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Fungicide resistance of *Phytophthora palmivora* causing durian diseases in eastern and southern Thailand and the *in vitro* alternative control by cajeput leaf extracts

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Abstract The results showed that 24 and 25 of 40 collected isolates of *Phytophthora palmivora* were resistant to metalaxyl and mancozeb, respectively with the 50% effective concentration (EC_{50}) higher than 100 mg/L. Meanwhile, all isolates were resistant to fosetyl-al with EC_{50} higher than 1000 mg/L. Moreover, mapping between the survey areas and all *Phytophthora* isolates with the resistant levels to the 3 tested fungicides was depicted. Interestingly, 20 of 40 isolates showed multiple fungicide resistance to all tested fungicides with different modes of action, thereby selecting for the *in vitro* evaluation of the cajeput extract effect. Lower than expected, only 9 out of 20 multiple fungicide resistance isolates were shown to be significantly sensitive to the 10000, 20000 and 40000 ppm of cajeput extract based on paper disc assay.

Keywords: Fungicide resistance, *Phytophthora palmivora*, Cajeput extract, Durian rot disease, Alternative control

Introduction

Durian is one of the most important economic crops in Thailand, and it is mostly cultivated in the eastern and southern regions. However, the main problem of durian production in both regions is *Phytophthora palmivora*. It can infect all growth stages of the durian tree, which causes rot symptoms on roots, base stems, branches, and fruits (Department of Agriculture Thailand, 2018). Therefore, most growers in Thailand have selected different chemical fungicide groups, such as metalaxyl in phenylamides, fosetyl-al in phosphonates, and mancozeb in dithiocarbamates to control *P. palmivora* as well as to delay the occurring of fungicide resistance. However, using fungicides for a long time have many negative impacts which both directly and indirectly to farmers,

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consumers, and the environment. Furthermore, the fungicide resistance of plant pathogens may occur in orchards (Chung *et al*., 2006; Ishii, 2006; Kongtragoul, 2018; He *et al*., 2019; Kongtragoul *et al*., 2021; Puig *et al*., 2021). Moreover, some reports showed chemical fungicides failed to control diseases because the fungi may resist the fungicides. Recently, the fungicide resistance problem of plant pathogenic fungi has been increasing worldwide, especially with fungi that cause economic crop diseases, such as in China (He *et al*., 2019), Japan (Chung *et al*., 2006), America (Puig *et al*., 2021), and Australia (Vawdrey *et al*., 2015), Venezuela. (Ramdial *et al*., 2017). In Thailand, there are also reports of fungicide resistance that cause diseases in some economic crops. For example, Kongtragoul (2018) indicated that *P. palmivora* isolated from durian fruit rot in the Chumphon market area exhibited fungicides resistance of more than 30 percent of the isolated fungi. It was found that *P. infestan*s isolated from potatoes in northern Thailand showed metalaxyl resistance in 90% of all fungi isolated (Chiampiriyakul *et al*., 2011). In addition, there are quite a number of reports on fungicide resistance in some species of *Phytophthora* (Vawdrey *et al*., 2015; Puig *et al*., 2021; Elansky *et al*., 2007; Earnshaw and Shattock, 2012; Wang *et al*., 2013). From fungicide-resistance problems in the above-mentioned, alternative practices such as plant extract are one of a good choice for controlling plant pathogens exhibiting fungicide resistance and being safety and environmentally friendly.

Cajeput (*Melaleuca cajuputi*) is a medicinal plant and an important indigenous plant in southern Thailand. It has properties to relieve symptoms in the respiratory system. It can be applied externally to relieve aches, sprains, or even repel insects (Sutrisno *et al*., 2018). In the past, Thai villagers used the boiled leaves as a herbal remedy, especially for extracting them to make green oil. As a result, cajeput was pushed to be an important plant in Thailand to increase the potential for utilization and upgraded to another important economic tree in the south. In addition, only a few studies is reported that the essential oil from the leaves of the cajeput leaves was effective against *Alternaria* spp., *Fusarium* spp., and *Aspergillus* spp. (Bharat and Praveen, 2016; Pawar and Thaker, 2007; Thanaboripat *et al*., 2007) while the crude methanol extract from cajeput leaves effectively inhibited *Fusarium* sp., *Colletotrichum* sp., *Phytophthora* sp., and *Pythium* sp. (Montri *et al*., 2010).

Therefore, the research was aimed to survey, collect, identify and confirm pathogenicity of *Phytophthora* isolates causing durian diseases from the areas previously subjected to different fungicides pressure in eastern and southern Thailand, to determine the occurrences of the fungicide resistance, to map the multiple fungicide resistance of *Phytophthora* isolates, and to evaluate the

effect of the ethanolic extract from Cajeput leaf on the growth of multiple fungicide resistance *Phytophthora* sp. as an alternative control measure.

Materials and methods

Sampling, isolation, and identification of Phytophthora sp.

Fruit, stem, and root tissues of durian ("Monthong" variety) showing rot symptoms were collected from durian orchard areas using a chemical fungicide or ineffective chemical fungicide in eastern and southern parts of Thailand. Five locations within the distance of 5 km apart were assigned by the provincial agricultural extension officers. The geographic locations of the samplings were shown in Table 1. *Phytophthora* spp. were isolated from the disease tissue using baiting or tissue transplanting technique on V8-selective agar media (Jeffers and Martin, 1986; Ferguson and Jeffers, 1999) and maintained on V8 agar for further study.

The morphological identification of the *Phytophthora* species was based on IDphy: an international online resource in website https://idtools.org/id/phytophthora/results.php?terms=palmivora (Abad *et al*., 2011) and the published description (Drenth and Sendall, 2001). A basic key for *Phytophthora palmivora* is recorded as a colony with no distinctive pattern (uniform, radial, stellate, chrysanthemum and rosette), and variations in shapes of sporangia (caducous with short pedicel; globose, ovoid, obpyriform, ellipsoid and irregular shapes (27–70 L x 21–46 W μm) originated in simple sympodial sporangiophores.

