CHAPTER 3

Phenotypic Assay Detection of Carbendazim-resistant *Colletotrichum* spp. Causing Mango Anthracnose

3.1 Introduction

Carbendazim is a broad spectrum benzimidazole with a systemic activity (Davidse, 1986), and has been used for anthracnose control in mango orchards (Prakash, 2004; Pike, 2007; Duamkhanmanee, 2008). The chemical is a very specific inhibitor of microtubule assembly that acts by binding to the beta-subunits of beta-tubulin and interfering with microtubule formation in mitosis of nuclear division (Davidse, 1986; Steffens *et al*., 1996; Ma and Michailides, 2005). However, its long-term overuse may have induced mutant isolates on the target site in the beta-tubulin, and subsequently increased in population of fungicide-resistant isolates, thereby causing a major problem for growers trying to control anthracnose (Staub, 1991; Brent and Hollomon, 1998; Ma and Michailides, 2005; Damicone and Smith, 2009; Deising *et al*., 2008). Deising *et al*. (2008) reported that two years of intensive benzimidazole use in the field induced development of fungicide resistance in the apple scab fungus, *Venturia inaequalis*, and grey mold fungus, *Botrytis cinerea*. Brent and Hollomon (1998) and Damicone and Smith (2009) estimated a high level risk of pests developing resistance to benzimidazoles.

The incidence of acquired fungicide resistance in the field has become an important factor in limiting the efficacy for disease control strategies in Thailand. Furthermore, the production cost of fungicides has increased due to

growers applied in higher dosage at a greater frequency. Several studies have reported the phenotypic responses of benzimidazole-resistant pathogens in Thailand (Farungsang and Farungsang, 1992; Farungsang *et al*., 1994), India (Kumar *et al*., 2007), South Africa (Sanders *et al*., 2000), Korea (Yoon, *et al*., 2008), Malaysia (Sariah, 1989), and England (Taggart *et al*., 1999). The resistance of *C. gloeosporioides* both phenotypic and genetic mutation levels was also reported for leguminous weeds in the USA (Buhr and Dickman, 1994), various fruit crops in Japan (Chung *et al*., 2006), pepper and strawberry in Korea (Kim *et al*., 2007), the herbaceous ornamental perennial genus *Limonium* in Israel (Maymon *et al*., 2006), citrus in the USA and Brazil (Peres *et al*., 2004), and mango in China (Ru-Lin and Jun-Sheng, 2007). In many cases benzimidazole resistance is correlated with point mutations of particular nucleotides in the beta-tubulin genes, especially the *TUB2* gene (Buhr and Dickman, 1994).

The objectives of this chapter were as follows:-

1. To evaluate carbendazim-resistance of *Colletotrichum* spp. isolates from different mango cultivars by phenotypic responses against carbendazim fungicide.
2. To evaluate the pathogenicity.
3. To identify *Colletotrichum* species causing mango anthracnose by sequence analyses of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA), and their relationship.

3.2 Materials and Methods

3.2.1 Carbendazim-resistant phenotype assay

Carbendazim resistance was studied for detection all isolates of *Colletotrichum* spp. by phenotypic assay. The resistibility of each isolate to carbendazim was observed from the growth of colony. A 5 mm diameter mycelial disc was cut from the margin of each colony and transferred onto PDA supplemented with carbendazim at a concentration of 0.1, 1, 10, 100, 500 and 1,000 mg/l, respectively. Carbendazim was added to PDA after autoclaving and the plates were incubated at room temperature.

After inoculation at room temperature, the diameter of each colony was measured and its growth percentage calculated. The medium without carbendazim served as a control. Five replications were maintained for each treatment. The growth of each isolate on the carbendazim concentration was based on the following categories:

- = The culture was able to grow at <10% compared with the control.
+ = The culture was able to grow at ≥10 - 35% compared with the control.
++ = The culture was able to grow at >35 - 65% compared with the control.
+++ = The culture was able to grow at >65 - 90% compared with the control.
++++ = The culture was able to grow at >90% compared with the control.

The level of resistance to carbendazim was evaluated and grouped into four representative phenotype reactions which highly resistant (Car^{HR}) and able to grow on carbendazim at ≥ 500 mg/l; moderately resistant (Car^{MR}) at ≤ 100 mg/l; weakly

resistant (Car^{WR}) at ≤ 10 mg/l; and sensitive (Car^{S}) at ≤ 1 mg/l as described in Table 3.1. The hyphal strands from the end of the fungal colony were removed aseptically in order to observe the morphological changes under a microscope for abnormalities.

Table 3.1 Phenotype-resistant levels of *Colletotrichum* spp. to carbendazim fungicide

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

[1/] = Field-use recommended concentration
✓ = Percentage of growth ≥ 10% compared with the control
X = Percentage of growth < 10% compared with the control

Sources: Farungsang and Farungsang (1992), Farungsang *et al*. (1994) and Koenraadt *et al*. (1992); Peres *et al*. (2004)

3.2.2 Pathogenicity test

Colletotrichum spp. isolates were randomly selected to represent different carbendazim-resistant phenotypes of each mango cultivar for pathogenicity testing. Wound inoculation was performed for pathogenicity test on the fruit and leaves of the mango cv. 'Namdokmai', which were washed thoroughly under tap water before being surface-sterilized with 10% Clorox for 1 min, and air-dried. Then they were placed in a plastic box. The experiment was done by using factorial in completely randomized design (CRD) with three replications. A circular inoculation site, with a

5 mm diameter, was marked on the surface of the fruit and leaf. The wound was created by a 2 mm deep puncture for 5 points using a sterile needle. Mycelium disc (5 mm diameter) of each isolate *Colletotrichum* spp. from a seven-day culture was cut at peripheral colony and transferred to the wounded area. The controls were inoculated with PDA discs only. Moistened paper towels were placed in the plastic box containing the inoculated fruits and leaves and incubated at room temperature. The lesion diameters were measured after three days of incubation.

3.2.3 Identification of *Colletotrichum* spp. using species specific primers and partial sequence of internal transcribed spacer regions

DNA extraction

All isolates from the pathogenicity test of *Colletotrichum* spp. were extracted genomic DNA for analysis. Additional information for taxonomic determination was obtained by sequence comparison of the internal transcribed spacer (ITS) of rDNA regions after partial amplification of the region by polymerase chain reaction (PCR). Each isolate was grown on PDA for 14 days at room temperature (28-30^{O}C). Approximately 100 mg of mycelia in each isolate was ground to a fine powder in liquid nitrogen, with a mortar and pestle. Genomic DNA was extracted using a NucleoSpin® kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions.

Determination of DNA concentration

DNA was quantified by taking a spectrophotometric absorbance (A) reading at wavelengths (λ) of 260 nm and 280 nm. Readings at 260 nm facilitated the calculation of nucleic acid in the sample, whereby an A_{260} of 1 corresponded to approximately 50 µg/ml of dsDNA. The A_{260} nm/A_{280} nm ratio provided an estimated purity, with pure preparation of DNA at an A_{260} nm/A_{280} nm coefficient of between 1.8 and 2.0.

$$\text{concentration of dsDNA solution (µg/ml)} = 50 \times A_{260}$$

Agarose gel electrophoresis

A standard 1% (w/v) agarose gel prepared in Tris-Acetate-EDTA (TAE) electrophoresis buffer was used for the analysis of total DNA preparations from fungal isolates and PCR amplicons. Agarose powder (Research Organics, Inc.) was added to TAE buffer and microwaved for 2 min to dissolve the powder. Ethidium bromide (0.005%) was added to the cooling solution and poured into a tray with a comb inserted to form sample slots. The agarose gel was allowed to solidify for approximately 30 min before the comb was removed and the gel immersed in an electrophoresis tank containing TAE buffer. The samples were loaded into individual slots in the gel and ran at 80 volts and maximum current for 50 min, before viewing and photographing under UV light.

Amplification of the ITS region

Extracted DNA was used as the template for PCR amplification with the species-specific primer designed from the *ITS1* region of *C. gloeosporioides* of CgInt (5'-GGC CTC CCG CCT CCG GGC GG-3') and conserved primer ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (Figure 3.1.) (White *et al*., 1990; Mills *et al*., 1992; Freeman *et al*., 2000). PCR reactions were performed in a total volume of 50 µl, containing 10 to 100 ng of genomic DNA, 5 µl of 10X PCR buffer (iNtRON Biotechnology, Seoul, Republic of Korea), 25 mM of $MgCl_2$ (iNtRON Biotechnology), 10 mM of dNTPs (iNtRON Biotechnology), 50 pmol of each primer, and 1 unit of *Taq* polymerase (Fermentas, Vilnius, Lithuania). All PCR reactions were carried out in a PTC-100^{TM} programmable thermal controller (MJ Research, MA, USA) with a hold of 5 min at 95°C, followed by 30 cycles of 1 min at 95°C, 54°C (T_m) and 72°C, and a final extension for 5 min at 72°C. The PCR product was separated using electrophoresis on 1% agarose gel (Research Organics, OH, USA), with a 100-bp sharp DNA marker (RBC Bioscience, Taipei, Taiwan) as a standard size.

Sequencing and phylogenetic analysis

PCR products were purified by ethanol precipitation and direct-sequence on both strands using cycle sequencing with CgInt and ITS4 primers. Sequences of PCR products were obtained from both strands using the dideoxy chain termination method, with an ABI-PRISM Dry Termination Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA), and automated fluorescent DNA sequencer

(Model 310, Applied Biosysterms) following the manufacturer's instructions. The BioEdit version 7.0.9.0 software (Hall, 1999) was used to assemble, edit, and generate high-quality sequences. Sequence similarity analyses were performed using the Basic Local Alignment Search Tool (BLAST) in GenBank or NCBI databases.

