
Evaluation of Carbendazim Resistance Levels of *Botrytis cinerea* Causing Gray Mold of Grape in Chiang Mai Province, Northern Thailand

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Mekwilai, T. and Nalumpang, S. (2017). Evaluation of Carbendazim Resistance Levels of *Botrytis cinerea* Causing Gray Mold of Grape in Chiang Mai Province, Northern Thailand. International Journal of Agricultural Technology 13(2): 169-182.

Gray mold caused by *Botrytis cinerea* is one of the most important diseases of grape and causes significant yield losses. Eighty-two isolates of *B. cinerea* causing gray mold disease in grape berries and strawberries were collected from markets and orchards in Chiang Mai province, northern Thailand. The carbendazim resistance of *B. cinerea* was evaluated according to inhibition of mycelia growth in PDA plates amended with different concentrations of the fungicide. All *B. cinerea* isolates were classified as highly resistant (HR) to carbendazim. In further studies, analysis of the β -tubulin gene sequence revealed amino acid replacements in the HR mutants compared to the wild-type strain. The result indicated that two benzimidazole resistance mutations occurred at codon 198. The first one was, a glutamic acid (GAG) to alanine (GCG) replacement, (E198A). The second mutation was, a glutamic acid (GAG) to valine (GTG) replacement at the same amino acid position, (E198V). Both mutations resulted in high resistance to carbendazim.

Keywords: *Botrytis cinerea*, carbendazim, high resistance, β -tubulin gene

Introduction

Grape is one of the most economically important fruit crops with a cultivated area of 8 million ha around the world (Vivier and Pretorius, 2002). Vineyards often face epidemics of disease. The most economically important postharvest disease is gray mold caused by the fungus *Botrytis cinerea* Pers. (teleomorph *Botryotinia fuckeliana* (deBary) Whetzel). *Botrytis cinerea* is a necrotrophic pathogen attacking over 200 different plant species (Jarvis, 1980). The pathogen can attack many plant organs including leaves, stems and fruit, often with heavy losses after harvest. In many vineyards around the world, gray mold disease causes significant yield losses; losses in both the quality and quantity of grape berries for wine production due to *B. cinerea* have

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been estimated at 2 billion USD per year (Elad *et al.*, 2007), and the cost to control *B. cinerea* was 780 million USD in 2002 (Genescope, 2002).

Infection of grape often occurs at bloom time, followed by a period of latency, during which the pathogen is present (latent) inside the berry without causing disease symptoms, generally until grape berries begin to ripen (McClellan *et al.*, 1973; Nair *et al.*, 1995; Pezet *et al.*, 1986). The most common and destructive phase of this disease is infection of ripe berries. Infected berries first appear soft and watery. The berries of white cultivars become brown and shriveled, and those of purple cultivars develop a reddish color. Under high relative humidity and moisture, infected berries usually become covered with a gray growth of fungus mycelium. The fungus can also cause a blossom blight that can result in significant crop loss early in the season (Elad *et al.*, 2007).

Effective gray mold control is usually based on fungicide application but chemical control of the pathogen has been impaired by the development of resistance to the intensively used site-specific, systemic benzimidazole fungicides, such as carbendazim benomyl, and thiophanate-methyl (Beever *et al.*, 1989; Yourman *et al.*, 1999). The evolution of fungicide resistance has become a major problem worldwide because the fungus is known to rapidly adapt to its environment. Fungicide resistance in the field is particularly common, where fungicide application is frequent and indiscriminate.

The first report of benzimidazole resistance in *B. cinerea* was on cyclamen in Dutch greenhouses after 2 years of use (Bollen and Scholten, 1971). A similar situation occurred in European vineyards after only three to four seasons (Leroux and Clerjeau, 1985; Smith, 1988). Benzimidazoles are a group of broad-spectrum systemic fungicides which interact with tubulin, especially β -tubulin. Benzimidazoles inhibit the assembly of microtubules by binding to β -tubulin sub-units of the fungus (Davidse and Ishii, 1995). In most cases, mutations of amino acids at positions 198 and 200 in the β -tubulin gene confer benzimidazole resistance in field isolates of many plant pathogens (Alpertini *et al.*, 1999; Koenraadt *et al.*, 1992; Ma and Michailides, 2005; Yarden and Katan, 1993).

The current study was conducted to determine the prevalence of carbendazim resistance in *B. cinerea* causing post-harvest gray mold disease of grape and strawberry in Chiang Mai province, northern Thailand, and the relationship between fungicide resistance and alteration of the β -tubulin gene sequence of resistant strains of *B. cinerea*. It was hoped that further research on the effective prevention and control of gray mold of grapes would be facilitated.