Pathogenicity test of Phytophthora spp.

In addition to morphological identification, the pathogenicity of collected *Phytophthora* sp. was carried out by a detached durian leaf test in order to confirm and determine the level of ability of the collected pathogen. All isolates of *Phytophthora* sp. were cultured on V8 agar for 5 days. Then, six mycelial discs (5 mm diameter) were inoculated on the durian leaf ("Monthong" variety) with 5 replications. The inoculated durian leaves were incubated in a moist plastic box for 3 days. The diameter of the lesion was measured, and then disease severity was assessed. The disease severity was rated up to 5 levels of the lesion size as 0 = no symptom; 1= lesion size 1-5 mm; 2 = lesion size 6-10 mm; 3 = lesion size 11-20 mm; 4 = lesion size > 20 mm.

Determination of fungicide resistance in Phytophthora sp.

Three fungicides in this study (metalaxyl, mancozeb, and fosetyl-aluminum) that have the different modes of action were selected from the list of fungicides commonly used by durian orchard growers. The multiple fungicide-resistant assays were performed by analyzing mycelial growth on agar plates with responsible for resistance to all three fungicides.

Metalaxyl (35% a.i.) and mancozeb (80% a.i.) were tested at 0, 0.1, 1, 10 and 100 mg/mL while fosetyl-Al (80% a.i.) was tested at 0, 0.1, 1, 10, 100 and 1000 mg/mL. The molten V8 agar was amended with each of the tested fungicides at a desired final concentration and poured into a 9 cm diameter plate. Then, a 5 mm diameter of mycelial disc of *Phytophthora* sp. was placed on the prepared V8 agar plate. V8 agar without the tested fungicides was used as a control. All tested plates were incubated at room temperature (30±3 ℃) for 5 days. After that, all colony diameters of tested *Phytophthora* sp. were measured, and the percentage of mycelial growth inhibition were calculated using the following formula as mean of control diameter - mean of treatment diameter/mean of control diameter ×100.

To determine the fungicide-resistance level, the growth inhibition percentages of each isolate *Phytophthora* sp. were plotted as probit versus the $\log_{10}$ of the fungicide concentration (mg/mL) and analyzed by linear regression. The regression equation was used to appraise an EC_{50} to inhibit the mycelial growth of each isolate. Then, the EC_{50} value of each *Phytophthora* sp. isolate was determined for fungicide-resistant levels. Metalaxyl and mancozeb were sensitive :S (EC_{50} < 1 mg/mL), moderately resistant :MR (EC_{50} 1 – 100 mg/mL), resistant :R (EC_{50} > 100 mg/mL). Whereas fosetyl-Al was sensitive :S (EC_{50} < 10 mg/mL), moderately resistant :MR (EC_{50} 10 – 1000 mg/mL) and resistant :R (EC_{50} > 1000mg/mL). Furthermore, the isolates of *Phytophthora* sp. were classified as multiple fungicide resistant fungi with R level to 2 or 3 tested fungicides.

Effect of cajeput ethanolic extract on Phytophthora sp. exhibiting multiple fungicide resistance

Multisite fungicides play an increasing role in spray programs to prevent or delay the fungicide resistance, however multiple fungicide resistance could probably emerge from this situation. To control the multiple fungicide resistance fungi by an alternative measure, the experiment was conducted to determine the effects of 3 concentrations (10000, 20000, and 40000 ppm) of cajeput ethanolic extract on the mycelial growth of multiple fungicide resistant

Phytophthora sp. by paper disc diffusion method. The 3 tested concentrations were within the same range of concentrations commonly used for other plant crude extract by other researchers (Mostafa *et al*., 2018; Abah and Egwari, 2011; Gonelimali *et al*., 2018). The isolates of *P. palmivora* that resisted to all 3 tested fungicides (R level) were screened as multiple fungicide resistance strains to be used in this experiment.

For plant extract preparation, *Melaleuca cajuputi* (cajeput) leaves were collected in Chumphon province, Thailand. Plant leaves were washed with tap water and dried in the open air. The leaves were then dried in hot air oven (Memmert, Germany) at 50℃. A blender was used to grind the dried leaf. The Soxhlet method was used to extract the ground leaves. The ethanolic solvent of obtained crude extract was removed by a rotary evaporator (Buchi, Brazil). Then the ethanolic crude extract of *Melaleuca cajuputi* were kept in the refrigerator at 4 ℃for further study.

The *Phytophthora* sp. isolates showing multiple fungicide resistance were cultured on V8 agar for 5 days. Then, a mycelial disc (5 mm diameter) was cut at margin of colony and inoculated at the center of a new V8 agar plate. Soon after, four sterilized paper discs were placed around the mycelial disc with 2 cm apart. The 3 concentrations of cajeput extract were diluted with 10% of tween20 for the final concentrations of 10000, 20000, and 40000 ppm, and then 30 µL of each concentration was dropped on the paper disc. The 10% of tween20 without the extract was used as the control. A randomized completely block design (RCBD) with 3 replications was carried out in this experiment. All tested plates were incubated at room temperature (30±3 ℃). The radius of mycelial growth of *Phytophthora* was measured. The percentage of inhibition effect was calculated as following the formula: inhibition effect (%) = radius of control treatment – radius of extract treatment/ radius of control treatment × 100.

Results

Isolation and identification of Phytophthora sp.

A total of 40 isolates were isolated from symptomatic tissue of durian in the eastern and southern parts of Thailand. In the eastern, 6 and 14 isolates were found in Trat and Chanthaburi, respectively, whereas 20 isolates were found in Chumphon, the southern part (Table 1). Then, the morphological characteristics were used for identification and divided into 2 groups. First group included 5 isolates showing cottony mycelial colony, ellipsoid and obpyriform sporangium (27-30 × 39-60 µm) with semi-papillae. Second group comprised 35 isolates

having slightly petaloid colony, ovoid and limoniform sporangium (size of 26-33 ×40-58 um) with papillae.

