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

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**Abstract** 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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beenestimated 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 $\beta$-tubulin. Benzimidazoles inhibit the assembly of microtubules by binding to $\beta$-tubulin sub-units of the fungus (Davidse and Ishii, 1995). In most cases, mutations of amino acids at positions 198 and 200 in the $\beta$-tubulin gene confer benzimidazole resistance in field isolates of many plant pathogens (Altertini *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 $\beta$-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 ºC. After single spore isolation, mycelial discs of *B. cinerea* were maintained in mineral oil at 5 ºC 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 ºC 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 ºC 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 µl volumes and contained 10-100 ng of DNA template, 5 µl 10x PCR buffer, 5 µl dNTPs, 1 µl primer Bcb-F, 1µl primer Bcb-R, 1 µl DNA template, 3 µl $MgCl_2$, 33 µl distilled water, and 1 µl Taq DNA Polymerase. The PCR program was 94 ºC x 5 min, (94 ºC x 1 min, 53 ºC x 1 min, 72 ºC x 1 min) x 35 cycles, followed by final extension at 72 ºC 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 µg/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

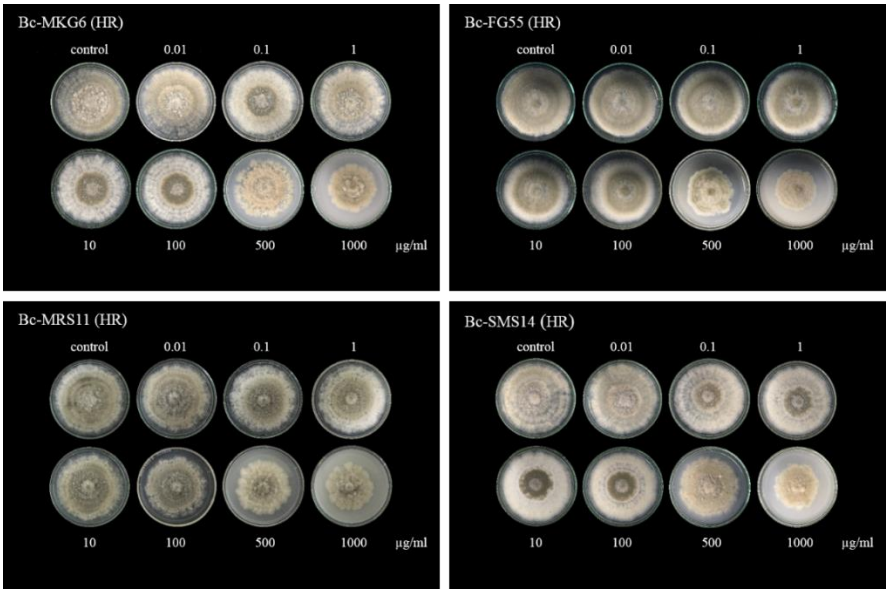


**Figure 1.** Carbendazim-resistant isolates of *Botrytis cinerea* on potato dextrose agar (PDA) amended with carbendazim at 0 (control), 0.01, 0.1, 1, 10, 100, 500, and 1,000 µg/ml; (HR) - highly resistant phenotype

***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).

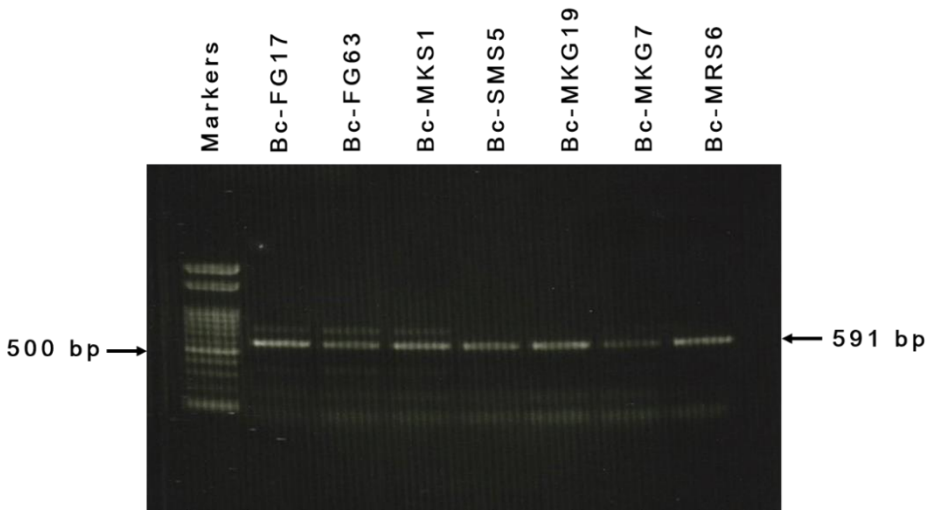


**Figure 2.** PCR amplification of the *β*-tubulin gene from isolates of *Botrytis cinerea* causing gray mold disease; PCR products were generated using the Bcb-F and Bcb-R primers. Markers are from the 100 bp Ladder.

| 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 | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT |
| | | H Q L V E N S D **A** T F C I D N E A |
| Bc-MKG3 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT |
| | | H Q L V E N S D **A** T F C I D N E A |
| Bc-MKG4 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT |
| | | H Q L V E N S D **A** T F C I D N E A |
| Bc-MKG7 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT |
| | | H Q L V E N S D **A** T F C I D N E A |
| Bc-MKG8 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT |
| | | H Q L V E N S D **A** T F C I D N E A |
| Bc-MKG9 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT |
| | | H Q L V E N S D **A** T F C I D N E A |
| Bc-MKG10 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT |
| | | H Q L V E N S D **A** T F C I D N E A |
| Bc-MKG11 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT |
| | | H Q L V E N S D **A** T F C I D N E A |
| Bc-MKG12 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT |
| | | H Q L V E N S D **A** T F C I D N E A |
| Bc-MKG14 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT |
| | | H Q L V E N S D **A** T F C I D N E A |
| Bc-MKG18 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT |
| | | H Q L V E N S D **A** T F C I D N E A |
| Bc-MKG19 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT |
| | | H Q L V E N S D **A** T F C I D N E A |
| Bc-MKG20 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT |
| | | 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 | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG17 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG19 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG21 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG22 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG40 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG42 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG43 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG46 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG48 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG49 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG51 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG55 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG58 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG61 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG62 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG63 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG66 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG67 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG69 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG70 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG72 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG73 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-FG75 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-MRS1 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-MRS2 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-MRS3 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>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 | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-MRS8 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-MRS10 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-MKS1 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-MKS2 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-SMS3 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-SMS4 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-SMS5 | HR | CATCAATTGGTTGAGAACTCTGACG**C**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **A** T F C I D N E A |
| Bc-SMS14 | HR | CATCAATTGGTTGAGAACTCTGACG**T**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **V** T F C I D N E A |
| Bc-SMS33 | HR | CATCAATTGGTTGAGAACTCTGACG**T**GACCTTCTGTATCGATAACGAGGCT<br>H Q L V E N S D **V** T F C I D N E A |

**Figure 3.** Continued

## Discussion

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.

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