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Molecular Analysis of *Botrytis cinerea* Causing Ginseng Grey Mold Resistant to Carbendazim and the Mixture of Carbendazin Plus Diethofencarb

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A total of 23 isolates of *Botrytis cinerea* causing the grey mold were collected from infected ginseng in several fields of Korea. The sensitivity to carbendazim and the mixture of carbendazim plus diethofencarb was determined through a mycelial inhibition test on PDA amended with or without fungicides. *B. cinerea* isolates were classified as 3 phenotypes, which were the first phenotype resistant to both of carbendazim and the mixture (Car^RMix^R), the second one resistant to carbendazim and sensitive to the mixture (Car^RMix^S), and the last one sensitive to both of them (Car^SMix^S). Carbendazim resistance correlated with a single mutation in β-tubulin gene of *B. cinerea* amplified with primer pair tubkjhL and tubkjhR causing a change of glutamate to alanine at amino acid position 198. Furthermore, the substitution of valine for glutamate led the resistance to carbendazim and the mixture at the same position of amino acid. PCR-restriction fragment length polymorphism (PCR-RFLP) analysis using the restriction endonuclease, *Tsp45I* and *BstUI* allowed differentiation of the PCR fragment of β-tubulin gene of Car^SMix^S isolates from that of Car^RMix^R and Car^RMix^S isolates. This method will aid in a fast detection of resistance of carbendazim and the mixture of carbendazim plus diethofencarb in *B. cinerea* in ginseng field.

Keywords : *Botrytis cinerea*, beta-tubulin gene, carbendazim, fungicide resistance, mixture of carbendazim plus diethofencarb, PCR-RFLP

Botrytis cinerea, the major pathogen on many crops causing severe economic damage, has been managed by sanitation and other cultural practices to avoid introducing the pathogen, and fungicide application to prevent or limit disease spread. Because host resistance is not available for many crops, fungicides traditionally have played an important role in managing gray mold caused by *B. cinerea*. In many cases, *B. cinerea* populations are repeatedly exposed to modern specific fungicides such as benzimidazoles which prevent the formation of microtubules by inhibiting α and β-tubulin polymerization and ultimately lead to reduced or restricted cell division and results in abnormal cell function and growth, creating selection pressure for the development of fungicide resistance. Resistance to benzimidazole fungicides has been described from a number of crops in the greenhouse and in the field (LaMondia and Douglas, 1997; Lennox and Spotts, 2003; Yourman and Jeffers, 1999). To manage the population of *B. cinerea* resistant to benzimidazole fungicides, the fungicide mixture was commercialized, which was composed with carbendazim as a benzimidazole and diethofencarb included into *N*-phenylcarbamate. Diethofencarb was reported to be negatively cross-resistant with carbendazim, for which it showed the specifically high activity against fungal isolates resistant to benzimidazole fungicides (Elad et al., 1988; Kato et al., 1984). In the field, however, chemical control of gray mold with the mixture of carbendazim plus diethofencarb has suffered from the development of resistance by *B. cinerea* to the mixture (Katan et al., 1989; Leroux et al., 1999; Yarden and Katan, 1993). Also, in Korea, the mixture of carbendazim plus diethofencarb had been applied to control the gray mold of ginseng plants in many fields, following the development of *B. cinerea* resistant to the fungicide mixture (Kim et al., 2007).

Recently advanced molecular techniques provided the understanding for the mechanism of fungicide resistance in filamentous fungi (Ma and Michailides, 2005; Michailides et al., 2005). Through molecular technique for sequencing of each β-tubulin gene in sensitive- and resistant isolates, it was reported in most cases that resistance was correlated with point mutation in those genes, which result in altered amino acid sequences at fungicide-binding site (Leroux et al., 2002). Jung et al. (1992) have demonstrated that ben A is the structural gene for β-tubulin in *Aspergillus nidulans* and the mutations in β-tubulin was responsible for the benomyl resistant mutations. Similarly, Yarden and Katan (1993) also reported three base pair mutations among the strains of grapes gray mold pathogen. The codon 198 encoding glutamic acid in wild type was changed to alanine in strain high resistant to just benzimidazole fungicide, and

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was changed to lysine in strain high resistant to benzimidazole fungidcide and resistant to diethofencarb, while the substitution of tyrosine for phenylalanine at the codon 200 of β-tubulin provided resistance to diethofencarb as well as benzimidazole fungicides moderately.