Table 1. Source of *Phytophthora* spp. causing "Monthong" durian disease

Province	District	Location No.	GPS	Isolate
Trat	Muang	Tr2	12º7'11" N; 102º41'27" E	Tr2-F10, Tr2-F11
		Tr3	12º8'14"N; 102º40'38"E	Tr3-F1, Tr3-F2, Tr3-F4, Tr3-F5
Chanthaburi	Khlung	Ch1	12º32'22"N; 102º16'34"E	Ch1-F1, Ch1-F2, Ch1-F3, Ch1-F5, Ch1-F6
		Ch2	12º31'59" N; 102º15'42" E	Ch2-F1, Ch2-F2, Ch2-F3, Ch2-F4, Ch2-F5
		Ch3	12º31'33" N; 102º15'12" E	Ch3-F5
		Ch4	12º32'15" N; 102º16'15" E	Ch4-F4
		Ch5	12º33'00" N; 102º16'0" E	Ch5-F4
	Nayaiam	Ch6	12º41'17" N; 101º55'51" E	Ch6-F5
Chumphon	Thasae	Ch1	10º50'09.7" N; 99º06'49.3" E	Cp1-F8, Cp1-F10, Cp1-F14, Cp1-F17, Cp1-F20, Cp1-F23, Cp1-F24, Cp1-F27, Cp1-F28
	Thasae	Ch2	10º52'31" N; 99º9'6" E	Cp2-F31, Cp2-F33, Cp2-F35, Cp2-F38, Cp2-F39, Cp2-F40, Cp2-F41, Cp2-F44, Cp2-F47, Cp2-F49, Cp2-F55

Pathogenicity test of Phytophthora sp.

Pathogenicity of all *Phytophthora* spp. isolates (40 isolates) were conducted by detached "Monthong" durian leaf test. Disease severity was categorized into 5 levels of lesion scores. The result showed that all the collected isolates of *Phytophthora* spp. were proven to be the causal agents of durian disease (disease severity score 1-4). Most of the eastern and southern isolates caused the disease severities which fell into the category of lesion score 2, and followed by score 3. Meanwhile, we found 4 isolates only from eastern part causing lesion score 4 (Table 3).

Table 2. Morphological characteristics of isolated *Phytophthora* sp. causing "Monthong" durian disease

Isolate	Colony pattern	Sporangium			Morphological identification
		Shape	Width × Length (µm)	Papillae	
Ch4-F4, Ch5-F4, Ch6-F5, Tr3-F5, Cp2-F49	cottony	obpyriform, basal plug conspicuous	27-30 × 39-60	semi-papillate	*P. palmivora*
Ch1-F1, Ch1-F2, Ch1-F3, Ch1-F5, Ch1-F6, Ch2-F1, Ch2-F2, Ch2-F3, Ch2-F4, Ch2-F5, Ch3-F5, Tr2-F10, Tr2-F11, Tr3-F1, Tr3-F2, Tr3-F4, Cp1-F8, Cp1-F10, Cp1-F14, Cp1-F17, Cp1-F20, Cp1-F23, Cp1-F24, Cp1-F27, Cp1-F28, Cp2-F31, Cp2-F33, Cp2-F35, Cp2-F38, Cp2-F39, Cp2-F40, Cp2-F41, Cp2-F44, Cp2-F47, Cp2-F55	slightly petaloid	limoniform, ovoid	26-33 × 40-58	papillate	*P. palmivora*

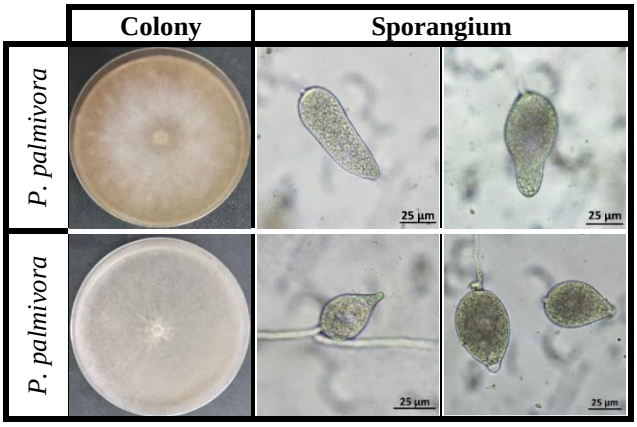


Figure 1. Representative of colony and sporangium of *P. palmivora*. grown on V8 agar

Determination of fungicide resistance of Phytophthora sp.

A total of 40 isolates of *Phytophthora* sp. were obtained successfully for testing fungicide resistance. Our findings in laboratory confirmed the occurrence and existence of fungicide resistance of *Phytophthora* sp. in the survey areas and the number of isolates were shown to resist to the 3 tested fungicides. There were 18 out of 20 eastern isolates and 7 out of 20 southern isolates showing resistance to

metalaxyl at EC_{50} > 100 mg/mL while 17 out of 20 eastern isolates and 8 out of 20 southern isolates resisting to mancozeb at EC_{50} > 100 mg/mL (Table 4). Interestingly, all 40 isolates from both areas showed resistance to fosetyl-Al at EC_{50} > 1000 mg/mL. Regarding sensitive level, it was pointed out that there were only 12 and 6 out of 40 isolates which were tested sensitive to metalaxyl and mancozeb, respectively.

