Journal of Agricultural Technology 2010 Vol.6(2): 365-378
Available online http://www.ijat-rmutto.com
ISSN 1686-9141

Point mutations in the beta-tubulin gene conferred carbendazim-resistant phenotypes of *Colletotrichum gloeosporioides* causing 'Nam Dok Mai' mango anthracnose

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Nalumpang, S., Miyamoto, Y., Miyake, C., Izumi, Y., Akitmitsu, K., and Kongtragoul, P. (2010). Point mutations in the beta-tubulin gene conferred carbendazim-resistant phenotypes of *Colletotrichum gloeosporioides* causing 'Nam Dok Mai' mango anthracnose. Journal of Agricultural Technology 6(2): 365-378.

Fifty-nine naturally-infected isolates of *Colletotrichum gloeosporioides* causing 'Nam Dok Mai' mango anthracnose disease were collected from markets and orchards in Thailand; consisting of 6 isolates (10.17%) from leaves and 53 isolates (89.83%) from fruits. In preliminary studies conducted *in vitro* with potato dextrose agar amended with carbendazim at various concentrations: 0.1, 1, 10, 100, 500 and 1,000 mg/l. The phenotype-resistant levels evaluation was grouping into four representative phenotypes of reactions as highly resistance (HR; ≥ 500 mg/l), moderately resistance (MR; ≤ 100 mg/l), weakly resistance (WR; ≤ 10 mg/l) and sensitive (S; ≤ 1 mg/l). The result showed 49 isolates (83.05%) were HR phenotypes; consisting of 2 isolates (3.39%) from leaves and 47 isolates (79.66%) from fruits, and 10 isolates (16.95%) were S phenotypes; consisting of 4 isolates (6.78%) from leaves and 6 isolates (10.22%) from fruits. The differences in the carbendazim-resistant phenotypes were conspicuous in sequence analysis of the second beta-tubulin (*TUB2*) gene compared with *C. gloeosporioides* f. sp. *aeschynomene* (accession No. U14138). HR phenotypes were revealed a single nucleotide mutation; an adenine (A) to cytosine (C) transversion, resulting in a substation of codon 198, which encodes glutamic acid (GAG) in S phenotypes, was converted to a codon for alanine (GCG) which is closely associated with conferring carbendazim-resistant phenotype. This indicates that careful management of carbendazim fungicides applications is necessary to achieve effective control.

Key words: anthracnose, beta-tubulin gene, carbendazim resistance, *Colletotrichum gloeosporioides*, mango (*Mangifera indica*)

Introduction

Mango fruit cv. 'Nam Dok Mai' (*Mangifera indica* L.) is one of the important economic fruit crops in Thailand because of its good smell,

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delicious taste, excellent flavor, and attractive fragrance (Singh *et al.* 2008). And Thailand is one of the major producers and exporters of this mango cultivar (Office of Agricultural Economics, Department of Agriculture, 2008). However, one of the constraints of markets is disease, especially anthracnose disease caused by fungus *Colletotrichum gloeosporioides*. It causes a problem after harvest due to disease expression starting at the ripening stage. These cultivar mangos are highly susceptible to this disease and can be infected as latent infection in high levels compared with other cultivar (Sangchote, 1987). In order to control this disease, in over time, benzimidazole fungicides such as carbendazim, benomyl and thiabendazole have been widely used to manage the mango anthracnose, because farmers believed that the chemical fungicides are able to control plant diseases better than other methods. In fact, the chemical fungicides effectively suppressed and controlled a wide variety of plant diseases at beginning; however, a consequence of a long term utilization of chemical fungicides, particularly systemic fungicides, reduced the significant of fungicide effects to the disease pathogens. Because the pathogens often become resistance to chemical fungicides, and the increase in number of these fungicide resistant isolates give the main problems for the farmers (Farungsang and Farungsang, 1992; Farungsang *et al*., 1994; Steffen *et al*., 1996; Yoon *et al*., 2008). The appearance of fungicide resistance has become an important factor in limiting the efficacy and useful lifetime of important disease control strategies, and therefore the cost spending for the fungicides also increase because the farmers are forced to increase the dosage of the chemical fungicide. Therefore, this resistance may also be an important aid to our understanding, at a molecular level, of the fungicidal mechanism of action.

To effectively control this disease, it is necessary to determine the resistibility of isolates of *C. gloeosporioides* causing anthracnose disease to fungicides. The objectives of this study were to examine resistance of *C. gloeosporioides* isolates obtained from 'Nam Dok Mai' mango to the carbendazim fungicide using phenotypic response and to sequence the partial second beta-tubulin (*TUB2*) gene which has been reported to be responsible for benzimidazole resistance (Orbach *et al.* 1986; Koenraadt *et al*., 1992; Yarden and Katan, 1993; Buhr and Dickman, 1994; Ma and Michailides, 2005).