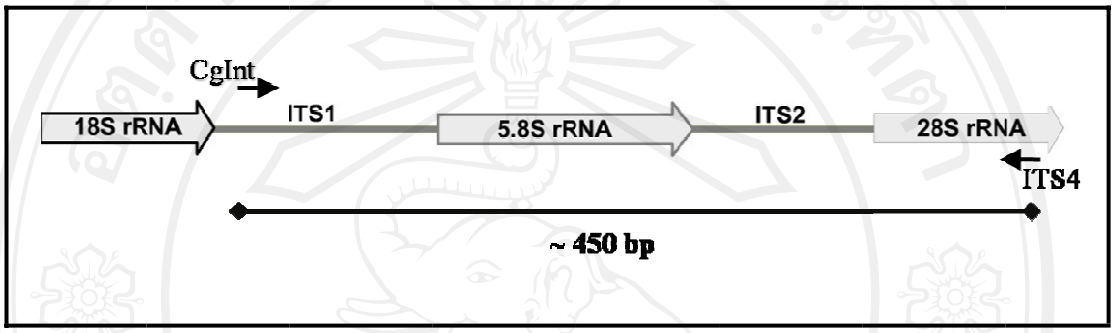


Figure 3.1 Diagrammatic representation of *Colletotrichum* spp. ribosomal DNA. The species-specific primer positions, CgInt and ITS4, in highly conserved 18S and 28s ribosomal gene sequences flanking the spacer regions, are indicated by arrows. Sizes of the ITS regions, and putative amplicon resulting from CgInt/ITS4 PCR amplification, are stated as nucleotide base pairs, 450 bp.

DNA sequences were aligned with the CLUSTAL W program (Thompson *et al*., 1997) and BioEdit. The phylogenetic tree was obtained from the data using one of three equally parsimonious trees obtained through 1000 replications of a heuristic search with random, stepwise sequence addition using Phylogenetic Analysis Using Parsimony (PAUP*) version 4.0 beta 10 (Swofford, 2002) and TreeView software. Additional ITS sequences of *Colletotrichum* spp. were retrieved from GenBank.

3.3 Results

3.3.1 Carbendazim-resistant phenotype assay

The one hundred and fifty isolates of *C. gloeosporioides* from Chapter 2 were tested for carbendazim resistance by growth assays on PDA amended with carbendazim at concentrations of 0.1, 1, 10, 100, 500, and 1000 mg/l, respectively. Table 3.2 gives details of each isolate response to carbendazim. The isolates were classified into four representative phenotype reactions as follows: highly resistant (Car^{HR}; ≥ 500 mg/l), moderately resistant (Car^{MR}; ≤ 100 mg/l), weakly resistant (Car^{WR}; ≤ 10 mg/l), and sensitive (Car^{S}; ≤ 1 mg/l). All isolates, 112 (74.7%) were classified as the Car^{HR} phenotype, of which 18 (12%) and 94 isolates (62.7%) were obtained from leaf and fruit samples, respectively. Thirty seven isolates (24.7%) were classified as the Car^{S} phenotype, of which 28 (18.7%) and 9 (6%) were obtained from leaf and fruit samples, respectively. Only 0.6% isolates were classified as the Car^{MR} phenotype. No isolates were classified as weak resistance (Car^{WR}) (Figure 3.2 and 3.3).

Table 3.2 Carbendazim-resistant assay of *Colletotrichum gloeosporioides* causing mango anthracnose on potato dextrose agar amended with carbendazim

No.	Isolate code	Carbendazim concentrations (mg/l)[1/]						Phenotype-resistant level[2/]
		0.1	1	10	100	500	1,000	
1. Chaokhunthip cultivar (CKT) = 1 isolate								
Leaf								
1.	CKT_L044	++++	+++	++	++	++	++	HR
2. Chokanan cultivar (CAN) = 23 isolates								
Leaf								
1.	CAN_L080	-	-	-	-	-	-	S
2.	CAN_L081	-	-	-	-	-	-	S
3.	CAN_L090	++++	++++	++++	++++	+++	++	HR
4.	CAN_L105	-	-	-	-	-	-	S
Fruit								
5.	CAN_F093	+++	+++	+++	+++	+++	++	HR
6.	CAN_F094	++++	++	++	++	++	+	HR
7.	CAN_F095	++++	++++	++++	++++	+++	+++	HR
8.	CAN_F097	++++	++++	++++	++++	++++	+++	HR
9.	CAN_F098	++++	++++	++++	++++	+++	+++	HR
10.	CAN_F123	++++	++++	++++	++++	++++	+++	HR
11.	CAN_F125	-	-	-	-	-	-	S
12.	CAN_F139	++++	++++	+++	++++	++	+++	HR
13.	CAN_F140	++++	++++	+++	+++	++	++	HR
14.	CAN_F141	++++	++++	+++	+++	++	++	HR
15.	CAN_F142	++++	+++	+++	+++	+++	+++	HR
16.	CAN_F143	++++	+++	+++	++	++	++	HR
17.	CAN_F144	++++	++++	+++	++++	++	+	HR
18.	CAN_F145	++++	+++	+++	++	++	++	HR
19.	CAN_F146	++++	++++	++++	++++	+++	+++	HR
20.	CAN_F147	++++	++++	+++	++	+++	++	HR
21.	CAN_F148	++++	++++	+++	++	+++	+++	HR
22.	CAN_F149	++++	+++	+++	+++	++	++	HR
23.	CAN_F150	++++	++++	+++	++	++	+++	HR
3. Farlun cultivar (FL) = 4 isolates								
Leaf								
1.	FL_L079	-	-	-	-	-	-	S
2.	FL_L111	-	-	-	-	-	-	S
Fruit								
3.	FL_F003	-	-	-	-	-	-	S
4.	FL_F066	++++	++++	++++	+++	+++	++	HR

[1/]Five replications, Percentage of growth: -, +, ++, +++, ++++; >10%, 10-34%, 35%-64%, 65%-89%, ≤ 90%, respectively.
[2/]Resistant response against carbendazim on PDA: HR = highly resistant (≥ 500 mg/l), MR = moderately resistant (≤ 100 mg/l), S = sensitive (≤ 1 mg/l), respectively.

Table 3.2 continued

No.	Isolate code	Carbendazim concentrations (mg/l)[1/]						Phenotype-resistant level[2/]
		0.1	1	10	100	500	1,000	
4. Kaew cultivar (K) = 8 isolates								
Leaf								
1.	K_L053	-	-	-	-	-	-	S
2.	K_L120	-	-	-	-	-	-	S
3.	K_L124	-	-	-	-	-	-	S
Fruit								
4.	K_F069	++++	+++	+++	+++	++	++	HR
5.	K_F099	+++	+++	+++	+++	++	++	HR
6.	K_F100	+++	+++	++	++	++	++	HR
7.	K_F101	++++	++++	++++	++++	++++	+++	HR
8.	K_F103	++++	+++	+++	++++	+++	+++	HR
5. Khiaomorakod cultivar(KMK) = 13 isolates								
Leaf								
1.	KMK_L041	++++	++++	++++	++++	+++	+++	HR
2.	KMK_L051	-	-	-	-	-	-	S
3.	KMK_L055	++++	+++	+++	+++	+++	+++	HR
4.	KMK_L058	++++	++++	+++	+++	+++	+++	HR
5.	KMK_L088	-	-	-	-	-	-	S
Fruit								
6.	KMK_F132	++++	++++	++++	+++	++	++	HR
7.	KMK_F133	++++	++++	++++	+++	++	++	HR
8.	KMK_F134	++++	++++	++++	+++	++	++	HR
9.	KMK_F135	++++	+++	++	++	++	++	HR
10.	KMK_F136	++++	+++	+++	++	++	+++	HR
11.	KMK_F137	++++	++++	+++	+++	++	+	HR
12.	KMK_F138	++++	++++	+++	++	++	++	HR
6. Khiaosawoey cultivar (KSW) = 4 isolates								
Leaf								
1.	KSW_L004	-	-	-	-	-	-	S
2.	KSW_L062	-	-	-	-	-	-	S
3.	KSW_L085	++	+	+	+	+	+	HR
4.	KSW_L119	+++	+++	+++	+++	+++	++	HR
7. Lin Nguhao cultivar (LNH) = 1 isolate								
Leaf								
1.	LNG_L031	-	-	-	-	-	-	S

[1/]Five replications, Percentage of growth: -, +, ++, +++, ++++; >10%, 10-34%, 35%-64%, 65%-89%, ≤ 90%, respectively.
[2/]Resistant response against carbendazim on PDA: HR = highly resistant (≥ 500 mg/l), MR = moderately resistant (≤ 100 mg/l), S = sensitive (≤ 1 mg/l), respectively.

