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Sensitivity of *Colletotrichum* spp. Isolated from Grapes in Korea to Carbendazim and the Mixture of Carbendazim Plus Diethofencarb

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Thirty-six isolates of *Colletotrichum* spp. were obtained from infected grapes in two different locations of Korea; 18 isolates from Cheonahn, where carbendazim (MBC) and the mixture of MBC and diethofencarb (NPC) had been applied to control grape ripe rot, and 18 isolates from Cheongju, where no fungicides had been used. Sequences analysis of the internal transcribed spacer (ITS) and the β-tubulin gene identified 34 of the 36 isolates as *Colletotrichum gloeosporioides*. The remaining two isolates from Cheongju were identified as *C. acutatum*. Of the 18 isolates from Cheonahn, 12 were resistant to both MBC and the mixture (MBC+NPC), and six were sensitive to them. All *C. gloeosporioides* isolates from Cheongju, but not the two *C. acutatum* isolates, were sensitive to these fungicides. Sequence analysis of the β-tubulin gene in all isolates revealed that *C. gloeosporioides* resistant to MBC and MBC+NPC had a tyrosine instead of phenylalanine at the amino acid position 200. The appearance of resistance to MBC and the mixture in *C. gloeosporioides* correlated with the history of fungicide application in Korea.

Keywords **:** fungicide resistance, mixture of carbendazim plus diethofencarb, grape ripe rot, *Colletotrichum gloeosporioides*, *Colletotrichum acutatum*

Grape ripe rot, a serious *Colletotrichum* bunch rot disease that occurs on mature grapes as they ripen, was first reported in the United States in 1891 and has been found in most regions where grapes are grown, especially in warm and humid areas such as the southeastern USA (Pearson and Goheen, 1988). Initially, *C. gloeosporioides* was considered to be the causal organism of the disease. However, both *C. gloeosporioides* and *C. acutatum* were implicated in ripe-rot disease outbreaks on muscadine grapes in the United States (Daykin and Milholland, 1984; Kummuang et al., 1996). More recently, *C. acutatum* was found to be the causal organism for ripe rot in Korea along with *C. gloeosporioides* (Hong et al., 2008). In Japan, ripe rot has also been the most serious pre-harvest disease of grape berries with the main causal pathogens being *Glomerella cingulata* and *C. acutatum* (Ozoe et al., 1972; Yamamoto et al., 1999).

In Korea, several fungicides have been used to control grape ripe rot. Because unlike *C. gloeosporioides*, *C. acutatum* is resistant to certain fungicides such as benzimidazoles (BENs), correct taxonomic identification is therefore important in selecting appropriate fungicides for disease management (Chung et al., 2006; Kim et al., 2007; Peres et al., 2004). For approximately 30 years, BENs including carbendazim (MBC), benomyl, and thiabendazole have been widely and successfully used to protect many crops from phytopathogens (Russell, 1995). These agents inhibit nuclear division via disruption of microtubule assembly during mitosis by binding to the β-tubulin subunit (Davidse and Flach, 1977). However, The extended use of these compounds has resulted in the selection of resistant pathogen genotypes, which can remain dominant in a population for several years after discontinued BEN use (Moorman and Lease, 1992). The first cases of resistance were reported in fungi with short life cycles, such as *Botrytis cinerea*, in grape vineyards (Leroux and Clerjeau, 1985). Due to the emergence of many types of resistant populations of phytopathogenic fungi in the field, the efficacy of these chemicals to control plant diseases has decreased (Bonnen and Hopkins, 1997; Maymon et al., 2006; Washington et al., 1992). In most reported cases of acquired resistance to BENs, point mutations in the β-tubulin gene were responsible (Koenraadt et al., 1992). Studies of the mutants generated in the laboratory have revealed a small number of amino acid substitutions in the β-tubulin gene that cause resistance (Fujimura et al., 1992).

