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Cross-Resistance to Benzimidazole Group and Mancozeb Fungicides in *Colletotrichum* spp. Causing Anthracnose Disease

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ABSTRACT

Anthracnose is considered to be one of the most serious diseases for major crops cultivated in Thailand. Although chemical fungicides and newer chemicals have been recommended and used for disease management, it is still difficult to control. Thus, the monitoring of fungicide resistance development is required and very important for the success of disease control strategies. In this study, a total of 34 isolates of *Colletotrichum* spp. were successfully collected from anthracnose diseased plants in central Thailand. Of these isolates, 16 were collected from mango, and 2 from crinum lily or orchid and were morphologically identified as *C. gloeosporioides,* while the other 16 isolates from chili were morphologically identified as *C. capsica.* Then, all isolates were evaluated *in vitro* against 3 fungicides to determine their sensitivity. The results showed that 25 isolates were cross-resistant to both benomyl and carbendazim, as the 50% effective concentration (EC_{50}) of each fungicide was >100 mg L^{-1}. All isolates were resistant to mancozeb. Nine out of 16 isolates from mango anthracnose were confirmed as *C. gloeosporioides* by the sequence of internal transcribed spacer (ITS) and 5.8S regions of rDNA. Eight cross-resistant isolates were not controlled by the treatment with 100 mg L^{-1} of benomyl or carbendazim on 'Nam Dok Mai' mango fruits, but sensitive isolate was better controlled at 79.33% and 85.11%, respectively. Our results indicated that cross-resistant strains are present in the populations of anthracnose fungi in central Thailand.

Keywords: Benzimidazole fungicides; Cross-resistance; Fungicide resistance

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1. Introduction

Thailand is one of the leading producers and exporters of various tropical fruits and vegetables worldwide [1]. During 2019-2020, the export value of Thai agriculture increased by 12 % from 3.8 billion dollars to 4.3 billion dollars, continuing the upward trend every year, according to the Office of Agricultural Economics database on export statistics [2]. However, fruit production and export are hindered by a number of fungal diseases which have a significant impact on quantity and qualily of produce. Anthracnose, caused by *Colletotrichum* spp., is one of the most important diseases of fruit crops due to its broad host range, and the severe devastation it causes on all parts of plant, at all growth stages [3, 4]. Moreover, this disease is the main disease that limits the export of mangoes from Thailand.

At present, several chemicals, mainly in the benzimidazole group with FRAC code 1 (namely benomyl, carbendazim, and thiabendazole), have been extensively used for pre- and post-harvest control of anthracnose [4, 5]. These fungicides kill fungal cells during mitosis by distorting the mitotic spindle; β-tubulin, a protein important in forming the cytoskeleton is targeted by the fungicides. They mostly inhibit the polymerization of β-tubulin by interacting with it directly, but other interactions also exist [5]. However, *Colletotrichum* spp. isolates that are less sensitive or resistant to these fungicides have been increasingly detected in Japan [6], Mexico [7], and Trinidad [8]. Similarly, in Thailand, *C. gloeosporioides* isolates causing anthracnose in mango were resistant to carbendazim [9]. The resistant strains have a point mutation at E198A/G/K or F200Y in the β-tubulin gene [5, 9]. In order to help develop proper strategies for achieving the effective control of anthracnose disease, the development of fungicide resistance must be understood and monitored.

The aims of this study were (i) to collect the *Colletotrichum* species causing anthracnose disease in central Thailand and identify them based on morphological characterization, (ii) to evaluate the *in vitro* resistance of anthracnose fungi to commercial fungicides, namely carbendazim, benomyl, and mancozeb by mycelial growth assay, (iii) to identify the species complex of *Colletotrichum* by internal transcribed spacer (ITS) and 5.8S regions of rDNA, and (iv) to further evaluate their fungicide resistance on detached mango fruits.

2. Materials and Methods

2.1 Collection and identification of the fungal pathogen of anthracnose disease

Thirty-four samples from naturally infected plants showing typical anthracnose symptoms (circular or angular sunken lesions with concentric rings of acervuli) were collected from orchards and local markets in central Thailand. Isolations were made from symptomatic plant tissue by the tissue transplanting technique and single spore isolation technique. Then, the fungi were transferred onto potato dextrose agar (PDA) and maintained as stock cultures at 4°C . Morphological identification of each isolate was done under light microscope. The identification of *Colletotrichum* species was based on the published description [10].