Materials and Methods

Isolation and cultivation of B. cinerea isolates

B. cinerea was isolated from infected grape berries and strawberries in Chiang Mai province, northern Thailand. The fungus was identified under a compound microscope and conidia were transferred by needle to potato dextrose agar (PDA). All isolates were incubated at 25 °C. After single spore isolation, mycelial discs of *B. cinerea* were maintained in mineral oil at 5 °C until use.

Evaluation of fungicide resistance of B. cinerea

Carbendazim resistance was assessed by observing the mycelial growth of the fungus on PDA amended with carbendazim at 0, 0.01, 0.1, 1, 10, 100, 500 (manufacturer's recommended rate) and 1000 µg/ml (adapted from Yarden and Katan, 1993; Leroux *et al.*, 2002). A 5-mm-diameter mycelial plug was cut from the margin of actively growing cultures of the fungus and transferred onto the amended and control PDA. Three replicated plates were used for each fungicide concentration. Petri plates were incubated at 25 °C for 10-14 d. The diameter of each colony was measured and its growth reduction percentage was calculated by comparison with the control. The level of resistance to carbendazim was evaluated and grouped into four representative phenotype resistance levels (Table 1).

Table 1 Phenotype resistance levels of *Botrytis cinerea* to carbendazim fungicide
(Koenraadt *et al.*, 1992; Peres *et al.*, 2004)

Resistance levels	Carbendazim concentration (µg/ml)							
	0	0.01	0.1	1	10	100	500*	1000
Sensitive (S)	✓ ^{1/}	✓	✓	✓	X ^{2/}	X	X	X
Weakly resistant (WR)	✓	✓	✓	✓	✓	X	X	X
Moderately resistant (MR)	✓	✓	✓	✓	✓	✓	X	X
Highly resistant (HR)	✓	✓	✓	✓	✓	✓	✓	✓

* = The field recommendation rate

^{1/}✓ = Percentage of growth ≥ 10% compared with the control

^{2/X} = Percentage of growth < 10% compared with the control

DNA Extraction

Botrytis cinerea mycelial discs were cultured on PDA at 25 °C for 2 wk. Mycelia were collected and genomic DNA was extracted using a NucleoSpin® kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions and the CTAB method (Hortigenetics Research (S.E. Asia) Co., Ltd. Chiang Mai, Thailand). The quantity and quality of genomic DNA were measured using a NanoDrop Spectrophotometer (Thermo Scientific, Inc.).

Sequence analysis of the β -tubulin gene from *B. cinerea* strains

The PCR primer pair Bcb-F (5'-CACTGAGGGTGCTGAGCTTGT-3') and Bcb-R (5'-GAAGCGGCCATCATGTTCTTA-3') according to Zhang *et al.* (2010) was used to amplify a 525 bp β -tubulin gene fragment from *B. cinerea* (GenBank accession number U27198). All PCR reactions were performed in 50 μ l volumes and contained 10-100 ng of DNA template, 5 μ l 10x PCR buffer, 5 μ l dNTPs, 1 μ l primer Bcb-F, 1 μ l primer Bcb-R, 1 μ l DNA template, 3 μ l MgCl₂, 33 μ l distilled water, and 1 μ l Taq DNA Polymerase. The PCR program was 94 °C x 5 min, (94 °C x 1 min, 53 °C x 1 min, 72 °C x 1 min) x 35 cycles, followed by final extension at 72 °C x 10 min. PCR products were analyzed by 1% agarose gel electrophoresis and observed by UV illumination after staining with 2.5% ethidium bromide. Sequence data were compared with the data of the β -tubulin gene in the GenBank database and analyzed by using GENETYX software, version 11.

Results

Isolation and cultivation of *B. cinerea* isolates

Isolates of *B. cinerea* from grape and strawberry were collected from markets and orchards in Chiang Mai province, northern Thailand. A total of 82 isolates of *B. cinerea* were collected, 64 from grape and 18 from strawberry; 24 isolates were obtained from markets and supermarkets, 42 from Fang District, 11 from Mae-Rim District and five from Sa-Maung District.

