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Metalaxyl Resistance of *Phytophthora palmivora* Causing Durian Diseases in Thailand

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Abstract: Thailand is the leading producer and exporter of durians worldwide. Serious diseases in durians include root rot, stem rot, and fruit rot, which are caused by *Phytophthora palmivora*, *P. nicotianae*, and *Pythium cucurbitacearum*, respectively. Thai farmers have applied fungicides for more than 20 years to control rot, but it remains difficult to control. Thus, the monitoring of fungicide-resistance development in pathogens is important for disease management. Pathogens were isolated from naturally infected durians between 2016 and 2017 in southern Thailand. The sequences of the internal transcribed spacer (ITS) and 5.8S regions of rDNA were used for the identification of their species. Seventeen out of twenty isolates were confirmed to be *P. palmivora*. All the isolates were tested for mycelium-growth sensitivity to metalaxyl, azoxystrobin, and dimethomorph. The results showed that nine isolates were resistant to metalaxyl with the 50% effective concentration (EC_{50}) higher than 100 mg L^{-1}. By contrast, all the isolates were sensitive to both azoxystrobin and dimethomorph, with EC_{50} < 1 mg L^{-1}. Metalaxyl-resistant isolates were not controlled (−25.6% to 22.2%) by the treatment of the detached leaves of 'Monthong' durian with 100 mg L^{-1} metalaxyl prior to inoculation, but all the metalaxyl-sensitive and moderately metalaxyl-resistant isolates were better controlled (33.0% to 62.6%). These results clearly indicate that metalaxyl-resistant strains are present in the populations of *P. palmivora* in Thailand.

Keywords: azoxystrobin; dimethomorph; fungicide resistance; metalaxyl; oomycete pathogen; *Phytophthora*

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1. Introduction

The durian (*Durio zibethinus*), known as the "king of Thai fruits", is one of the most popular fruits in the region and thus attracts a premium price. Thailand is the leading producer of durians, producing 95% of the world's supply, and was the largest exporter in 2016, with 402,700 tons [1]. Eighty percent of the durians in Thailand are an important export commodity [2–4]. However, root rot, stem rot, and fruit rot diseases, which have been shown to be caused by several *Phytophthora* species, including *P. palmivora*, *P. nicotianae*, and *Pythium cucurbitacearum*, are the key limiting factors for durian production [5,6]. They are serious pathogens because the crop losses and control costs are estimated to be in the range of 20–25% of production [5]. In regions with high rainfall, such as southern Thailand, durians grow in an environment that is conducive to outbreaks of *Phytophthora* diseases [5,7,8]. Fruit infection commonly occurs in orchards, usually resulting in serious decay and a 10–25% loss of durian fruits after harvest or during transport to a market [5]. Oomycete fungicides have been used extensively for controlling crop losses. There was an increase in the imports of fungicides from approximately 10,988 tons (154 million USD) in 2014 to approximately 21,004 tons (687 million USD) in 2018 [9,10]. Various fungicide groups, such as phenylamides (PAs), quinone outside inhibitors (QoIs), and carboxylic

acid amides (CAAs), can be applied to control these pathogens. These fungicides are sprayed more than 20 times per year during the pre-harvest period [10]. In southern Thailand, durian farmers have used PAs, particularly metalaxyl, more widely than QoIs and CAAs. Although these fungicides effectively suppress and control diseases, their long-term use may lead to the development of pathogen resistance, which may significantly reduce their effectiveness. The increase in fungicide-resistant strains in the pathogen populations has been resulting in serious economic problems for farmers [11–13]. The incidence of fungicide resistance in the field has become an important factor limiting the efficacy of disease-control strategies. Spending on fungicides has also increased because few farmers know that fungicide-resistant strains exist, meaning that they still use the same fungicides [14]. Furthermore, the production costs for crops have increased as growers apply fungicides at higher dosages and greater frequency than before. The side effects of fungicides may come with risks, such as serious hazards to humans and the environment.