2.2 Fungicide resistance test using traditional mycelial growth assay

All isolates of *Colletotrichum* spp. were cultured on PDA for 3-4 days. The fungicide resistance assay was done using the poison food technique on petri dishes containing PDA medium mixed with each fungicide (carbendazim, benomyl, and mancozeb) at different concentrations 0, 0.1, 1, 10, 100 mg L^{-1}, with 4 replications for each assay. The agar plug of *Colletotrichum* spp. was placed at the center of the culture medium then incubated at 30°C.

Mycelial growth inhibition was evaluated when the control fungus had reached the edge of the plate. Growth inhibition (GI) was calculated using the following formula: $(C_1 - C_2)/C_1 \times 100$, where C_1: diameter of the colony on the control plate and C_2: diameter of the colony on PDA medium mixed with each fungicide plate. The 50% effective dose (EC_{50}) for each fungicide was determined using a log-linear model in Microsoft excel software. Isolates of tested fungi with EC_{50} values < 10 mg L^{-1} were considered sensitive (S). Isolates with EC_{50} values between 10-100 mg L^{-1} were considered moderately resistant (MR). Finally, isolates with EC_{50} values greater than 100 mg L^{-1} were considered resistant ®. The fungicide- resistance categorization levels were modified from Chung *et al*. and Torres-Calzada *et al*. [6, 7].

2.3 Identification of *Colletotrichum* species complex by sequence analysis

To confirm the identity of the 9 isolates of *Colletotrichum* species from mango anthracnose (8 isolates as R and 1 isolate as S), the genomic DNA of all isolates was extracted. The extracted DNA was used as the template for amplification with ITS1 (5' TCCGTAGGTGAACCT GCGG 3') and ITS4 (5' TCCTCCGCT TATTGATATGC '3) of the internal transcribed spacer (ITS) of rDNA regions using polymerase chain reaction (PCR) [11]. The PCR products were directed sequence using cycle sequencing with ITS1 and ITS4 primers by Axil Scientific Pte Ltd., Singapore. Sequence similarity analyses were performed using the Basic Local Alignment Search Tool (BLAST) in the GenBank NCBI database.

2.4 Fungicide-resistance test on detached mango fruits

Cross-resistant isolates were further studied on detached 'Nam Dok Mai' mango fruits. The mango fruits were washed thoroughly using sterilized water before being surface sterilized with 10% Clorox® and then air-dried. A wounded inoculation site about 1.5 mm in diameter was marked on the surface of the fruits. Each wound (two wounds/fruit) was punctured with a sterile needle. The wounded fruits were soaked with and without 100 mg L^{-1} benomyl or carbendazim for 10 min. Mycelial discs of 0.5 mm diameter were cut with a sterilized cork borer and transferred upside down to the wounded area of mango fruits. The inoculated fruits were incubated in a moist plastic box at room temperature (28-30ºC) for 4 days.

The diameter of the lesion that appeared as brown rot around the wounded area was measured after 4 days of incubation, and the percentage of inhibition (PI) was calculated using the following formula:

$$\frac{\textit{lesion diameter on untreated} - \textit{lesion diameter on fungicide treated}}{\textit{lesion diameter on untreated}} \times 100.$$

The experiment was arranged in CRD with 4 replications. Data were processed using Statistix 8 analytical software. Mean of treatment was compared by the least significant difference (LSD) at $P \leq 0.05$.

3. Results and Discussion

3.1 Collection and identification by morphology

Plant samples showing typical anthracnose symptoms were collected from orchards and fresh markets in Cha Choeng Sao, Samut Prakarn, Bangkok, and Chonburi in 2018 (Figs. 1A, 1B, 1C, 1D). A total of 34 isolates were obtained; this included 16 isolates from mango (C1m02, C1m03, C1m04, C1m05, C1m06, C1m07, C1m08, C1m09, C1m10, C1m31, C1m33, C1m34, C1m35, C1m36, C1m37, C1m38), 16 isolates from chili (C2c11, C2c12, C2c13, C2c16, C2c17, C2c18, C2c41, C2c42, C2c43, C2c44, C2c45, C2c46, C2c47, C2c48, C2c49, C2c50), 1 isolate from crinum lily (C1cl39), and 1 isolate from orchid (C1o40). The results revealed that all isolates had white, gray-colored colonies with a dark and gray conidial mass in the

center. The isolates of *C. gloeosporioides* produced hyaline cylindrical conidia (Fig. 1E), and the isolates of *C. capsici* produced hooked shaped conidia (Fig. 1F). The collection and location of each isolate, as well as morphological identification are shown in Table 1.