The morphological characterization of all isolate was done by growing on PDA for 14 d. The colony characteristics of all isolates were divided into three categories: dark gray colony (20.73 %), greyish-brown colony (51.22 %) and light gray colony (28.05 %). The initial color of colonies on PDA was white, then became greyish-brown, dark gray or light gray. Conidia were

usually produced over the surface of the medium. In some isolates sclerotia were abundant. Sclerotia were superficial or deeply imbedded in the agar and adherent to the bottom of the petri dish. The hyphae were branched, septate, hyaline to brown. Conidiophore arising directly from the mycelium, 2 μm or longer, apically branching and bearing numerous conidia. Conidia unicellular, ellipsoidal or ovoid, colorless to pale brown, smooth, 6-18 x 3.5-12 μm .

Evaluation of fungicide resistance of *B. cinerea*

The 82 isolates of *B. cinerea* were tested for carbendazim resistance by growth assays on PDA amended with carbendazim at concentrations of 0, 0.01, 0.1, 1, 10, 100, 500 and 1000 $\mu\text{g}/\text{ml}$. The isolates were classified into four representative phenotype reactions, highly resistant (HR), moderately resistance (MR), weakly resistance (WR), and sensitive (S). All, 82 isolates collected were classified as highly resistant (HR) to carbendazim (Table 2 and Figure 1).

Table 2 Carbendazim-resistant isolates of *Botrytis cinerea* causing gray mold disease

Isolate	Host	Origin (District/location, Province)	Resistance Level
Bc-MKG1	Grape	Tesco Lotus Super Market, CM ^{1/}	HR ^{2/}
Bc-MKG2	Grape	Tesco Lotus Super Market, CM	HR
Bc-MKG3	Grape	Tesco Lotus Super Market, CM	HR
Bc-MKG4	Grape	Tesco Lotus Super Market, CM	HR
Bc-MKG5	Grape	Tesco Lotus Super Market, CM	HR
Bc-MKG6	Grape	Tesco Lotus Super Market, CM	HR
Bc-MKG7	Grape	Tesco Lotus Super Market, CM	HR
Bc-MKG8	Grape	Tesco Lotus Super Market, CM	HR
Bc-MKG9	Grape	Tesco Lotus Super Market, CM	HR
Bc-MKG10	Grape	Tesco Lotus Super Market, CM	HR
Bc-MKG11	Grape	Tesco Lotus Super Market, CM	HR
Bc-MKG12	Grape	Tesco Lotus Super Market, CM	HR
Bc-MKG13	Grape	Tesco Lotus Super Market, CM	HR
Bc-MKG14	Grape	Tesco Lotus Super Market, CM	HR
Bc-MKG15	Grape	Tesco Lotus Super Market, CM	HR
Bc-MKG16	Grape	Tesco Lotus Super Market, CM	HR
Bc-MKG17	Grape	Big C Super Market, CM ^{1/}	HR ^{1/}
Bc-MKG18	Grape	Big C Super Market, CM	HR
Bc-MKG19	Grape	Big C Super Market, CM	HR
Bc-MKG20	Grape	Big C Super Market, CM	HR
Bc-MKG21	Grape	Big C Super Market, CM	HR

^{1/}CM =Chiang Mai Province, Thailand

^{2/}HR = highly resistant

Table 2 Continued

Isolate	Host	Origin (District/location, Province)	Resistance Level
Bc-MKG22	Grape	Big C Super Market, CM	HR
Bc-FG17	Grape	Fang, CM	HR
Bc-FG18	Grape	Fang, CM	HR
Bc-FG19	Grape	Fang, CM	HR
Bc-FG20	Grape	Fang, CM	HR
Bc-FG21	Grape	Fang, CM	HR
Bc-FG22	Grape	Fang, CM	HR
Bc-FG40	Grape	Fang, CM	HR
Bc-FG41	Grape	Fang, CM	HR
Bc-FG42	Grape	Fang, CM	HR
Bc-FG43	Grape	Fang, CM	HR
Bc-FG44	Grape	Fang, CM	HR
Bc-FG45	Grape	Fang, CM	HR
Bc-FG46	Grape	Fang, CM	HR
Bc-FG47	Grape	Fang, CM	HR
Bc-FG48	Grape	Fang, CM	HR
Bc-FG49	Grape	Fang, CM	HR
Bc-FG50	Grape	Fang, CM	HR
Bc-FG51	Grape	Fang, CM	HR
Bc-FG52	Grape	Fang, CM	HR
Bc-FG53	Grape	Fang, CM	HR
Bc-FG54	Grape	Fang, CM	HR
Bc-FG55	Grape	Fang, CM	HR
Bc-FG56	Grape	Fang, CM	HR
Bc-FG57	Grape	Fang, CM	HR
Bc-FG58	Grape	Fang, CM	HR
Bc-FG59	Grape	Fang, CM	HR
Bc-FG60	Grape	Fang, CM	HR
Bc-FG61	Grape	Fang, CM	HR
Bc-FG62	Grape	Fang, CM ^{1/}	HR ^{2/}
Bc-FG63	Grape	Fang, CM	HR
Bc-FG64	Grape	Fang, CM	HR
Bc-FG65	Grape	Fang, CM	HR
Bc-FG66	Grape	Fang, CM	HR
Bc-FG67	Grape	Fang, CM	HR
Bc-FG68	Grape	Fang, CM	HR
Bc-FG69	Grape	Fang, CM	HR
Bc-FG70	Grape	Fang, CM	HR
Bc-FG71	Grape	Fang, CM	HR
Bc-FG72	Grape	Fang, CM	HR
Bc-FG73	Grape	Fang, CM	HR
Bc-FG74	Grape	Fang, CM	HR
Bc-FG75	Grape	Fang, CM	HR