Table 3.2 Continued

No.	Isolate code	Carbendazim concentrations (mg/l)[1/]						Phenotype-resistant level[2/]
		0.1	1	10	100	500	1,000	
8. Mahacharnok cultivar (MCN) = 9 isolates								
Leaf								
1.	MCN_L013	-	-	-	-	-	-	S
2.	MCN_L049	+++	-	-	-	-	-	S
3.	MCN_L052	++++	++++	++++	+++	+++	+++	HR
4.	MCN_L056	++++	++++	++++	++++	++++	+++	HR
5.	MCN_L059	-	-	-	-	-	-	S
6.	MCN_L070	-	-	-	-	-	-	S
7.	MCN_L075	-	-	-	-	-	-	S
8.	MCN_L089	++++	-	-	-	-	-	S
9.	MCN_L121	-	-	-	-	-	-	S
9. Mankhunsi cultivar (MKS) = 2 isolates								
Leaf								
1.	MKS_L034	++++	++++	++++	++++	++++	++++	HR
2.	MKS_L086	-	-	-	-	-	-	S
10. Namdokmai cultivar (NDM) = 59 isolates								
Leaf								
1.	NDM_L057	-	-	-	-	-	-	S
2.	NDM_L067	-	-	-	-	-	-	S
3.	NDM_L068	-	-	-	-	-	-	S
4.	NDM_L071	-	-	-	-	-	-	S
5.	NDM_L078	+++	+++	+++	+++	+++	++	HR
6.	NDM_L096	-	-	-	-	-	-	S
Fruit								
7.	NDM_F001	++++	++++	++++	++++	++++	+++	HR
8.	NDM_F002	++++	+++	+++	+++	++	++	HR
9.	NDM_F005	++++	++++	++++	++++	+++	+++	HR
10.	NDM_F006	+	-	-	-	-	-	S
11.	NDM_F007	+++	+++	+++	++	++	++	HR
12.	NDM_F008	-	-	-	-	-	-	S
13.	NDM_F009	++++	++++	++++	++++	+++	++	HR
14.	NDM_F010	++++	+++	+++	++	++	++	HR
15.	NDM_F011	++++	+++	++	++	++	++	HR
16.	NDM_F012	++++	+++	+++	+++	++	++	HR
17.	NDM_F014	++	+	+	+	+	+	HR
18.	NDM_F015	++++	++++	++++	++++	+++	+++	HR
19.	NDM_F016	++++	++++	++++	++++	+++	+++	HR
20.	NDM_F018	++++	++++	++++	++++	++++	+++	HR
21.	NDM_F019	-	-	-	-	-	-	S
22.	NDM_F020	++++	++++	++++	++++	+++	+++	HR
23.	NDM_F021	+++	+++	+	+	+	+	HR

[1/]Five replications, Percentage of growth: -, +, ++, +++, ++++; >10%, 10-34%, 35%-64%, 65%-89%, ≤ 90%, respectively.
[2/]Resistant response against carbendazim on PDA: HR = highly resistant (≥ 500 mg/l), MR = moderately resistant (≤ 100 mg/l), S = sensitive (≤ 1 mg/l), respectively.

Table 3.2 Continued

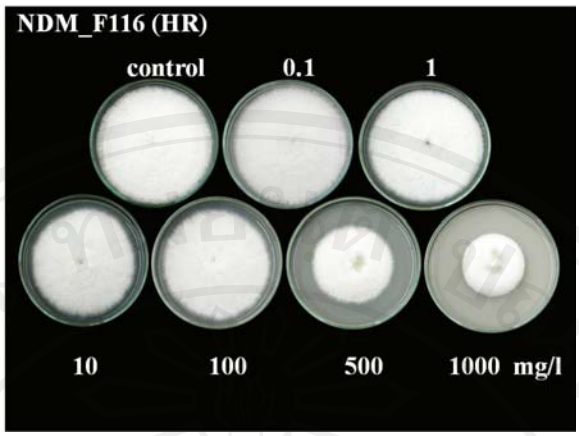
No.	Isolate code	Carbendazim concentrations (mg/l)[1/]						Phenotype-resistant level[2/]
		0.1	1	10	100	500	1,000	
24.	NDM_F022	+++	++++	++++	++++	+++	+++	HR
25.	NDM_F023	++++	++++	++++	+++	+++	+++	HR
26.	NDM_F024	++++	++++	++++	+++	+++	+++	HR
27.	NDM_F025	++++	-	-	-	-	-	S
28.	NDM_F026	++	++	+	+	+	+	HR
29.	NDM_F027	+++	+++	+++	+++	+++	+++	HR
30.	NDM_F028	++++	++	++	++	++	++	HR
31.	NDM_F029	++++	++++	++++	++++	+++	+++	HR
32.	NDM_F035	++++	++++	++++	++++	+++	++	HR
33.	NDM_F036	++++	+++	+++	+++	+++	+++	HR
34.	NDM_F037	++++	++++	++++	++++	++++	++++	HR
35.	NDM_F038	++++	++++	++++	++++	+++	+++	HR
36.	NDM_F039	++++	++	++++	+++	+++	+++	HR
37.	NDM_F042	++++	++++	+++	++++	+++	+++	HR
38.	NDM_F043	++++	++	++	+++	++	++	HR
39.	NDM_F045	++++	++++	++++	++++	+++	+++	HR
40.	NDM_F046	+++	++++	++++	++++	+++	+++	HR
41.	NDM_F050	++++	++++	++++	++++	++++	+++	HR
42.	NDM_F061	+++	++++	++++	++++	++	++	HR
43.	NDM_F063	++++	+	+	+	-	-	MR
44.	NDM_F072	++++	++++	++++	++++	++++	++++	HR
45.	NDM_F104	++++	++++	+++	++++	+++	+++	HR
46.	NDM_F106	++++	++++	++++	++++	+++	+++	HR
47.	NDM_F107	++++	++++	++++	++++	++++	+++	HR
48.	NDM_F108	-	-	-	-	-	-	S
49.	NDM_F109	++++	++++	++++	++++	+++	+++	HR
50.	NDM_F110	++++	++++	++++	++++	+++	+++	HR
51.	NDM_F112	++++	++++	++++	++++	+++	+++	HR
52.	NDM_F115	++++	++++	++++	++++	+++	++	HR
53.	NDM_F116	++++	++++	++++	++++	+++	+++	HR
54.	NDM_F117	++++	++++	++++	+++	+++	++	HR
55.	NDM_F118	-	-	-	-	-	-	S
56.	NDM_F127	++++	++++	++++	++++	+++	+++	HR
57.	NDM_F128	++++	++++	++++	++++	+++	+++	HR
58.	NDM_F129	++++	+++	+++	+++	+++	++	HR
59.	NDM_F130	++++	++++	+++	++++	+++	+++	HR
11. Naree Luemrang cultivar (NLR) = 2 isolates								
Leaf								
1.	NLR_L047	++++	++++	+++	++++	+++	+++	HR
2.	NLR_L048	+	-	-	-	-	-	S

[1/]Five replications, Percentage of growth: -, +, ++, +++, ++++; >10%, 10-34%, 35%-64%, 65%-89%, ≤ 90%, respectively.
[2/]Resistant response against carbendazim on PDA: HR = highly resistant (≥ 500 mg/l), MR = moderately resistant (≤ 100 mg/l), S = sensitive (≤ 1 mg/l), respectively.

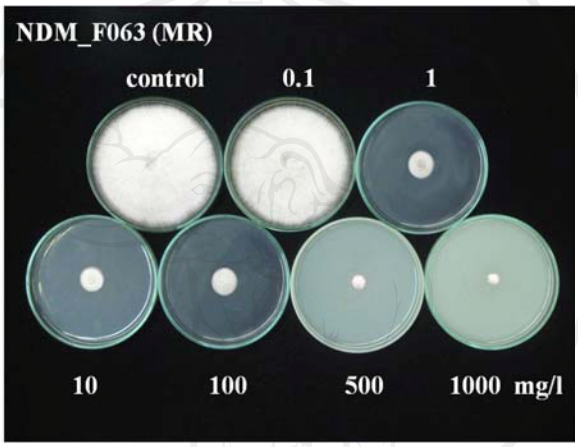
Table 3.2 Continued

No.	Isolate code	Carbendazim concentrations (mg/l)[1/]						Phenotype-resistant level[2/]
		0.1	1	10	100	500	1,000	
12. Okrong cultivar (OR) = 2 isolates								
Leaf								
1.	OR_L040	-	-	-	-	-	-	S
Fruit								
2.	OR_F126	++++	++++	++++	++++	+++	+++	HR
13. Phetbanlat cultivar (PBL) = 13 isolates								
Fruit								
1.	PBL_F030	++	++	+	+	+	+	HR
2.	PBL_F033	+++	+++	+++	++++	++++	++	HR
3.	PBL_F054	++	++++	++++	++++	+++	+++	HR
4.	PBL_F064	++++	++++	+++	+++	+++	+++	HR
5.	PBL_F076	++++	++++	++++	++++	++++	++++	HR
6.	PBL_F077	++++	++++	+++	+++	++	++	HR
7.	PBL_F083	++++	++++	++++	++++	++++	+++	HR
8.	PBL_F084	++++	++++	++++	++++	++++	+++	HR
9.	PBL_F091	++++	++++	++++	++++	+++	+++	HR
10.	PBL_F092	++++	++++	++++	++++	++++	+++	HR
11.	PBL_F102	-	-	-	-	-	-	S
12.	PBL_F113	++++	+++++	++++	++++	+++	+++	HR
13.	PBL_F131	++++	++++	++++	++++	++++	+++	HR
14. Phimsen cultivar (PS) = 6 isolates								
Leaf								
1.	PS_L032	-	-	-	-	-	-	S
2.	PS_L082	++	+	+	+	+	+	HR
Fruit								
3.	PS_F073	++++	++++	++++	+++	+++	+++	HR
4.	PS_F074	++++	++	++	+++	++	++	HR
5.	PS_F114	++++	++++	++++	++++	+++	+++	HR
6.	PS_F122	++++	++++	++++	++++	+++	++	HR
15. Raet cultivar (R) = 1isolate								
Leaf								
1.	R_L087	-	-	-	-	-	-	S
16. Salaya cultivar (SLY) = 1 isolate								
Leaf								
1.	SLY_L017	-	-	-	-	-	-	S
17. Talapnak cultivar (TLN) = 2 isolates								
Leaf								
1.	TLN_L060	-	-	-	-	-	-	S
2.	TLN_L065	+++	+++	+++	+++	+++	++	HR

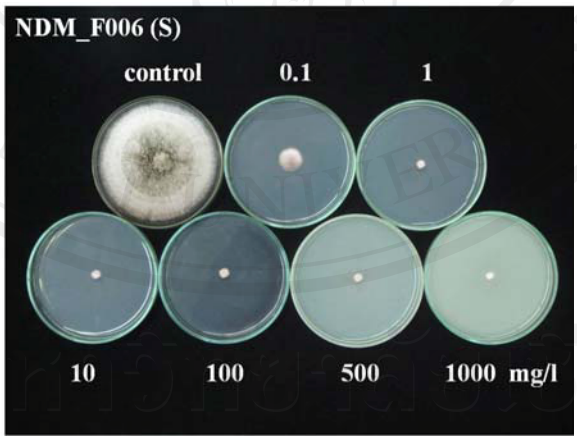
[1/]Five replications, Percentage of growth: -, +, ++, +++, ++++; >10%, 10-34%, 35%-64%, 65%-89%, ≤ 90%, respectively.
[2/]Resistant response against carbendazim on PDA: HR = highly resistant (≥ 500 mg/l), MR = moderately resistant (≤ 100 mg/l), S = sensitive (≤ 1 mg/l), respectively.