Anthracnose has historically affected the quality and quantity of many crops in Thailand. The fungal pathogens were isolated from the symptomatic tissue on some plants collected from markets and orchards. They were identified by morphology as either *C. gloeosporioides* or *C. capsici* based on criteria by Sutton [10]. Similarly, *C. gloeosporioides* and *C. capsici* were reported as the causal agent of anthracnose disease in mango and chili [3-4].

Table 1. List of *Colletotrichum* spp. isolates causing anthracnose of mango, chili, crinum lily, and orchid.

Host	Isolate code	Location	Species
mango	C1m02	Orchard, Chachoengsao	*C. gloeosporioides*
	C1m03	Orchard, Chachoengsao	*C. gloeosporioides*
	C1m04	Orchard, Chachoengsao	*C. gloeosporioides*
	C1m05	Local market, Bangkok	*C. gloeosporioides*
	C1m06	Orchard, Samutprakarn	*C. gloeosporioides*
	C1m07	Orchard, Samutprakarn	*C. gloeosporioides*
	C1m08	Orchard, Samutprakarn	*C. gloeosporioides*
	C1m09	Orchard, Samutprakarn	*C. gloeosporioides*
	C1m10	Orchard, Samutprakarn	*C. gloeosporioides*
	C1m31	Orchard, Bangkok	*C. gloeosporioides*
	C1m33	Orchard, Bangkok	*C. gloeosporioides*
	C1m34	Orchard, Bangkok	*C. gloeosporioides*
	C1m35	Local market, Bangkok	*C. gloeosporioides*
	C1m36	Local market, Bangkok	*C. gloeosporioides*
	C1m37	Orchard, Bangkok	*C. gloeosporioides*
	C1m38	Orchard, Bangkok	*C. gloeosporioides*
chili	C2c11	Local market, Bangkok	*C. capsici*
	C2c12	Local market, Bangkok	*C. capsici*
	C2c13	Local market, Bangkok	*C. capsici*
	C2c16	Local market, Bangkok	*C. capsici*
	C2c17	Local market, Bangkok	*C. capsici*
	C2c18	Local market, Bangkok	*C. capsici*
	C2c41	Orchard, Bangkok	*C. capsici*
	C2c42	Local market, Bangkok	*C. capsici*
	C2c43	Local market, Bangkok	*C. capsici*
	C2c44	Local market, Bangkok	*C. capsici*
	C2c45	Local market, Bangkok	*C. capsici*
	C2c46	Local market, Bangkok	*C. capsici*
	C2c47	Local market, Bangkok	*C. capsici*
	C2c48	Local market, Bangkok	*C. capsici*
	C2c49	Local market, Bangkok	*C. capsici*
	C2c50	Local market, Bangkok	*C. capsici*
crinum lily	C1cl39	Private house, Bangkok	*C. gloeosporioides*
orchid	C1o40	Private house, Chonburi	*C. gloeosporioides*

3.2 Fungicide resistant test based on mycelial growth assay

Fungicide resistance in the 34 isolates was determined *in vitro* using mycelial growth assays and grouped into 3 representative responses by EC_{50} shown in Table 2. Twenty-five isolates were classified as R to both benomyl and carbendazim, and 6 isolates were classified as S to both fungicides. However, 3 isolates were classified as different phenotypes to both fungicides, including 3 isolates as MR to only benomyl. For mancozeb, 100% of the isolates were classified as R.

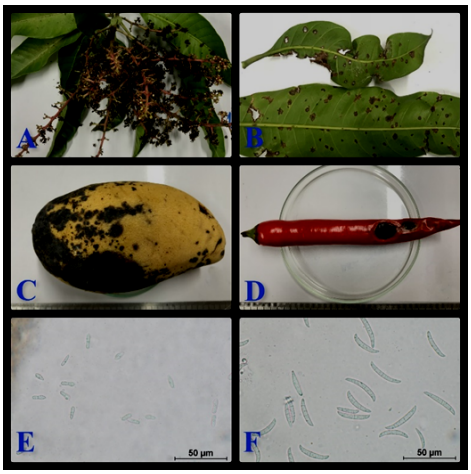

Fig. 1. Anthracnose symptoms on naturally infected mango inflorescence (A), mango leaves (B), mango fruits (C), and chili fruit (D); Representative conidia of *Colletotrichum gloeosporioides* (E) and *C. capsici* (F) isolates causing anthracnose disease.

