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Research article

Fungicide resistance in *Colletotrichum* species causing durian anthracnose in eastern Thailand

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Abstract

Importance of the work: Durian (*Durio zibenthinus* Murr.) is one of the most popular and exceptionally high-value fruit crops globally. *Colletotrichum* species are the causal pathogens of durian anthracnose, making managing fungicide resistance in the field mandatory.
Objectives: To evaluate the sensitivity of *Colletotrichum* species on various fungicides currently used in durian orchards in eastern Thailand.
Materials and Methods: In total, 15 *Colletotrichum* isolates were tested for sensitivity to several fungicide classes: Quinone outside Inhibitors (QoIs), DeMethylation Inhibitors (DMIs) and multi-site fungicides were tested on culture medium at concentrations of 0.1, 1, 10, 100 and 1,000 µg/mL. The 50% mycelial growth inhibition (EC_{50}) was analyzed and used to classify sensitive or resistant phenotypes. The selected isolates were evaluated for their fungicide sensitivity to spore germination and on detached durian leaves.
Results: In all 15 tested *Colletotrichum* isolates, the mycelial growth was sensitive to difenoconazole and prochloraz (EC_{50} range of 0.02–45.5 µg/mL). Additionally, the EC_{50} values varied significantly for pyraclostrobin (0.09 to over 100 µg/mL). Of these isolates, six showed sensitivity to pyraclostrobin. Unfortunately, 11 and 6 isolates exhibited resistance to chlorothalonil and mancozeb, respectively. Furthermore, both pyraclostrobin and mancozeb exhibited similar sensitivity phenotypes in mycelial growth and spore germination inhibition across all tested isolates. Notably, difenoconazole had higher EC_{50} values (74.42–316.75 µg/mL) to spore germination inhibition. Pyraclostrobin, difenoconazole and mancozeb effectively controlled disease severity in sensitive isolates on detached durian leaves. In contrast, these fungicides had no effect on the fungicide-resistant isolates.
Main finding: A population of *Colletotrichum* resistant to pyraclostrobin, chlorothalonil and mancozeb was identified in durian orchards. Fortunately, the pathogen remains susceptible to two other fungicides (difenoconazole and prochloraz). Thus, these latter two fungicides should be recommended for use or rotation with other mode-of-action fungicides in the anthracnose disease management program for durians in eastern Thailand.

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Introduction

Durian (*Durio zibenthinus* Murr.), belonging to the family Malvaceae, is one of the most popular and exceptionally high-value fruit crops globally, with Thailand's dominance in durian production and exports being exceptional—in 2023, more than 90% of global durian exports were supplied by Thailand (Food and Agriculture Organization, 2023). Thailand's durian cultivation is concentrated in the eastern region of the country, including Chanthaburi, Rayong and Trat Provinces. However, anthracnose disease, caused by species of the fungus *Colletotrichum*, is a major concern. This pathogen can infect durian leaves during all growth stages, resulting in spots that gradually expand and merge, eventually causing the affected leaves to lose their photosynthetic capacity. Currently, limited studies on fungicides have been used to inform the management of durian anthracnose disease in the field. Only Namsawang et al. (2019) reported that the fungicide pyraclostrobin could effectively control post-harvest anthracnose in durians. Furthermore, *Colletotrichum* species are also the main pathogen causing anthracnose diseases in several economically important crops worldwide, such as chili, mango, strawberry, pear, peach, soybean and olive (Tashiro et al., 2019; Wu et al., 2019; Kongtragoul et al., 2020; Usman et al., 2022; Shi et al., 2023).

Several classes of fungicides have been approved and registered for controlling diseases caused by *Colletotrichum* species, including DeMethylation Inhibitors (DMIs), Quinone outside Inhibitors (QoIs) and multi-site fungicides such as mancozeb, organic sulphur and chlorothalonil (Moral et al., 2018; Piccirillo et al., 2018; Dowling et al., 2020; Gao et al., 2021). Unfortunately, prolonged application of the same fungicide leads to the development of resistant fungal populations, resulting in disease control failure. Notably, many studies have demonstrated differences in fungicide resistance among *Colletotrichum* species. For example, Nalumpang et al. (2010) reported carbendazim resistance in *C. gloeosporioides* causing mango anthracnose in Thailand, with resistant isolates showing point mutations at the beta-tubulin gene related to this fungicide's mode of action. Recently in Thailand, reports have documented fungicide resistance across diverse species of *Colletotrichum* affecting various crops. For example, *C. gloeosporioides* in mangoes, *C. scovillei* in chilies and *C. siamense* in oranges were resistant to the QoI fungicide azoxystrobin, the DMI fungicide carbendazim and the multi-site fungicide mancozeb (Kongtragoul et al., 2020; Kongtragoul et al., 2022). Additionally, *C. truncatum*, isolated from soybean anthracnose, has shown insensitivity to several fungicides, including carbendazim, azoxystrobin, difenoconazole and penthiopyrad (Poti et al., 2024).