Fungicide-resistant *Phytophthora* strains have been reported in many countries, such as the United Kingdom [15], Cameroon [16], China [17], Estonia [18], Mexico [19,20], Morocco [21], Poland [22], Russia [23], Uganda [24], and the United States [25–28]. The Fungicide Resistance Action Committee (https://www.frac.info/ (accessed on 10 January 2019)) in 2018 reported field resistance to metalaxyl among *Phytophthora* species, including *P. cactorum*, causing crown rot in strawberries; *P. capsici*, causing stem rot in lima bean pods; *P. cinnamomi*, causing root rot in avocados; *P. erythroseptica*, causing pink rot in potatoes; *P. infestans*, causing late blight in potatoes; *P. melonis*, causing foot rot in cucurbits; *P. nicotianae*, causing root rot in ornamentals; and *P. porri*, causing white tip in leeks [29]. In Thailand, metalaxyl resistance was first reported in *P. infestans* isolates, causing late blight of potatoes in the northern part of the country [30]. It is necessary to monitor the sensitivity of pathogens to fungicides for the effective control of crop disease. Because only limited information on the resistance of *Phytophthora* spp. to fungicides in Thailand is available, the monitoring of resistance is very important for the development of durian disease-management strategies. The objectives of this research were to (1) collect isolates of *Phytophthora* spp. from naturally infected trees in durian orchards, (2) analyze their internal transcribed spacer (ITS) and 5.8S regions of rDNA to identify the species, and (3) evaluate their sensitivity to metalaxyl (PA), azoxystrobin (QoI), and dimethomorph (CAA).

2. Materials and Methods

2.1. Pathogen Collection and Fungicides

Fruit rot and stem rot samples of durian that showed natural infection were collected from commercial durian orchards in Chumphon and Ranong Provinces, southern Thailand. *Phytophthora*-selective PAR(PH)-V8 medium was prepared as follows: a basal medium (Campbell's® V8 juice, 100 mL; $CaCO_3$, 1.5 g; agar, 15 g; and distilled water, 900 mL) was cooled to ~50 °C after sterilization and antibiotics (10 mg of pimaricin, 200 mg of ampicillin, and 10 mg of rifamycin) and fungicides (66.7 mg of pentachloronitrobenzene and 50 mg of hymexazol) were added (https://fhm.fs.fed.us/sp/sod/misc/culturing_species_phytophthora.pdf accessed on 18 January 2020). The tissues from the transplanting procedure, in which the plant tissues between diseased and healthy areas were cut into pieces of approximately 5 × 5 mm and surface sterilized by soaking them in 10% Clorox® solution for 1–2 min, were then rinsed in sterile distilled water, and blotted dry on sterile paper towels. The dried tissues were placed on PAR (PH)-V8 selective medium and incubated at a room temperature (RT) of approximately 28–30 °C. All of the isolates obtained were used in this study (Table 1). To test sensitivity, commercial formulations of the following fungicides were used in the experiments: metalaxyl (a.i. (active ingredient), 25%), azoxystrobin (a.i., 25%), and dimethomorph (a.i., 50%).

Table 1. Sources of *Phytophthora* isolates used in this study.

Year of Isolation	Isolate Code	Host Tissue	Location
2016	D001	Fruit	Thale Sap Sub-District, Pathio District, Chumphon.
	DS_T024	Stem	Thale Sap Sub-District, Pathio District, Chumphon.
	DF_Z030	Fruit	Tham Sing Sub-District, Muang Chumphon District, Chumphon.
	DS_B032	Stem	Tham Sing Sub-District, Muang Chumphon District, Chumphon.
	DF_M034	Fruit	Khron Sub-District, Sawi District, Chumphon.
	DF_PA01	Fruit	Pak Chan Sub-District, Kra Buri District, Ranong.
	DF_S053	Fruit	Numcha Sub-District, Sawi District, Chumphon.
	DF_S055	Fruit	Numcha Sub-District, Sawi District, Chumphon.
	DF_N014	Fruit	Thale Sap Sub-District, Pathio District, Chumphon.
2017	DF_K012	Fruit	Numcha Sub-District, Sawi District, Chumphon.
	DF_M035	Fruit	Khron Sub-District, Sawi District, Chumphon.
	DF_P027	Fruit	Thale Sap Sub District, Pathio District, Chumphon.
	DF_P075	Fruit	Thale Sap Sub-District, Pathio District, Chumphon.
	DF_PA01/2	Fruit	Pak Chan Sub-District, Kra Buri District, Ranong.
	DF_S065	Fruit	Numcha Sub-District, Sawi District, Chumphon.
	DS_T024/2	Stem	Thale Sap Sub-District, Pathio District, Chumphon.
	DS_T026	Stem	Thale Sap Sub-District, Pathio District, Chumphon.
	DF_M050	Fruit	Khron Sub-District, Sawi District, Chumphon.
	DS_B033	Stem	Tham Sing Sub-District, Muang Chumphon District, Chumphon.
	DF_CH04	Fruit	Na Kha Sub-District, Lang Suan District, Chumphon.

