Resistance to Quinone-outside Inhibitor (QoI) Fungicides in *Colletotrichum* Species Isolated from Anthracnose Disease Occurring in Thailand

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Abstract

Colletotrichum species are plant pathogens causing anthracnose diseases in many crops that result in significant reduction of quality and quantity of the crop products. Management of these diseases has become increasingly difficult recently due to the development of fungicide resistance in pathogens. Thus, monitoring for fungicide sensitivity in the pathogen is important to manage these diseases. The isolates of *Colletotrichum* species were isolated from anthracnose disease on various crops collected during 2016-2017 in central and southern Thailand. They were tested for their mycelium growth sensitivity to azoxystrobin, a quinone-outside inhibitor (QoI) fungicide and their internal transcribed spacer (ITS) and 5.8S regions of rDNA were analyzed. The results showed that eleven out of twenty four isolates of *Colletotrichum* species were resistant to azoxystrobin. They were able to grow on PDA amended with 100 mg/l azoxystrobin in the presence of salicylhydroxamic acid (SHAM) at 100 mg/l. Moreover, two isolates of azoxystrobin-resistant *Colletotrichum* spp. produced much larger lesions than azoxystrobin-sensitive isolates on mango fruits after treatment with 100 mg/l azoxystrobin. This is the first report on the occurrence of azoxystrobin resistance in *Colletotrichum* species present in Thailand and will contribute to the management of these important diseases in the future.

Keywords: anthracnose, azoxystrobin, *Colletotrichum*, fungicide resistance, mango, QoIs
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1. Introduction

Anthracnose diseases, caused by *Colletotrichum* species are major diseases of many crops in Thailand because the weather conditions in this country are favorable to disease development. They destroy both pre- and post-harvest products of economic crops such as chili [1], coffee [2], orchid [3], banana and mango [4]. The management of anthracnose diseases has relied largely on various As a result, QoI fungicides, which inhibit mitochondrial respiration at the ubiquinol oxidation center of the cytochrome *bc1* enzyme complex (complex III) have been introduced to control groups of

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chemical fungicides such as quinone-outside inhibitor (QoI), sterol demethylation inhibitor (DMI), methyl benzimidazole carbamate (MBC) fungicides during the growing season for a long time [5, 6]. They are systemic fungicides with single-site mode of actions which have high risk for the development of resistance in fungal populations [7]. In fact, resistance of *Colletotrichum* species, the cause of mango anthracnose, to the benzimidazole fungicide carbendazim has been reported formerly in Thailand [8, 9].

As a result, QoI fungicides, which inhibit mitochondrial respiration at the ubiquinol oxidation center of the cytochrome *bc1* enzyme complex (complex III), have been introduced to control anthracnose [10-12]. These fungicides inhibit electron transfer which leads to preventing the production of ATP in fungal cells [10-12].

Currently, azoxystrobin and trifloxystrobin, members of the QoI fungicides, are recommended for the control of mango and chili anthracnose as alternative fungicides of carbendazim in Thailand [5, 6]. They were imported into Thailand with the cost of approx. 5,264,773 US dollars a year which was ranging the top ten fungicides by value in 2017 [13]. Furthermore, the QoI fungicide resistance in several *Colletotrichum* species has been reported on various crops from many countries such as *C. graminicola* from creeping bent grass in Japan and annual bluegrass in the United States [14], *C. gloeosporioides* from strawberry anthracnose in Japan [15], *C. cereale* causing turfgrass anthracnose in California and the southern United States [16, 17], *C. siamense* from peach and blueberry anthracnose in South Carolina [18], *C. acutatum* from strawberry anthracnose in Florida [19], *C. gloeosporioides* causing orange anthracnose in Italy [20], *C. truncatum* from physic nut, papaya, and pepper in Mexico [21] and *C. acutatum* and *C. gloeosporioides* from boxthorn and apple in Korea [22]. However, it has never been reported in Thailand. Consequently, the monitoring of QoI fungicide resistance development in *Colletotrichum* species is necessary for anthracnose disease management.

The objectives of this research were to (i) collect isolates of *Colletotrichum* species causing anthracnose from naturally infected crops, (ii) evaluate their sensitivity to azoxystrobin and (iii) analyze their internal transcribed spacer (ITS) and 5.8S regions of rDNA to identify species complex. The overall impact of this research is expected to help determining the management strategies for anthracnose diseases in the future.