Besides Thailand, fungicide resistance in *Colletotrichum* species has been reported in other countries worldwide. In China, several studies have documented extensive fungicide resistance across various modes of action in *Colletotrichum* species in many plantation areas. These studies have highlighted resistance to different classes of fungicides, including QoI fungicides (azoxystrobin, picoxystrobin and pyraclostrobin), DMI fungicides (difenoconazole, flusilazole and tebuconazole), Methyl Benzimidazole Carbamates fungicides (carbendazim and thiabendazole), Succinate Dehydrogenase Inhibitor fungicides (boscalid and penthiopyrad) and multi-site fungicides such as mancozeb (Wu et al., 2019; Liang et al., 2020; Wang et al., 2020; Wei et al., 2020; Shi et al., 2021; Ishii et al., 2022; Shi et al., 2023). Countries, including Japan, India and Spain, have reported the emergence of fungicide-resistant *Colletotrichum* species to various fungicides that include carbendazim, copper oxychloride, propiconazole, thiophanate-methyl and trifloxystrobin (Chung et al., 2006; Kumar et al., 2007; Moral et al., 2018). However, there has been no reported information regarding fungicide resistance in *Colletotrichum* species causing anthracnose in durian.

Therefore, it is necessary for durian anthracnose management to evaluate resistance to current fungicides to aid in the control of *Colletotrichum* species in Thailand. The objectives of the current research were: 1) to identify the *Colletotrichum* species causing durian anthracnose in eastern Thailand based on internal transcribed spacer (ITS) regions of rDNA sequences; 2) to evaluate the fungicide resistance of *Colletotrichum* isolates on culture medium; and 3) to investigate the efficiency of selected fungicides in controlling the disease on detached durian leaves. The outcomes of this research should make an important contribution to the effective control of durian anthracnose disease.

Materials and Methods

Pathogen isolation and pathogenicity test

Durian leaves showing anthracnose symptoms were collected from 10 durian orchards in eastern Thailand (Chanthaburi, Rayong and Trat Provinces). The tissue transplanting technique was used to isolate the fungal cultures. Briefly, the margins of infected and healthy tissues were

cut into 5 mm pieces, surface-sterilized using 1% sodium hypochlorite for 7 min and washed twice with sterile distilled water. Diseased tissues were cultured on potato dextrose agar (PDA; BD™ Difco Laboratories; 38800 Le Pont de Claix, France) Petri dishes and incubated at room temperature (25 ± 2°C) for 7 days. The mycelia cultured from diseased tissues were preserved on slant agar for pathogenicity testing. Briefly, a mycelial plug (5 mm in diameter, aged 7 d) of each isolate was inoculated on a fresh durian leaf in a moist plastic box. The inoculated leaves were incubated at room temperature (25 ± 2°C) for 7 d. All isolates were assessed for disease severity using a 0 to 4 scale (slightly modified from Apithanasakulngeon et al., 2023): 0 = no symptoms; 1 = lesion size 1−5 mm; 2 = lesion size 6−10 mm; 3 = lesion size 11−20 mm; and 4 = lesion size >20 mm. Each symptomatic sample was re-isolated using the tissue transplanting technique. Subsequently, a single-spore isolation technique was used to purify the isolates, which were then preserved on PDA slants for further use.

Molecular identification

For DNA extraction, *Colletotrichum* mycelia of each isolate were transferred to malt broth medium and cultured on a shaker at 150 rpm for 2–3 d. The hyphal balls were transferred into 1.5 µL microcentrifuge tubes and added with 200 µL of solution A (0.1 M NaCl, 0.2 M sucrose, 0.01 M Ethylenediaminetetraacetic acid (EDTA) and 0.03 M Tris-HCl). All samples were ground using a polypropylene pestle and added with 200 µL of solution B (50 mM Tris-HCl, 50 mM EDTA and 2.5% sodium dodecyl sulfate). All tubes were incubated at 65°C for 30 min. After that, 192 µL of solution C (potassium acetate and acetic acid) was added, followed by 200 µL of a chloroform-to-isoamyl alcohol ratio of 24:1(Alexander et al., 2007), and mixed well. The mixture was centrifuged at 14,000 revolutions per minute (rpm) for 15 min to separate the genomic DNA and protein precipitation.

The genomic DNA of each isolate was amplified using polymerase chain reaction (PCR) based on the primer pair ITS1 (5’ TCCGTAGGTGAACCTGCGG 3’) and ITS4 (5’ TCCTCCGCTTATTGATATGC 3’), according to White et al. (1990). The DNA amplification was conducted in a PCR Thermal Cycler (MiniCycler PTC-150; 1145 Atlantic Avenue Alameda, CA 94501, USA). The procedure consisted of 35 cycles, with a temperature profile of initial denaturation at 95°C for 5 min, denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 5 min. After PCR, gel electrophoresis was carried out to visualize the amplification products on 1.5% agarose gel in 1.5x Tris-acetate EDTA buffer and stained with RedSafe™. The DNA sequences of the PCR products were analyzed by U2Bio Thailand Co., Ltd (Bangkok, Thailand). The sequencing results confirmed the species of each isolate using the nucleotide basic local alignment search tool (Nucleotide BLAST) in the National Center for Biotechnology Information on the GenBank database website (https://www.ncbi.nlm.nih.gov/).