2.2. DNA Extraction, PCR Amplification, and Sequence Analysis

To identify the species, isolates of *Phytophthora* that formed zoosporangia were cultured on potato dextrose agar (PDA) plates at 25 °C, and total DNA was extracted as described by Saitoh et al. [31] with slight modifications [32]. A small piece of agar medium with actively growing mycelium (approximately 1 cm^2 in size) was transferred into a 1.5 mL Eppendorf tube containing 500 µL of lysis buffer (200 mM Tris-HCl, 50 mM ethylenediaminetetraacetic acid (EDTA), 200 mM NaCl, and 1% *n*-lauroylsarcosine sodium salt; pH 8.0) and homogenized using a plastic pestle and electric drill. The mixture was incubated at room temperature for 10 min and then centrifuged at 13,000 rpm for 5 min at 4 °C, and the supernatant (300 µL) was transferred to a fresh tube. After mixing the supernatant with 750 µL of ethanol to induce precipitation, the DNA was pelleted by centrifugation at 13,000 rpm for 2 min at 4 °C. The pellet was washed with 70% ethanol, air-dried in a laminar air flow bench and dissolved in 50 µL of Tris-EDTA (TE) buffer containing 10 mM Tris-HCl and 1 mM EDTA (pH 8.0). To amplify the rDNA-ITS (ITS1-5.8S-ITS2) regions from total DNA, the PCR primers ITS5 and ITS4 were used [33]. The 50 µL PCR mixture contained 1 µL of total DNA, a set of forward and reverse primers (a 0.2 µM concentration for each) and premixed Go Taq Green Master Mix (Promega, Madison, WI, USA). PCR was performed in a Mastercycler nexus gradient (Eppendorf, Hamburg, Germany) programmed for 1 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 52 °C, 2 min at 72 °C, a final extension for 10 min at 72 °C, and holding at 10 °C. The PCR products were separated by electrophoresis on a 1.5% agarose gel in 89 mM Tris-borate (pH 8.0) + 2 mM EDTA (TBE) buffer and stained with GelRed™ (Biotium, Hayward, CA, USA). The PCR products were cleaned using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) according to the instructions supplied by the manufacturer. Sequencing was conducted at Macrogen Japan Corp. (Kyoto, Japan) using the same primers employed for PCR. After sequencing, the nucleotide sequences were analyzed using the National Center for Biotechnology Information (NCBI)/GenBank database using basic local alignment search tools (BLAST).

2.3. Fungicide Sensitivity Tests on Culture Medium

The sensitivities to metalaxyl, azoxystrobin, and dimethomorph of all the tested isolates were assessed using a mycelial growth assay performed on agar culture plates. Mycelial discs, 4 mm in diameter, were cut from actively growing colony margins and

transferred upside down onto clarified V8 juice agar amended with 0, 0.1, 1, 10, and 100 mg L^{-1} (a.i.) of metalaxyl, dimethomorph, or azoxystrobin with *n*-propyl gallate (PG) at 1 mM as the alternative oxidase (AOX) inhibitor (3 replications). Fungicides were added to the medium after autoclaving, and the plates were incubated at room temperature (28–30 °C). After incubation for 3 days, the colony diameter of the isolates grown on the fungicide-amended and unamended medium was recorded, and the percentage of mycelial growth inhibition by the fungicides was calculated after subtracting 4 mm from the colony diameter. The growth inhibition (%) value for each of the fungicide treatments was calculated using the formula given as: [(mean colony diameter on the control medium − mean colony diameter on the medium with fungicide)/(mean colony diameter on the control medium) × 100].