Numerous cases of resistance to BENs have been reported for several species of *Colletotrichum* in various crops (Kim et al., 2007; Maymon et al., 2006; Peres et al., 2004; Sanders et al., 2000; Wong et al., 2008; Yang and TeBeest, 1995). Although BENs have been widely used to

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control grape ripe rot caused by *C. gloeosporioides* and *C. acutatum* in Korea, there have been no studies on the sensitivity of populations of two pathogen species to BENs. The focus of this study was to determine the incidence of resistance to carbendazim (MBC) and the mixture of MBC and diethofencarb (NPC) among *Colletotrichum* isolates obtained from two main grape production areas in Korea, Cheonahn and Cheongju, and characterize the β-tubulin gene in resistant isolates.

Materials and Methods

Fungal isolates. The *Colletotrichum* isolates used in this study were prepared from diseased grapes collected at Cheonahn vineries, where MBC and the mixture (MBC+ NPC) had been used to control grape ripe rot, and at Cheongju vineries, where no fungicides had been used. A pure culture originated from a single conidium was prepared as follows: the infected grape fruits were incubated at 25°C for 24 h in plastic boxes (200×250×70 mm) containing two layers of paper towels wetted with 50 ml of sterilized water at the bottom. The conidia that formed on the lesions were scraped and suspended in sterilized water. After adjusting the concentration of the conidia suspension to 100-200 conidia/ml, 100 μl were spread on potato dextrose agar (PDA; Difco, Franklin Lakes, NJ, USA) containing 300 μg/ml of streptomycin. The plates were incubated at 25°C for 3 days. The apex of the growing hyphae from one colony was detached from the agar plate and transferred to PDA. All isolates were grown on PDA at 25°C and maintained on PDA slants at 4°C. A new culture was activated by transferring an agar block from stocks on fresh PDA. In total, 36 *Colletotrichum* isolates were used in this study. To measure mycelial growth, isolates were incubated on PDA at 25 and 30°C for 5 days; each colony diameter was then measured.

Fungicide sensitivity tests. MBC (a.i. 25%, WP), a member of BENs, and the MBC/NPC (a.i. 25+ 25%, WP) were used to assess the sensitivity of *Colletotrichum* isolates. Sterile distilled water was used to dissolve commercial preparation of each fungicide. The fungicides were adjusted to the indicated concentrations and were added to the medium just before pouring into the Petri plates. Using a cork borer, an agar block containing mycelia of each isolate was cut from the edge of the colony, which was grown on PDA in dark at 25°C for 7 days, and transferred to PDA containing the fungicide. The diameter of the growing colony was measured after incubation at 25°C in dark for 7 days. The inhibitory activity of the fungicides was estimated based on the effective concentration required to induce 50% effect (EC_{50}) and minimum inhibitory concentration (MIC) values. The fungicide sensitivity tests were replicated two times with 5 replicates.

Sequence analysis. Fungal genomic DNA was extracted from mycelia obtained from the PDA cultures grown for 10 days at 25°C using the modified method of Kim et al. (2007). For the amplification of the ITS region, the primers ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') and ITS4-3 (5'-TCC TCC GCT TAT TGA TAT GCT TAA G-3') were used. The β-tubulin gene was amplified using the primers TB2L (5'-GYT TCC AGA TYA CCC ACT CC-3') and TB2R (5'- TGA GCT CAG GAA CRC TGA CG-3'). Amplification was conducted in a total reaction volume of 25 μl using a PCR kit (Bioneer Inc., Daejeon, Korea). The parameters used were as follows: a 2-min hold at 95°C followed by 40 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. For species level identification of the *Colletotrichum* isolates, PCR was performed with primers specific to either *C. gloeosporioides* or *C. acutatum*. The primers CgInt (5'-GGC CTC CCG CCT CCG GGC GG-3') and Ca1-1 (5'-CAG GGG AAG CCT CTC GCG GGC CT-3') were designed based on sequence similarities in the ITS1 region between *C. gloeosporioides* and *C. acutatum* (Kim et al., 2008; Mills et al., 1992). CgInt (specific for *C. gloeosporioides*) or Ca1-1 (specific for *C. acutatum*) was used in combination with the primer ITS4-3, which anneals to both *C. gloeosporioides* and *C. acutatum*. Amplification was conducted with at least three replicates. The amplicons of ITS or β-tubulin gene were column-purified using a PCR purification kit (Atman Biosciences, Uiwang, Korea) and sequenced at Eugentech Inc. (Taejon, Korea). The nucleotide sequences were analyzed using both EditSeq and MegAlign programs (DNASTAR Inc., Madison, WI, USA).