Fungicides

The fungicides used were commercial formulations. Two classes of single-site fungicides were used: 1) QoIs, exemplified by pyraclostrobin (Seltima® 10 CS; BASF Espanola S.L.; Barcelona, Spain), and DMIs, represented by difenoconazole (Kenji® 25 EC; I C P Ladda Intertrade Co. Ltd.; Nakhon Pathom, Thailand) and prochloraz (Garratt® 45 EW; I C P Ladda Intertrade Co. Ltd.; Nakhon Pathom, Thailand). Also used were the multi-site fungicides: chlorothalonil (Chlorothalonil® 75 WP; Limin Chemical Co., Ltd.; Zhejiang, China) and mancozeb (Top Gun® 80 WP; I C P Ladda Intertrade Co. Ltd.; Nakhon Pathom, Thailand). These fungicides were selected based on their current usage for suppressing durian anthracnose disease in Thailand (Table 1). For the QoI experiment, the reagent salicylhydroxamic acid (SHAM, 99%; Alfa Aesar®; Ward Hill, MA 01835, USA) was dissolved in an acetone-to-methanol ratio of 1:1 to create the stock solution for inhibiting the alternative respiration pathway of the pathogens (Kumar et al., 2020). Normally, when testing a QoI Class fungicide, SHAM must always be mixed in the control treatment plate.

Table 1 Fungicides currently used for controlling anthracnose disease in durian orchards in Thailand

Fungicide	Recommended concentration (µg/mL)	Fungicide class	Mode of action/target site	FRAC group
Pyraclostrobin (10% w/v SC)	1,500	Quinone outside Inhibitor	Complex III cytochrome bc1 at the QoI site	11
Difenoconazole (25% w/v EC)	1,000	DeMethylation Inhibitor	Sterol biosynthesis in membranes	3
Prochloraz (45% w/v EC)	1,000	DeMethylation Inhibitor	Sterol biosynthesis in membranes	3
Chlorothalonil (75% WP)	1,000	Chloronitrile	Multi-site contact activity	M05
Mancozeb (80% WP)	2,000	Dithiocarbamate	Multi-site contact activity	M03

FRAC = Fungicide Resistance Action Committee, sourced from Fungicide Resistance Action Committee (2018); w/v = weight per volume.

Fungicide sensitivity tests for mycelial growth of Colletotrichum species

The poisoned food technique was used to evaluate the sensitivity of the mycelial growth of the 15 *Colletotrichum* isolates to the fungicides. Each fungicide was mixed with PDA medium at final concentrations of 0, 0.1, 1, 10, 100, and 1,000 µg/mL. Simultaneously, 100 µg/mL of SHAM were added to the QoI-amended PDA plate at each concentration, as well as to the corresponding control PDA plate. After that, a mycelial plug (0.5 cm diameter) of each *Colletotrichum* isolate was cut from the actively growing colony margin and transferred to the center of the prepared PDA plates. The plates were incubated at room temperature (25 ± 2°C) for 7 d and the colony diameter of the pathogen was recorded based on measurements along two perpendicular directions to the colony perimeter. This experiment was conducted with four replicates. The percentage mycelial growth inhibition was calculated using the formula described in Tongsri et al. (2023) and shown in Equation 1:

$$\text{Mycelial growth inhibition (\%)} = (D_c - D_t) / D_c \quad (1)$$

where: D_c is the colony diameter of the control plates and D_t is the colony diameter of fungicide-amended plates, both in centimeters.

The values for the effective concentration to inhibit mycelial growth by 50% (EC_{50}) were investigated based on probit analysis in the SPSS Statistics 21.0 software (IBM Corp.; Redmond, WA, USA). The EC_{50} value of each isolate was categorized according to the level of fungicide sensitivity (Table 2).

Sensitivity tests for spore germination of Colletotrichum species to selected fungicides

Five representative *Colletotrichum* isolates (that had produced abundant spores and demonstrated sensitivity to three different modes of action fungicides in the mycelial growth assay) were selected for the spore germination test. A 500 µL sample of spore suspension (1×10^6 spores/mL) of each selected isolate was mixed with each selected fungicide in final concentrations of 0, 0.1, 1, 10, 100, or 1,000 µg/mL in a 1.5 mL test tube, mixed well and incubated at room temperature (25 ± 2°C) for 30 min. SHAM at 100 µg/mL was supplemented in each concentration of QoI fungicide and its control treatments. The mixture (30 µL) was dropped onto water agar plates, the spores were uniformly distributed using a sterile triangular glass rod, and then incubated at room temperature (25 ± 2°C) for 8 hr. Germinated spores (100 spores of each isolate) were counted under a light compound microscope. This experiment was conducted with three replicates and the EC_{50} values were analyzed. The recorded observation considered the length of the germ tube, which exceeded one-half the width of the spore. The percentage spore germination inhibition was calculated using Equation 2:

$$\text{Spore germination inhibition (\%)} = (G_c - G_t) / G_c \times 100 \quad (2)$$

where G_c is the number of germinated spores in the control treatment and G_t is the number of germinated spores in the fungicide treatment.

Sensitivity tests on detached durian leaves to selected fungicides

The three effective fungicides used in the spore germination test were evaluated for their efficacy in controlling disease caused by five *Colletotrichum* isolates on detached durian leaves. Healthy leaves were cut from trees of the durian cultivar ‘Monthong’ at 10 d after leaf expansion. The detached leaves were surface-sterilized in 1% sodium hypochlorite for 5 min, rinsed twice with sterile distilled water and dried. Then, separate leaf samples were soaked in each tested fungicide at the recommended concentration (Table 1) for 5 min. The control treatment involved immersing the leaves in distilled water instead of using a fungicide. After drying, the treated leaves were punctured with a sterilized needle.