Materials and methods

Isolation of Colletotrichum gloeosporioides from 'Nam Dok Mai' mango anthracnose

Naturally-infected fruits and leaves of 'Nam Dok Mai' mango were collected from markets and orchards in Thailand. Isolations were made by

cutting small sections about 5x5 mm from lesions and asymptomatic tissues, wetting the sections briefly for 1 min in 70% ethanol, surface disinfecting in 1% sodium hypochlorite for 2-3 min, and rinsing in sterile distilled water. Sections tissues were placed on potato dextrose agar (PDA) media plates and incubated at room temperature. Those plates were observed daily until the mycelium grows and subculture to the new PDA media plates. Cultures were prepared by plating each strain on PDA media plate at room temperature for 5 days productions of mycelial plugs.

Carbendazim resistibility assays

Screening resistibility of all *C. gloeosporioides* isolates to carbendazim were tested using mycelial growth assays. Each isolate was cultured on PDA media plates at room temperature. Mycelial plugs, 5 mm diameter, was cut from the margins of colonies and transferred onto carbendazim supplemented with PDA media at the concentration of 0, 0.1, 1, 10, 100, 500 and 1,000 mg/l. Carbendazim was added to PDA after autoclaving. After inoculation at room temperature, the diameter of each colony was measured and the percentages of growth were calculated and data expressed as percentage of the control. Values obtained were categorized as phenotypes carbendazim resistibility was evaluated into 4 levels shown in Table 1.

Table 1. Phenotype-resistant levels of *Colletotrichum gloeosporioides* to carbendazim at various concentrations: 0.1, 1, 10, 100, 500 and 1,000 mg/l amended with potato dextrose agar (Modified from Farungsang and Farungsang (1992); Farungsang *et al*. (1994); Koenraadt *et al*. (1992) and Peres *et al*. (2004)).

Phenotype-resistant levels	Carbendazim concentration (mg/l)					
	0.1	**1**	**10**	**100**	**500***	**1,000**
Sensitive (S)	✓	X	X	X	X	X
	✓	✓	X	X	X	X
Weakly resistance (WR)	✓	✓	✓	X	X	X
Moderately resistance (MR)	✓	✓	✓	✓	X	X
Highly resistance (HR)	✓	✓	✓	✓	✓	X
	✓	✓	✓	✓	✓	✓

* = the field recommendation rate
✓ = the percentage of growth $\geq$ 10% compared with the control
X = the percentage of growth < 10% compared with the control

Partial sequencing of the second beta-tubulin (TUB2) gene

DNA extraction and PCR amplification

Some isolates of field carbendazim-resistant *C. gloeosporioides* were selected to represent different phenotypes. Genomic DNA was extracted and

purified followed NucloSpin® Plant Kit (MACHREY-NAGEY) was used as the protocol described by the company. Primers TB2L (5'-GTT TCC AGA TCA CCC ACT CC-3') and TB2R (5'-TGA GCT CAG GAA CAC TGA CG-3') (Peres *et al*., 2004) were used to amplify a portion of the partial *TUB2* where carbendazim resistance mutations occurred. Amplification of partial *TUB2* sequences were carried out in a total reaction volume of 50 μl. Polymerase chain reaction (PCR) reaction mixtures contained 1 μl of purified genomic DNA, 5 μl of 10X PCR buffer (iNtRON Biotechnology, Inc.), 25 mM $MgCl_2$ (iNtRON Biotechnology, Inc.), 10 mM dNTPs (iNtRON Biotechnology, Inc.), 50 *p*moles each primer, and 1 unit of *Taq* polymerase (Fermentas). All PCR reactions were carried out in PTC-100^{TM} programmable thermal controller (MJ Research, INC.) with a hold of 5 min at 95 ^{O}C, followed by 30 cycles of 1 min at 95 ^{O}C, 1 min at 35 ^{O}C, and 1 min at 72 ^{O}C, and a final extension for 5 at 72 ^{O}C. PCR products were separated by electrophoresis on 1% agarose gels (Research Organics, INC) with 100-bp sharp DNA maker (RBC Bioscience, Corp.) as a size standard.

DNA sequencing and alignment

Purified PCR products were direct-sequenced on both strands using cycle sequencing with TUB2L and TUB2R primers. Sequence of PCR products were obtained from both strands by the dideoxy chain termination method (Sanger *et al.* 1977) using an ABI PRISM Dye Termination Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA) and an automated fluorescent DNA sequencer (Model 310, Applied Biosystems) following the manufacturer's instructions. DNA sequences were aligned with the BioEdit version 5.0.6 software was used to assemble, edit, and generate high-quality sequences. Using Blast, we searched GenBank, NCBI database for sequences that were similar to those isolates in our study. Alignment of sequences was performed with the implemented ClustalX software automated alignment tool, and alignments were refined manually.