As well as understanding the mechanism of fungicide resistance, advances in molecular biology have provided detecting fungicide resistant genotype rapidly once the mechanisms have been elucidated at a molecular level (Ma and Michailides, 2005). Several molecular techniques, such as polymerase chain reaction (PCR), PCR-restriction fragment length polymorphism (PCR-RFLP), and allele-specific PCR (AS-PCR), have been used to detect fungicide-resistant populations in several plant pathogens. Because PCR-RFLP is a general technique to detect a point mutation in target genes that alters a restriction enzyme site, it has been used to detect phytopathogenic fungi resistant to several fungicide groups (Ma et al., 2003; Ma et al., 2005; Oshima et al., 2006; Saito et al., 2008).

In this study, we investigated the sensititivity of *B. cinrea* isolates collected from ginseng plants to carbendazim and fungicidal mixture of carbendazim plus diethofencarb by in vitro mycelial growth inhibition test, and the possible resistance mechanisms of carbendazim and the mixture of carbendazim plus diethofencarb. Furthermore, a PCR-RFLP method was developed to detect isolates of *B. cinerea* resistant to fungicides, based on point mutation at the codon of β-tubulin gene.

Materials and Methods

Fungal isolates. Field isolates of *B. cinerea* were collected from infected leaves of Korean ginseng plant sampled from several fields located in Gyeonggi, Chungbuk, Chungnam, Jeonbuk, and Gyeongbuk during 2005 to 2006. Pieces of diseased leaves were placed into a plate containing two layers of filter paper (diameter, 9 cm) wetted with 10 mL of distilled water at the bottom. To induce sporulation of *B. cinerea*, plates were kept at 22°C for 2 days. Under a microscope a spore was taken out with sterile Pasteur pipette, and inoculated on PDA (PDA; Difco Laboratories, Detroit, MI) amended with 300 µg/ml streptomycin sulfate. Isolates of *B. cinerea* were grown on PDA at 22°C, and maintained on PDA slants at 4°C until used.

Sensitivity testing of *Botrytis cinerea*. The used fungicides were carbendazim (a.i. 60%, WP) and the mixture of carbendazim plus diethofencarb (a.i. 50%, WP). To prepare fungicide-amended medium, fungicides, which were suspended with sterile distilled water (SDW) at indicated concentrations, were added to PDA just before pouring in plates. Final concentrations of fungicides in PDA were adjusted to 0.032, 0.16, 0.8, 4 and 20 µg/ml. Mycelial disc (diameter, 5 mm) were taken from the margins of the colonies of *B. cinerea* grown at 22°C for 3 days, and transferred on PDA amended with fungicides. After incubation at 22°C for 4 days, colony diameters of each isolates were evaluated on PDA with or without the fungicide. Effective concentration inhibiting mycelial growth by 50% (EC_{50}) was determined with inhibition ratio (%) of mycelial growth at each concentration calculated relative to growth on PDA without fungicide.

DNA extraction, PCR, and sequence analysis. Fungal genomic DNA was extracted as previously described (Kim et al., 2007). The PCR primer pair tubkjhL (5'-ACT GAG GGT GCT TGA GCT TGT-3') and tubkjhR (5'-TCA ACT CTG GAA CGG TGA CA-3') was used to amplify the β-tubulin gene fragment from *B. cinerea*. The PCR reaction was performed in 50-µl reaction mixtures containing 50 ng of genomic DNA, 10 pmole/µl of each primer, 2 mM of each dNTP, 15 mM $MgCl_2$, 10x Taq buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3 (25°C), 1% Triton X-100), and 5 unit/µl of Taq polymerase (Enzynomics, South Korea). The PCR amplification was performed by using following parameters: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 61.2°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min. The amplified products were column-purified with PCR DNA purification kit (GeneAll Biotechnology Co. Ltd. Seoul, Korea) and cycle-sequenced by the National Instrumentation Center for Environmental Management (NICEM; Seoul, Korea). The sequences of nucleotides and amino acid were analyzed by PHYDIT version 3.1 and chromas version 2.31.

PCR-Restriction Fragment Length Polymorphism (PCR-RFLP). For PCR-RFLP analysis, a 20-µl aliquot of PCR products amplified by the primer pair tubkjhL and tubkjhR was digested by 10 unit of *Tsp45I* and *BsyUI* (Fermentas Life Science) at 37°C for 8 h, inactivated at 65°C for 20 min according to the manufacturer's protocol, and analyzed on 3% agarose gel.