Table 2 Baseline sensitivity of fungicides for mycelial growth of *Colletotrichum* spp. based on other published research.

Fungicide	Phenotype (EC_{50}, µg/mL)			References
	Sensitive	Moderately resistant	Resistant	
Pyraclostrobin	<50	50–100	>100	Usman et al. (2022)
Difenoconazole	<50	50–100	>100	Zhang et al. (2017)
Prochloraz	<50	50–100	>100	Zhang et al. (2017)
Chlorothalonil	<80	80–120	>120	Moral et al. (2018)
Mancozeb	<80	80–120	>120	Zhang et al. (2017)

EC_{50} = effective concentration to inhibit mycelial growth by 50%.

Then, a mycelial plug (5 mm in diameter, aged 7 d) of each isolate was inoculated onto each leaf at the wound site (two wound sites per leaf). Inoculated leaves were incubated in moist chambers at room temperature (25 ± 2°C) for 5 d. This experiment was conducted with five replicates. The lesion sizes were recorded and the percentage disease reduction was calculated using Equation 3:

$$\text{Disease reduction (\%)} = (L_c - L_t) / L_c \times 100 \quad (3)$$

where L_c is the lesion size in the control treatment and L_t is the lesion size in the fungicide treatment.

Results

Pathogen isolation, pathogenicity test, and molecular identification

In the orchard, anthracnose disease on durian leaves exhibits water-soaked margins, enlarged lesions, turning almost completely brown, and producing a large number of acervuli with a nested circle pattern, which differ from those observed in artificial inoculation, where typically the lesions are more circular (Peralta-Ruiz et al., 2023). However, when performing re-isolation, the recovered fungus exhibited colony characteristics and spore morphology identical to the original ones initially isolated (Fig. 1).

The colony characteristics of the five representative fungal isolates causing anthracnose disease on the PDA medium had distinct morphological features. Their conidial shapes were similar, and the conidium lengths were in the range 10.68–15.71 µm (Table 3 and Fig. 2).

The pathogens were classified using sequencing and nucleotide BLAST analysis based on the conserved ITS region of the ITS1/ITS4 primers. All 15 isolates were identified as belonging to the genus *Colletotrichum*. Most of the isolates (11 out of 15) were identified as *C. gloeosporioides*, with two other isolates (Ch_Tm06 and Tr_Mu05) being identified as *C. siamense* and the remaining two isolates (Ry_Kl04 and Ch_Kh02) being identified as *Colletotrichum* sp.—however, these latter two could not be identified to the species level based on the ITS sequences alone. The ITS sequences of all isolates were submitted to the GenBank database under the accession numbers PP808952–PP808966 (Table 4).

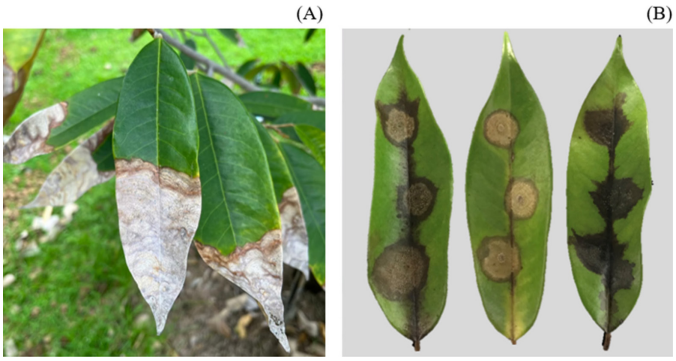

Fig. 1 Anthracnose disease on durian leaves: (A) advanced symptoms occurring in orchards; (B) circular symptoms resulting from artificial inoculation.

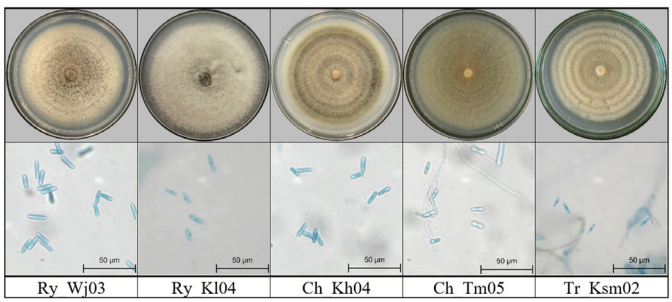

Fig. 2 Culture and conidium morphology of five representative *Colletotrichum* isolates collected from durian leaf anthracnose.

Table 3 Morphological characteristics of five representative *Colletotrichum* species derived from durian anthracnose.