Results

Isolation of Colletotrichum gloeosporioides from 'Nam Dok Mai' mango anthracnose

Colletotrichum spp. was isolated from naturally-infected leaves and fruits of 'Nam Dok Mai' mango collected from markets and orchards in Thailand (Fig. 1). Isolation was made by tissue transplanting technique. The mycelium grows and then subculture to the new PDA plate. Cultures were prepared by plating each strain onto PDA media plate at room temperature for 7-10 days. Fifty-nine *Colletotrichum* spp. isolates were successfully isolated, consisting of 6 isolates (10.17%) from leaves and 53 isolates

(89.83%) from fruits. Studies on morphology characteristic were carried out by examinations of their characteristics of colonies and conidia. The results showed that aerial mycelium of the colonies of all isolates are white and grey. They form cylindrical conidia (4.2-5.1 x 15.4-20.6 μm) and also some isolates produced slimy spore mass and/or sclerotium (Fig. 2). These morphology characteristics were identical with that of *C. gloeosporioides* referred by Sutton (1980).

Fig. 1. Naturally-infected leaves or fruits of 'Nam Dok Mai' mango.

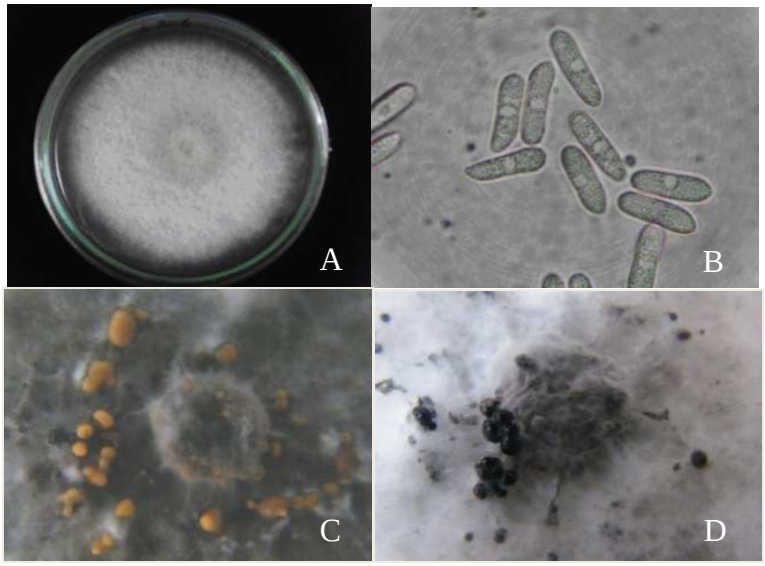

Fig. 2. Characterizations of *Colletotrichum gloeosporioides* causing 'Nam Dok Mai' mango anthracnose; (A) Colony on PDA 10 days, (B) Conidia (X100), (C) Slimy spore mass, (D) Sclerotia.

Carbendazim resistibility assays

Starter cultures were prepared by incubating each *C. gloeosporioides* isolates on PDA plates for 3-4 days. Mycelial plugs, (5 mm diameter) were cut from starter plate. The carbendazim-resistant test was conducted to each strain on PDA amended with carbendazim at various concentrations: 0.1, 1, 10, 100, 500 and 1,000 mg/l, unamended PDA served as control. The result

showed that 49 isolates were highly resistant (HR) phenotypes; consisting 2 isolates from leaves and 47 isolates from fruits. Four isolates were sensitive (S) phenotypes; consisting 2 isolates from leaf and 2 isolates from fruits. None showed weakly resistance (WR) and moderately resistance (MR) phenotypes in this examination (Table 2, Fig. 3).

Table 2. The phenotypes of carbendazim-resistant *Colletotrichum gloeosporioides* causing 'Nam Dok Mai' mango anthracnose base on Table 1.

Mango parts	No. of isolates of carbendazim-resistant phenotypes				Total
	Sensitive (S)	Weakly resistance (WR)	Moderately resistance (MR)	Highly resistance (HR)	
Leaves	4 (6.78%)	0	0	2 (3.39%)	6 (10.17%)
Fruits	6 (10.22%)	0	0	47 (79.66%)	53 (89.83%)
Total	10 (18.60%)	0	0	49 (83.05%)	59 (100%)

Partial sequencing of the second beta-tubulin (TUB2) gene

Partial *TUB2* gene sequences from representative of HR and S phenotypes of *C. gloeosporioides* from 'Nam Dok Mai' mango anthracnose were 430 bp in lenght. The nucleotides at 878-1,308 and amino acid at codon 147-289 sequences of *TUB2* gene from the thirteen HR phenotypes and four S phenotypes were compared with wild type *C. gloeosporioides* f. sp. *aeschynomene* (accession No. U14138) (Buhr and Dickman, 1994). In this study, there were both silent and missense mutation. In missense mutation, the single nucleotide point mutation which resulted in deduced amino acid altered was observed at some codons in *TUB2* fragment, but the single nucleotide point mutation occurred at 1,032; an adenine (A) to cytosine (C) transversion, resulting in a substation of amino acid at codon 198; glutamic acid (GAG) in all S phenotypes, was converted to a codon for alanine (GCG) in all HR phenotypes which is closely associated with conferring carbendazim-resistant phenotype (Fig. 4).