Results

Sensitivity of *B. cinerea* isolates to fungicides in *in vitro* test. A total of 23 *B. cinerea* isolates were used for fungicide resistance tests on media including carbendazim and the mixture of carbendazim plus diethofencarb. The fungal isolates were classified as 3 groups with regard to the sensitivity to fungicides, which are carbendazim/mix-

Table 1. Sensitivity of *Botrytis cinerea* isolates collected from ginseng between 2005 and 2006 to fungicides and mutations at codon 198 of β-tubulin gene

Isolates	Location	Response[a] to fungicide	EC_{50} (μg/ml)		Amino acid at codon 198
			carbendazim	mixture[b]	
BB1-3	Bongwha	Car^RMix^R	>10	0.98	Valine
KA1-2	Anseong	Car^RMix^R	>10	1.90	Valine
KA1-6	Anseong	Car^RMix^R	>10	1.05	Valine
KP6	Paju	Car^RMix^R	>10	0.98	Valine
YP1	Yecheon	Car^RMix^R	>10	1.25	Valine
YP3	Yecheon	Car^RMix^R	>10	1.48	Valine
YP4	Yecheon	Car^RMix^R	>10	0.86	Valine
YP6	Yecheon	Car^RMix^R	>10	1.36	Valine
YP9	Yecheon	Car^RMix^R	>10	0.87	Valine
BB1-9	Bongwha	Car^RMix^S	>10	<0.5	Alanine
KA2-4	Anseong	Car^RMix^S	>10	<0.5	Alanine
KA2-5	Anseong	Car^RMix^S	>10	<0.5	Alanine
KP1	Paju	Car^RMix^S	>10	<0.5	Alanine
KY1	Yeoncheon	Car^RMix^S	>10	<0.5	Alanine
YP5	Yecheon	Car^RMix^S	>10	<0.5	Alanine
KY3	Yeoncheon	Car^SMix^S	<0.032	<0.5	Glutamate
YC1	Yeoncheon	Car^SMix^S	0.02	<0.5	Glutamate
YD1	Yeoncheon	Car^SMix^S	0.02	<0.5	Glutamate
YD11	Yeoncheon	Car^SMix^S	0.01	<0.5	Glutamate
YD12	Yeoncheon	Car^SMix^S	<0.032	<0.5	Glutamate
YD2	Yeoncheon	Car^SMix^S	0.05	<0.5	Glutamate
YD3	Yeoncheon	Car^SMix^S	0.05	<0.5	Glutamate
YD9	Yeoncheon	Car^SMix^S	0.02	<0.5	Glutamate

[a] Responses to fungicides were classified with 3 phenotypes such as Car^RMix^R, Car^RMix^S, and Car^SMix^S. Isolates of Car^RMix^R phenotype showed up to 10 μg/ml of EC_{50} value against carbendazim and up to 0.8 μg/ml of EC_{50} value against the mixture of carbendazim plus diethofencarb. While isolates of Car^RMix^S phenotype showed up to 10 μg/ml of EC_{50} value against carbendazim, EC_{50} values of them was below 0.5 μg/ml against the mixture. EC_{50} values of Car^SMix^S phenotype isolates were below 0.05 and 0.5 μg/ml against carbendazim and the mixture, respectively.
[b] The mixture was composed with carbendazim (a.i. 25%) and diethofencarb (a.i. 25%).

ture-resistant isolates (Car^RMix^R), carbendazim-resistant and mixture-sensitive isolates (Car^RMix^S), and both fungicide-sensitive isolates (Car^SMix^S). As showing in Table 1, EC_{50} values of Car^RMix^R isolates were showed up to 10 μg/ml against carbendazim, and ranged from 0.86 to 1.90 μg/ml against the mixture. Both of them, Car^RMix^S and Car^SMix^S isolates, showed less than 0.5 μg/ml of EC_{50} values against the mixture. In respect to the sensitivity to carbendazim, EC_{50} values of Car^SMix^S isolates ranged from 0.032 to 0.05 μg/ml, while those of Car^RMix^S isolates showed more than 10 μg/ml.

Molecular analysis of the β-tubulin gene in *B. cinerea*. To determine whether point mutation in the deduced amino acid sequences were related to resistance to carbendazim and the mixture of carbendazim plus diethofencarb, the nucleotide sequence of β-tublin were analyzed among Car^RMix^R, Car^RMix^S and Car^SMix^S isolates. In the 23 isolates analyzed, resistance was correlated with single nucleotide substitutions, conferring changes just amino acid 198 (Table 1). Isolate YP9 resistant to both fungicides (Car^RMix^R) had GTG at codon 198 of β-tubulin gene (Fig. 1). Car^RMix^S isolates had GCG, and Car^SMix^S isolates had GAG at the same codon. No other polymorphisms in the sequences of β-tubulin amplified by PCR using primers, tubkjhL and tubkjhR, were detected in any of the isolates. Thus, codon 198, which encodes glutamic acid in Car^SMix^S isolates, was substituted by valine in Car^RMix^R isolates, and codon 198 encoding glutamic acid was substituted by alanine in Car^RMix^S isolates.