(a)

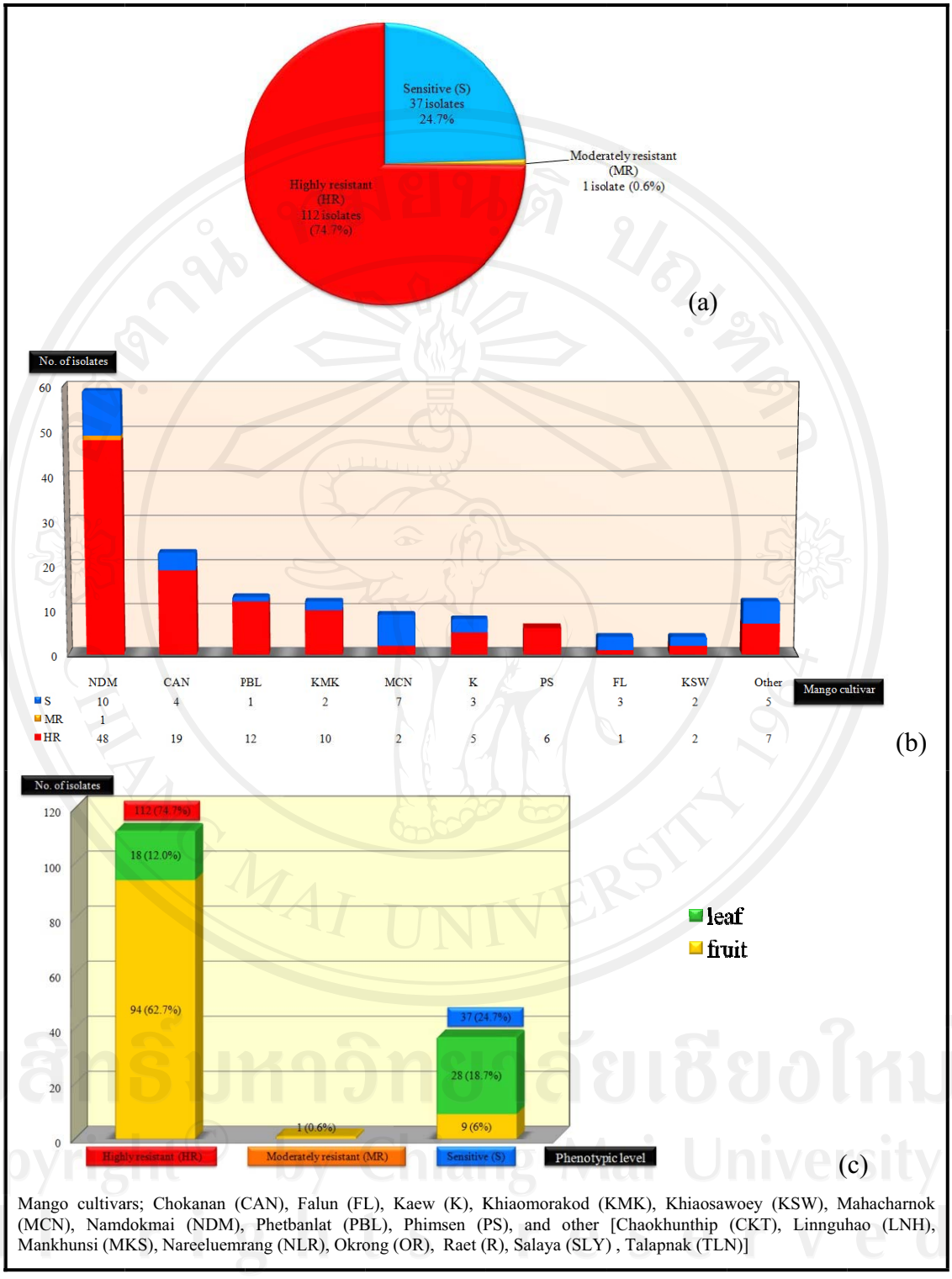


(b)



(c)

Figure 3.2 The carbendazim resistibility assays of *Colletotrichum gloeosporioides* causing mango anthracnose on potato dextrose agar amended with carbendazim; highly resistant (Car^{HR}) (a), moderately resistant (Car^{MR}) (b), sensitive (Car^{S}) (c).



Mango cultivars; Chokanan (CAN), Falun (FL), Kaew (K), Khiaomorakod (KMK), Khiaosawoey (KSW), Mahacharnok (MCN), Namdokmai (NDM), Phetbanlat (PBL), Phimsen (PS), and other [Chaokhunthip (CKT), Linnguhao (LNH), Mankhunsi (MKS), Nareeluemrang (NLR), Okrong (OR), Raet (R), Salaya (SLY) , Talapnak (TLN)]

Figure 3.3 Isolate number of carbendazim-resistant *Colletotrichum gloeosporioides* causing mango anthracnose; grouped by level of phenotype-resistance (a), mango cultivar (b), leaf or fruit (c).

The colony characteristics of Car^{HR} isolates grown on PDA amended with carbendazim at 500 mg/l gave a normal colony. Isolates classified as Car^{MR} and Car^{S} could not grow with this concentration. However, isolate growth of Car^{MR} and Car^{S} on PDA amended with carbendazim at 100 mg/l showed abnormality of mycelia under compound microscope observation, thus the growth was reduced and colony abnormality when compared to normal mycelia growth on PDA alone (Figure 3.4).

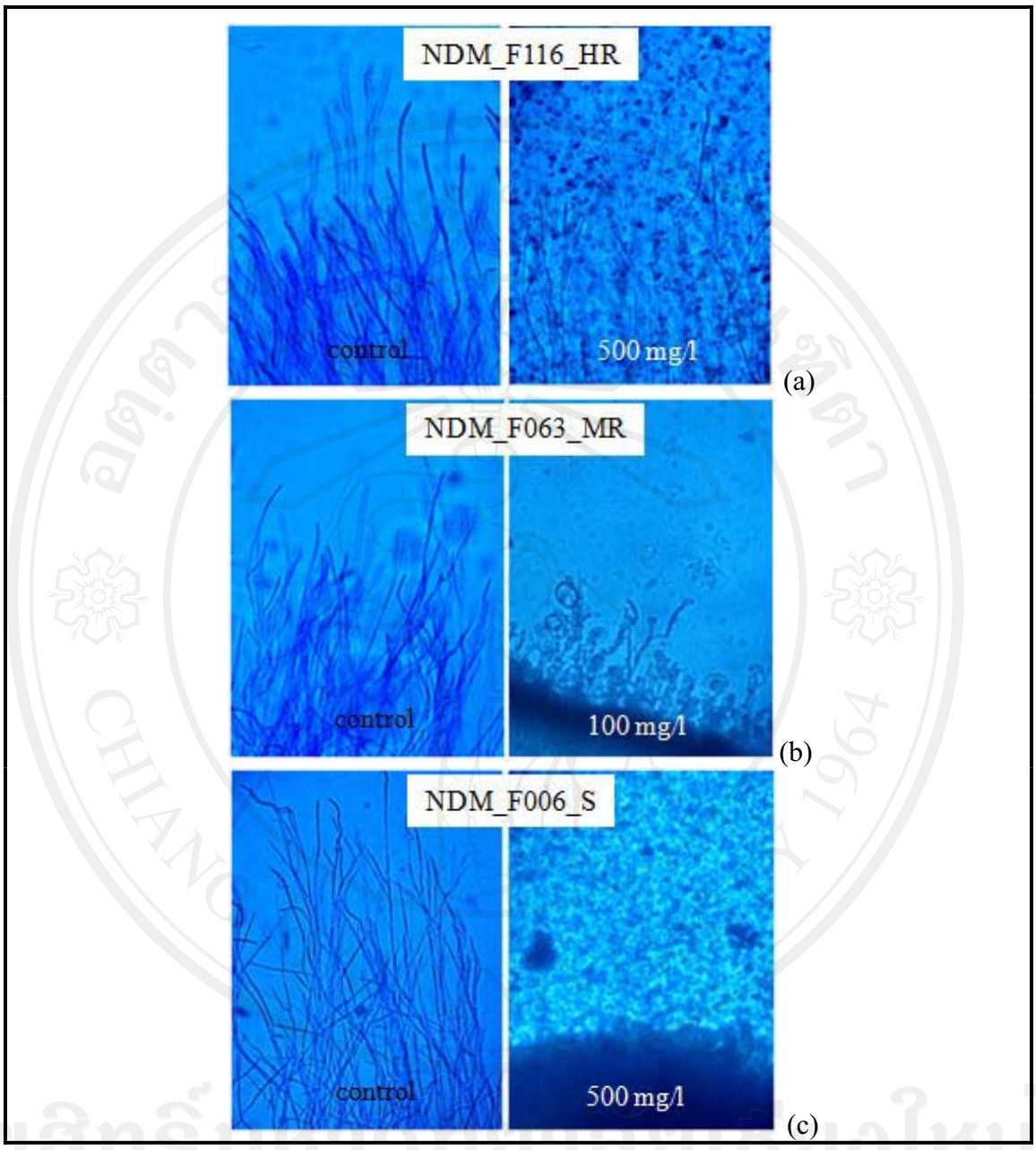


Figure 3.4 The mycelial characterisation of carbendazim-resistant *Colletotrichum gloeosporioides*; on only potato dextrose agar (PDA) as the control (left column), in comparison with PDA amended with carbendazim (X400) (right column); highly resistant (Car^{HR}) on 500 mg/l (a), moderately resistant (Car^{MR}) on 100 mg/l (b), sensitive (Car^{S}) on 500 mg/l (c).