Isolate	Colony characteristics	Conidial shape and length[1]
Ry_Wj03	Mycelium thick; darker concentric zone margin of colony regular; orange conidial mass	Cylindrical; straight; nonseptate; hyaline; a few slightly curved; base truncate; 15.71 ± 1.62 µm
Ry_Kl04	Mycelium thick and fluffy; colony white at first; becomes grayish overtime	Cylindrical; straight; nonseptate; hyaline; gradually tapered to each end; 14.95 ± 2.24 µm
Ch_Kh04	Mycelium moderate; at first colony white and becoming light gray; pinkish-to-grayish conidial mass	Cylindrical; straight; nonseptate; hyaline; gradually slight curved; ends broadly rounded; 14.63 ± 1.82 µm
Ch_Tm05	Mycelium thin; dull yellow toward the center; yellowish conidial mass across the colony	Cylindrical; straight; apex obtuse; nonseptate; hyaline; base truncate; 12.1 ± 1.51 µm
Tr_Ksm02	Mycelium moderate; colony pinkish-orange; yellowish conidial mass across the colony	Short; straight; nonseptate; hyaline; fusiform with obtuse to slightly rounded ends; 10.68 ± 2.05 µm

[1] = values (± SE) for length of conidia based on at least 20 conidia.

Table 4 Location, disease severity and GenBank accession number of 15 *Colletotrichum* isolates used in this study.

Isolate name	Location	Disease severity score	Species	Accession no.
Ry_Wj03	Rayong	3	C. gloeosporioides	PP808952
Ry_Wj04	Rayong	4	C. gloeosporioides	PP808953
Ry_Kl02	Rayong	3	C. gloeosporioides	PP808954
Ry_Kl04	Rayong	3	*Colletotrichum* sp.	PP808955
Ch_Kh02	Chanthaburi	4	*Colletotrichum* sp.	PP808956
Ch_Kh04	Chanthaburi	4	C. gloeosporioides	PP808957
Ch_Po02	Chanthaburi	3	C. gloeosporioides	PP808958
Ch_Tm05	Chanthaburi	3	C. gloeosporioides	PP808959
Ch_Tm06	Chanthaburi	4	*C. siamense*	PP808960
Ch_Tm08	Chanthaburi	4	C. gloeosporioides	PP808961
Ch_Ki05	Chanthaburi	4	C. gloeosporioides	PP808962
Tr_Mu03	Trat	3	C. gloeosporioides	PP808963
Tr_Mu05	Trat	4	*C. siamense*	PP808964
Tr_Br02	Trat	3	C. gloeosporioides	PP808965
Tr_Ksm02	Trat	4	C. gloeosporioides	PP808966

Location = provinces in Thailand.

Disease severity: 0 = no symptoms; 1 = lesion size 15 mm; 2 = lesion size 6–10 mm; 3 = lesion size 11–20 mm; 4 = lesion size >20 mm (slightly modified from Apithanasakulngeon et al., 2023).

Fungicide sensitivity tests for mycelial growth of Colletotrichum species

The EC_{50} values and phenotype classification for mycelial growth inhibition of the 15 *Colletotrichum* isolates against different fungicides are shown in Tables 5 and 6. The results revealed that the fungal isolates responded differently to the three tested fungicides (pyraclostrobin, chlorothalonil and mancozeb). With pyraclostrobin, the fungi had EC_{50} values in the range 0.09 µg/mL to >100 µg/mL. In addition, 6 out of the 15 isolates were sensitive to pyraclostrobin, while the remaining isolates showed moderate resistance (1 isolate) or were resistant (8 isolates). In the multi-site fungicide experiment, the EC_{50} values of chlorothalonil and mancozeb were <80 µg/mL, which were categorized as sensitive for 4 and 9 isolates, respectively. The remaining isolates were resistant to both fungicides, with EC_{50} values >120 µg/mL. Fortunately, all isolates of the pathogens had sensitivity toward the DMI fungicides (difenoconazole and prochloraz), with EC_{50} values in the range 0.02–45.5 µg/mL.

Table 5 EC_{50} values of mycelial growth inhibition for 15 *Colletotrichum* isolates against five different fungicides.

Isolate	EC_{50} (µg/mL) for mycelial growth inhibition				
	Pyraclostrobin	Chlorothalonil	Mancozeb	Difenoconazole	Prochloraz
Ry_Wj03	0.59	>120	2.77	0.03	0.01
Ry_Wj04	>100	>120	>120	0.39	0.15
Ry_Kl02	34.45	>120	11.87	6.72	0.14
Ry_Kl04	>100	>120	>120	13.71	0.14
Ch_Kh02	0.09	0.69	>120	45.5	0.29
Ch_Kh04	61.68	3.66	2.54	12.09	0.24
Ch_Po02	>100	>120	>120	5.55	0.14
Ch_Tm05	46.26	>120	9.62	3.87	0.29
Ch_Tm06	>100	>120	23.05	0.47	0.32
Ch_Tm08	>100	>120	>120	0.13	0.07
Ch_Ki05	>100	22.8	27.03	0.02	0.24
Tr_Mu03	>100	>120	11.61	10.98	0.1
Tr_Mu05	>100	>120	19.36	0.14	0.01
Tr_Br02	33	>120	>120	1.03	0.07
Tr_Ksm02	0.16	1	15.08	26.69	0.42

EC_{50} = concentration of fungicide required to inhibit mycelial growth of fungus by 50%.

Table 6 Phenotype classification of five different fungicides regarding their effect on mycelial growth of 15 *Colletotrichum* isolates.