2.4. Data Analysis for Fungicide Sensitivity

The values of mycelial growth inhibition (%) were plotted as probits versus the $\log_{10}$ of the fungicide concentration (mg L^{-1}) and analyzed by linear regression. The regression equation was used to appraise a 50% effective concentration (EC_{50}) for each fungicide's inhibition of the mycelial growth of each isolate. The EC_{50} values were used to form three categories for sensitivity assays. The isolates with EC_{50} values < 1 mg L^{-1} (metalaxyl and dimethomorph) and <10 mg L^{-1} (azoxystrobin) were considered sensitive (S); isolates with EC_{50} values of 1 to 100 mg L^{-1} (metalaxyl), 1 to 10 mg L^{-1} (dimethomorph), and 10 to 100 mg L^{-1} (azoxystrobin) were classified as moderately resistant (MR); and isolates with EC_{50} values greater than 10 mg L^{-1} (dimethomorph) and 100 mg L^{-1} (metalaxyl and azoxystrobin) were considered resistant (R) [34,35].

2.5. Fungicide Sensitivity Tests on Detached Durian Leaves

The *P. palmivora* isolates from each metalaxyl-resistant group, Met^R (DS_B032, DF_P027, DF_P075, and DF_M050), Met^{MR} (DF_M034, DF_PA01, DF_N014, and DF_S053), and Met^S (DF_S055), were selected, and their metalaxyl sensitivity on detached leaves of the durian variety 'Monthong' was determined in vivo. Fifty-four durian leaves were washed thoroughly using sterilized water before being surface sterilized with 10% Clorox® and air-dried. A wounded inoculation site, with a diameter of 1.5 mm, was marked on the surface of the leaves with a digital Vernier caliper. Each wound (six wounds/leaf) was punctured with a sterile needle. The wounded leaves were soaked in water with and without 100 mg L^{-1} metalaxyl, the recommended concentration in practice, for 5 min. All the tested isolates were previously cultured on clarified V8 juice agar at 25 °C for 5 days. The mycelial discs, 5 mm in diameter, were cut with a sterilized cork borer and transferred upside down to the wounded site of the durian leaves. The inoculated leaves were incubated in a moist plastic box at room temperature. The diameter of the lesion that appeared as brown rot around the wounded site was measured 4 days after incubation, and the percentage of disease control by the fungicide was calculated with the following formula: [(Mean lesion diameter on water treated leaves—Mean lesion diameter on metalaxyl treated leaves)/Mean lesion diameter on water treated leaves] × 100. The experiment was arranged in completely randomized design (CRD) with 3 replications. Data were subjected to Statistix 8 analytical software. Mean of treatment was compared by least significant difference (LSD) at $p \leq 0.05$.

3. Results

3.1. Species Identification by rDNA-ITS Sequence Analysis

A total of 17 out of 20 isolates tested (Table 1) were identified as *P. palmivora*. In comparison with the sequence of the NCBI accession number KY475630, the identity of the sequences was 97% to 100%, except for isolate DS_T024, which showed a 95% alignment with the rDNA-ITS region of *P. palmivora*. The sequences of the ITS segments of the 15 isolates have been deposited in DDBJ under the accession numbers LC510501 to LC510515, respectively (Figure S1).

3.2. Fungicide Sensitivity Tests on Culture Medium

The sensitivity of *P. palmivora* isolates to metalaxyl, azoxystrobin, and dimethomorph is shown in Table 2. The EC_{50} values of metalaxyl for the nine isolates were >100 mg L^{-1}, and these isolates were determined to be metalaxyl-resistant (Met^{R}). The EC_{50} values of metalaxyl were between 1 and 100 mg L^{-1} for seven moderately resistant (Met^{MR}) isolates, while four isolates were sensitive (Met^{S}) at <1 mg L^{-1}. Moreover, all the isolates were sensitive (S) to azoxystrobin in the presence of PG and dimethomorph, for which the EC_{50} values were <1 mg L^{-1}.

Table 2. Sensitivity of *Phytophthora* isolates to metalaxyl, azoxystrobin, and dimethomorph on clarified V8 juice agar.