PCR-RFLP analysis for detection of fungicide resistance in *B. cinerea*. It was obtained an approximate 550 bp product from all the isolates by PCR with primer pair, tubkjhL and tubkjhR. The restriction endonuclease *Tsp45I* recognized the sequence GTGAC in the PCR product amplified from 3 phenotypes of *B. cinerea* isolates. *Tsp45I* digested the 550 bp PCR product from Car^RMix^R isolates

KA2-4 ^{251} GAGAACTCTGAC**GCG**ACCTT ^{270}

YP5 ^{251} GAGAACTCTGAC**GCG**ACCTT ^{270}

YD12 ^{251} GAGAACTCTGAC**GAG**ACCTT ^{270}

YC1 ^{251} GAGAACTCTGAC**GAG**ACCTT ^{270}

Fig. 1. Comparison of partial nucleotide sequence of the β-tubulin gene amplified with PCR primer pair tubkjhL and tubkjhR from *Botrytis cinerea* isolates. Isolate YP9 belonged into Car^{R}Mix^R phenotype group. Isolate KA2-4 and isolate YP5 were representative of Car^{R}Mix^S phenotype group, while YD12 and YC1 were Car^{S}Mix^S.

into three fragments 51, 212, 267 bp in length on agarose gel, while it did the PCR product from Car^{R}Mix^S and Car^{S}Mix^S isolates into two fragments 51 and 522 bp (Fig. 2). In contrast, *BstUI*, which recognize the sequence CGCG, digested PCR product from Car^{R}Mix^S isolates three fragments 125, 138, 267 bp, except for each Car^{R}Mix^R and Car^{S}Mix^S isolates. Car^{R}Mix^S and Car^{S}Mix^S isolates had two fragments 138 and 389 bp in length after digesting the product by using the endonuclease.

Discussion

In this study, we observed 3 resistant groups to carbendazim and fungicide mixture of carbendazim plus diethofencarb, such as Car^{R}Mix^R, Car^{R}Mix^S and Car^{S}Mix^S group, in *B. cinerea* isolates collected in Korea. Comparison of the nucleotide sequence of β-tubulin gene among 3 groups revealed that Car^{R}Mix^R isolates of *B. cinerea* had only a single mutation of GAG in Car^{S}Mix^S isolates to GTG, which caused a change of glutamic acid (GAG) to a valine (GTG) at codon position 198 of β-tubulin gene. In contrast, glutamic acid at codon 198 was changed to an alanine in Car^{R}Mix^S isolates of *B. cinerea*. Resistance to benzimidazoles has been described for many organisms including fungi and nematodes and is due to a reduction of the binding affinity of the fungicide to β-tubulin (Coles et al., 1992, 2006; Davidse, 1986; Elard et al., 1999). The loss of binding affinity has been associated with one or several single point mutations in the β-tubulin gene which cause changes in several amino acids probably located at fungicide's binding site (Davidse, 1986; Leroux et al., 2002). The most common single point mutations described to be associated with benzimidazole resistance are located at codon 50 (McKay et al., 1998), 198 and 200 (Yaden and Katan, 1993), and 240 (Albertini et al., 1999) of the β-tubulin gene. Likewise, a single point mutation at codon 198, leading the substitution of valine for glutamate in Car^{R}Mix^R group of *B. cinerea* and alanine in Car^{R}Mix^S group, was correlated with fungicide resistance in this study.