Isolate	Phenotype				
	Pyraclostrobin	Chlorothalonil	Mancozeb	Difenoconazole	Prochloraz
Ry_Wj03	S	R	S	S	S
Ry_Wj04	R	R	R	S	S
Ry_Kl02	S	R	S	S	S
Ry_Kl04	R	R	R	S	S
Ch_Kh02	S	S	R	S	S
Ch_Kh04	MR	S	S	S	S
Ch_Po02	R	R	R	S	S
Ch_Tm05	S	R	S	S	S
Ch_Tm06	R	R	S	S	S
Ch_Tm08	R	R	R	S	S
Ch_Ki05	R	S	S	S	S
Tr_Mu03	R	R	S	S	S
Tr_Mu05	R	R	S	S	S
Tr_Br02	S	R	R	S	S
Tr_Ksm02	S	S	S	S	S

S = sensitive; MR = moderately resistant; R = resistant.

Sensitivity tests for spore germination of Colletotrichum species to selected fungicides

Fungicides (pyraclostrobin, mancozeb and difenoconazole), each with a different mode of action, were selected for spore germination inhibition testing on five representative isolates (Table 7). With pyraclostrobin, the fungi had a wide range of EC_{50} values (0.05–907.75 µg/mL), with three isolates (Ry_Wj03, Ch_Tm05 and Tr_Ksm02) with outstandingly low EC_{50} values (0.05–5.59 µg/mL). These low values suggested a high sensitivity to pyraclostrobin, which correlated with their mycelial growth inhibition test results (sensitive to mycelial growth). The remaining two isolates (Ry_Kl04 and Ch_Kh04) demonstrated resistance to this fungicide, with high EC_{50} values (>100 µg/mL) that was consistent with their performance in the mycelial growth inhibition tests (resistant to mycelial growth).

Table 7 EC_{50} values of spore germination inhibition for five *Colletotrichum* isolates against three different fungicides.

Isolate	Mycelial growth test and EC_{50} (µg/mL) for spore germination inhibition					
	Pyraclostrobin		Difenoconazole		Mancozeb	
Ry_Wj03	S^{My}	0.05	S^{My}	0.02	S^{My}	1.52
Ry_Kl04	R^{My}	120.79	S^{My}	158.54	R^{My}	362.64
Ch_Kh04	R^{My}	907.75	S^{My}	316.75	S^{My}	46.35
Ch_Tm05	S^{My}	5.59	S^{My}	227.16	S^{My}	50.47
Tr_Ksm02	S^{My}	0.14	S^{My}	74.42	S^{My}	13.95

Result of mycelial growth inhibition testing: S^{My} = sensitive to mycelial growth; R^{My} = resistant to mycelial growth.

In the difenoconazole test, only the Ry_Wj03 isolate showed susceptibility to the fungicide, with a very low EC_{50} value of 0.02 µg/mL that was consistent with its result from the mycelial growth inhibition test (sensitive to mycelial growth). Most isolates had high EC_{50} values (74.42–316.75 µg/mL), indicating their resistance to difenoconazole. However, these results contrasted with their performance in the mycelial growth inhibition tests (sensitive to mycelial growth).

In the mancozeb test, the EC_{50} values covered a wide range (1.52–362.64 µg/mL). Only the Ry_Kl04 isolate showed potential resistance to spore germination inhibition, with a high EC_{50} value (362.64 µg/mL), contrary to its result in the mycelial growth inhibition test (sensitive to mycelial growth).

Sensitivity tests on detached durian leaves to selected fungicides

Three fungicides (pyraclostrobin, difenoconazole and mancozeb) were tested for disease control of the five representative *Colletotrichum* isolates on detached durian leaves (Table 8; Fig. 3). In tyraclostrobin showed good efficacy against the three fungicide-sensitive isolates (Pyr^S; Ry_Wj03, Ch_Tm05, and Tr_Ksm02), achieving reductions in the range 73.4–100%. However, it did not effectively control the two fungicide-resistant isolates (Pyr^R; Ry_Kl04 and Ch_Kh04).

Based on the efficacy test of difenoconazole, 4 out of the 5 tested *Colletotrichum* fungicide-susceptible isolates (Dif^S; Ry_Wj03, Ry_Kl04, Ch_Kh04 and Ch_Tm05) were greatly suppressed, with suppression levels in the range 75.0–93.5%. Unfortunately, the remaining single fungicide-sensitive isolate (Dif^S; Tr_Ksm02) had much lower control (40% reduction).

Table 8 Reduction in anthracnose disease severity on detached durian leaves after individual soaking in three different fungicides at 7 d after inoculation.

Isolate	Disease severity reduction (%)		
	Pyraclostrobin (1,500 µg/mL)	Difenoconazole (1,000 µg/mL)	Mancozeb (2,000 µg/mL)
Ry_Wj03	$Pyr^{S/}$ 86.3	Dif^{S} 84.9	Man^{S} 48.9
Ry_Kl04	Pyr^{R} -106.0[2/]	Dif^{S} 93.5	Man^{R} -1.7
Ch_Kh04	Pyr^{R} 27.2	Dif^{S} 81.5	Man^{S} 80.6
Ch_Tm05	Pyr^{S} 73.4	Dif^{S} 75.0	Man^{S} 15.1
Tr_Ksm02	Pyr^{S} 100	Dif^{S} 40.0	Man^{S} 45.2

Fungicide names with superscript S = susceptible to that fungicide and with superscript R = resistant to that fungicide.
Minus sign (-) indicates smaller lesion size in control treatment than in fungicide treatment.