Year of Isolation	Isolate Code	EC_{50} (mg L^{-1})			Sensitivity Type [1]		
		Metalaxyl	Azoxystrobin	Dimethomorph	Metalaxyl	Azoxystrobin	Dimethomorph
2016	D001	>100	<0.1	0.3	R	S	S
	DS_T024	>100	0.17	0.4	R	S	S
	DF_Z030	<0.1	<0.1	0.3	S	S	S
	DS_B032	>100	<0.1	0.3	R	S	S
	DF_M034	2.3	0.2	<0.1	MR	S	S
	DF_PA01	11.7	0.2	0.2	MR	S	S
	DF_S053	3.9	0.1	0.1	MR	S	S
	DF_S055	<0.1	0.3	<0.1	S	S	S
	DF_N014	1.4	<0.1	<0.1	MR	S	S
2017	DF_K012	<0.1	<0.1	0.1	S	S	S
	DF_M035	4.9	<0.1	<0.1	MR	S	S
	DF_P027	>100	<0.1	0.3	R	S	S
	DF_P075	>100	<0.1	0.3	R	S	S
	DF_PA01/2	5.49	<0.1	0.86	MR	S	S
	DF_S065	7.86	<0.1	0.35	MR	S	S
	DS_T024/2	>100	<0.1	0.37	R	S	S
	DS_T026	>100	<0.1	0.29	R	S	S
	DF_M050	>100	<0.1	0.43	R	S	S
	DS_B033	>100	<0.1	0.35	R	S	S
	DF_CH04	<0.1	<0.1	0.34	S	S	S

[1] R = resistant, MR = moderately resistant, S = sensitive.

3.3. Fungicide Sensitivity Tests on Detached Durian Leaves

The inoculation tests confirmed that the Met^{R}, Met^{MR}, and Met^{S} isolates of *P. palmivora* were pathogenic to the durian leaves. Initial symptoms appeared as dark-brown necrotic lesions 2 days after inoculation. Four days after inoculation with Met^{R}, Met^{MR}, and Met^{S} isolates, large brown lesions developed on the wounded leaves, regardless of treatment with metalaxyl or water. The mean lesion diameter on the leaves treated with 100 mg L^{-1} metalaxyl (11.32 mm) was not significantly different from that in the water controls (12.32 mm) in the Met^{R} group. However, the mean lesion diameters of the Met^{MR} and Met^{S} groups showed a significant difference between the leaves treated with 100 mg L^{-1} metalaxyl and water controls. Moreover, the percentage of disease control showed a significant difference between isolates. Metalaxyl showed only 22.2% disease control at a maximum against the group of Met^{R} isolates, which was much less than the 33.0% to 62.6% and 61.5% control against the group of Met^{MR} and Met^{S} isolates, respectively (Table 3).

Table 3. Lesion diameters and disease control (%) on detached leaves after treatment with 100 mg L^{-1} metalaxyl or water and inoculation with *Phytophthora palmivora* isolates for 4 days.

Phenotype [1]	Isolate Code	Mean Lesion Diameter (mm) [2]		Disease Control (%) [3]
		Water	Metalaxyl 100 mg L^{-1}	
Met^{R}	DS_B032	12.79	11.64	8.99 c
	DF_P027	12.41	11.41	8.06 c
	DF_P075	16.80	13.07	22.20 c
	DF_M050	7.30	9.17	−25.61 d
	Mean	12.32 A	11.32 A	
Met^{MR}	DF_M034	6.39	3.64	43.06 b
	DF_PA01	12.60	4.71	62.62 a
	DF_N014	7.66	5.13	33.02 bc
	DF_S053	10.28	4.31	58.07 ab
	Mean	9.23 A	4.44 B	
Met^{S}	DF_S055	11.33 A	4.36 B	61.52 a

[1] Met^{R} = metalaxyl-resistant, Met^{MR}= moderately metalaxyl-resistant, and Met^{S} = metalaxyl-sensitive. [2] Mean lesion diameter of water controls and metalaxyl treatments in each phenotype followed by a distinct uppercase letter was significantly different, with $p = 0.05$ or lower. [3] Disease-control values followed by distinct lowercase letters in the same column were significantly different, with $p = 0.05$ or lower.