2. Materials and Methods

2.1 Fungicides and Pathogen collection

Commercial formulation of azoxystrobin (Amistar®, 25% active ingredient (a.i.) SC) was used in this study. The diluted suspensions of azoxystrobin were prepared in sterile distilled water and added to the medium after autoclaving. It is well known that an alternative respiration pathway is activated in fungal cells after treatment with QoI fungicides and isolates are able to grow on fungicide amended culture medium [14-17]. Therefore, it is a common practice to include salicylhydroxamic acid (SHAM) as alternative oxidase (AOX) inhibitors in the medium to suppress alternative respiration [15]. SHAM was dissolved in methanol and added to azoxystrobin amended potato dextrose agar (PDA).

Chili fruits, orange fruits, mango fruits inflorescences, and leaves, crinum lily leaves and orchid leaves showing symptoms of anthracnose disease naturally infected were selected from local markets, orchards and private houses in Chumphon, Saraburi, Prachuap Khiri Khan, Bangkok, Cha Choeng Sao, Samut Prakarn and Chon Buri provinces during 2016-2017. Fungal isolation was made by cutting a small section of anthracnose infected portion, which was surface sterilized with 10% Clorox® (8.25% a.i. sodium hypochlorite) for 1-2 min, rinsed in sterilized distilled water, and blotted

dry on sterile paper towels in a laminar flow hood. The dried tissues were then placed on the plates of water agar, and incubated at room temperature of approx. 28-30ºC. The pure culture of isolates was maintained on PDA slants. All isolates used in this study are listed in Table 1.

Table 1. Source list of *Colletotrichum* isolates used in this study.

Year of isolation	Isolate code	Host	Location
2016	CC004	chili_fruits	Local market, Chumphon
	CC005	chili_fruits	Local market, Chumphon
	CC007	chili_fruits	Local market, Chumphon
	CO010	orange_fruits	Local market, Chumphon
2017	CM_S013	mango_fruits	Orchard, Saraburi
	CM_C019	mango_fruits	Local market, Prachuap Khiri Khan
	CM_C020	mango_fruits	Local market, Prachuap Khiri Khan
	CM_002	mango_fruits	Local market, Bangkok
	C1_m02	mango_inflorescences	Orchard, Cha Choeng Sao
	C1_m05	mango_fruits	Local market, Bangkok
	C1_m06	mango_fruits	Orchard, Samut Prakarn
	C1_m07	mango_fruits	Orchard, Samut Prakarn
	C1_m08	mango_fruits	Orchard, Samut Prakarn
	C1_m10	mango_fruits	Orchard, Samut Prakarn
	C1_m31	mango_fruits	Orchard, Bangkok
	C1_m32	mango_fruits	Orchard, Bangkok
	C1_m33	mango_fruits	Orchard, Bangkok
	C1_m34	mango_fruits	Orchard, Bangkok
	C1_m35	mango_fruits	Local market, Bangkok
	C1_m36	mango_fruits	Local market, Bangkok
	C1_m37	mango leaves	Orchard, Bangkok
	C1_m38	mango leaves	Orchard, Bangkok
	C1_cl39	crinum lily leaves	Private house, Bangkok
	C1_or40	orchid leaves	Private house, Chon buri

2.2 Sensitivity of *Colletotrichum* isolates to azoxystrobin in mycelial growth assay

The sensitivity of 24 *Colletotrichum* isolates to azoxystrobin was assessed by mycelial growth assay. Each isolate of *Colletotrichum* spp. was previously grown on PDA plates at 25ºC for 3 days. Mycelial discs (4 mm in diameter) were cut from actively growing colony margins and transferred onto PDA amended with azoxystrobin at 0, and 100 mg (a.i.)/l and SHAM at 100 mg/l (3 replications). After incubation at 25ºC for 3 days, the diameter of colony was recorded and the percentage of mycelial growth inhibition by fungicide treatment was calculated as follows: [(mean of colony diameter on the control medium – mean of colony diameter on the medium with fungicide)/(mean of colony diameter on the control medium) × 100].