^{1/}CM =Chiang Mai Province, Thailand^{2/}HR =highly resistant

Table 2 Continued

Isolate	Host	Origin (District/location, Province)	Resistance Level
Bc-MRS1	Strawberry	Mae-Rim, CM	HR
Bc-MRS2	Strawberry	Mae-Rim, CM	HR
Bc-MRS3	Strawberry	Mae-Rim, CM	HR
Bc-MRS4	Strawberry	Mae-Rim, CM	HR
Bc-MRS5	Strawberry	Mae-Rim, CM	HR
Bc-MRS6	Strawberry	Mae-Rim, CM	HR
Bc-MRS7	Strawberry	Mae-Rim, CM	HR
Bc-MRS8	Strawberry	Mae-Rim, CM	HR
Bc-MRS9	Strawberry	Mae-Rim, CM	HR
Bc-MRS11	Strawberry	Mae-Rim, CM	HR
Bc-MKS1	Strawberry	Meung-Mai Market, CM	HR
Bc-MKS2	Strawberry	Meung-Mai Market, CM	HR
Bc-SMS3	Strawberry	Sa-Maung, CM	HR
Bc-SMS4	Strawberry	Sa-Maung, CM	HR
Bc-SMS5	Strawberry	Sa-Maung, CM	HR
Bc-SMS14	Strawberry	Sa-Maung, CM	HR
Bc-SMS33	Strawberry	Sa-Maung, CM	HR

¹/CM =Chiang Mai Province, Thailand

²/HR = highly resistant

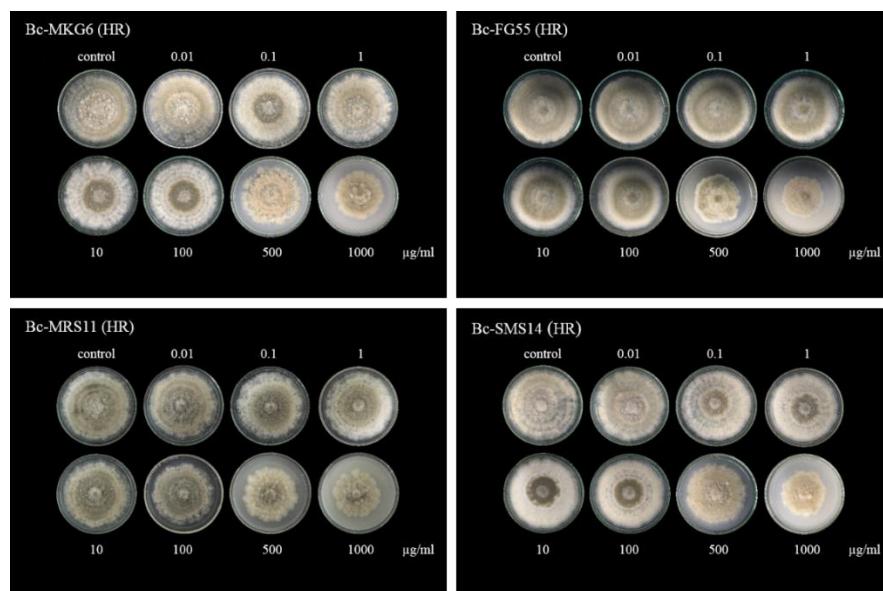


Figure 1 Carbendazim-resistant isolates of *Botrytis cinerea* on potato dextrose agar (PDA) amended with carbendazim at 0 (control), 0.01, 0.1, 1, 10, 100, 500, and 1,000 $\mu\text{g}/\text{ml}$; (HR) - highly resistant phenotype

Sequence analysis of β -tubulin gene from *B. cinerea* strains

Fifty isolates of *B. cinerea* with high resistance to carbendazim were randomly selected for identification through molecular techniques.