Table 3. Pathogenicity test of *Phytophthora* sp. on "Monthong" durian leaf at 3 days after inoculation

Lesion score[1]	Symptom	Eastern isolate	Southern isolate
1		Tr3-F4	Cp2-F49
2		Ch1-F1, Ch1-F2, Ch1-F3, Ch2-F3, Ch2-F4, Ch3-F5, Tr2-F11, Tr3-F2,	Cp1-F8, Cp1-F10, Cp1-F14, Cp1-F17, Cp1-F20, Cp1-F24, Cp1-F28, Cp1-F31, Cp1-F35, Cp1-F38, Cp1-F39, Cp1-F40, Cp2-F41, Cp2-F44, Cp2-F47, Cp2-F55
3		Ch1-F5, Ch1-F6, Ch2-F1, Ch2-F2, Ch2-F5, Tr2-F10, Tr3-F1	Cp1-F23, Cp1-F27, Cp1-F33
4		Ch4-F4, Ch5-F4, Ch6-F5, Tr3-F5	

[1] Lesion scores are 0 = no symptom, 1= lesion size 1-5 mm, 2 = lesion size 6-10 mm, 3 = lesion size 11-20 mm, 4 = lesion size > 20 mm.

Table 4. EC_{50} values of *Phytophthora palmivora* isolates from eastern and southern parts of Thailand in response to metalaxyl, mancozeb, and fosetyl-aluminum by mycelial growth test

Resistance level	Metalaxyl[1]		Mancozeb[1]		Fosetyl-aluminium[2]	
	Eastern isolate	**Southern isolate**	**Eastern isolate**	**Southern isolate**	**Eastern isolate**	**Southern isolate**
Resistance	*Ch1-F1**	Cp1-F8,	*Ch1-F1*	Cp1-F14,	*Ch1-F1*	Cp1-F8,
	Ch1-F2	*Cp1-F20*,	*Ch1-F2*	Cp1-F17,	*Ch1-F2*	Cp1-F10,
	Ch1-F3	Cp1-F23,	*Ch1-F3*	*Cp1-F20*,	*Ch1-F3*	Cp1-F14,
	Ch1-F5	Cp1-F28,	*Ch1-F5*	Cp1-F27,	*Ch1-F5*	Cp1-F17,
	Ch1-F6	*Cp2-F39*,	*Ch1-F6*	Cp2-F33,	*Ch1-F6*	*Cp1-F20*,
	Ch2-F1	*Cp2-F40*,	*Ch2-F1*	*Cp2-F39*,	*Ch2-F1*	Cp1-F23,
	Ch2-F2	*Cp2-F49*	*Ch2-F2*	*Cp2-F40*,	*Ch2-F2*	Cp1-F24,
	Ch2-F3		*Ch2-F3*	*Cp2-F49*	*Ch2-F3*	Cp1-F27,
	Ch2-F4		*Ch2-F4*		*Ch2-F4*	Cp1-F28,
	Ch2-F5		*Ch2-F5*		*Ch2-F5*	Cp2-F31,
	Ch4-F4,		*Ch4-F4*		Ch3-F5,	Cp2-F33,
	Ch5-F4,		*Ch5-F4*,		*Ch4-F4*,	Cp2-F35,
	Ch6-F5,		*Ch6-F5*,		*Ch5-F4*,	Cp2-F38,
	Tr2-F10,		Tr2-F11,		*Ch6-F5*,	Cp2-F39,
	Tr3-F1,		*Tr3-F1*,		Tr2-F10,	Cp2-F40,
	Tr3-F2,		*Tr3-F4*,		Tr2-F11,	Cp2-F41,
	Tr3-F4,		*Tr3-F5*,		*Tr3-F1*,	Cp2-F44,
	Tr3-F5				Tr3-F2,	Cp2-F47,
					Tr3-F4,	*Cp2-F49*,
					Tr3-F5	Cp2-F55
	No.= 18	No.= 7	No.= 17	No.= 8	No.= 20	No.= 20
Moderate resistance		Cp1-F10, Cp2-F47, Cp2-F55	Ch3-F5 Tr2-F10, Tr3-F2,	Cp1-F8, Cp1-F10, Cp1-F28, Cp2-F44, Cp2-F47, Cp2-F55		
	No.= 0	No.= 3	No.= 3	No.= 6	No.= 0	No.= 0
Sensitive	Ch3-F5 Tr2-F11,	Cp1-F14, Cp1-F17, Cp1-F24, Cp1-F27, Cp2-F31, Cp2-F33, Cp2-F35, Cp2-F38, Cp2-F41, Cp2-F44		Cp1-F23, Cp1-F24, Cp2-F31, Cp2-F35, Cp2-F38, Cp2-F41		
	No.= 2	No.= 10	No.= 0	No.= 6	No.= 0	No.= 0

[1]Resistance to metalaxyl and mancozeb were determined at 0.1-100 ppm concentration (Kongtragoul *et al*., 2021).
[2]Resistance to fosetyl-Al was determined at 0.1-1000 ppm concentration (Neema *et al*., 1988).
*Italic isolates (16 eastern and 4 southern isolates) were screened as *P. palmivora* showing multiple fungicide resistance to all 3 tested fungicides.

Furthermore, the multiple fungicide resistance with R level to the 2 or 3 tested fungicides were detected in this population. Ten and 20 out of 40 isolates exhibited multiple fungicide resistance to the 2 and 3 tested fungicides, respectively (Table 4 and Figure 2). The mapping of all fungicide resistance levels including multiple fungicide resistance of *P. palmivora* occurred in the survey areas was created (Table 4 and Figure 3). Overall, it depicted that the most incidences of fungicide resistance to the each tested fungicide as well as the multiple fungicide resistance were rather distributed in the eastern survey area of Thailand.

Effect of cajeput ethanolic extract on Phytophthora sp. exhibiting multiple fungicide resistance

Out of 40 collected isolates, 20 isolates (16 isolates from eastern part and 4 isolates from southern part) were screened from the above experiment as the multiple fungicide resistance isolates. To manage these isolates by cajeput extract, Its extract efficacy was *in vitro* assessed and revealed that the cajeput leaf extract possessed the antifungal potency (Table 5 and Figure 4). However, its inhibitory effect at all tested concentrations (10000, 20000, and 40000 ppm) was only pronounced against the 7 out of 20 multiple fungicide resistance isolates while there were 2 isolates being inhibited by the extract at 2 concentrations (20000 and 40000 ppm). On the contrary, our expected inhibition effect of the extract shifted to non inhibition (on 3 isolates) and growth stimulation (on 8 isolates).