Benzimidazoles are broad-spectrum fungicides that have been registered commercially in many countries for use on more than 70 crops, including cereal grains, grapes, fruits, and vegetables [3]. These fungicides effectively suppress and control a wide range of anthracnose diseases, but their efficacy has declined over time, most likely due to the development of fungicide resistance. Our results showed that 25 out of the 34 isolates of *Colletotrichum* spp. revealed cross- resistant response to both benomyl and carbendazim. Many other

studies have also indicated that repeated applications of benzimidazole fungicides enhance the development of resistant strains [12-14]. Moreover, the high EC_{50} of the isolates for mancozeb expresses the importance of modification in plant management. This fungicide is one of the most widely used for controlling this disease in the field. *Colletotrichum* resistance to mancozeb has been previously reported in apples, grapes, and rubber [15-17].

Table 2. Benomyl, carbendazim, and mancozeb resistance assay of *Colletotrichum* spp. isolates on potato dextrose agar amended with fungicide.

Isolate code	EC_{50} (mg L^{-1})*			Response**		
	Benomyl	Carbendazim	Mancozeb	Benomyl	Carbendazim	Mancozeb
C1m02	>100	>100	>100	R	R	R
C1m03	>100	>100	>100	R	R	R
C1m04	>100	>100	>100	R	R	R
C1m05	>100	>100	>100	R	R	R
C1m06	>100	>100	>100	R	R	R
C1m07	>100	>100	>100	R	R	R
C1m08	>100	>100	>100	R	R	R
C1m09	>100	>100	>100	R	R	R
C1m10	0.41	0.13	>100	S	S	R
C1m31	>100	>100	>100	R	R	R
C1m33	>100	>100	>100	R	R	R
C1m34	>100	>100	>100	R	R	R
C1m35	87.95	>100	>100	MR	R	R
C1m36	16.23	3.68	>100	MR	S	R
C1m37	0.21	0.89	>100	S	S	R
C1m38	3.16	<0.1	>100	S	S	R
C2c11	>100	>100	>100	R	R	R
C2c12	>100	>100	>100	R	R	R
C2c13	>100	>100	>100	R	R	R
C2c16	>100	>100	>100	R	R	R
C2c17	>100	>100	>100	R	R	R
C2c18	>100	>100	>100	R	R	R
C2c41	>100	>100	>100	R	R	R
C2c42	<0.1	<0.1	>100	S	S	R
C2c43	9.98	6.65	>100	S	S	R
C2c44	>100	>100	>100	R	R	R
C2c45	>100	>100	>100	R	R	R
C2c46	>100	>100	>100	R	R	R
C2c47	>100	>100	>100	R	R	R
C2c48	>100	>100	>100	R	R	R
C2c49	<0.1	<0.1	>100	S	S	R
C2c50	>100	>100	>100	R	R	R
C1cl39	12.55	2.06	>100	MR	S	R
C1o40	>100	>100	>100	R	R	R

*EC_{50} = the concentration of the fungicide at which fungal development is inhibited by 50%.

** S = sensitive (EC_{50} values < 10 mg L^{-1}), MR = moderately resistant (EC_{50} values between 10-100 mg L^{-1}), R = resistant (EC_{50} values greater than 100 mg L^{-1})

3.3 Identification by ITS

Nine out of the 16 mango isolates were previously identified as *C. gloeosporioides* by morphological characteristics and then confirmed with the molecular technique. DNA from each sample amplified with ITS1 and ITS4 primers was sequenced and compared with known DNA sequences in the NCBI databases. The nucleotide sequences in all isolates ranged in size from 574-577 bp. The nucleotide sequences of isolate C1m03, C1m05, C1m08, and C1m10 were identified as *C. gloeosporioides* which showed 99-100% identity. Moreover, isolates C1m02, C1m04, C1m06, C1m07, and C1m09 showed 100% identity with *Glomerella cingulata*. Sequence comparison between morphology and molecular identification are shown in Table 3. Therefore, we demonstrated that 9 of the *C. gloeosporioides* isolates from mango anthracnose expressed cross-resistance, and sensitive responses were confirmed by DNA sequence [3]. Therefore, *C. gloeosporioides* are the anamorph stage of *G. cingulata*. The sequence of 5 mentioned isolates showed 100% identity with *G. cingulata* and showed 99% identity with *C. gloeosporioides* as well (not shown in Table 3).