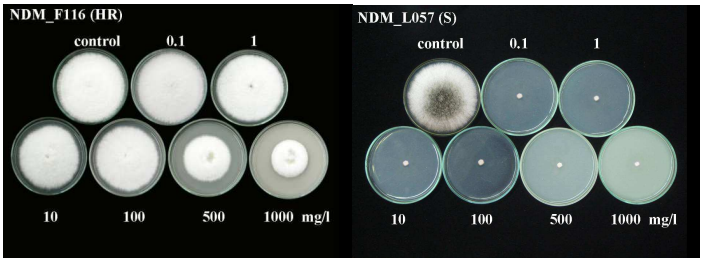

Fig. 3. The carbendazim-resistant phenotypes of highly resistance (HR≥ 500 mg/l) and sensitive (S≤ 1 mg/l,) *Colletotrichum gloeosporioides* isolates on PDA amended with carbendazim at control, 0.1, 1, 10, 100, 500 and 1000 mg/l.

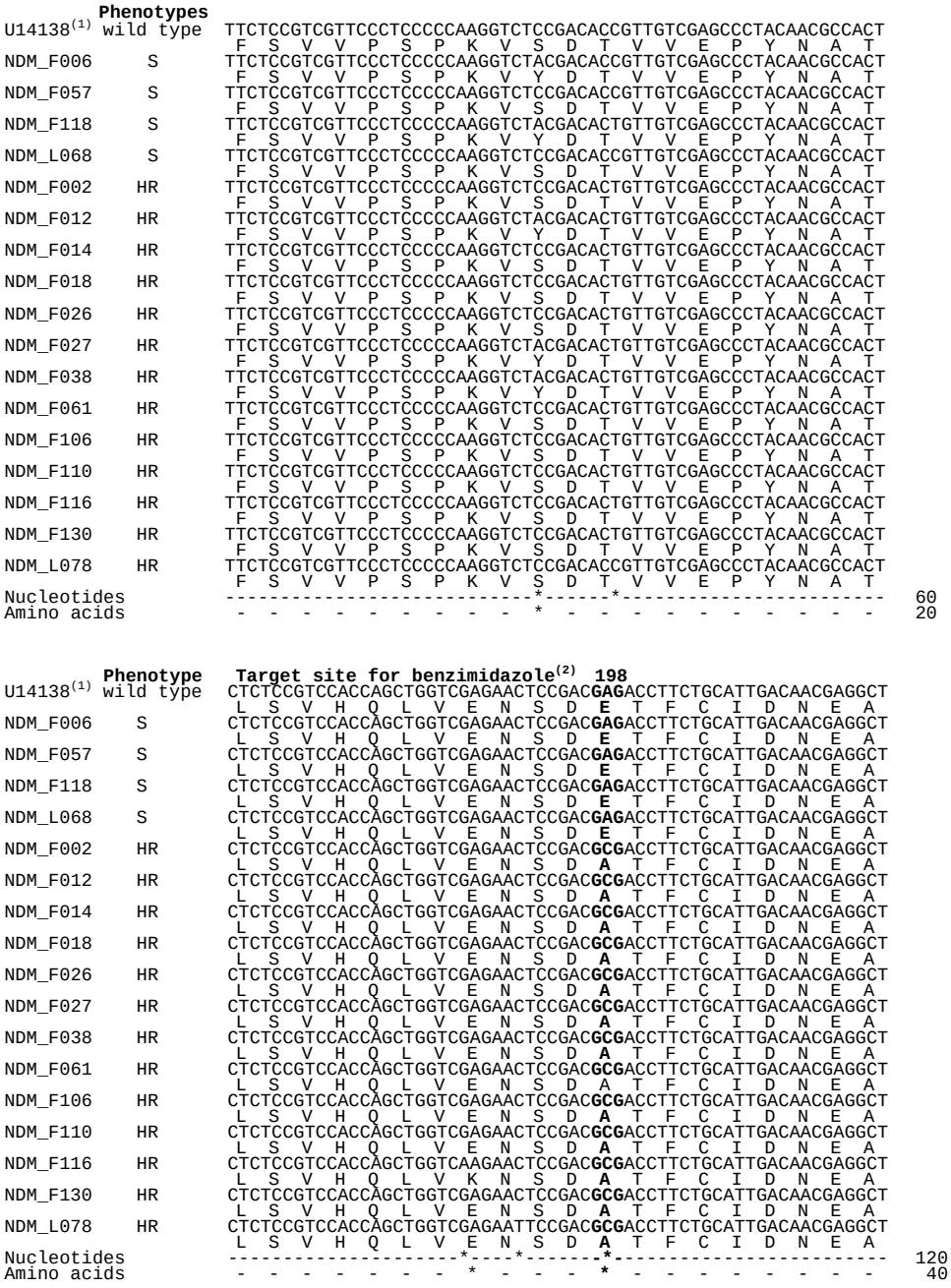

Fig. 4. Comparison of deduced nucleotide sequences and amino acids in *TUB2* gene of *Colletotrichum gloeosporioides* f. sp. *aeschynomene*[(1)] between carbendazim-resistant *C. gloeosporioide*s isolates causing 'Nam Dok Mai' mango anthracnose. [(1)]Buhr and Dickman (1994), [(2)]Peres *et al.* (2004).

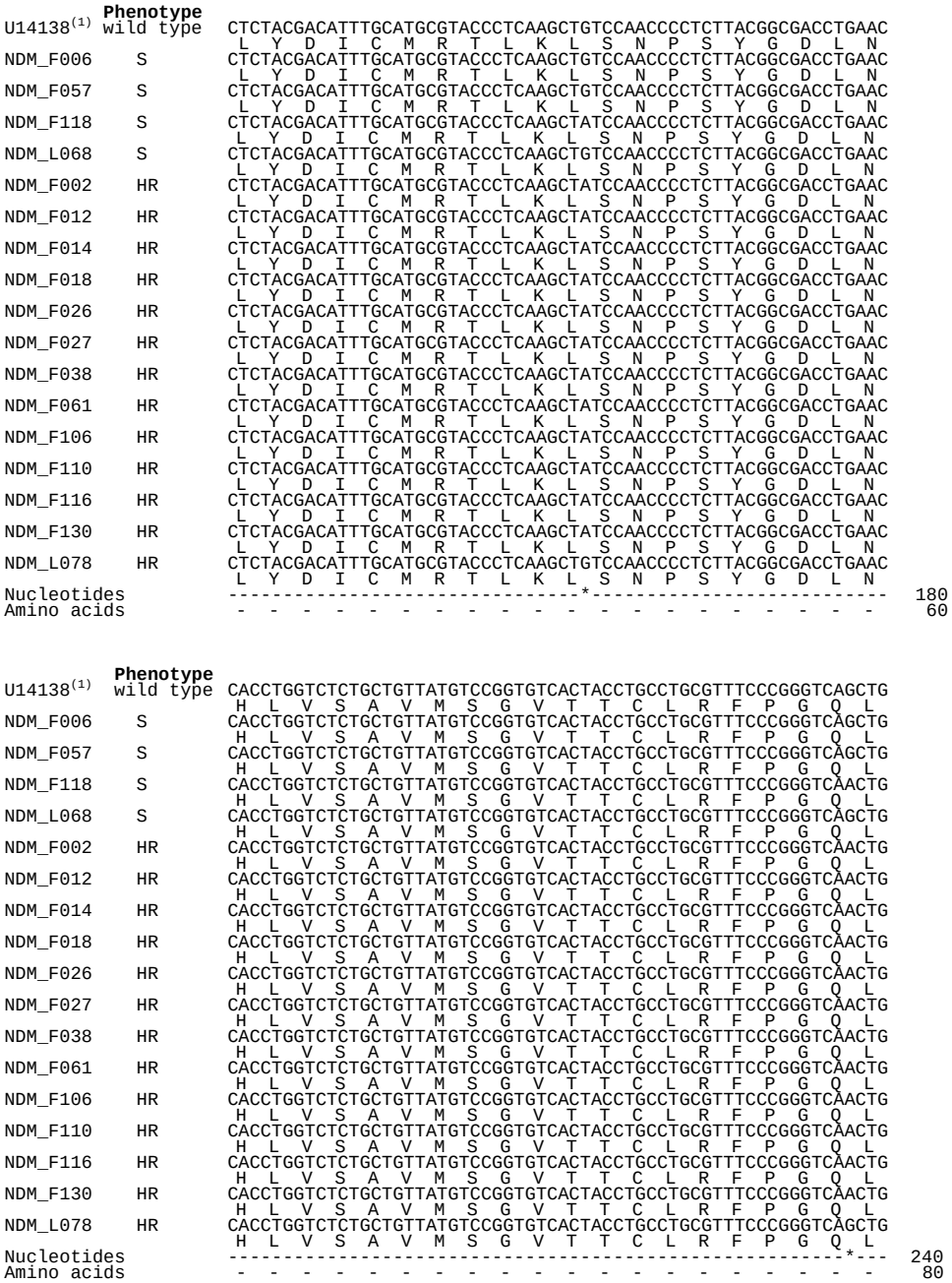

Fig. 4. (continued) Comparison of deduced nucleotide sequences and amino acids in *TUB2* gene of *Colletotrichum gloeosporioides* f. sp. *aeschynomene*[(1)] between carbendazim-resistant *C. gloeosporioide*s isolates causing 'Nam Dok Mai' mango anthracnose. [(1)]Buhr and Dickman (1994), [(2)]Peres *et al.* (2004).