Results

Fungicide sensitivity. Percent inhibition of mycelial growth of the *C. gloeosporioides* isolates GCSG3, GCSG7, GCJY3, and GCJY13, which were sensitive to the mixture (MBC +NPC) at 0.1 μg ml^{-1}, ranged from 70.8 to 93.5%; mycelial growth was completely inhibited at higher concentrations (Fig. 1). The *C. gloeosporioides* isolates (GCSG8 and GCSG10) resistant to the mixture were not affected by concentrations lower than 10.0 μg ml^{-1}; the inhibition ratios of mycelial growth were 87.9 and 75.1% at 10.0 μg ml^{-1}, respectively. In contrast, the degrees of inhibition of the *C. acutatum* isolates GCJY17 and GCJY18 were 55.9 and 61.8% at 1.0 μg ml^{-1}, but higher concentrations of fungicide did not further reduce mycelial growth to more than 70%.

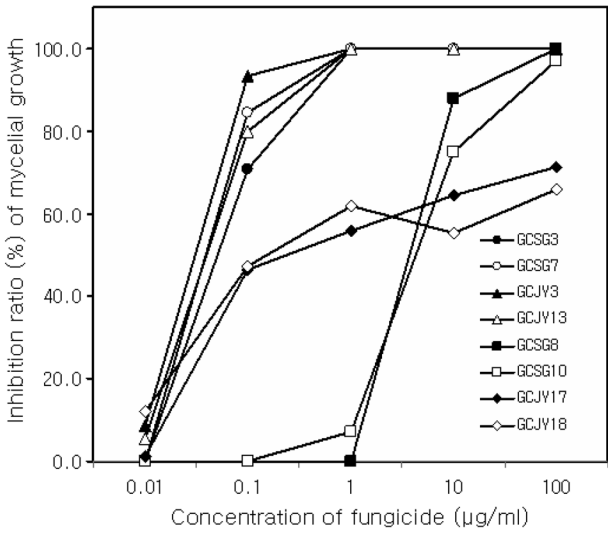

Fig. 1. Responses of the *Colletotrichum* spp. isolates from Cheonahn (GCSG3, GCSG7, GCSG8, and GCSG10) and Cheongju to increasing concentrations of the fungicidal mixture of carbendazim plus diethofencarb in potato dextrose agar. GCSG3, GCSG7, GCJY3, and GCJY13 were sensitive to the mixture, while GCSG8 and GCSG10 resistant to the fungicide(s). GCJY17 and GCJY18 were *C. acutatum.* The isolates were evaluated after 7 days of growth at 25°C. The inhibition ratio (%) of mycelial growth is calculated as follows:
(1−colony diameter in PDA with the fungicide/colony diameter in PDA without the fungicide)×100

As shown in Table 1, the 18 isolates of *C. gloeosporioides* from the Kyoho cultivar grown in Cheonahn could be divided into two groups, one that was sensitive to MBC and the mixture and the other that was resistant to both fungicides. In the former group, the calculated EC_{50} values of MBC ranged from 0.016 to 0.056 μg ml^{-1} with a mean of 0.033 μg ml^{-1}; and the calculated EC_{50} values of the mixture ranged from 0.045 to 0.105 μg ml^{-1} with a mean of 0.083 μg ml^{-1}. In contrast, for the isolates in the latter group, the EC_{50} values of MBC ranged from 1.167 to 28.566 μg ml^{-1}, with a mean of 7.291 μg ml^{-1}, while the EC_{50} values of the mixture ranged from 1.868 to 5.034 μg ml^{-1}, with a mean of 2.999 μg ml^{-1}. These two groups of isolates could also be separated based on MIC values. While the MIC values of the isolates included in the sensitive group ranged from 0.1 to 1.0 μg ml^{-1}, those in the resistant group ranged from 10.0 to more than 100 μg ml^{-1}.