Discussion

Fungicide resistance is the naturally occurring, inheritable adjustment in the ability of individual fungus in a population to survive from a plant protection product treatment (Ishii, 2006) while fungal isolates that are resistant to one fungicide and to other closely-related fungicides are described as cross resistance (van den Bosch and Gilligan, 2008). At present, fungicide resistance and cross-resistance continue to generate disease control problems in many crops worldwide (Hollomon, 2015; Kongtragoul, 2018). In Thailand, multiple fungicide (single-site and multi-site fungicides) have been used in spray program especially in durian orchard to prevent or delay the fungicide resistance for many years. Only a few report on fungal resistance to different fungicide groups (multiple fungicide resistance) has been reported so far (Kongtragoul, 2018). Therefore, our research was first conducted to determine the emerging fungicide resistance in populations of *Phytophthora* sp. causing durian disease in the eastern and southern areas of Thailand and a possible

alternative control of *Phytophthora* isolates showing multiple fungicide resistance was *in vitro* evaluated using cajeput leaf extract.

A total of 40 isolates of *Phytophthora* sp. were collected from fruit, stem and root rot symptomatic tissues of durian from the orchards previously subjected to different fungicide pressures. Although, their morphological characteristics were described and categorized into 2 groups (mentioned earlier), all the isolates were identified as *Phytophthora palmivora* since this fungus had a variation of colony patterns and sporangium shapes (Phung *et al.*, 2015; *Drenth* and Sendall, 2011; Rodriguez-Polanco *et al*., 2020; Puig *et al*., 2021). Besides, there were reports on *P. palmivora* in Thailand (Phung *et al*., 2015; Suksiri *et al*., 2018) and in other countries (Latifah *et al*., 2018; Perrine-Walker, 2020; Rodriguez-Polanco *et al*., 2020; Puig *et al*., 2021). Moreover, all isolates from the eastern and southern parts were pathogenicity proven and confirmed as causal agents of "Monthong" durian diseases. Our results were in line with Ritmontree and Kongtragoul (2021) and Suksiri *et al*. (2018).

In vitro determination of fungicide resistance of *Phytophthora* sp. confirmed the multiple fungicide resistance to the 3 tested fungicides occurring in the survey areas from eastern and southern parts of Thailand. In the metalaxyl (single site) sensitivity, our findings revealed the 18 out of 20 eastern isolates and 7 out of 20 southern isolates showing resistance at EC_{50} > 100 mg/mL. This agreed with Kongtragoul (2018) who found 3 of 10 isolates of *P. palmivora* exhibiting resistance to metalaxyl while 9 out of 17 isolates collected from Chumphon, southern part of Thailand showing resistance to this fungicide as well (Kongtragoul *et al*., 2021). In the case of mancozeb (multi site) and fosetyl-Al (single site), we found 17 out of 20 eastern isolates and 8 out of 20 southern isolates being resistant to mancozeb at EC_{50} > 100 mg/mL and all 40 isolates exhibiting resistance to fosetyl-Al at EC_{50} > 1000 mg/mL. The above-stated results of fungicide resistance were very interesting and noteworthy since they were not in agreement with the others reported earlier that *P. palmivora* (10 isolates) from the same province (Chumphon) in the southern part of Thailand were still sensitive to both mancozeb and fosetyl-Al in that time (Kongtragoul, 2018). In addition to this contradiction of result, there were 7 isolates of *P. palmivora* (causal agent of black pod disease in cacao in Hawaii) showing their sensitivity to mancozeb and fosetyl-Al (Puig *et al*., 2021). This phenomenon shown in our study implies the rising threat of fungicide resistance in *P. palmivora* to all tested fungicides especially mancozeb and fosetyl-Al in Chumphon province, the southern part of Thailand. Additionally, the applications of the fungicide overdose for controlling *Phytophthora* spp. would increase the occurrence of resistant isolates (Earnshaw and Shattock, 2012; Wang *et al*., 2013; Kongtragoul, 2018; Kongtragoul *et al*., 2021).