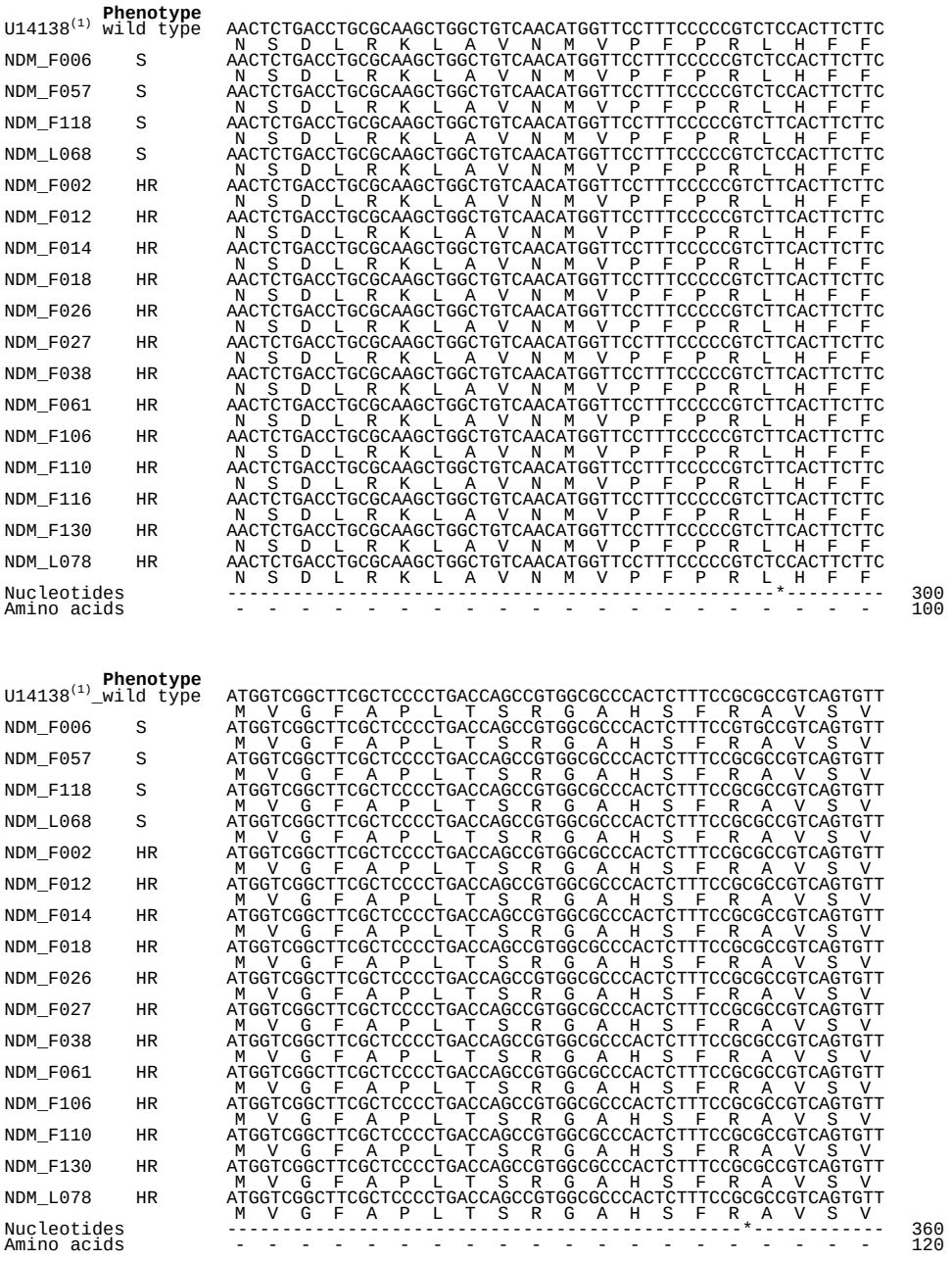

Fig. 4. (continued) Comparison of deduced nucleotide sequences and amino acids in *TUB2* gene of *C. gloeosporioides* f. sp. *aeschynomene*[(1)] between carbendazim-resistant *C. gloeosporioide*s isolates causing 'Nam Dok Mai' mango anthracnose. [(1)]Buhr and Dickman (1994), [(2)]Peres *et al.* (2004).