3.3.2 Pathogenicity test

Fifty-eight *C. gloeosporioides* isolates from leaves and fruits expressed Car^{HR}, Car^{MR} and Car^{S} were randomly selected to be representative of carbendazim-resistant phenotypes for pathogenicity test (Table 3.3).

Table 3.3 Selected isolates from the phenotype of carbendazim-resistant *Colletotrichum gloeosporioides* from leaf and fruit of each mango cultivar

Mango cultivar	Isolate code of carbendazim-resistant phenotypes[1/]		
	Car^{HR}	Car^{MR}	Car^{S}
1. Chaokhunthip (CKT)	CKT_L044	-	-
2. Chok Anan (CAN)	CAN_F095 CAN_F146	-	CAN_F125 CAN_L080 CAN_L105
3. Farlun (FL)	FL_F066	-	FL_F003 FL_L079
4. Kaew (K)	K_F103	-	K_L120
5. Khiaosawoey (KSW)	KSW_L085	-	KSW_L062
6. Khiaomorakod (KMK)	KMK_F135 KMK_L058	-	KMK_L088
7.Lin Nguhao (LNG)	-	-	LNG_L031
8. Mahacharnok (MCN)	MCN_L056	-	MCN_L059 MCN_L070 MCN_L121
9. Mankhunsi (MKS)	-	-	MKS_L086
10. Namdokmai (NDM)	NDM_F002 NDM_F012 NDM_F014 NDM_F018 NDM_F026 NDM_F027 NDM_F038 NDM_F061 NDM_F106 NDM_F110 NDM_F116 NDM_F130 NDM_L078	NDM_F063	NDM_F006 NDM_F118 NDM_L057 NDM_L067 NDM_L068 NDM_L071 NDM_L096
11. Naree Luemrang (NLR)	NLR_L047	-	NLR_L048
12. Okrong (OR)	OR_F126	-	OR_L040
13. Phetbanlat (PBL)	PBL_F033 PBL_F076 PBL_F131	-	PBL_F102
14. Phimsen (PS)	PS_F114 PS_L082	-	PS_L032
15. Raet (R)	-	-	R_L087
16. Salaya (SLY)	-	-	SLY_L017
17. Talapnak (TLN)	TLN_L065	-	TLN_L060

[1/] Phenotype-resistant levels; highly resistant (Car^{HR}), moderately resistant (Car^{MR}), sensitive (Car^{S}): ≥ 500 µg/ml, ≤ 100 µg/ml, ≤ 1 µg/ml, respectively.

The pathogenicity test confirmed that Car^{HR}, Car^{MR} and Car^{S} isolates of *C. gloeosporioides* from leaf and fruit of various mango cultivars were pathogenic to both the fruits and leaves of the mango cv. 'Namdokmai'. Initial symptoms appeared as dark-brown necrotic lesions at 2 days after incubation in a plastic box with moist conditions at room temperature. The Car^{HR}, Car^{MR} and Car^{S} isolates produced typical symptoms on the fruits and leaves, i.e. limited dark-brown necrotic lesions. Four days after inoculation, the lesions which inoculated with Car^{HR}, Car^{MR} and Car^{S} developed dark-brown, necrotic, circular, and sunken on the fruits (Figure 3.5) that showed an average diameter of 10.19±1.09 mm, 8.33±0.33 mm, and 10.80±0.98 mm, respectively (Table 3.4). Similarly, dark-brown, circular, necrotic lesions developed after 4 days inoculation on the leaves (Figure 3.6) which lesions showed an average diameter of 9.75±0.92 mm, 11.00±1.53 mm and 9.73±0.78 mm for the Car^{HR}, Car^{MR} and Car^{S} group, respectively (Table 3.4). There was not observed any lesions in the control group.

The result of this experiment showed a significant difference (P=0.01) in pathogenicity test between isolates and inoculation on leaves and fruits when compared to the control.

Table 3.4 Lesion diameter of anthracnose symptoms on fruits and leaves of the mango cv. 'Namdokmai' inoculated with carbendazim-resistant *Colletotrichum* spp. for 4 days

Phenotype	Isolate code	Lesion diameter (mm)	
		on fruit	on leaf
Highly resistant (Car^{HR})	1. CKT_L044	12.50a-g[1/]	8.00n-p
	2. CAN_F095	8.67k-p	8.33m-p
	3. CAN_F146	12.00b-i	8.67k-p
	4. FL_F066	8.33m-p	11.00d-m
	5. KMK_F135	10.00f-p	12.67a-f
	6. KMK_L058	12.67a-f	10.67e-n
	7. K_F103	13.00a-e	8.00n-p
	8. KSW_L085	10.67e-n	10.00f-p
	9. MCN_L056	9.50h-p	8.67k-p
	10. NDM_F002	9.33i-p	9.00j-p
	11. NDM_F012	8.50l-p	9.00j-p
	12. NDM_F014	10.00f-p	9.33i-p
	13. NDM_F026	8.33m-p	8.67k-p
	14. NDM_F027	8.67k-p	9.67g-p
	15. NDM_F018	11.17c-m	10.00f-p
	16. NDM_F038	10.83d-n	10.00f-p
	17. NDM_F061	9.33i-p	9.33i-p
	18. NDM_F106	9.67g-p	10.00f-p
	19. NDM_F110	14.00a-c	9.67g-p
	20. NDM_F116	9.00j-p	11.00d-m
	21. NDM_F130	7.50p	10.67e-n
	22. NDM_L078	10.67e-n	10.00f-p
	23. NLR_L047	13.67a-d	10.00f-p
	24. OR_F126	8.50l-p	10.67e-n
	25. PBL_F033	9.00j-p	9.67g-p
	26. PBL_F076	11.33c-l	9.67g-p
	27. PBL_F131	9.67g-p	10.33e-p
	28. PS_F114	9.83f-p	10.67e-n
	29. PS_L082	11.67c-j	10.00f-p
	30. TLN_L065	7.67op	9.00j-p
Moderately resistant (Car^{MR})	1. NDM_F063	8.33m-p	11.00d-m

[1/]Average of three replications. Means followed by the same letter are not significantly different by Duncan's new Multiple Range Test (DMRT) at P=0.01.

Table 3.4 Continued

Phenotype	Isolate code	Lesion diameter (mm)[1/]	
		on fruit	on leaf
Sensitive (Car^S)	1. CAN_F125	15.17a	8.33m-p
	2. CAN_L080	8.67k-p	9.00j-p
	3. CAN_L105	11.00d-m	8.33m-p
	4. FL_F003	8.00n-p	9.33i-p
	5. FL_L079	14.67ab	10.00f-p
	6. K_L120	8.50l-p	10.33e-p
	7. KMK_L088	9.00j-p	9.33i-p
	8. KSW_L062	12.67a-f	8.00n-p
	9. LNG_L031	11.67c-j	9.00j-p
	10. MCN_L059	10.00f-p	9.67g-p
	11. MCN_L070	10.00f-p	10.00f-p
	12. MCN_L121	12.00b-i	10.00f-p
	13. MKS_L086	8.50l-p	10.67e-n
	14. NDM_F006	9.00j-p	10.67e-n
	15. NDM_F118	10.50e-o	12.67a-f
	16. NDM_L057	10.67e-n	9.33i-p
	17. NDM_L067	12.33a-h	9.67g-p
	18. NDM_L068	8.33m-p	10.00f-p
	19. NDM_L071	11.00d-m	9.00j-p
	20. NDM_L096	9.00j-p	9.67g-p
	21. NLR_L048	10.50e-o	10.00f-p
	22. OR_L040	9.17i-p	10.00f-p
	23. PBL_F102	12.67a-f	10.33e-p
	24. PS_L032	12.67a-f	9.67g-p
	25. R_L087	10.33e-p	11.33c-l
	26. SLY_L017	14.00a-c	9.67g-p
	27. TLN_L060	11.50c-k	8.67k-p
CV (%)		18.17	

[1/]Average of three replications. Means followed by the same letter are not significantly different by Duncan's new Multiple Range Test (DMRT) at P=0.01.