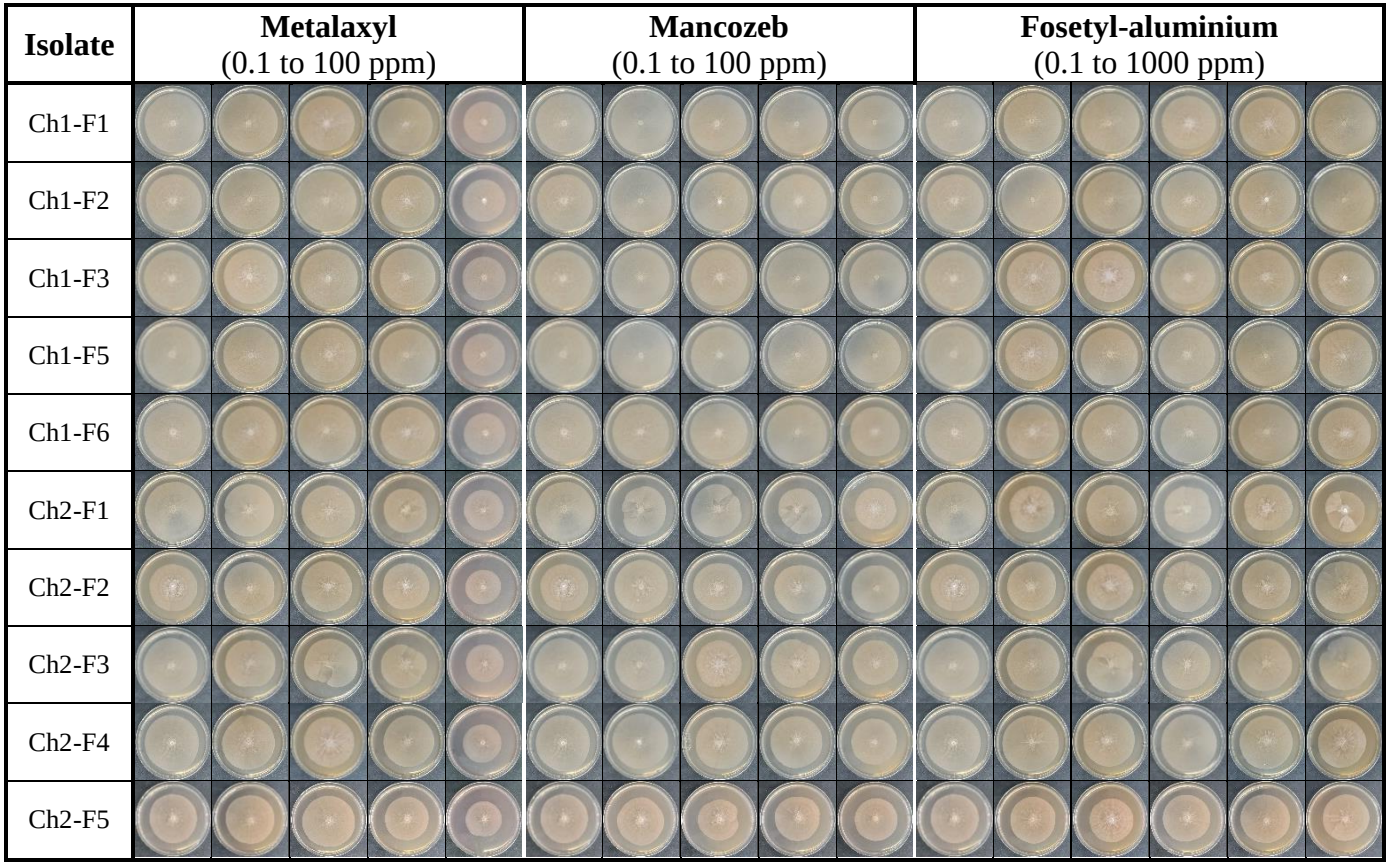


Figure 2. The phenotype of *Phytophthora palmivora* isolates showing multiple fungicide resistance to all tested fungicides

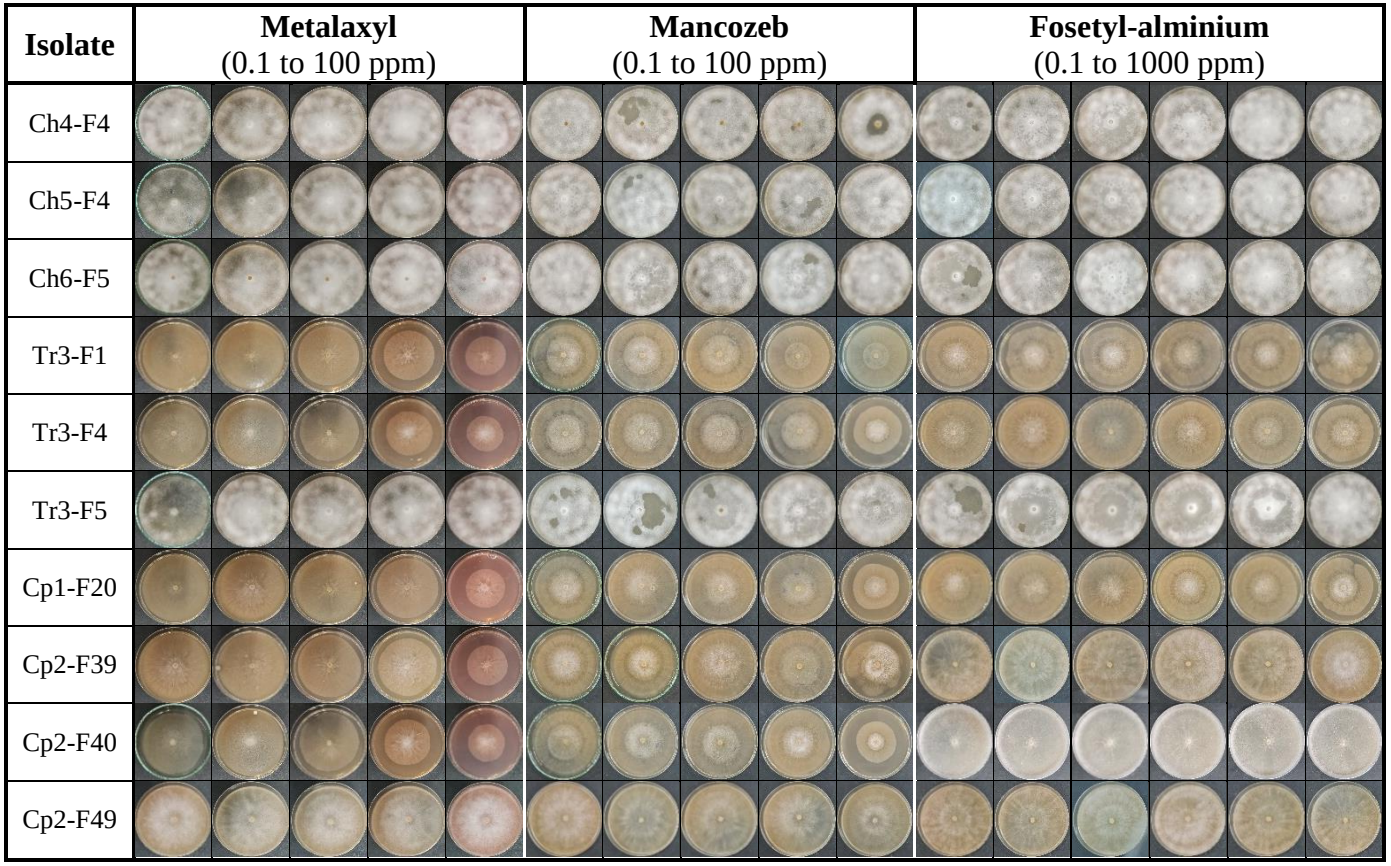


Figure 2. (Continue)