Table 3. Identification comparing between morphology and molecular of *Colletotrichum* spp. isolates.

Isolate	Morphology	Identification			
		Molecular	Sequence length (bp)	Accession number	Identity (%)
C1m02	*C. gloeosporioides*	*G. cingulata*	574	AB233339	100
C1m03	*C. gloeosporioides*	*C. gloeosporioides*	574	KC816034	99
C1m04	*C. gloeosporioides*	*G. cingulata*	574	AB233339	100
C1m05	*C. gloeosporioides*	*C. gloeosporioides*	575	GQ865569	99
C1m06	*C. gloeosporioides*	*G. cingulata*	574	AB233339	100
C1m07	*C. gloeosporioides*	*G. cingulata*	574	AB233339	100
C1m08	*C. gloeosporioides*	*C. gloeosporioides*	574	KC816034	100
C1m09	*C. gloeosporioides*	*G. cingulata*	574	AB233339	100
C1m10	*C. gloeosporioides*	*C. gloeosporioides*	577	GU066671	99

3.4 Fungicide resistance test on mango fruits

Based on cross-resistance to benomyl and carbendazim, 2 groups of isolates (8 isolates of cross-resistant and 1 isolate of sensitive phenotypes) were used on inoculated mango fruits after being treated with each fungicide. The results showed that the PI of benomyl and carbendazim on the 8 cross-resistant isolates was significantly different compared with that on the sensitive isolate. Eight cross-resistant isolates (C1m02, C1m03, C1m04, C1m05, C1m06, C1m07, C1m08, and C1m09) produced brown lesions on the mango fruit pretreated with 100 mg L^{-1} benomyl and carbendazim. However, sensitive isolate (C1m10) significantly differed in PI (79.33% to benomyl and 85.11% to carbendazim) (Table 4, Fig. 2). All of them were *C. gloeosporioides*. Moreover, it was thus confirmed that both carbendazim and benzimidazole were not effective at 100 mg L^{-1} against resistant isolates on mango fruits. Therefore, our results indicate that benzimidazole treatment provides insufficient anthracnose control in mango.

A similar cross- resistance between benzimidazole fungicides was found in other reports on *Botrytis cinerea*, *Aspergillus nidulans*, and *Saccharomyces cerevisiae* [18, 19].

Table 4. Inhibition percentage on detached fruits after treated with 100 mg L^{-1} benomyl or 100 mg L^{-1} carbendazim or control and inoculated with *Colletotrichum gloeosporioides* isolates for 5 days.

Response	Isolates	Benomyl (100 mg L^{-1})	Carbendazim (100 mg L^{-1})
Ben^R, Car^R	C1m02	15.86c	-4.89f
	C1m03	5.95cd	1.57ef
	C1m04	3.35cd	6.02def
	C1m05	9.95c	11.65cdef
	C1m06	15.71c	26.72bcd
	C1m07	45.79b	43.82b
	C1m08	-15.77e	32.44bc
	C1m09	-12.83de	16.82cde
Ben^S, Car^S	C1m10	79.33a	85.11a

Means with the different letters in the same column were significantly different by least significant difference (LSD) at $p < 0.05$, Ben^R = benomyl resistant, Car^R = carbendazim resistant, Ben^S = benomyl sensitive, Car^S = carbendazim sensitive.