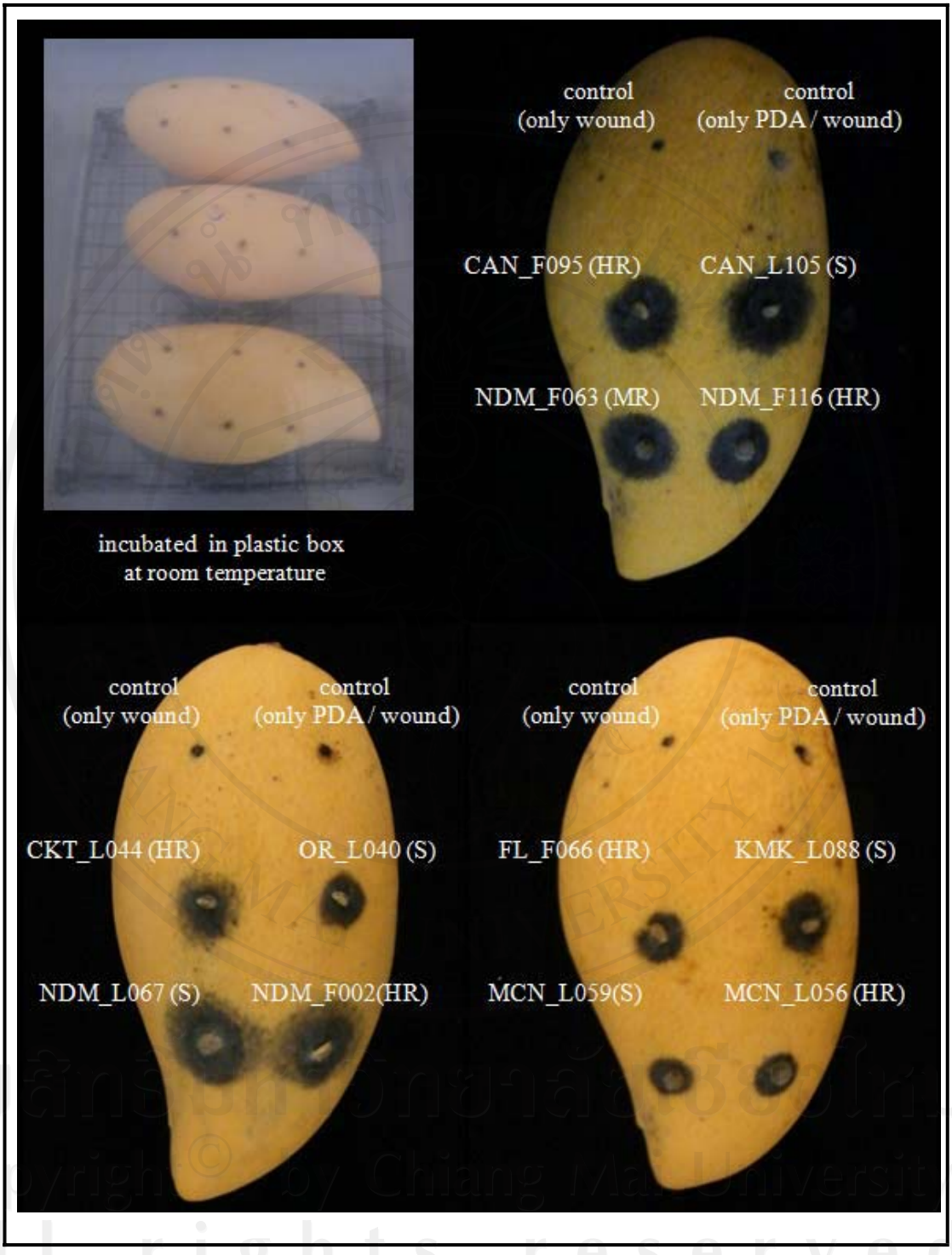


Figure 3.5 Lesions on fruits of mango cv. 'Namdokmai' inoculated with carbendazim-resistant *Colletotrichum gloeosporioides* isolates for 4 days.

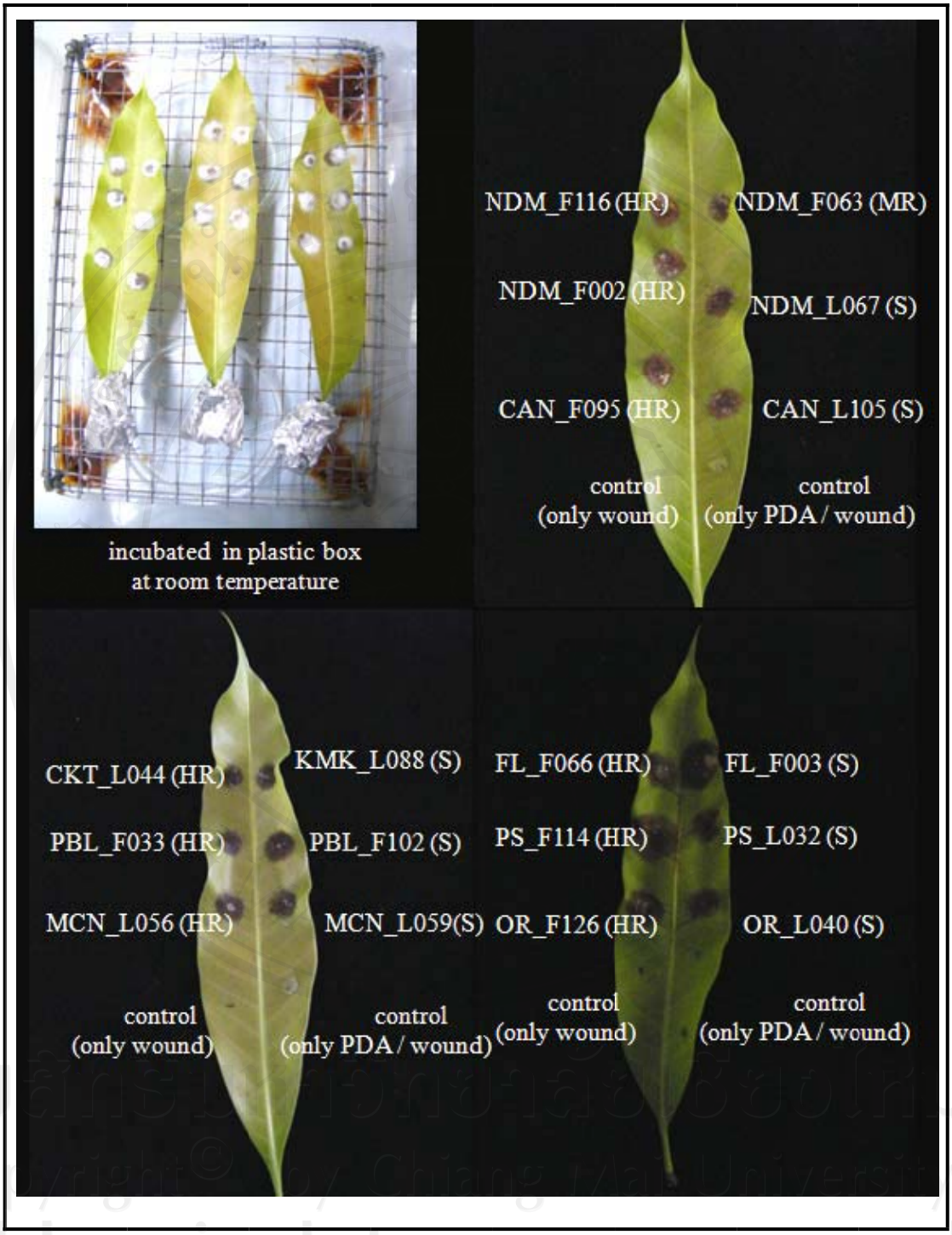


Figure 3.6 Lesions on leaves of mango cv. 'Namdokmai' inoculated with carbendazim-resistant *Colletotrichum gloeosporioides* isolates for 4 days.

3.3.3 Molecular identification of *Colletotrichum gloeosporioides*

PCR products of the ITS gene

The pathogenic isolates (30, 1 and 27 isolates of the Car^{HR}, Car^{MR} and Car^{S} group) were confirmed species identification as *C. gloeosporioides* by molecular techniques. DNA from each isolate was amplified when the PCR reaction was performed using CgInt and ITS4 primers. The corresponding PCR region amplified the ITS rDNA sequence and the PCR product region was approximately 450 bp for all samples. No PCR products were produced in any of the reactions with *Colletotrichum capsici* controls (Figure 3.6). High-quality sequences of 430 nucleotides for each isolate were used for analysis (Appendix 1). Comparisons of these sequences with GenBank (http://www.ncbi.nlm.nih.gov) and NCBI databases showed that all of them were similar to *C. gloeosporioides* (Table 3.5).