2.3 Azoxystrobin sensitivity on inoculated mango fruits

Azoxystrobin sensitivity of the isolates was determined on mango fruits. Thirty six mango fruits were washed thoroughly using sterilized water before being surface sterilized with 10% Clorox®

and air-dried. Two 1 × 1.5 mm (diameter × depth) wounded inoculation sites were marked on the top (4 cm from stalk) and the bottom (4 cm from apex) of the fruits with a sterile needle. The wounded mango fruits were soaked with 100 mg/l azoxystrobin or sterilized water for 5 min. All tested isolates were previously cultured on PDA at 25ºC for 4 days. The mycelial discs of 5 mm diameter were cut with a sterilized cork borer and transferred upside down onto the wounded site of mango fruits (3 replications). The inoculated mango fruits were incubated in a moist plastic box at room temperature (28-30ºC) for 4 days. The lesion zone appeared as brown rot around the wounded site was measured after 4 days of incubation and the percentage of disease control was calculated from [(mean lesion diameter on water treated mango fruits – mean lesion diameter on azoxystrobin treated mango fruits)/ mean lesion diameter on water treated mango fruits] × 100. For statistical analysis, one-way ANOVA was performed and followed by least significant difference test. Probability values ($P<0.05$) were considered statistically significant.

2.4 Isolation of total DNA

The isolates of *Colletotrichum* were grown on PDA at 25ºC for 5 days. The total DNA of each isolate was extracted from mycelia as described by Saitoh *et al.* [23] with slight modifications [24]. A small piece about 1 cm^2 in size of agar medium with actively growing mycelia was transferred in an Eppendorf tube (1.5 ml size) containing 500 µl of lysis buffer (50 mM of ethylenediamine-tetraacetic acid (EDTA), 200 mM of NaCl, 200 mM of Tris-HCl, 1% *n*-lauroylsarcosine sodium salt, pH 8.0). They were homogenized using a plastic pestle and an electric drill. The mixture was incubated for 10 min at room temperature and then centrifuged at 13,000 rpm at 4°C for 5 min. The 300 µl of supernatant was transferred to a fresh tube. After mixing with 750 µl of ethanol, the DNA was precipitated by centrifugation at 13,000 rpm at 4°C for 2 min. The pellet was washed with 70% ethanol and then air dried in a laminar floor bench. The dried pellet was dissolved in 50 µl of Tris-EDTA (TE) buffer containing 1 mM of EDTA (pH 8.0) and 10 mM of Tris-HCl.

2.5 PCR experiments and sequencing

To amplify the rDNA-ITS (ITS1-5.8S rDNA-ITS2) regions from total DNA, the PCR primers ITS5 and ITS4 [25] were used. A total volume of 50 µl of PCR reaction mixtures contained a set of forward and reverse primers (0.2 µM for each), premixed Go Taq Green Master Mix (Promega, Madison, WI) and 1 µl of total DNA. PCR reactions were performed in a Mastercycler nexus gradient (Eppendorf, Hamburg, Germany) programmed at 94°C for 1 min, followed by 35 cycles at 94°C for 1 min, 52°C for 1 min, 72°C for 2 min, a final extension at 72°C for 10 min and holding at 10°C.

The PCR products were separated by electrophoresis on a 1.5% agarose gel in 2 mM EDTA + 89 mM Tris-borate (TBE) buffer (pH 8.0) and stained with GelRed™ (Biotium, Hayward, CA). They were cleaned up using ExoSAP-IT (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. Sequencing in both directions was conducted at Macrogen Japan Corp. (Kyoto, Japan) using the same primers employed for PCR. The nucleotide sequences were analysed with the database of National Center for Biotechnology Information (NCBI)/GenBank using basic local alignment search tools (BLAST).

3. Results and Discussion

3.1 Azoxystrobin sensitivity of mycelial growth

Azoxystrobin sensitivity was examined based on mycelial growth inhibition on fungicide amended PDA plates. As shown in Table 2, ten isolates (C1_m02, C1_m10, C1_m31, C1_m32, C1_m33, C1_m34, C1_m35, C1_m36, C1_m37, and C1_m38) of *Colletotrichum* spp. from mango anthracnose and one isolate (C1_cl39) from crinum lily anthracnose grew on the plate containing azoxystrobin at 100 mg/l in the presence of SHAM at 100 mg/l and they were determined as resistant (R) according to Avila-Adame *et al*. [14] and Torres-Calzada *et al*. [21]. In contrast, three isolates from chili anthracnose (CC004, CC005, and CC007), eight isolates from mango anthracnose (CM_S013, CM_C019, CM_C020, CM_002, C1_m05, C1_m06, C1_m07 and C1_m08) and two isolates from orange and orchid anthracnose (CO010 and C1_or40) were sensitive (S) to azoxystrobin because their growth was completely inhibited at 100 mg/l azoxystrobin with 100 mg/l SHAM [14]. To our knowledge, this is the first documentation of azoxystrobin resistance in *Colletotrichum* species in Thailand. Historically, azoxystrobin was used as an alternative control agent to manage anthracnose diseases in commercial chili farms and mango orchards because these diseases were poorly controlled by other fungicides. As a result, the use of this fungicide has also caused a problem in disease control. The same problem of anthracnose disease control has occurred in many other countries on various crops [14-22].