Fungal isolates from Cheongju consisted of 16 of *C. gloeosporioides* and two of *C. acutatum*. All *C. gloeosporioides* isolates were sensitive to MBC and the mixture, showing EC_{50} values of ranging from 0.001 to 0.090 μg ml^{-1}; those of the mixture ranged from 0.002 to 0.063 μg ml^{-1}. The 16 *C. gloeosporioides* isolates showed MIC values ranging from 0.01 to 1.0 μg ml^{-1}. The two *C. acutatum* isolates, GCJY17 and GCJY18, were resistant to both fungicides, showing EC_{50} of 1.039 and 4.932 μg ml^{-1} (MBC) and 1.368 and 1.362 μg ml^{-1} (fungicide mixture), respectively. For both isolates, MIC values were >100 μg ml^{-1}.

Species identification based on the nucleotide sequences. Amplification and sequencing of the ITS region and the β-tubulin gene was conducted for all isolates used in this study as well as two additional isolates representing *C. gloeosporioides* KACC40690 and *C. acutatum* JC24 from pepper, to determine their species identity. Of the 36 isolates, 34 were identified as *C. gloeosporioides* by both the ITS region and the β-tubulin gene, while two isolates, GCJY17 and GCJY18, were identified as *C. acutatum* (Fig. 2). Moreover, species-specific PCR amplification also confirmed the identification of all 36 isolates (Table 1). The *C. gloeosporioides*-specific CgInt primer in conjunction with the ITS4-3 primer amplified a 466-bp fragment from DNA of the representative isolate (KACC40690) of *C. gloeosporioides* from pepper, and from the 34 *Colletotrichum* isolates from grape used in this study. However, no product was amplified with DNAs of *C. acutatum* JC24 or that of the two isolates, GCJY17 and GCJY18. The primers Ca1-1 and ITS4-3 specific to *C. acutatum* amplified a 499-bp fragment from JC24 and two grape isolates, but not from *C. gloeosporioides* KACC40690 and the other *C. gloeosporioides* isolates.

Mutation of the β-tubulin gene. Amplification of the β-tubulin gene with the primers TB2L and TB2R produced amplicons of 500 bp from the 34 *C. gloeosporioides* and two *C. acutatum* isolates (Table 2). Among the 35 *C. gloeosporioides* (including KACC40690), 12 were intermediately resistant to MBC and MBC+NPC. All of the *C. gloeosporioides* isolates resistant to the fungicides had a single mutation (from TTC to TAC) in the codon corresponding to amino acid residue 200, which resulted in the substitution of phenylalanine by tyrosine. No additional mutations were found. In *C. acutatum* JC24 from pepper and in the two isolates of *C. acutatum* from grape, GCJY17 and 18, no substitutions were observed at any codons, similar to *C. gloeosporioides* isolates sensitive to fungicides.

Discussion

Colletotrichum isolates, collected from grapes showing the typical symptom of anthracnose in two regions of Korea (Cheonahn and Cheongju), were identified as *C. gloeosporioides* and *C. acutatum* based on results of ITS and the β-tubulin gene sequences and species-specific diagnostic

Table 1. Source of *Colletotrichum* isolates used in this study, sensitivity to carbendazim (MBC) and to the mixture of MBC plus diethofencarb (NPC), and response to species-specific primers