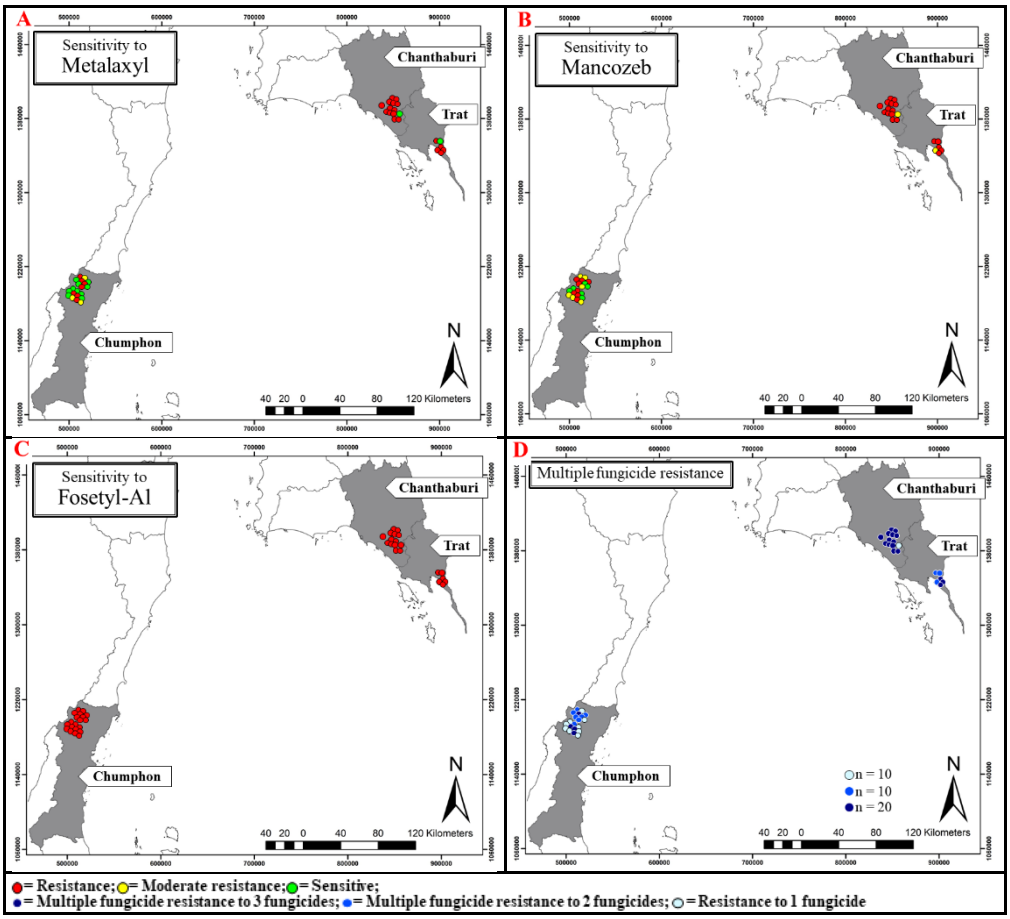


Figure 3. Mapping of *P. palmivora* isolates showing sensitivity levels to each tested fungicide (A-C) and showing multiple fungicide resistance to 2 or 3 fungicides (D) in survey area

Moreover, we found 20 and 10 out of 40 isolates of *P. palmivora* showing multiple fungicide resistance to 3 and 2 tested fungicides, respectively. It would be pointed out that our notable observation on this multiple fungicide resistance development was in line with the works of Liu *et al*. (2016), Kongtragoul (2018) also stating the multiple fungicide resistance in phytopathogens of Thailand, and China, respectively.

Furthermore, another example of multiple fungicide resistance occurred with other fungus such as *Alternaria alternata* developing its multiple fungicide resistance to mancozeb and difenoconazole with different mode of action (Yang *et al*., 2019). Accordingly, it would be hypothesized that mechanisms such as antimicrobial compounds efflux and detoxification that limit intercellular accumulation of natural/ synthetic chemicals in pathogens might account for the multiple fungicide resistance (Yang *et al*., 2019; Steffens *et al*., 1996). In regard to the distribution mapping of the incidences of fungicide resistance and multiple fungicide resistance of *P. palmivora*, it

clearly demonstrated that most of the isolates showing resistance to the 3 tested fungicides (metalaxyl, mancozeb and fosetyl-Al) and multiple fungicide resistance distributed in the survey areas of eastern part of Thailand. Such a mapping was earlier used to depict and describe the distribution of resistant strains in rice blast fungus in Japan (Ishii, 2006) as well as the incidence of G143A mutation conferring resistance to QoI fungicides in European populations of *Zymoseptoria tritici* in 2003 (Lucas *et al*., 2015).