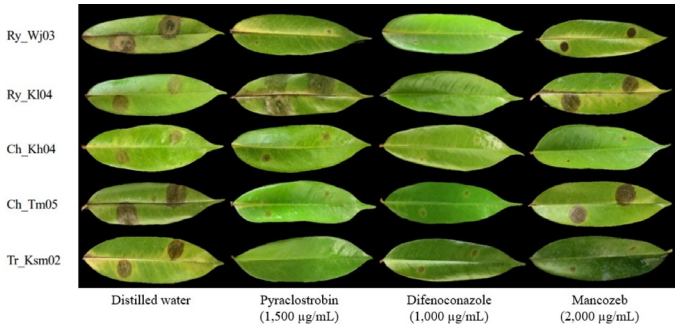

Fig. 3 Disease symptom characteristics on representative detached durian leaves after treatments with different fungicides at recommended concentrations.

With mancozeb, Ch_Kh04, a fungicide-sensitive isolate (Man^{S}), had the best control among the tested isolates, achieving an 80.6% reduction in disease severity. However, three isolates (Ry_Wj03, Ch_Tm05 and Tr_Ksm02), which were also fungicide-sensitive, had much lower effective control percentages (15.1–48.9%). Specifically, the Ry_Kl04 isolate labeled as Man^{R} (mancozeb-resistant), did not show any effects of being inhibited when treated with mancozeb.

Discussion

Anthracnose disease, caused by *Colletotrichum* spp., poses major challenges to cultivating durian in all growing areas, impacting both their yield and quality. In the current study, the causal agents of durian anthracnose were predominantly identified as *C. gloeosporioides*, based on the analysis of nucleotide sequences from the ITS regions of rDNA. Sutthisa et al. (2024) provided additional confirmation based on morphological features that the genus *Colletotrichum* was indeed a causative agent of durian leaf anthracnose in Thailand. Additionally, Tongsri et al. (2023) added support to the identification of *C. gloeosporioides*, focusing on morphological analysis, as a causative agent of durian fruit anthracnose in eastern Thailand. Furthermore, Armand et al. (2023) provided molecular identification of several *Colletotrichum* species associated with durian fruit rot in northern Thailand, including *C. durionigenum*, *C. gigasporum*, *C. pandanicola* and *C. truncatum*.

The current study highlighted that isolates of *Colletotrichum* associated with durian had varying sensitivity to the three tested fungicides (pyraclostrobin, chlorothalonil and mancozeb). Notably, pyraclostrobin showed resistance to the mycelial growth of most of the tested *Colletotrichum* isolates, which was consistent with findings from other studies by Ali et al. (2019), Rogério et al. (2022), Usman et al. (2022), which reported resistance in various *Colletotrichum* species such as *C. gloeosporioides*, *C. fructicola*, and *C. truncatum*. The resistance mechanism to pyraclostrobin involves a specific mutation in the *cytochrome b* at the G143A mutation point, which is the binding site for QoI fungicides, with the presence of the G143A mutation preventing the binding of pyraclostrobin to the cytochrome b protein, thereby conferring resistance to the fungicide (Hu et al., 2015; Cortaga et al., 2023). Supporting this, Hu et al. (2023) discovered that *Colletotrichum* isolated from strawberry anthracnose also showed resistance to pyraclostrobin associated with the G143A mutation. This resistance mechanism led to cross-resistance to other QoI fungicides such as azoxystrobin and kresoxim-methyl. Resistance to QoI fungicides has been documented in several studies involving other fungal pathogens such as *Corynespora cassiicola*, *Pyricularia oryzae*, *Pyrenophora tritici-repentis* and *Magnaporthe oryzae* (Li et al., 2021; Sautua and Carmona, 2021; D'Ávila et al., 2022; Peng et al., 2022).

Based on the current results, the pyraclostrobin-sensitive phenotype against mycelial growth was aligned with low EC_{50} values for spore germination inhibition of *Colletotrichum* representative isolates and vice versa. These findings agreed with those reported by Gao et al. (2017), who observed that pyraclostrobin remained effective against mycelial growth and spore germination of *C. acutatum* causing chili anthracnose. In contrast, other QoI fungicides, such as azoxystrobin and picoxystrobin, were unable to effectively suppress *Colletotrichum* growth. In another study consistent with these findings, Kumar et al. (2020) showed that picoxystrobin,

which belongs to the QoI fungicides, exhibited resistance in terms of inhibiting spore germination of *Colletotrichum* species.