Species	Isolate	Source region	Species-specific primer[a]		MBC		Mixture (MBC+NPC)	
			CgInt	Ca1-1	EC_{50} (μg/ml)	MIC (μg/ml)	EC_{50} (μg/ml)	MIC (μg/ml)
C. gloeosporioides	GCSG2	Cheonahn	+	–	0.045	0.1-1.0	0.098	0.1-1.0
C. gloeosporioides	GCSG3	Cheonahn	+	–	0.016	0.1-1.0	0.091	0.1-1.0
C. gloeosporioides	GCSG5	Cheonahn	+	–	0.018	0.1-1.0	0.089	0.1-1.0
C. gloeosporioides	GCSG7	Cheonahn	+	–	0.006	0.1-1.0	0.068	0.1-1.0
C. gloeosporioides	GCSG11	Cheonahn	+	–	0.054	0.1-1.0	0.045	0.1-1.0
C. gloeosporioides	GCSG13	Cheonahn	+	–	0.056	0.1-1.0	0.105	0.1-1.0
C. gloeosporioides	GCSG1	Cheonahn	+	–	3.299	>100.0	3.953	>100.0
C. gloeosporioides	GCSG4	Cheonahn	+	–	4.346	10.0-100.0	3.036	10.0-100.0
C. gloeosporioides	GCSG6	Cheonahn	+	–	5.086	10.0-100.0	1.868	10.0-100.0
C. gloeosporioides	GCSG8	Cheonahn	+	–	3.918	10.0-100.0	2.122	10.0-100.0
C. gloeosporioides	GCSG9	Cheonahn	+	–	4.806	10.0-100.0	3.042	10.0-100.0
C. gloeosporioides	GCSG10	Cheonahn	+	–	2.997	10.0-100.0	3.638	>100.0
C. gloeosporioides	GCSG12	Cheonahn	+	–	1.167	10.0-100.0	5.034	10.0-100.0
C. gloeosporioides	GCSG14	Cheonahn	+	–	4.549	10.0-100.0	3.621	10.0-100.0
C. gloeosporioides	GCSG15	Cheonahn	+	–	2.134	10.0-100.0	3.769	10.0-100.0
C. gloeosporioides	GCSG16	Cheonahn	+	–	21.434	>100.0	3.327	>100.0
C. gloeosporioides	GCSG17	Cheonahn	+	–	5.190	10.0-100.0	3.869	10.0-100.0
C. gloeosporioides	GCSG18	Cheonahn	+	–	28.566	>100.0	4.709	>100.0
C. gloeosporioides	GCJY1	Cheongju	+	–	0.024	0.1-1.0	0.041	0.1-1.0
C. gloeosporioides	GCJY2	Cheongju	+	–	0.022	0.1-1.0	0.031	0.1-1.0
C. gloeosporioides	GCJY3	Cheongju	+	–	0.040	0.1-1.0	0.025	0.1-1.0
C. gloeosporioides	GCJY4	Cheongju	+	–	0.090	0.1-1.0	0.039	0.1-1.0
C. gloeosporioides	GCJY5	Cheongju	+	–	0.006	0.1-1.0	0.021	0.1-1.0
C. gloeosporioides	GCJY6	Cheongju	+	–	0.005	0.1-1.0	0.031	0.1-1.0
C. gloeosporioides	GCJY7	Cheongju	+	–	0.004	0.1-1.0	0.063	0.1-1.0
C. gloeosporioides	GCJY8	Cheongju	+	–	0.004	0.01-0.1	0.023	0.01-0.1
C. gloeosporioides	GCJY9	Cheongju	+	–	0.001	0.01-0.1	0.005	0.01-0.1
C. gloeosporioides	GCJY10	Cheongju	+	–	0.004	0.1-1.0	0.036	0.1-1.0
C. gloeosporioides	GCJY11	Cheongju	+	–	0.001	0.01-0.1	0.002	0.01-0.1
C. gloeosporioides	GCJY12	Cheongju	+	–	0.003	0.01-0.1	0.010	0.01-0.1
C. gloeosporioides	GCJY13	Cheongju	+	–	0.016	0.1-1.0	0.051	0.1-1.0
C. gloeosporioides	GCJY14	Cheongju	+	–	0.037	0.01-0.1	0.005	0.01-0.1
C. gloeosporioides	GCJY15	Cheongju	+	–	0.023	0.1-1.0	0.034	0.1-1.0
C. gloeosporioides	GCJY16	Cheongju	+	–	0.022	0.1-1.0	0.031	0.1-1.0
C. acutatum	GCJY17	Cheongju	–	+	1.039	>100.0	1.368	>100.0
C. acutatum	GCJY18	Cheongju	–	+	4.932	>100.0	1.362	>100.0