3.2 Azoxystrobin sensitivity on inoculated mango fruits

Based on the differential azoxystrobin sensitivity of mycelial growth in the presence of SHAM, two groups of isolates were used for inoculation onto detached mango fruits. Two azoxystrobin-resistant isolates, C1m02 and C1m10, produced brown lesions on mango fruits pretreated with 100 mg/l azoxystrobin with no significant differences in their diameter from those in non-treated control (disease control: -5.41 and 8.37%, respectively). However, four azoxystrobin-sensitive isolates, C1m05, C1m06, C1m07, and C1m08, significantly differed in lesion diameter when compared with the non-treated control, which showed the disease control of 55.38% - 87.31% (Table 3 and Figure 1). It was thus confirmed that azoxystrobin was not effective at 100 mg/l against resistant isolates on mango fruits. A similar QoI resistance was found in other reports on anthracnose in strawberry [15], annual bluegrass and creeping bent grass [17]. These results indicated that QoI fungicides will not successfully control anthracnose if azoxystrobin-resistant isolates are widely distributed in the field.

3.3 rDNA-ITS sequence analysis

The PCR products from each isolate (except the isolate C1_m34) amplified using the ITS5 and ITS4 primers were sequenced directly and analysed with the NCBI database. The results showed that the nucleotide sequences of all isolates indicated that the causal agent of anthracnose diseases in chili, mango, crinum lily-leaf, and orchid-leaf identified as *Colletotrichum* species. The chili isolates CC004 was *C. acutatum* with 99% identity, CC005 was associated with *C. acutatum* and *C. scovillei* with 100% identity, and CC007 was associated with *C. aeschynomenes*, *C. acutatum*, *C. boninense*, *C. fructicola*, *C. gloeosporioides*, *C. siamense* , *C. nupharicola*, and *C. viniferum* with 99-100% identity [Table 4]. Three species, *C. capsici*, *C. gloeosporioides* and *C. acutatum* were the major causal agents of chili anthracnose in Thailand and other countries in Southeast Asia [1]. Some species including in our results, such as *C. scovillei* and *C. siamense* have been shown to cause

Table 2. Mycelial growth inhibition of *Colletotrichum* species on potato dextrose agar plates at 100 mg/l azoxystrobin amended with 100 mg/l SHAM and phenotype classification.

Isolate code	Growth inhibition of azoxystrobin at 100 mg/l (%)	Phenotype
CC004	100	S
CC005	100	S
CC007	100	S
CO010	100	S
CM_S013	100	S
CM_C019	100	S
CM_C020	100	S
CM_002	100	S
C1_m02	57	R
C1_m05	100	S
C1_m06	100	S
C1_m07	100	S
C1_m08	100	S
C1_m10	55.2	R
C1_m31	65	R
C1_m32	75.6	R
C1_m33	77.8	R
C1_m34	70.0	R
C1_m35	68.3	R
C1_m36	62.2	R
C1_m37	63.4	R
C1_m38	70.8	R
C1_cl39	60.4	R
C1_or40	100	S

R = resistant (the mycelia were able to grow on 100 mg/l azoxystrobin with 100 mg/l SHAM)
S = sensitive (the mycelia were not able to grow on 100 mg/l azoxystrobin with 100 mg/l SHAM)

Table 3. Anthracnose lesion diameter and disease control on detached mango fruits after treatment with azoxystrobin prior to pathogen inoculation.

Response to azoxystrobin	Isolate code	Lesion diameter (mm)*		Disease control (%)
		Control	Azoxystrobin at 100 mg/l	
Resistant	C1m02	18.5a	19.5a	-5.41
	C1m10	21.5a	19.7a	8.37
Sensitive	C1m05	19.7a	2.5b	87.31
	C1m06	19.5a	8.7b	55.38
	C1m07	23.5a	5.2b	77.87
	C1m08	17.7a	3.5b	80.23

*Values expressed are means with 3 replications. The different letters in the same row are significantly different as determined by least significant difference test at $P<0.05$.