	Phenotype		
U14138[(1)]	wild type	CCTGAGCTCA	
		P E L	
NDM_F006	S	CCTGAGCTCA	
		P E L	
NDM_F057	S	CCTGAGCTCA	
		P E L	
NDM_F118	S	CCTGAGCTCA	
		P E L	
NDM_L068	S	CCTGAGCTCA	
		P E L	
NDM_F002	HR	CCTGAGCTCA	
		P E L	
NDM_F038	HR	CCTGAGCTCA	
		P E L	
NDM_F061	HR	CCTGAGCTCA	
		P E L	
NDM_F106	HR	CCTGAGCTCA	
		P E L	
NDM_F110	HR	CCTGAGCTCA	
		P E L	
NDM_F116	HR	CCTGAGCTCA	
		P E L	
NDM_F130	HR	CCTGAGCTCA	
		P E L	
NDM_L078	HR	CCTGAGCTCA	
		P E L	
Nucleotides		----------	430
Amino acids		- - -	143

Fig. 4. (continued) Comparison of deduced nucleotide sequences and amino acids in *TUB2* gene of *C. gloeosporioides* f. sp. *aeschynomene*[(1)] between carbendazim-resistant *C. gloeosporioide*s isolates causing 'Nam Dok Mai' mango anthracnose. [(1)]Buhr and Dickman (1994), [(2)]Peres *et al.* (2004).

Discussion

C. gloeosporioides causing 'Nam Dok Mai' mango anthracnose, according to their differential carbendazim-resistant phenotypes. HR phenotypes of *C. gloeosporioides* were developed naturally under conditions of continuously applied fungicide that resistance in field. It showed that continuous application enhanced fungal pathogen development against chemical fungicides as reported by many researchers (Sariah, *et al*., 1989; Farungsang and Farungsang, 1992; Farungsang *et al*., 1994; Steffen *et al*., 1996; Sander *et al.*, 2000; Kim *et al.*, 2007 and Kumar *et al.*, 2007). The appearance of fungicide resistance is a key factor in limiting the efficacy and lifetime of important disease control strategies. This is the worldwide problem of farmer. Therefore, resistance may also be an important aid to our understanding, at a molecular level, of the fungicidal mechanism of action.

Carbendazim fungicide act by inhibition of tubulin biosynthesis (Davidse, 1973 and Ma and Michailides, 2005). Several researchers have reported that fungicide-resistant mutations of almost all fungi are closely associated with the single nucleotide mutation, and results in the mutation of amino acid as well as the structure of fungicide binding point in the *TUB2* (Fujimura *et al*., 1992 and Gafur *et al*., 1998). These mutations that confer fungicide resistance have been identified in the *TUB2* homologs from several fungi. This region of the gene was amplified because every identified mutation which confers fungicide resistance in the phytopathoginic fungi (Table 4). In this study, we were analysis of partial sequences of the *TUB2* gene in *C. gloeosporioides* from 'Nam Dok Mai' mango in Thailand that is responsible for carbendazim resistance showed that the typical single

nucleotide mutation converting codon 198 caused HR phenotypes. Only the amino acid mutation at residue 198 was closely correlated with all HR phenotypes. The amino acid mutation of codon 198 in the *TUB2* gene has been identified in fungicide-resistant fungi such as *Botrytis cinerea* causing gray mold disease of a number of crops in Israel (Yarden and Katan, 1993), *C. gloeosporioides* causing postbloom fruit drop disease of citrus in Sao Paulo, Brazil and Florida, United States (Peres *et al*., 2004) or causing anthracnose diseases of fruit crops in Japan (Chung *et al*., 2006) or causing anthracnose disease of *Limonium* spp. in Israel (Maymon *et al*., 2006) or causing anthracnose disease of mango in south China (Ru-lin and Jun-sheng, 2007), *C. gloeosporioides* f. sp. *aeschynomene* from northern jointvetch (Buhr and Dickman, 1994), *Monilinia fructicola* causing brown rot of stone fruits in California (Ma *et al*., 2003), *Penicillium expansum* causing blue mold disease of stored apples in north America (Sholberg *et al*., 2005), *Venturia inaeqalis* causing scab disease of apple in Michigan and other plant pathogenic fungi (Koenraadt *et al*., 1992). Besides, different mutation points such as codon 50 in *Fusarium moniliforme* (Yan and Dickman, 1996) or 200 in *C. gloeosporioides* (Chung *et al*., 2006), *P. aurantiogriseum Venturia inaeqalis V. pirina* (Koenraadt *et al*., 1992). There were the different codons in the *TUB* gene may result in different resistance levels to chemical fungicide (Koenraadt *et al*., 1992; Albertini *et al*., 1999 and Chung *et al*., 2006). In the present study, *C. gloeosporioides* highly resistant phenotypes to carbendazim also had the amino acid substitution of glutamic (GAG) with alanine (GCG) at codon 198. Therefore, we conclude that mutations in codon 198 of the *TUB2* gene confer phenotype of carbendazim resistance in *C. gloeosporioides*. However, the fungicide resistance may result from single or multiple gene mutation. Resistant phenotypes typically arise from a very low natural rate of genetic mutation, and these isolates are less affected or not inhibited at all by a labeled application rate of this fungicide (Ma and Michailides, 2005). This indicates that careful management of chemical fungicides applications is necessary to achieve effective control.