[a] CgInt was the species-specific primer for *C. gloeosporioides*, and Ca1-1 for *C. acutatum.*

PCR. Only two of them corresponded to *C. acutatum*, both of which were isolated from Cheongju. Isolates of *C. gloeosporioides* isolated from Cheonahn, where MBC and the mixture had been applied to control grape ripe rot, differed greatly in their sensitivity to MBC and the mixture and could be divided into two groups. Whereas all isolates of *C. gloeosporioides* from Cheongju, which were not exposed to the application of fungicides (MBC and the mixture), were highly sensitive to fungicides used in this study. The sensitive group had EC_{50} values of less than 0.1 μg/ml, and the resistant group had values greater than 10 μg/ml. This difference was also observed in MIC values, which were less than 1.0 μg/ml for the sensitive group, but higher than 10 μg/ml for the resistant group. Isolates of *C. acutatum* were insensitive to the fungicides tested; although 1.0 μg/ml of the two fungicides reduced colony diameter by about 60% compared to untreated controls (Fig. 1), higher concentrations of fungicides did not further reduce colony

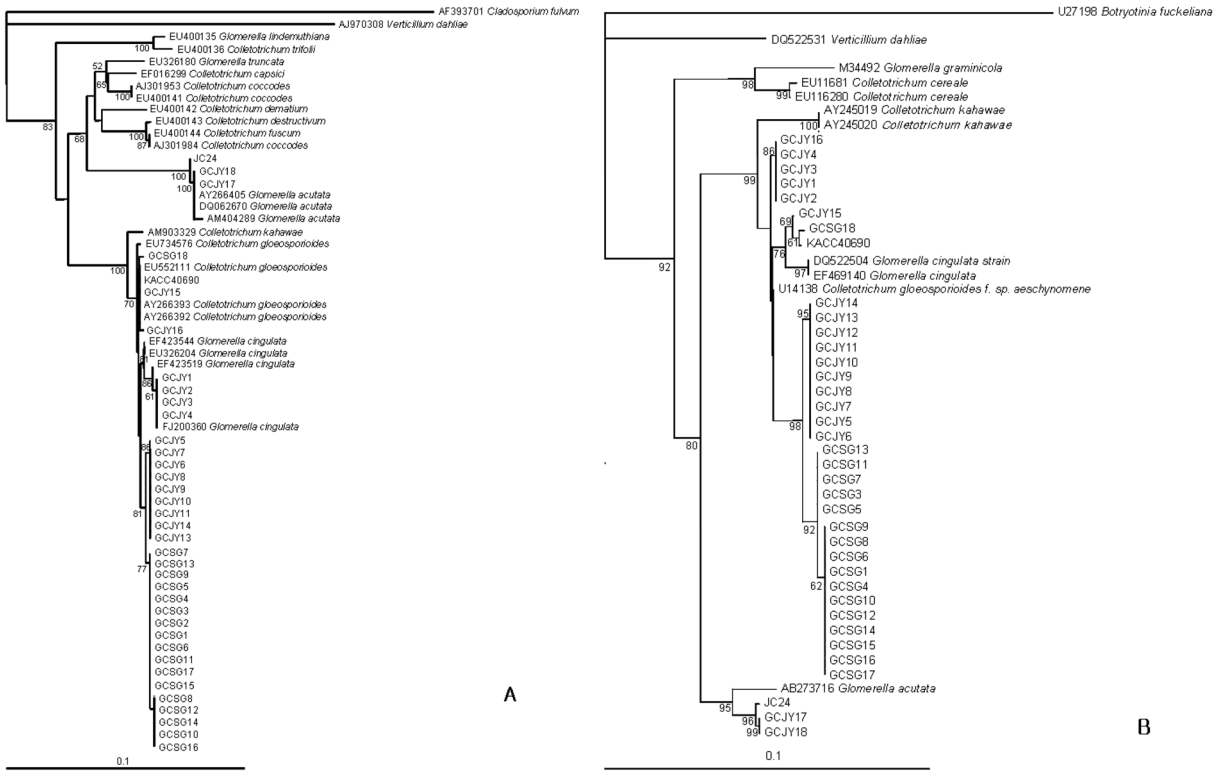

Fig. 2. ITS (A) and β-tubulin gene sequence (B)-based tree generated using the Neighbor-joining method. Numbers at the branch node indicate the confidence values from bootstrap analysis using 1,000 replications.