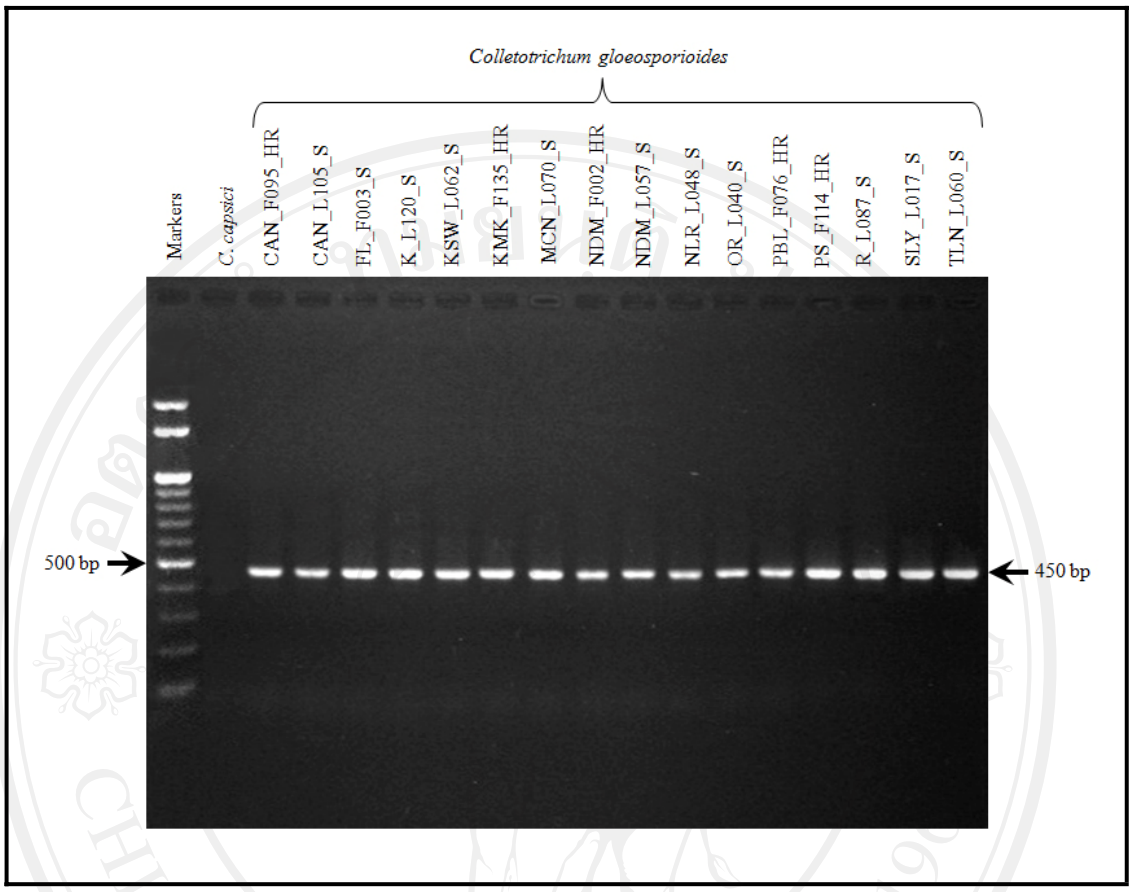


Figure 3.7 Amplification of the partial ITS region for species identification of various mango anthracnose pathogen isolates by PCR using species-specific primers of CgIT and ITS4. Markers are the 100 bp ladder, and the arrow on the left side indicates the 500 bp position. The arrow on the right marks the group-specific band. *Colletotritrichum capsici* (control).

Table 3.5 The blast result of rDNA ITS sequences (430 bp) from *Colletotrichum gloeosporioides* causing mango anthracnose and their most closely related sequences in GenBank during October 2010

Isolate code	Blast result in GenBank sequences					
	Accession number	Host	Country	Similarity (%)	bp change /gap	References
Highly resistant (Car^{HR}) 30 isolates						
CKT_L044	HM575266	Citrus	China[1/]	98.60	0 / 6	Unpublished
CAN_F095	AY177316	Mango	South Africa[2/]	98.61	0 / 6	Sanders and Korsten (2003)
CAN_F146	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
FL_F066	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
K_F103	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
KMK_F135	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
KMK_L058	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
KSW_L085	HM575266	Citrus	China	98.60	0 / 6	Unpublished
MCN_L056	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
NDM_F002	EF025937	Mango	India[3/]	98.17	0 / 8	Unpublished
NDM_F012	EF025937	Mango	India	98.17	0 / 8	Unpublished
NDM_F014	EF025937	Mango	India	98.17	0 / 8	Unpublished
NDM_F018	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
NDM_F026	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
NDM_F027	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
NDM_F038	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
NDM_F061	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
NDM_F106	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
NDM_F110	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
NDM_F116	AY177316	Mango	South Africa	98.38	1 / 6	Sanders and Korsten (2003)
NDM_F130	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
NDM_L078	HM575266	Citrus	China	98.60	0 / 6	Unpublished
NLR_L047	HM575266	Citrus	China	98.60	0 / 6	Unpublished
OR_F126	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
PBL_F033	EF025937	Mango	India	98.17	0 / 8	Unpublished
PBL_F076	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
PBL_F131	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
PS_F114	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
PS_L082	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
TLN_L065	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
Moderately resistant (Car^{MR})1 isolate						
NDM_F063	AY177316	Mango	South Africa	98.38	1 / 6	Sanders and Korsten (2003)
Sensitive (Car^{S}) 27 isolates						
CAN_F125	AY177316	Mango	South Africa	98.38	1 / 6	Sanders and Korsten (2003)
CAN_L080	HM575266	Citrus	China	98.60	0 / 6	Unpublished
CAN_L105	HM575266	Citrus	China	98.60	0 / 6	Unpublished

[1/]Submitted (19-MAY-2010) College of Agriculture and Biotechnology, Zhejiang University, Institute of Biotechnology, Kaixuan Road 268, Hangzhou, Zhejiang 310029, China.

[2/]Submitted (11-NOV-2002) Microbiology and Plant Pathology, University of Pretoria, Lynnwood Rd., Pretoria, Gauteng 0002, South Africa.

[3/]Submitted (20-SEP-2006) Plant Pathology, Indian Institute of Horticultural Research, Hessarghatta Lake Post, Bangalore, Karnataka 560 089, India.

Table 3.5 Continued

Isolate code	Blast result in GenBank sequences					
	Accession number	Host	Country	Similarity (%)	bp change /gap	References
FL_F003	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
FL_L079	EF025937	Mango	India	98.17	0 / 8	Unpublished
K_L120	EF025937	Mango	India	98.17	0 / 8	Unpublished
KMK_L088	HM575266	Citrus	China	98.60	0 / 6	Unpublished
KSW_L062	HM575266	Citrus	China	98.60	0 / 6	Unpublished
LNG_L031	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
MCN_L059	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
MCN_L070	HM575266	Citrus	China	98.60	0 / 6	Unpublished
MCN_L121	HM575266	Citrus	China	98.60	0 / 6	Unpublished
MKS_L086	EF025937	Mango	India	98.17	0 / 8	Unpublished
NDM_F006	HM575266	Citrus	China	98.60	0 / 6	Unpublished
NDM_F118	EF025937	Mango	India	98.17	0 / 8	Unpublished
NDM_L057	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
NDM_L067	EF025937	Mango	India	98.17	0 / 8	Unpublished
NDM_L068	HM575266	Citrus	China	98.60	0 / 6	Unpublished
NDM_L071	HM575266	Citrus	China	98.60	0 / 6	Unpublished
NDM_L096	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
NLR_L048	HM575266	Citrus	China	98.60	0 / 6	Unpublished
OR_L040	HM575266	Citrus	China	98.60	0 / 6	Unpublished
PBL_F102	EF025937	Mango	India	98.17	0 / 8	Unpublished
PS_L032	AY177316	Mango	South Africa	98.61	0 / 6	Sanders and Korsten (2003)
R_L087	HM575266	Citrus	China	98.60	0 / 6	Unpublished
SLY_L017	EF025937	Mango	India	98.17	0 / 8	Unpublished
TLN_L060	HM575266	Citrus	China	98.60	0 / 6	Unpublished

[1/]Submitted (19-MAY-2010) College of Agriculture and Biotechnology, Zhejiang University, Institute of Biotechnology, Kaixuan Road 268, Hangzhou, Zhejiang 310029, China.

[2/]Submitted (11-NOV-2002) Microbiology and Plant Pathology, University of Pretoria, Lynnwood Rd., Pretoria, Gauteng 0002, South Africa.

[3/]Submitted (20-SEP-2006) Plant Pathology, Indian Institute of Horticultural Research, Hessarghatta Lake Post, Bangalore, Karnataka 560 089, India.

Phylogenetic analysis

ITS sequences, including three *C. gloeosporioides* (accession no. EU37122, DQ185438, DQ084498) and three *C. acutatum* (accession no. GU183326, FJ972601, AY376510) retrieved from GenBank, were included for comparison in this study.

These sequences were published in Mycologia (accession no. EU37122, AY376510), Phytopathology (accession no. DQ185438, DQ084498), and Fungal Diversity (accession no. GU183326, FJ972601). Furthermore, DQ185438 and DQ084498 of *C. gloeosporioides* isolates were detected as benomyl-resistant and -sensitive, respectively (Table 3.6).

Table 3.6 ITS sequences used in phylogenetic analyses from GenBank

Pathogen	Isolate code	Host	Location	Accession no.	References
C. gloeosporioides	IMI 356878	Orange	Italy	EU371022	Cannon *et al*. (2009)
	L11 (Benomyl-resistant)	Statice	Israel	DQ185438	Maymon *et al*. (2006)
	P1 (Benomyl-sensitive)			DQ084498	
C. acutatum	27048	Mango	Australia	GU183326	Shivas and Tan (2009)
	BRIP 28519	Papaya	Australia	FJ972601	Prihastuti *et al*. (2009)
	IMI 117617	Papaya	Australia	AY376510	Lubbe *et al*. (2004)

Nucleotide sequences in the ITS region of the 58 representative isolates from Car^{HR}, Car^{MR}, and Car^{S} phenotypes were compared with those in published ITS sequences of *C. gloeosporioides* and *C. acutatum* reference isolates. Multiple sequence alignment was used to infer the phylogenetic tree, as shown in Figure 3.7. All of the *C. gloeosporioides* in this study were clustered together and the bootstrap value of 100% showed the polyphyly for the species. There also was supported for *C. gloeosporioides* forming a distinct cluster from *C. acutatum*. Separate dendrograms of each species were constructed to facilitate the presentation of genetic relationships among species. However, differences existed in the ITS sequence of the isolates these could not be associated with carbendazim resistance.