The DNA of each isolate was amplified by PCR using the Bcb-F and Bcb-R primers. The PCR product region was approximately 591 bp for all samples (Figure 2).

The *benA* sequences (accession number U27198), including wild-type sequences (accession number Z69263) and benomyl-sensitive sequences (accession number X73133) of the *B. cinerea* β -tubulin gene retrieved from GenBank were used for comparison. The nucleotide sequence of a 591 bp segment of the β -tubulin gene of *B. cinerea*, was used for analysis of each isolate.

Comparison and alignment of the β -tubulin gene sequence of each isolate indicated that amino acid changes in the highly benzimidazole resistant mutants occurred at position 198. Two benzimidazole resistance mutations were found at codon 198. The first one, a glutamic acid (GAG) to alanine (GCG) replacement and the second, a glutamic acid (GAG) to valine (GTG) replacement occurred at the same position, both resulting in high carbendazim resistance (Figure 3).

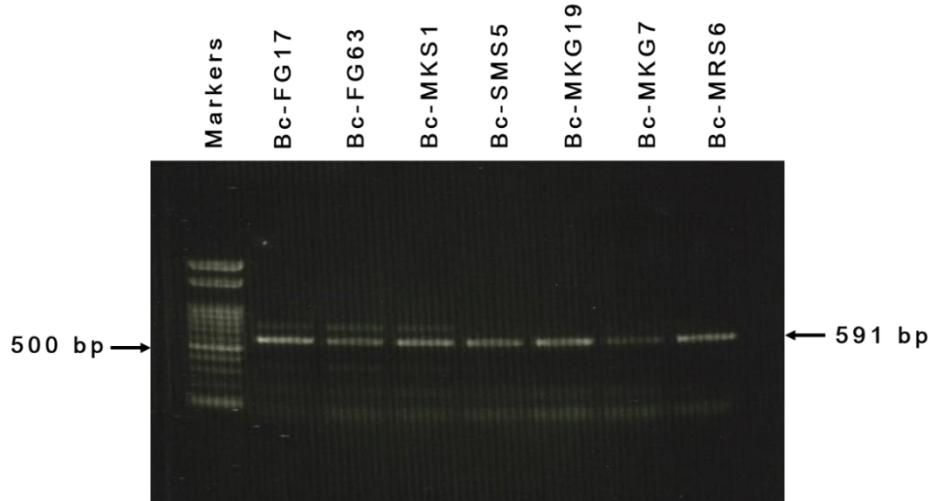


Figure 2 PCR amplification of the β -tubulin gene from isolates of *Botrytis cinerea* causing gray mold disease; PCR products were generated using the Bcb-F and Bcb-R primers. Markers are from the 100 bp Ladder.

Isolate code	Phenotype	Codon 198
Z69263	Wild-type	CATCAATTGGTTGAGAACTCTGACGAGACCTTCTGTATCGATAACGAGGCT H Q L V E N S D E T F C I D N E A
X73133	S	CATCAATTGGTTGAGAACTCTGACGAGACCTTCTGTATCGATAACGAGGCT H Q L V E N S D E T F C I D N E A
U27198	HR	CATCAATTGGTTGAGAACTCTGACGCGACCTTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-MKG1	HR	CATCAATTGGTTGAGAACTCTGACGCGACCTTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-MKG3	HR	CATCAATTGGTTGAGAACTCTGACGCGACCTTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-MKG4	HR	CATCAATTGGTTGAGAACTCTGACGCGACCTTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-MKG7	HR	CATCAATTGGTTGAGAACTCTGACGCGACCTTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-MKG8	HR	CATCAATTGGTTGAGAACTCTGACGCGACCTTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-MKG9	HR	CATCAATTGGTTGAGAACTCTGACGCGACCTTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-MKG10	HR	CATCAATTGGTTGAGAACTCTGACGCGACCTTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-MKG11	HR	CATCAATTGGTTGAGAACTCTGACGCGACCTTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-MKG12	HR	CATCAATTGGTTGAGAACTCTGACGCGACCTTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-MKG14	HR	CATCAATTGGTTGAGAACTCTGACGCGACCTTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-MKG18	HR	CATCAATTGGTTGAGAACTCTGACGCGACCTTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-MKG19	HR	CATCAATTGGTTGAGAACTCTGACGCGACCTTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-MKG20	HR	CATCAATTGGTTGAGAACTCTGACGCGACCTTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A