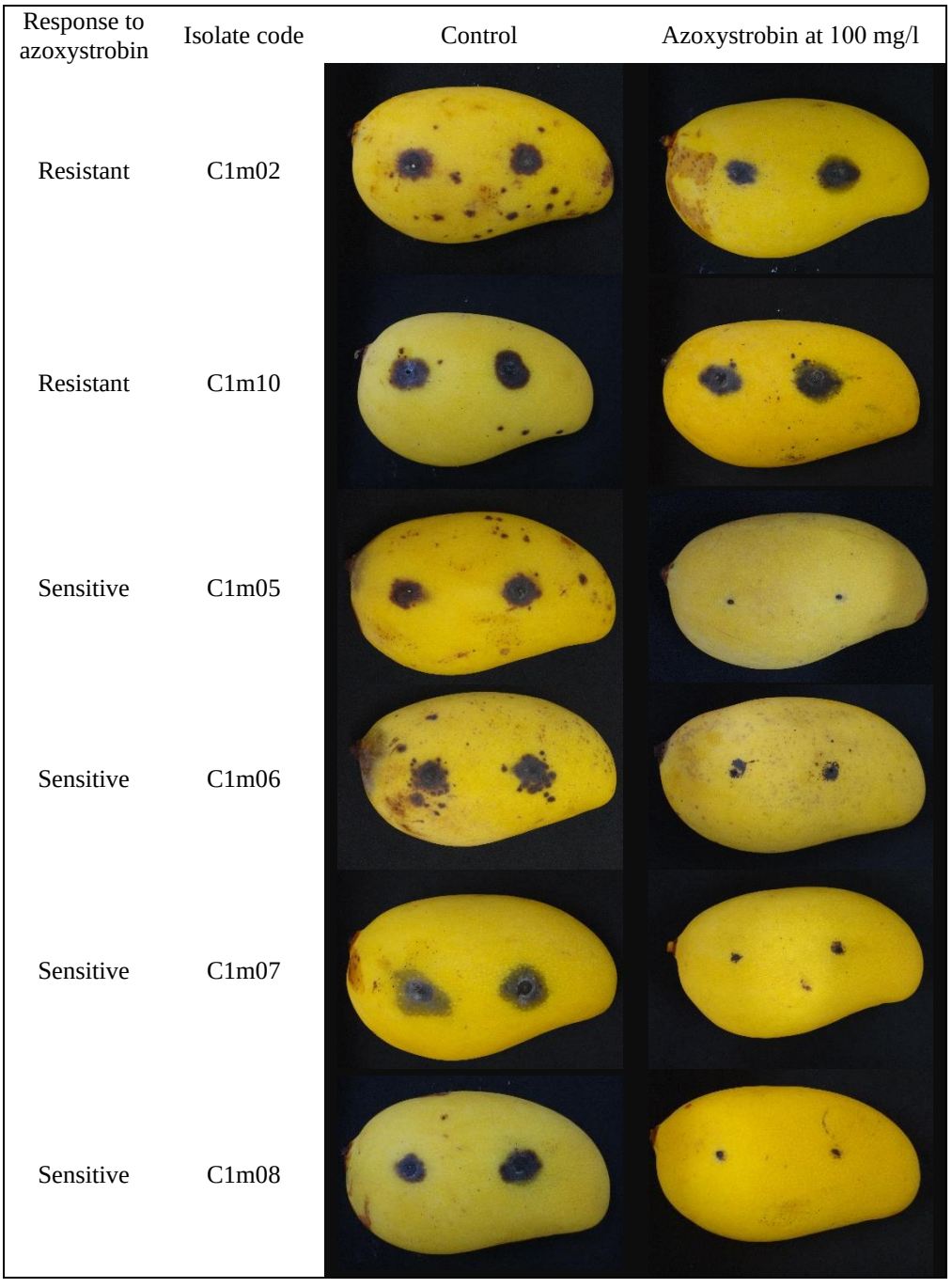

Figure 1. Comparative development of anthracnose lesion on detached mango fruits pretreated with 100 mg/l axozystrobin or untreated 5 days after inoculation with *Colletotrichum* isolates

Table 4 Percentage of identity of rDNA-ITS nucleotide sequence after NCBI BLAST analysis.