diameter. Recently, Nakaune and Nakano (2007) reported that benomyl resistance was due to enhanced expression of the *C. acutatum* tubulin (*CaTUB1*) gene in response to benomyl, a member of the BENs. They demonstrated that CaBEN1, a putative leucine zipper protein that regulates the expression of *CaTUB1*, was necessary for resistance to BENs. Although mutations conferring high levels of benomyl resistance appear to be absent in *C. acutatum* populations, most of the *C. acutatum* isolates showed inherently intermediate resistance to BENs. In fact, BENs were not effective in controlling pepper anthracnose caused by *C. acutatum* (Kang et al., 2005; Van Bach et al., 2004).

Prevalence of *C. gloeosporioides* isolates resistant to the BEN fungicides in the field correlated with historic usage of BENs. Koenraadt et al. (1992) identified strains of *Venturia inaequalis* resistant to benomyl in an orchard more than 10 years after benomyl use had been discontinued. In *Mycosphaerella fijiensis* from banana plantations where benomyl had been used for approximately 10 years to control black Sigatoka disease, 86% of isolates were resistant to benomyl, whereas no resistance was detected in isolates collected from plantations with no history of benomyl use (Romero and Sutton, 1998). In *C. cereale* causing turfgrass anthracnose, the difference in the prevalence of benomyl resistance among sampling sites also reflected the cumulative effects of benomyl use (Wong et al., 2008). Based on our results, the appearance of resistance to MBC and the mixture in *C. gloeosporioides* in Korea was related to historical use of these fungicides. Fungicides inhibiting tubulin assembly have been continuously used despite decreasing controlling activity against the disease in regions with a high prevalence of BEN-resistant isolates of *C. gloeosporioides*, because fungicide resistance in the pathogen populations has not been recognized. This emphasizes the importance of monitoring fungicide resistance for the development of disease control strategies.

The discovery that benomyl-resistant *Botrytis cinerea* showed sensitivity to NPCs raised the possibility that combinations of BENs and NPCs might be able to delay the development of resistance to BENs in phytopathogens, and to control plant diseases caused by populations composed of benzimidazole-resistant (BEN^R) and sensitive (BEN^S) isolates (Kato et al., 1984; Leroux and Gredt, 1989). In many countries, including Korea, the mixture was introduced to control plant diseases in fields where both BEN^R and BEN^S pathogens had been established. Although mixed populations of *B. cinerea* on grapes could be controlled by applying the mixture, unsatisfactory gray mold control was reported from a cucumber plot in Israel treated with the same

Table 2. Deduced amino acid substitutions in the sequence of the β-tubulin gene among the *Colletotrichum* isolates with different phenotypic responses to fungicides