Figure 3 Comparison of deduced nucleotide and amino acid sequences of the β -tubulin gene from a wild-type strain of *Botrytis cinerea* [(Wild-Type) Bentino *et al.*, 1998], a benomyl-sensitive strain [(S) Yarden and Katan, 1993], benomyl-resistant strain [(HR) Park *et al.*, 1996] and the carbendazim-resistant phenotypes of *B. cinerea* isolates causing gray mold disease in northern Thailand. Amino acid mutations are indicated by bold letters.

Isolate code	Phenotype	Codon 198
Bc-MKG22	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG17	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG19	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG21	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG22	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG40	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG42	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG43	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG46	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG48	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG49	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG51	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG55	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG58	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG61	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG62	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG63	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG66	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG67	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG69	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG70	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG72	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG73	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-FG75	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-MRS1	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-MRS2	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-MRS3	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A

Figure 3 Continued

Isolate code	Phenotype	Codon 198
Bc-MRS7	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-MRS8	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-MRS10	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-MKS1	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-MKS2	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-SMS3	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-SMS4	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-SMS5	HR	CATCAATTGGTTGAGAACTCTGACCGCACCTCTGTATCGATAACGAGGCT H Q L V E N S D A T F C I D N E A
Bc-SMS14	HR	CATCAATTGGTTGAGAACTCTGACGTGACCTCTGTATCGATAACGAGGCT H Q L V E N S D V T F C I D N E A
Bc-SMS33	HR	CATCAATTGGTTGAGAACTCTGACGTGACCTCTGTATCGATAACGAGGCT H Q L V E N S D V T F C I D N E A

Figure 3 Continued

Discussions

Benzimidazole-resistant *B. cinerea* isolates have been found in numerous crops throughout the world (Stehmann and de Waard 1996). In Chiang Mai, Thailand, *B. cinerea* isolates collected from grape and strawberry showed 100% high resistance to carbendazim.

Resistance to benzimidazole fungicides has been reported in many fungal species. In most cases, resistance is associated with point mutations in the β -tubulin gene which result in altered amino acid sequences at the benzimidazole binding site (Davidson *et al.*, 2006; Koenraadt *et al.*, 1992; Ma *et al.*, 2003; Maymon *et al.*, 2006). Most field-resistant isolates of plant pathogenic fungi show codon changes that seem to be restricted to positions 50 (McKay *et al.*, 1998), 198, 200 (Albertini *et al.*, 1999; Koenraadt *et al.*, 1992), and 240 (Albertini *et al.*, 1999).

Analysis of the sequence of the β -tubulin gene revealed amino acid replacements in the highly benzimidazole resistant mutants compared to the wild-type strain. Two benzimidazole resistance mutations at codon 198 were identified in isolates with the carbendazim HR phenotype. The first one was a glutamic acid (GAG) to alanine (GCG) replacement (E198A) in highly carbendazim-resistant isolates. The second mutation was a glutamic acid (GAG) to valine (GTG) replacement (E198V) at the same amino acid position. The glutamic acid to alanine mutation at codon 198 was also found in the resistant strain Ben-26 and two benzimidazole resistant field isolates (Ben-F1,

Ben-F2) of *B. cinerea* (Ziogas *et al.*, 2009). The role of this mutation in benzimidazole resistance has previously been recognised in *B. cinerea* (Yarden and Katan 1993; Leroux *et al.*, 2002), *Neurospora crassa* (Orbach *et al.*, 1986; Jung *et al.*, 1992), *Penicillium expansum* (Fujimura *et al.*, 1992), *Venturia inaequalis* and *V. pirina* (Fujimura *et al.*, 1992), *Penicillium aurantiogriseum* (Koenraadt *et al.*, 1992) and *Monilinia fructicola* (Ma *et al.*, 2003). The GAG to GTG point mutation at codon 198 has been reported in *Penicillium* spp. (Sholberg *et al.*, 2005) and *V. inaequalis* (Koenraadt *et al.*, 1992), both resulting in high resistance to benzimidazoles.