Host	Plant organ	Isolate code	Identity (%)	Species
chili	fruits	CC004	99	*C. acutatum*
		CC005	100	*C. acutatum, C. scovillei*
		CC007	99-100	*C. acutatum, C. aeschynomenes, C. boninense, C. fructicola, C. gloeosporioides, C. nupharicola, C. siamense, C. viniferum*
mango	fruits	CM_S013	99-100	*C. gloeosporioides*
		CM_C019	99	*C. asianum C. gloeosporioides*
		CM_C020	99	*C. asianum C. gloeosporioides*
		CM_002	99-100	*C. gloeosporioides*
		C1_m05	99-100	*C. asianum, C. fructicola, C. gloeosporioides*
		C1_m06	100	*C. asianum, C. gloeosporioides*
		C1_m07	99-100	*C. asianum, C. gloeosporioides*
		C1_m08	99-100	*C. asianum, C. boninese, C. gloeosporioides*
		C1_m10	99-100	*C. gloeosporioides C. queenslandicam, C. siamense, C. tropicale,*
		C1_m31	99-100	*C. alatae, C. gloeosporioides, C. siamense, C. tropicale, Glomerella cingulata,*
		C1_m32	99-100	*C. alatae, C. gloeosporioides, C. siamense, C. tropicale G. cingulata*
		C1_m33	99	*C. gloeosporioides, C. siamense, C. tropicale*
		C1_m35	99-100	*C. queenslandicam, G. cingulata*
		C1_m36	99-100	*C. gloeosporioides, C. siamense*
	leaves	C1_m37	99-100	*C. gloeosporioides, C. musae*
		C1_m38	100	*C. gloeosporioides, C. musae*
	Inflo-rescences	C1_m02	99-100	*C. asianum, C. gloeosporioides*
Crinum lily	leaves	C1_cl39	99-100	*C. alienum, C. endomangiferae C. gloeosporioides, C. siamense, G. cingulata*
orange	fruits	CO010	99	*C. siamense, C. queenslandicum, C. gloeosporioides*
orchid	leaves	C1_or40	98-99	*C. coelogynes, C. cordylinicola, C. tropicicola*

anthracnose of chili in Thailand, Brazil, China and Malaysia [26, 27]. *C. fructicola* was reported to be associated with chili anthracnose in China, India, and Malaysia [26, 27] and *C. viniferum* associated with chili anthracnose in China [26, 27]. However, 24 *Colletotrichum* species have been identified as the pathogen of chili anthracnose from different countries and regions [26, 27].

In mango anthracnose, the isolates CM_S013 and CM_002 were *C. gloeosporioides* with 99-100% identity. Moreover, five isolates (CM_C019, CM_C020, C1_m02, C1_m06, and C1_m07) were associated with *C. asianum* and *C. gloeosporioides* with 99-100% identity. Moreover, other species were identified with 99-100% identity including *C. alatae*, *C. boninense*, *C. fructicola*, *C. musae*, *C. queenslandicam*, *C. siamense*, and *C. tropicale* [Table 4]. Several *Colletotrichum* species such as *C. gloeosporioides* and *C. asianum* are well known as the pathogen of a mango anthracnose in Thailand and many countries of mango producers [29-33]. Moreover, *C. siamense* and *C.*

fructicola were reported from mango, too [29, 34, 35]. In this study, eleven isolates with azoxystrobin resistance were associated with several *Colletotrichum* species. High level of genetic variability among the *Colletotrichum* isolates from mango was reported by de Souza *et al*. [31]. However, comparison of the rDNA-ITS sequence alone is not always satisfactory to separate *Colletotrichum* species [36]. Therefore, the precise identification of *Colletotrichum* species needs to be conducted using multi locus phylogenetic analysis [28, 37] in the future.

4. Conclusions

The present study first revealed the development of QoI resistance in *Colletotrichum* species isolated from anthracnose-infected mango fruits and crinum lily in Thailand. The detection of resistant phenotypes in pathogen populations is the first step for effective disease control. The use of QoIs in mango should be carefully reconsidered in the management of anthracnose. It could contribute to prevent the further development of QoI-resistant pathogen populations by altering fungicide application programmes. Future studies should be focused on the wide-range of QoI resistance monitoring and identification of *Colletotrichum* species.

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