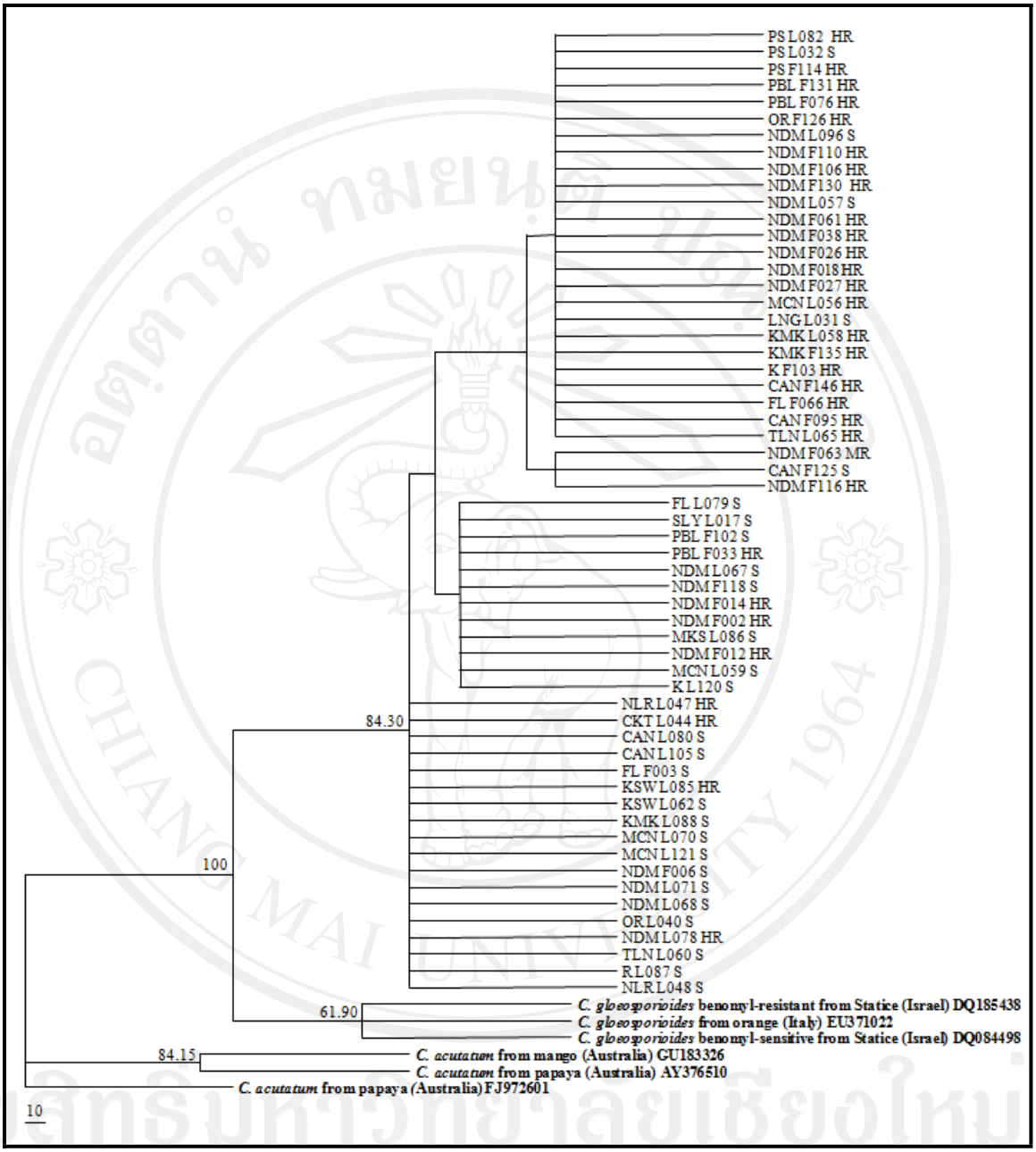


Figure 3.8 Phylogenetic tree based on the rDNA ITS sequence data representing relationships between *Colletotrichum gloeosporioides* isolates within the published sequence. Bootstrap values (>50%) from 1,000 replications of Unweighted Maximum Parsimony (UMP) analyses are shown above the internodes.

3.4 Discussion

Benzimidazole fungicides, *e.g.* benomyl, carbendazim and thiabendazole, have been used widely against the pathogens (Prior *et al*., 1992; Ploetz, 2003; Prakash, 2004; Akem, 2006; Pike, 2007). These fungicides effectively suppress and control a wide range of mango diseases, but their efficacy has declined over time, most likely due to the development of fungicide-resistance as reported for worldwide (Staub, 1991; Brent and Hollomon, 1998; Ma and Michailides, 2005; Deising *et al*., 2008; Damicone and Smith, 2009). Fungicide resistance is widely believed to be one of the reasons for increasing difficulties in disease control. The focus of many studies has therefore been determined the presence and severity of resistance to the single-site mode of active fungicides.

Carbendazim, as an inhibitor of tubulin biosynthesis, can control *C. gloeosporioides* in mango effectively (Davidse, 1986; Steffens *et al*., 1996; Ma and Michailides, 2005). It is a widely applied fungicide to control many fruit diseases in Thailand and is recommended to apply for a long time. The results of this study showed that about 75% of randomly selected isolates of *C. gloeosporioides* from various mango cultivars collected from markets and orchards revealed highly resistant (Car^{HR}) response to carbendazim. Many other studies also indicated that repeated applications of carbendazim enhance development of resistant isolates (Farungsang and Farungsang, 1992; Farungsang *et al*., 1994; Kumar *et al*., 2007; Kuo, 2001; Sanders *et al*, 2000; Sariah 1989; Taggart *et al*., 1999; Yoon *et al*., 2008). The highly resistant isolate of *C. gloeosporioides* is likely to develop under repeated carbendazim application in the field, which is the case in Thailand, where fungicide application has increased (Thapinta and Hudak, 2000). The development of fungicide-resistance

severely limits the efficacy and lifetime of current disease control strategies. To combat this, a strategy to develop a sufficient number of effective fungicides, which could be used alternately, might avoid producing resistant mango pathogens. Therefore, resistance may help in understanding at least the molecular level of the fungicidal mechanism.

In this study, the pathogenicity test confirmed that Car^{HR}, Car^{MR} and Car^{S} isolates of *C. gloeosporioides* from the leaf and fruit samples of various mango cultivars were pathogenic to both the fruit and leaves of the mango cv. 'Namdokmai' on which they produced dark-brown, necrotic, circular, and sunken lesions. Although differences existed in the pathogenicity of the isolates, they could not be associated with carbendazim resistance.

In this study, morphological characterisation indicated that the causal agent of mango anthracnose identified as *C. gloeosporioides* which agreement with previous reports from Sangchote (1987) and Farungsang *et al*. (1994). Moreover, PCR with primers specific for the species, followed by nucleotide sequencing of the amplicons, demonstrated that the causal agent was *C. gloeosporioides*. The blast result in GenBank (http://www.ncbi.nlm.nih.gov) showed that thirty (22 Car^{HR} isolates, 1 Car^{MR} isolate, and 7 Car^{S} isolates), seventeen (4 Car^{HR} isolates and 13 Car^{S} isolates) and eleven isolates (4 Car^{HR} isolates and 7 Car^{S} isolates) were closely related to *C. gloeosporioides* of the mango in South Africa from Microbiology and Plant Pathology in Pretoria University, citrus in China from College of Agriculture and Biotechnology in Zhejiang University and mango in India from Plant Pathology, Indian Institute of Horticultural Research, respectively. Sutton (1992) stated that *C. gloeosporioides* is a polymorphic species aggregate containing a number of

subgroups which varying degrees of pathogenicity, host-specificity and genetic homogeneity (Sutton, 1992). Its aggregate has been primarily defined by morphological methods (Sutton, 1992; Baxter *et al*., 1983) primarily through using characteristics of the conidia, which were considered to be cylindrical with rounded ends of less than 4.5 μm in diameter. Such features are sometime not considered reliable; especially as *Colletotrichum* species in culture frequently produce secondary conidia that are highly variable in size and shape (Sutton, 1992). Recently, applications of molecular techniques have been implemented to differentiate within and among *Colletotrichum* spp., including: species-specific PCR primers; random amplified polymorphic DNA (RAPD); arbitrarily primed PCR; and sequence analyses of the ITS regions of ribosomal DNA (Photita *et al*., 2005). Furthermore, differences existed in the ITS sequence of the isolates that could not be associated with carbendazim resistance. Therefore, Freeman *et al*. (1998) staled that correct and accurate identification ultimately leads to more effective disease control and management, e.g. the selection of appropriate fungicides. For example, in mixed populations of *Colletotrichum* species such as *C. gloeosporioides* and *C. acutatum*, higher resistibility of one species over another, to a certain fungicide (benzimidazole group), may cause a shift in population structure. Molecular techniques provide alternative methods, and are important tools in solving the problems of species delimitation.

3.5 Conclusion

It concluded that 75% of the carbendazim-resistant isolates were highly-resistant phenotype at 1,000 mg/l of carbendazim.

A pathogenicity test confirmed that Car^{HR}, Car^{MR} and Car^{S} isolates of *C. gloeosporioides* from the fruits and leaves of various mango cultivars were pathogenic to both fruits and leaves of the mango cv. 'Namdokmai'.

Molecular phylogeny confirmed that the causal agent is *C. gloeosporioides* in all carbendazim-resistant phenotypes.