Acknowledgments

This research is partially supported by the Center for Agricultural Biotechnology, Postgraduate Education and Research Development Office, Commission on Higher Education, Ministry of Education, Thailand. And we would like to thank are also due to Plant Pathology Laboratory, Faculty of Agriculture, Chiang Mai University, Thailand and Kagawa University, Japan.

Table 4. Point mutations of some phytopathoginic fungi at the second beta-tubulin (*TUB2*) gene causing the resistance to fungicide.

Phytopathoginic fungi	Amino acid		Growth on concentration of kind fungicides (phenotype)	Reference
	Substitution	Position		
Colletotrichum gloeosporioides	Glu (GAG)-to-Ala (GCG)	198	10 µg of benomyl/ml (resistant)	Peres *et al.* ,2004
			> 100 mg of thiophanate-methyl/l (highly resistant)	Chung *et al*., 2006
			1,000 µg of carbendazim/ml (high resistant)	Ru-lin and Jun-sheng, 2007
	Phe (TTC)-to-Tyr (TAC)	200	10-100 mg of thiophanate-methyl /l (intermediately resistant)	Chung *et al*., 2006
C. gloeosporioides f. sp. *aeschynomene*	Glu (GAG)-to-Ala (GCG)	198	1 µg of benomyl / ml (resistant)	Buhr and Dickman, 1994
Fusarium moniliforme	Tyr(ATC)-to-Asn (AAC)	50	1.5 µg of benomyl / ml (resistant)	Yan and Dickman, 1996
Monilinia fructicola	Glu (GAA)-to-Lys (AAA)	198	slow growth on 50 mg of benomyl/l (highly resistant)	Koenraadt *et al.*, 1992
Penicillium aurantiogriseum	Glu (GAG)-to-Ala (GCG)	198	rapid growth on 50 mg of benomyl/l (very high resistance)	
	Glu (GAG)-to-Lys (AAG)	198	slow growth on 50 mg of benomyl/l (highly resistant)	
	Phe (TTC)-to-Tyr (TAC)	200	5 mg of benomyl /l (medium resistance)	
P. digitatum	Glu (GAG)-to-Lys (AAG)	198	slow growth on 50 mg of benomyl/l (highly resistant)	
	Glu (GAG)-to-Val (GTG)	198	slow growth on 50 mg of benomyl/l (highly resistant)	
P. expansum	Glu (GAG)-to-Ala (GCG)	198	rapid growth on 50 mg of benomyl/l (very high resistance)	
	Glu (GAG)-to-Ala or Val (GCG or GTG)	198	1,000 of benomyl or thiabendazole/ml (highly resistant)	Sholberg *et al.*, 2005
P. puberrulum	Glu (GAG)-to-Ala (GCG)	198	rapid growth on 50 mg of benomyl/l (very high resistance)	Koenraadt *et al.*, 1992
	Glu (GAG)-to-Lys (AAG)	198	slow growth on 50 mg of benomyl/l (highly resistant)	
P. solitum	Glu (GAG)-to-Lys (AAG)	198	1,000 of benomyl or thiabendazole/ml (highly resistant)	Sholberg *et al.*, 2005
P. viridicatum	Glu (GAG)-to-Lys (AAG)	198	slow growth on 50 mg of benomyl/l (highly resistant)	Koenraadt *et al.*, 1992
Sclerotinia homoeocarpa	Glu (GAG)-to-Lys (AAG)	198	slow growth on 50 mg of benomyl/l (highly resistant)	
Venturia inaeqalis	Glu (GAG)-to-Ala (GCG)	198	rapid growth on 50 mg of benomyl/l (very high resistance)	
	Glu (GAG)-to-Lys (AAG)	198	slow growth on 50 mg of benomyl/l (highly resistant)	
	Phe (TTC)-to-Tyr (TAC)	200	5 mg of benomyl /l (medium resistance)	
V. pirina	Glu (GAG)-to-Ala (GCG)	198	rapid growth on 50 mg of benomyl/l (very high resistance)	
	Phe (TTC)-to-Tyr (TAC)	200	5 mg of benomyl /l (medium resistance)	

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(Received 15 October 2009; accepted 2 February 2010)