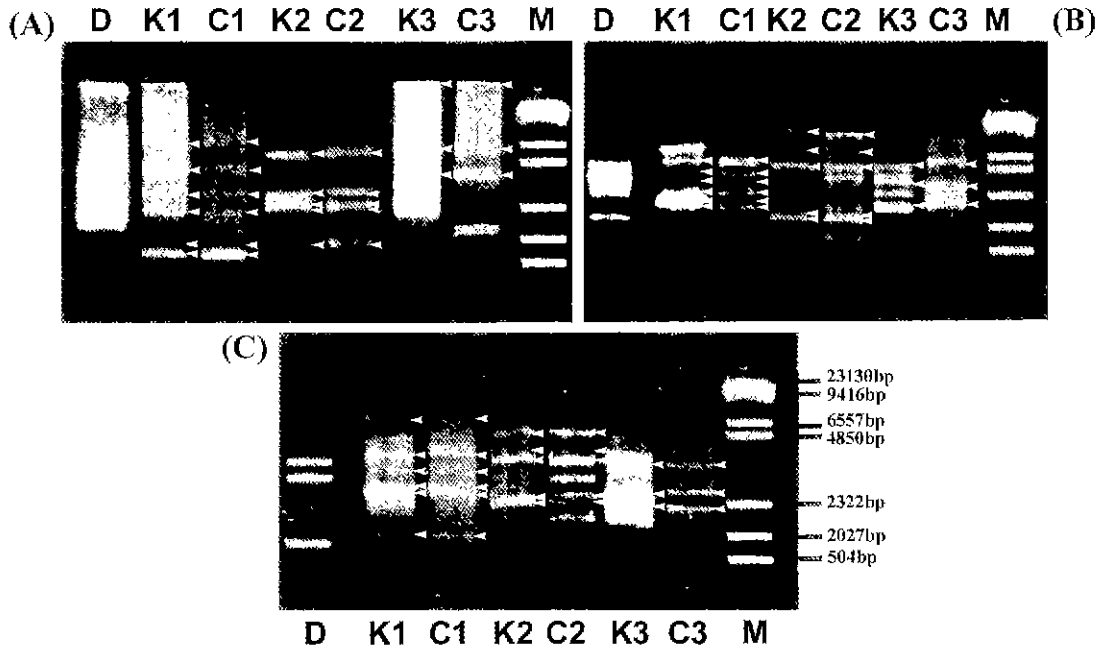

Fig. 2. RAPD patterns of *Collectotrichum* isolates from Korea and China using random primers. A panel: Primer A_5 (AGGGGTCTTG); B panel: Primer M_{20} (AGGTCTTGGG); C panel: Primer O_2 (ACCTTAGCGTC). M: *Hind* III-digested λ DNA was used as a molecular size marker. D: reference isolate, *Colletotrichum acutatum*

Table 4. Similarities of the *Colletotrichum* isolates of pepper anthracnose pathgen isolates from Korea and China generated by RAPD-PCR

Isolates	No. of DNA fragments	Similarity (%)[Y]						
		K1	K2	K3	C1	C2	C3	*C.a*[Z]
K1	7	-	14.28	15.38	85.71	28.57	15.38	15.38
K2	7	14.28	-	30.76	14.28	71.43	15.38	30.76
K3	6	15.38	30.76	-	15.38	15.38	50.0	16.67
C1	7	85.71	14.28	15.38	-	28 57	15.38	15.38
C2	7	28.57	71 43	15.38	28.57	-	14.28	15.38
C3	6	15.38	15.38	50.0	15.38	14.28	-	33.33
C.a[Y]	6	15.38	30.76	16.67	15.38	15.38	33.33	-

[Y] Numbers are the average calculated from pairwise comparisons done by using formula: $[(2\ N_{ab})/N_a + N_b] \times 100\%$ among representative pepper *Colletotrichum* isolates with primers A_5 (5' AGGGGTCTTG 3'), M_{20} (AGGTCTTGGG), O_2 (ACCTTAGCGTC) from Fig. 2 and primers P_{12} (AAGGGCGAGT) and O_4 (AAGTCCGCTC), O_{20} (ACACACGCTG).