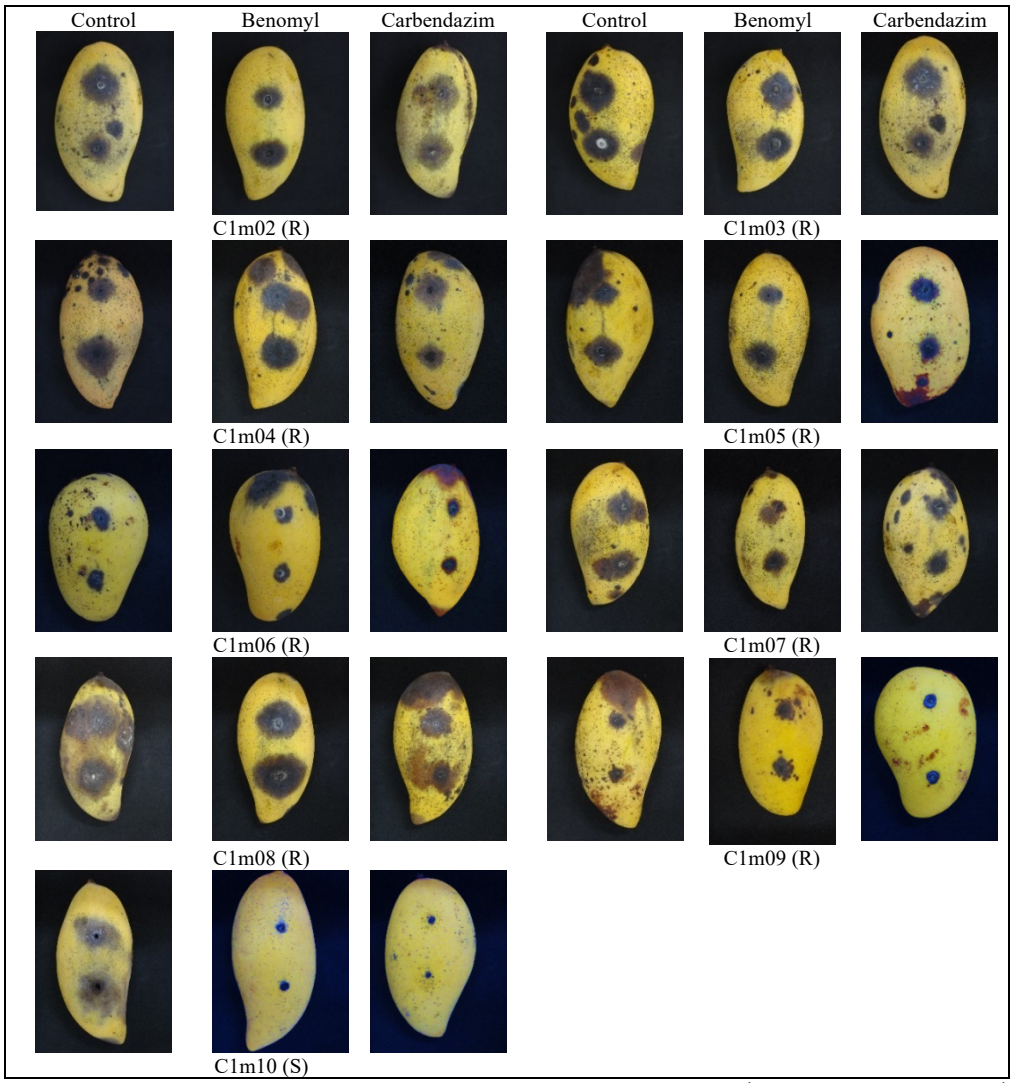

Fig. 2. Lesion on detached fruits after being treated with 100 mg L^{-1} benomyl or 100 mgL^{-1} carbendazim or control and inoculated with *Colletotrichum gloeosporioides* isolates for 5 days.

4. Conclusion

Thirty-four isolates of *Colletotrichum* spp. were successfully isolated from anthracnose disease in central Thailand; this included 18 isolates of *C. gloeosporioides* from mango, crinum lily, and orchid, as well as 16 isolates of *C. capsici* from chili. Among these, 25 isolates were shown to be cross-resistant between benomyl and carbendazim in the benzimidazole group whereas all isolates were proven to be resistant to mancozeb with EC_{50} > 100 mg L^{-1} as determined by mycelial growth assay. Moreover, 9 out of 16 isolates from mango were phenotypically identified and then confirmed with DNA sequences in the NCBI databases as *C. gloeosporioides*. Eight cross-resistant isolates could not be controlled by the treatment of 100 mg L^{-1} of benomyl or carbendazim on mango fruits. Hence, our study can confirm that cross-resistance in the benzimidazole group and mancozeb resistance are indeed present in central Thailand.

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