Fortunately, all 15 isolates of *Colletotrichum* tested in the current study had high susceptibility to mycelial growth inhibition when treated with DMI fungicides such as difenoconazole and prochloraz. The current study provided initial documentation of DMI fungicide sensitivity in *Colletotrichum* species responsible for durian anthracnose in Thailand. The results indicated difenoconazole and prochloraz consistently suppressed the mycelial growth of these fungi, demonstrating their effectiveness. Several reports have confirmed that many species of *Colletotrichum* displayed sensitivity in mycelial growth to various fungicides in the DMI class, including difenoconazole, prochloraz, epoxiconazole, prothioconazole and mefentrifluconazole (Zhang et al., 2020; Ishii et al., 2022; Mello et al., 2023). However, it has been observed that both *C. truncatum* and *C. gloeosporioides*, which cause chili anthracnose, exhibited resistance to the DMI fungicide tebuconazole. Additionally, these two fungi have shown positive cross-resistance to other DMI fungicides such as difenoconazole and propiconazole (Zhang et al., 2017; Wei et al., 2020). Furthermore, based on the results of the current study, notably, despite the isolates being sensitive to fungicides when tested using the mycelial growth inhibition assay, difenoconazole did not uniformly inhibit spore germination across all isolates. This situation may be described as the spore germination being more tolerant to the fungicide than mycelial growth (Chiocchio et al., 2000). As a result, for effective control of anthracnose disease using difenoconazole, it may be advisable to apply the fungicide prior to sporulation of the fungi. The mechanism of DMI fungicides is to inhibit sterol biosynthesis by targeting the CYP51 gene, which is crucial for producing ergosterol in fungal cells (Zhang et al., 2020; Bian et al., 2021; Kumar et al., 2021). Variation in the CYP51 protein leads to DMI resistance in *Colletotrichum* spp. (Zhang et al., 2017; Chen et al., 2018; Wang et al., 2020; Wei et al., 2020).

Multi-site fungicides are commonly applied in spray programs to manage diseases in durian orchards, while it is widely recognized that it is important to apply rotating applications to prevent fungicide resistance and to extend the efficacy of single-site fungicides (Fungicide Resistance Action Committee, 2024). There have been limited published reports on the study of fungal resistance to multi-site fungicides, including *Colletotrichum*. In the current research, *Colletotrichum* populations in durian orchards showed resistance to both chlorothalonil and mancozeb in terms of mycelial growth. This finding was not surprising as Nianwichai et al. (2022) documented that another pathogen causing durian leaf blight and stem rots (*Phytophthora palmivora*) also displayed resistance to mancozeb, which was also consistent with other studies, indicating that *Colletotrichum* spp. have shown high resistance to multi-site fungicides such as mancozeb and copper oxychloride (Kumar et al., 2007; Moral et al., 2018).

In addition, the resistance of multi-site fungicides in other genera of fungi has been documented by several investigations. For example, *Alternaria* spp. were resistant to chlorothalonil and mancozeb, while *Septoria tritici* was resistant to chlorothalonil by the mechanisms related to the overexpression of thiols (Fairchild et al., 2013; Yang et al., 2019). Noticeably, in the current trial involving mancozeb, the isolate that exhibited resistance (based on the inhibition of hyphal growth) was also resistant to spore germination of a representative isolate. This would make it easier to decide whether to use mancozeb to control anthracnose in durians in eastern Thailand. However, it is necessary to investigate the sensitivity of mancozeb against spore germination in multiple isolates to ensure consistent inhibition of spore germination.

The three fungicides (pyraclostrobin, difenoconazole and mancozeb), used at their recommended concentrations in the current study, effectively controlled disease on detached durian leaves caused by fungicide-sensitive isolates by >70%. However, when tested against fungicide-resistant isolates of *Colletotrichum*, the disease severity could not be controlled. This finding aligned with several studies, with Kongtragoul et al. (2020) reporting that the QoI fungicide azoxystrobin was effective against fungicide-sensitive isolates of *Colletotrichum* sp. on mango fruit but ineffective against resistant isolates. Additionally, Shetty et al. (2014) demonstrated that difenoconazole greatly suppressed grape anthracnose caused by *C. gloeosporioides*, assuming the pathogen was sensitive to the fungicide. Furthermore, another report by Patrice et al. (2021) found that mancozeb-treated cashew plants had reduced anthracnose severity caused by *C. gloeosporioides*, suggesting the pathogen was susceptible to mancozeb. Notably, in the current research, some sensitive isolates of *Colletotrichum* showed less effective disease control by difenoconazole and mancozeb (15.1–48.9% disease reduction), which may have been due to variability in the age of the durian leaves used for testing, as younger leaves might be more susceptible to disease, as described in soybean rust (Xavier et al., 2017), or it could have been due to the sedimentation of fungicides during the soaking of the leaves. Furthermore, in the pyraclostrobin

treatment, the isolate Ry_Kl04 had more evidence of symptoms than the untreated control, potentially due to the leaf being in a weaker condition or the overexpression of fungal genes that enhanced its survival under stress conditions (Wei et al., 2020; Usman et al., 2020; Cortaga et al., 2023). Additionally, it could have caused more damage to the leaves. However, this fungicide's efficacy in controlling durian anthracnose disease should be further evaluated under plant conditions.

In conclusion, in durian orchards in eastern Thailand, *C. gloeosporioides* is a major anthracnose pathogen, with most of its tested isolates resisting both fungicides currently used (pyraclostrobin and chlorothalonil). Some of the tested *Colletotrichum* isolates had resistance to mancozeb. However, none of the isolates tested for mycelial growth inhibition exhibited insensitivity or resistance to both the DMI fungicides (difenoconazole, and prochloraz). Thus, both of these could be recommended in the disease management program for rotation with fungicides using other modes of action. Furthermore, alternative multi-site fungicides should be tested for sensitivity to replace chlorothalonil and mancozeb. Monitoring fungicide sensitivity is a critical component of effective disease control in durian orchards.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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