[Z] *Colletotrichum acutatum*

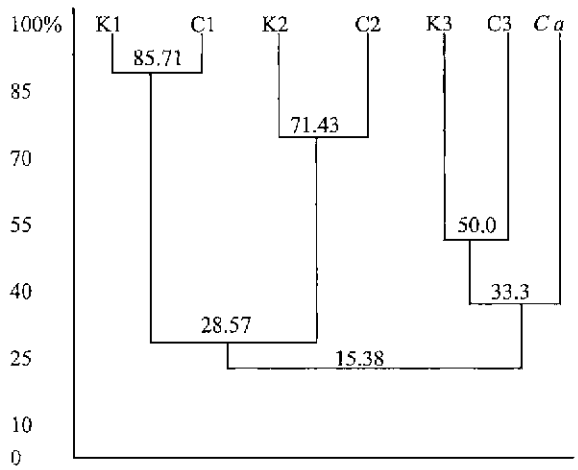

Fig. 3. Clustering analysis of RAPD fragments of the pepper anthracnose pathogen isolates from Korea and China. K1, K2, K3 and *C.a*: Korean isolates; C1, C2 and C3: Chinese isolates. *C.a*=*Colletotrichum acutatum* (reference isolate)

cnose on *Capsicum annuum* under names of genus *Colletotrichum, Gloeosporium* and *Glomerella.* He pointed out that most virulent pathogen was *Gloesporium piperatum.* Arx (1970), Simmond (1965), Wang & Li (1987) and Wu & Zhuang (1994a, b) considered *Gloeosporium piperatum* as a synonym *of Collectotrichum gloeosporioides.* Based on morphological chracters and pathogenicity, *G. piperatum* is apparently the same species as *C. gloeosporioides* described in this study, the predominant pathogen of pepper anthracnose in Korea and China. Simmonds (1965) identified two forms of *C. gloeosporoides* pathogenic to *Capsicum*, and proposed them as *C. gloeosporioides* var. *gloeosporioides* and *C. gloeosporioides* var. *minor. C. gloeosporioides* has been classified into G and R strains. The R strains infect only red pepper fruits, whereas the former infects both green and red fruits. These strains of *C. gloeosporioides* have been isolated from pepper fields in

Korea (Kim et al., 1986). In this study, we also found two morphologically distinct types of *C. gloeosporioides*. These two types could be distinguished based on presence or absence of setae, perithecia and sclerotia, mycelial growth rate and degree of virulence. The one without setae, sclerotia and perithecia, and with slow mycelial growth was *C gloeosporioides* (G), the most frequent and virulent pathogen, which has caused severe epidemics in Korea and China.

Simmonds (1965) has reported *C. nigarum* (a synonym of *C.c*). *C. coccdes* in this study was well distinguished from others, since this species produced abundant small black sclerotia on PDA medium, and had long slender, cylindrical conidia and round apperessoria. These morphological characteristics of *C. coccodes* were observed by Oh et al. (1988) in Korea. The results of this study showed that K1 and C1, K2 and C2 and K3 and C3 were each the same species. Based on morphological characteristics, no difference was found between Korea and China isolates.

The virulence of *C. gloeosporioides* seems to be specific mainly on fruits. Foliage and seedling anthracnose was attributed to *C. coccodes*. In this study, also pathogenicity of Korea isolates and China isolates in pepper lines did not show difference.

Molecular analyses with protein patterns, restriction fragment length polymorphism, polymerase chain reaction, and so forth have been conducted to differentiate isolates of some *Colletotrichum* species. RAPD-PCR has been useful for separating morphologically indistinguishable isolates of *Colletotrichum* spp or isolate of same species collected from different regions. (Wang and Li 1987, Adaskaver et al., 1989, Prusky et al. 1992) RAPD-PCR analysis has been applied to differentiate between isolates of *C. gloeosporioides* from strawberries and a variety of tropical fruits including avocados, mangos and papayas, and to assess the extent of variability within populations of *C. gloeosporioides* that infect tropical fruit. (Alahakoon et al., 1992 and 1994, Stanley 1996) In this study, we aimed to determine whether between Korea and China isolates were difference of genomic DNA. Results of the RAPD-PCR analysis indicate the varying levels of molecular diversity within and between *C. gloeosporioides* and *C. coccodes*. These results showed that geographical segregation did not much affect genetic diversity between K1 and C1 isolates, K2 and C2 isolates, and K3 and C3 isolates. But, indicated the varying levels of genetic diversity between G type and R type of *C. gloeosporioides*. We have shown the potential of RAPD-PCR for identifying and differentiating isolates.

In practical sense, studies on pepper anthracnose are described to focus on *C. gloeosporioides*, major pathogen responsible for recent anthracnose epidemics in Korea and China, also, need to RFLP analysis and sequencing of variable region of the ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) in order to elucidate the molecular variation of the pepper anthracnose fungi and determine the interrelationship among Korea isolates, K1, K2 and K3, and China isolates, C1, C2 and C3. Up to present, G and R types were not separated as to species, but both have been referred to as *C. gloeosporioides*, future classification studies on G and R type of *C. gloeosporioides* are desired to separate as to species.

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