In the current research, all isolates of *B. cinerea* possessed a high level of carbendazim resistance, associated with mutations at codon 198, and attributed to β -tubulin modifications. The modification of the β -tubulin gene in the pathogen can be passed on to progeny thus increasing the incidence of carbendazim resistance in the field populations of *B. cinerea*. Moreover, benzimidazole cross resistance has been reported (Damicone, 2009). Cross resistance occurs when pathogens resistant to one fungicide are also resistant to other fungicides that have the same site-specific mode of action. Therefore, detection of resistance levels in populations of phytopathogenic fungi in a field will help growers make proper decisions on resistance management programs to control diseases and prevent the risk of fungicide resistance development.

Acknowledgement

This research was supported by Hortigenetics Research (S.E. Asia) Co., Ltd. Chiang Mai, Thailand and Prof. Dr. Kazuya Akimitsu, Kagawa University, Japan.

References

Altertini, C., Gredt, M. and Leroux, P. (1999). Mutations of the β -tubulin gene associated with different phenotypes of benzimidazole resistance in the cereal eyespot fungi *Tapesia yallundae* and *Tapesia acuformis*. *Pesticide Biochemistry and Physiology* 64: 17-31.

Beever, R.E., Laracy, E.P. and Pak, H.A. (1989). Strains of *Botrytis cinerea* resistant to dicarboximide and benzimidazole fungicides in New Zealand vineyards. *Plant Pathology* 38: 427-437

Bentino, P.E., Have, A., van't Klooster, W.J. and van Kan, A.L.J. (1998). Fungal and plant gene expression during synchronized infection of tomato leaves by *Botrytis cinerea*. *European Journal of Plant Pathology* 104: 207-220.

Bollen, G.J. and Scholten, G. (1971). Acquired resistance to benomyl and some other systemic fungicides in a strain of *Botrytis cinerea* in cyclamen. *Netherlands Journal of Plant Pathology* 77: 83-90.

Damicone, J. and D. Smith. (2009). Fungicide resistance management. (On-line). Oklahoma Cooperative Extension Fact Sheets. Available:

<http://pods.dasnr.okstate.edu/docushare/dsweb/Get/Document-2317/F-7663web.pdf> (November 8, 2016).

Davidse, L.C. and Ishii, H. (1995). Biochemical and molecular aspects of the mechanisms of action of benzimidazoles, N-phenylcarbamates and N-phenylformamidoximes and the mechanisms of resistance to these compounds in fungi. In: Lyr H (ed.) Modern Selective Fungicides. Gustav Fisher Verlag, Jena, Germany. pp 305-322.

Davidson, R.M., Hanson, L.E., Franc, G.D. and Panella, L. (2006). Analysis of β -tubulin gene fragments from benzimidazole-sensitive and tolerant *Cercospora beticola*. Journal of Phytopathology 154: 321-328.

Elad, Y., Williamson, Tudzynski P. and Delen N. (2007). Botrytis: Biology, Pathology and Control. Springer, P.O. Box 17, 3300 AA Dordrecht, The Netherlands. 403 p.

Fujimura, M., Kmakura, T., Inoue, S. and Yamaguchi, I. (1992). Sensitivity of *Neurospora crassa* to benzimidazoles and N-phenylcarbamates: effect of amino acid substitutions at position 198 in β tubulin. Pesticide Biochemistry and Physiology 44: 165-167.

Genescope. (2002). *Botrytis cinerea* Estimated losses to vineyards in France. (Annual Report, UIPP, 2002).

Jarvis, W.R. (1980). Epidemiology. Pages 219-250 in: The Biology of Botrytis. J. R. Coley-Smith, K. Verhoeff, and W. R. Jarvis, eds. Academic Press, New York.

Jung, M.K., Wilder, I.B. and Oakley, B.R. (1992). Amino acid alterations in the *benA* (Beta-tubulin) gene of *Aspergillus nidulans* that confer benomyl resistance. Cell Motility and the Cytoskeleton 22: 170-174.

Koenraadt, H., Somerville, S.C. and Jones, A.L. (1992). Characterization of mutations in the beta-tubulin gene of benomyl-resistant field strains of *Venturia inaequalis* and other plant pathogenic fungi. Phytopathology 82: 1348-1354.

Leroux, P. and Clerjeau, M. (1985). Resistance of *Botrytis cinerea* and *Plasmopara viticola* to fungicides in French vineyards. Crop Protection 4: 137-160.

Leroux, P., Fritz, R., Debieu, D., Albertini, C., Lanen, C., Bach, J., Gredt, M. and Chapeland, F. (2002). Mechanisms of resistance to fungicides in field strains of *Botrytis cinerea*. Pest Management Science 58(9): 876-888.