Species	Isolate	MBC[a]	Mixture[b] (MBC+NPC)	200[th]
C. gloeosporioides	KACC40690	S	S	Phe
C. gloeosporioides	GCJY1	S	S	Phe
C. gloeosporioides	GCJY2	S	S	Phe
C. gloeosporioides	GCJY3	S	S	Phe
C. gloeosporioides	GCJY4	S	S	Phe
C. gloeosporioides	GCJY5	S	S	Phe
C. gloeosporioides	GCJY6	S	S	Phe
C. gloeosporioides	GCJY7	S	S	Phe
C. gloeosporioides	GCJY8	S	S	Phe
C. gloeosporioides	GCJY9	S	S	Phe
C. gloeosporioides	GCJY10	S	S	Phe
C. gloeosporioides	GCJY11	S	S	Phe
C. gloeosporioides	GCJY12	S	S	Phe
C. gloeosporioides	GCJY13	S	S	Phe
C. gloeosporioides	GCJY14	S	S	Phe
C. gloeosporioides	GCJY15	S	S	Phe
C. gloeosporioides	GCJY16	S	S	Phe
C. gloeosporioides	GCSG2	S	S	Phe
C. gloeosporioides	GCSG3	S	S	Phe
C. gloeosporioides	GCSG5	S	S	Phe
C. gloeosporioides	GCSG7	S	S	Phe
C. gloeosporioides	GCSG11	S	S	Phe
C. gloeosporioides	GCSG13	S	S	Phe
C. gloeosporioides	GCSG1	R	R	Tyr
C. gloeosporioides	GCSG4	R	R	Tyr
C. gloeosporioides	GCSG6	R	R	Tyr
C. gloeosporioides	GCSG8	R	R	Tyr
C. gloeosporioides	GCSG9	R	R	Tyr
C. gloeosporioides	GCSG10	R	R	Tyr
C. gloeosporioides	GCSG12	R	R	Tyr
C. gloeosporioides	GCSG14	R	R	Tyr
C. gloeosporioides	GCSG15	R	R	Tyr
C. gloeosporioides	GCSG16	R	R	Tyr
C. gloeosporioides	GCSG17	R	R	Tyr
C. gloeosporioides	GCSG18	R	R	Tyr
C. acutatum	JC24	R	R	Phe
C. acutatum	GCJY17	R	R	Phe
C. acutatum	GCJY18	R	R	Phe

[a] S was indicated a isolate sensitive to 1.0 μg/ml of carbendazim, and R did a isolate resistant to more than 10.0 μg/ml to the fungicide.
[b] S was indicated a isolate sensitive to 1.0 μg/ml of the mixture (MBC+NPC), and R did a isolate resistant to more than 10.0 μg/ml to the mixture.

mixture (Elad et al., 1988; Katan et al., 1989). Isolates of *B. cinerea* resistant to both carbendazim and diethofencarb (MBC^RNPC^R) have also been reported in France (Leroux and Gredt, 1989) and Italy (Faretra et al., 1989). In this study, all carbendazim-resistant (MBC^R) isolates of *C. gloeosporioides* appeared to be resistant to the mixture (MIX^R); we used this mixture to evaluate the resistance of *Colletotrichum* spp. isolates from grapes. The appearance of MBC^RMIX^R isolates of *C. gloeosporioides* might be due to selection pressure and fungicide application in the Cheonahn area.

Resistance to BEN fungicides could be conferred by single amino acid substitutions in the β-tubulin molecule (Jung et al., 1992). Our results suggested that the substitution of tyrosine for phenylalanine at amino acid residue 200 conferred resistance to MBC and to the mixture in 12 isolates of *C. gloeosporioides*. With regard to the relationship between a substitution of amino acid in β-tubulin and resistant phenotypes, the polar hydroxy (OH) group of the tyrosine at amino acid 200 might interfere with binding to the BEN fungicide, resulting in BEN resistance (Jung et al., 1992).

Several levels of resistance to BEN have been observed in field populations of phytopathogens, such as *V. inaequalis*, *B. cinerea*, and *C. gloeosporioides* (Koenraadt et al., 1992; Yarden and Katan, 1993; Peres et al., 2004). Generally, populations of *B. cinerea* not exposed to NPCs were composed almost exclusively of highly resistant strains ($Ben^{HR}NPC^S$). Ben^RNPC^R strains have been extremely rare in such populations and became apparent only after the application of the mixture (MBC+NPC) for control of gray mold. Yarden and Katan (1993) reported that the rarity of such strains might indicate the low fitness of Ben^RNPC^R strains. In this study, however, more than 70% of *C. gloeosporioides* isolates exposed to the mixture to control grape ripe rot in Cheonahn appeared to be resistant to both of MBC and the mixture. The results suggest that MBC^RMIX^R isolates of *C. gloeosporioides* obtained from Cheonahn show high fitness in the field. Further studies will be needed to monitor the fungicide resistance of *Colletotrichum* spp. causing grape ripe rot nationwide in Korea and to assess the field fitness of isolates resistant to the fungicidal mixture (MBC+NPC).

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