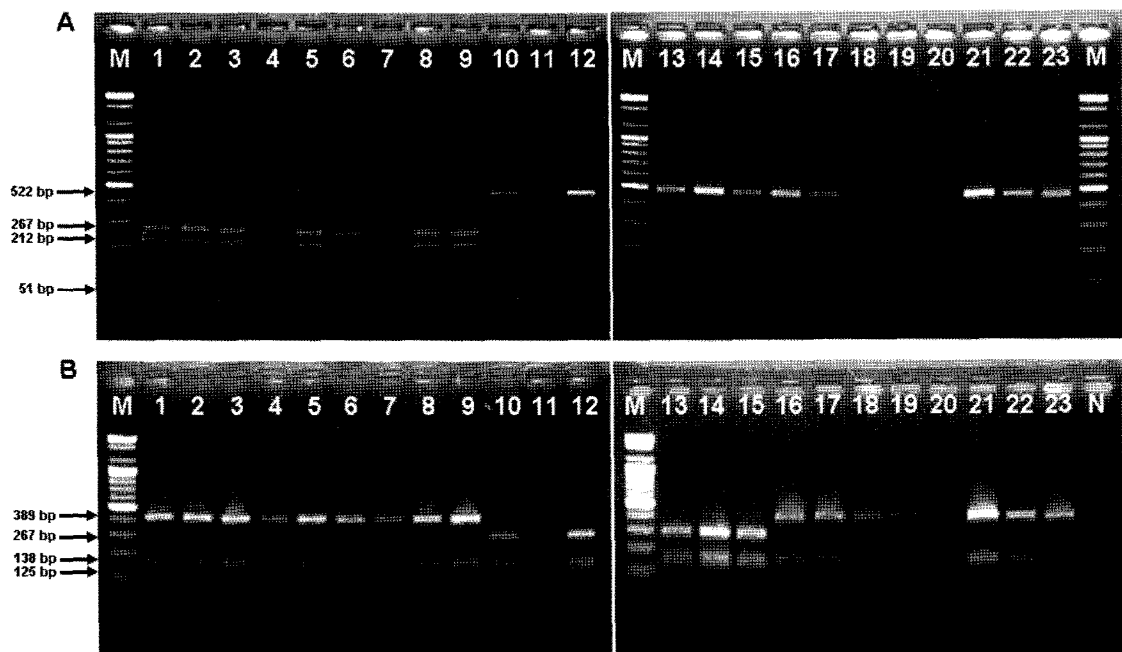

Fig. 2. *Tsp45I*-(A) or *BstUI*-(B) digestion of the amplified β-tubulin gene from the isolates resistant to both carbendazim- and the mixture (1-9), and to carbendazim only (10-15), and those sensitive to carbendazim- and the mixture (16-23). Markers (M) are the 1-kb DNA ladders. Numbers indicate each isolate of *B. cinerea*; 1, YP3; 2, KA1-2; 3, KP6; 4, KA1-6; 5, BB1-3; 6, YP6; 7, YP9; 8, YP4; 9, YP1; 10, KA2-4; 11, KP1; 12, KY1; 13, KA2-5; 14, BB1-9; 15, YP5; 16, YC1; 17, YD9; 18, YD2; 19, YD1; 20, YD3; 21, KY3; 22, YD11; 23, YD12; N, negative control.

A molecular based PCR-RFLP method was developed to detect *B. cinerea* isolates resistant to carbendazim and the mixture of carbendazim plus diethofencarb. Showing the band patterns digesting the PCR product of *B. cinerea* with endonucleases *Tsp45I* and *BstUI*, it is easy to differentiate fungicide-resistant phenotype from sensitive one. The fungicide-resistant phenotypes of Car^RMix^R, Car^RMix^S and Car^SMix^S identified by the PCR-RFLP method were consistent with those from *in vitro* fungicide resistance tests on the same samples, demonstrating the reliability of the PCR-RFLP method. Moreover, this method can save working time but may be a little bit more expensive due to higher cost of molecular supplies, compared to the traditional spore germination test or the mycelium growth test on a medium amended with fungicides (Ma et al., 2003). The high frequency occurring fungicide-resistant *B. cinerea* has been becoming a problem for disease control in many plants (LaMondia and Douglas, 1997; Myresiotis et al., 2007; Yourman and Jeffers, 1999). Additionally it is difficult to control the grey mold disease of ginseng plant, because ginseng plants are cultivated in the same place for 5-6 years. For the integrated control program of *B. cinerea*, it is needed to monitor the fungicide resistance of *B. cinerea* quickly in fields and to select effective fungicides. By using the molecular technique such as PCR-RFLP to monitor the pathogen resistant to fungicides, it will be possible to save the time, which will allow the development of quick resistance monitoring programs that will enable farmers to decide fungicides available for controlling grey mold disease in ginseng plant, based on the results of monitoring program.

In conclusion, PCR-RFLP method developed in this study is expected to have the advantage of rapid detection for *B. cinerea* isolates resistant to carbendazim and the fungicide mixture of carbendazim plus diethofencarb with high sensitivity and reliability. Further work, which is a DNA extraction method from a lot of infected plants of ginseng and a design of different primers, is needed to ensure that the PCR-RFLP method can be used in fields practically and identify all the resistant phenotypes known to occur in *B. cinerea* populations.

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