Ma, Z.H., Yoshimura, M.A. and Michailides, T.J. (2003). Identification and characterization of benzimidazole resistance in *Monilinia fructicola* from stone fruit orchards in California. Applied and Environmental Microbiology 69: 7145-7152.

Ma, Z., and Michailides, T.J. (2005). Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. Crop Protection 24: 853-863.

Maymon, M., Zveibil, A., Pivonia, S., Minz, D. and Freeman, S. (2006). Identification and characterization of benomyl-resistant and sensitive populations of *Colletotrichum gloeosporioides* from Statice (*Limonium* spp.). Phytopathology 96: 542-548.

McClellan, W.D. and Hewitt, W.B. (1973). Early Botrytis rot of grapes: Time of infection and latency of *Botrytis cinerea* Pers. in *Vitis vinifera* L. Phytopathology 63: 1151-1157.

McKay, G., Egan, J.D., Morris, E. and Brown, A.E. (1998). Identification of benzimidazole resistance in *Cladobotryum dendroides* using a PCR-based method. Mycological Research 102: 671-676.

Nair, N.G., Guilbaud-Oulton, S., Barchia, I. and Emmett, R. (1995). Significance of carryover inoculum, flower infection and latency on the incidence of *Botrytis cinerea* in berries of grapevines at harvest in New South Wales. Australian Journal of Experimental Agriculture 35: 1177-1180.

Orbach, M.J., Porro, E.B. and Yanofsky, C. (1986). Cloning and characterization of the gene for beta-tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker. *Molecular and Cellular Biology* 6: 2452-2461.

Park, S.Y., Jungt, J.O., Chung, Y.R. and Lee, W.C. 1996. Isolation and Characterization of a Benomyl-resistant Form of β -Tubulin-encoding Gene from the Phytopathogenic Fungus *Botryotinia fuckeliana*. *Molecular Cells* 7(1): 104-109.

Peres, A. R.N., Souza, N.L., Peever, T.L. and Timmer, L.W. (2004). Benomyl sensitivity of Isolates of *Colletotrichum acutatum* and *C. gloeosporioides* from citrus. *Plant Disease* 88(2): 125-130.

Pezet, R. and Pont, V. (1986). Infection florale et latence de *Botrytis cinerea* dans les grappes de *Vitis vinifera* (var. Gamay). *Revue Suisse Viticulture Arboriculture Horticulture* 18: 317-322.

Sholberg, P.L., Harlton, C., Haag, P., L'evseque, C.A., Gorman, D.O. and Seifert, K. (2005). Benzimidazole and diphenylamine sensitivity and identity of *Penicillium* spp. that cause postharvest blue mold of apples using β -tubulin gene sequences. *Postharvest Biology Technology* 36: 41-49.

Smith, C.M. (1988). History of benzimidazole use and resistance. In: Delp CJ (ed.) *Fungicide Resistance in North America*. American Phytopathological Society Press, St. Paul, Minnesota, USA. pp. 23-24.

Stehmann, C. and de Waard, M.A. (1996). Sensitivity of populations of *Botrytis cinerea* to triazoles, benomyl, and vinclozolin. *European Journal of Plant Pathology* 102: 171-180.

Vivier, M.A. and Pretorius, I.S. (2002). Genetically tailored grapevines for the wine industry. *Trends in Biotechnology* 20: 472-478.

Yarden, O., and Katan, T. (1993). Mutations leading to substitutions at amino acids 198 and 200 of beta-tubulin that correlate with benomyl resistance phenotypes of field strains of *Botrytis cinerea*. *Phytopathology* 83: 1478-1483.

Yourman, L.F. and Jeffers, S.N. (1999). Resistance to benzimidazole and dicarboximide fungicides in greenhouse isolates of *Botrytis cinerea*. *Plant Disease*. 83: 569-575.

Zhang, Q.C., Liu, H.Y. and Zhu, N.G. (2010). Detection and characterization of benzimidazole resistance of *Botrytis cinerea* in greenhouse vegetables. *European Journal Plant Pathology* 126: 509-515.

Ziogas, N.B., Nikou, D., Markoglou, N.A., Malandrakis, A.A. and Vontas, J. (2009). Identification of a novel point mutation in the β -tubulin gene of *Botrytis cinerea* and detection of benzimidazole resistance by a diagnostic PCR-RFLP assay. *European Journal Plant Pathology* 125: 97-107.

(Received 23 January 2017, accepted 28 February 2017)