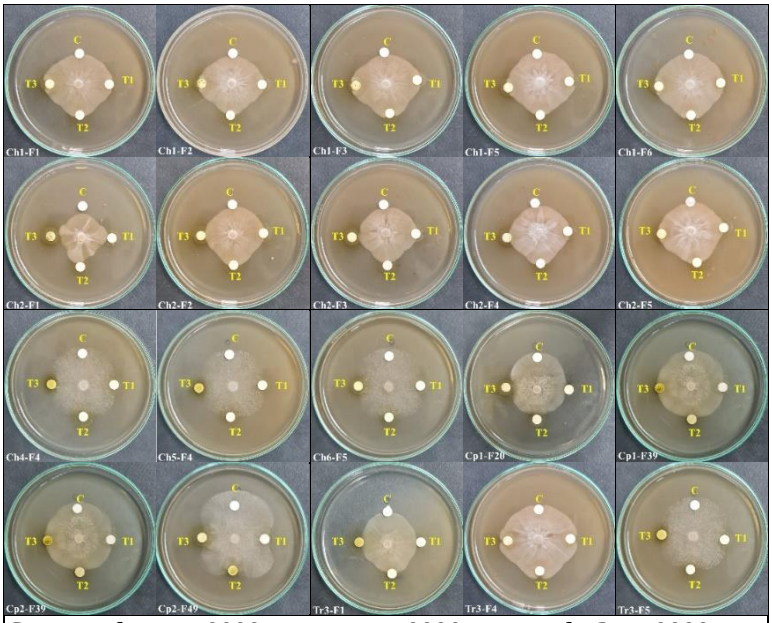
In vitro alternative control by cajeput leaf extracts on *P. palmivora* isolates showing multiple fungicide resistance, only 9 out of 20 tested isolates which was lower than expected were shown to be significantly sensitive to the cajeput extract. Our findings agreed with our previous work indicating strong inhibitory effect of cajeput leaf extract and bottle brush leaf extract on mycelial growth of *Alternaria* sp. (Somnuek *et al*., 2021). In addition, essential oil from cajeput leaf exhibited the inhibition effect on the mycelial growth of several plant pathogenic fungi such as *Alternaria* sp., *Fusarium* sp., and *Aspergillus* spp. (Pawar and Thaker, 2007; Thanaboripat *et al*., 2007, 2016; Bharat and Praveen, 2016). Regarding the above-mentioned antifungal activity of cajeput extract obtained in our study, the phytochemical compounds in the extract, namely phenolics, flavonoids, tannins and terpenoids (Al-Abd *et al*., 2015) were likely to be responsible for the antifungal potency of the extract on multiple fungicide resistance *P. palmivora*. Those phytochemical compounds could cause damage to the fungi probably by inhibiting enzymes, binding adhesins and proteins including degrading cell walls (Gurjar *te al*., 2012 and Tiwari *et al*., 2011).

In conclusion, a total of 40 *Phytophthora* isolates were isolated from symptomatic tissue of fruit, stem, and root rot of durian in areas of eastern and southern Thailand and identified by morphological characteristics as *Phytophthora palmivora*. All collected isolates were successfully proven by detached durian leaf test and confirmed to be the causal agents of durian diseases. Our *in vitro* findings confirmed the emerging fungicide resistance and multiple fungicide resistance in populations of *P. palmivora* causing durian diseases in the survey areas from eastern and southern parts of Thailand. Subsequently, the *in vitro* alternative control using ethanolic cajeput leaf extract exhibited its substantial potential to inhibit 9 out of 20 isolates of fungicide multiple fungicide resistance *P. palmivora*. It is pointed out that to alternate with an appropriate and effective plant extract (such as cajeput leaf extract) would be concerned one of the main resistance management strategies which currently recommended for controlling durian disease caused by *P. palmivora*. However, further research on detection of point mutation and confirmation on the phenotype of those 20 identified isolates having multiple fungicide resistance would be required using molecular technique. Consequently, these future data would be of great benefit for planning the alternative disease control measure in durian orchard by cajeput extract.

Table 5. Effect of ethanolic crude extract from cajeput leaf (*Melaleuca cajuputi*) on mycelial growth of the multiple fungicide resistant *Phytophthora palmivora* by paper disc diffusion technique

Tested isolate	Radius of colony growth (cm) on plant extract				Antifungal potency on[2/]
	Control	10,000 ppm	20,000 ppm	40,000 ppm	
Ch1-F1	2.00a[1/]	2.06a	2.00a	2.03a	-
Ch1-F2	1.80b	2.00a	2.03a	2.00a	-*
Ch1-F3	1.93b	2.10a	2.06a	2.13a	-*
Ch1-F5	1.90b	2.10a	2.06ab	2.06ab	-*
Ch1-F6	1.90b	2.06a	2.03a	2.03a	-*
Ch2-F1	1.43a	1.60a	1.66a	1.60a	-*
Ch2-F2	1.66b	1.86ab	2.00a	1.73b	-*
Ch2-F3	1.66b	2.00a	1.96a	1.83ab	-*
Ch2-F4	1.76b	2.03a	2.03a	2.00a	-*
Ch2-F5	1.90a	2.10a	2.03a	2.00a	-
Ch4-F4	2.56a	2.06b	1.80c	1.80c	+
Ch5-F4	2.60a	2.00a	1.93bc	1.76c	+
Ch6-F5	2.60a	2.20b	2.00bc	1.83a	+
Cp1-F20	2.46a	1.90b	1.70c	1.60d	+
Cp2-F39	2.40a	2.10b	2.00c	1.93c	+
Cp2-F40	2.16a	2.16a	2.16a	2.06a	-
Cp2-F49	3.06a	2.20b	2.20b	2.00b	+
Tr3-F1	2.06a	1.90b	1.90b	1.60c	+
Tr3-F4	2.03a	2.00a	1.90b	1.90b	+
Tr3-F5	2.76a	1.96b	1.90b	1.70c	+

[1/]Values are the average of five replications. Values in the same row followed by the same letter are not significantly different, as determined with Tukey HSK (P>0.05).
[2/]Antifungal potency: + = having effect, - = no effect, -* = stimulation effect



C =control, T1 =10000 ppm, T2 = 20000 ppm, and T3 = 40000 ppm

Figure 4. Paper disc diffusion technique of ethanolic crude extracts from cajeput leaf on the colony of multiple fungicide resistant *Phytophthora* sp.

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