4. Discussion

Phytophthora disease affects the quality and quantity of durians. It has been a major disease for more than 20 years in Thailand. In this study, most of the pathogen isolates collected from the naturally infected 'Monthong' cultivar in commercial orchards in southern Thailand were identified as *P. palmivora* according to the nucleotide sequences of the rDNA-ITS region. This situation is similar to that in other durian-producing countries such as Australia, Malaysia, the Philippines, Indonesia, and Vietnam, where *P. palmivora* has been reported to cause diseases [5,8]. The analysis of the rDNA-ITS regions was successfully used for the identification of the major species in the genus *Phytophthora*, including *P. palmivora* [36]. Phylogenetic relationships among the 50 *Phytophthora* species were also examined based on their rDNA-ITS sequences [37].

Chemical fungicides are still important for controlling this disease and, hence, maintaining durian production in Thailand. Fungicides are sprayed frequently and in increasing doses. In general, the field recommended rate of metalaxyl, azoxystrobin, and dimethomorph were applied for durian usually range from 20 to 40 g 20 L^{-1}, 5 to 10 mL 20 L^{-1}, 10 to 20 g 20 L^{-1}, respectively. Thus, monitoring the resistance development of this pathogen is essential for guiding farmers. In 2016–2017, the majority of the *P. palmivora* isolates collected from southern Thailand were found to be resistant to metalaxyl but sensitive to azoxystrobin and dimethomorph.

The detection of *P. palmivora* isolates resistant to metalaxyl indicated that this fungicide might not be effective in controlling *Phytophthora* diseases in durians. Moreover, Kongtragoul and Viriyaekkul [38] found that *Phytophthora* spp. caused leaf fall disease in para-rubber but were resistant to metalaxyl in the same area. In the durian orchards of southern Thailand, metalaxyl is sprayed regularly, approximately 2–3 times/month or more often during the rainy season from May to October, which results in the development of resistance to this fungicide. Metalaxyl was sprayed, but the control efficacy was low and resulted in decreased or complete loss of durian yields [38]. In these regions, metalaxyl has been used more frequently than azoxystrobin and dimethomorph in the past, which may explain the higher EC_{50} values of metalaxyl.

Azoxystrobin and dimethomorph-resistant isolates of *P. palmivora* were not found in this study. Similar research results have been reported for *P. infestans* sensitivity to azoxystrobin and dimethomorph in Russia between 1993 and 2003 [23], Mexico in 2000 [39], and some provinces in China between 2000 and 2008 [17,40]. However, resistance to

azoxystrobin in *P. capsici* causing pepper Phytophthora Blight in China [41], and the G143A mutation commonly responsible for high QoI resistance has been found in the cytochrome *b* gene of resistant isolates [42]. In this case, the resistance frequency was greater than 40% in the pathogen populations. Therefore, substantial attention needs to be continuously given to prevent or delay the development of fungicide resistance in the management of *P. palmivora* in durians. Durian growers should reduce the frequency of fungicide applications, mix single-site inhibitors such as QoIs and CAAs with multi-site contact fungicides, and/or rotate with fungicides with different modes of action. The application of metalaxyl should be well-designed to combine with alternate fungicides for disease control in orchards where metalaxyl-resistant strains have already appeared or are widely distributed. Currently, there are several reports on effective fungicides for managing *Phytophthora*. For example, Ramallo et al. [43] reported that potassium phosphite presents a complex mode of action against the *Phytophthora* brown rot of lemons in pre- and post-harvest applications. Moreover, durian growers should introduce integrated approaches with resistant cultivars, cultural practices, or biological control [5,44,45]. We conclude that metalaxyl use should be considered carefully, as it could increase the management costs for durian production in southern Thailand. In contrast, populations of *P. palmivora* are still sensitive to azoxystrobin and dimethomorph, which suggests that both fungicides can be used in this area for the time being. However, durian growers should use azoxystrobin and dimethomorph carefully because these fungicides also pose the risk of resistance development. Further studies are necessary to monitor the fungicide resistance in a wider range of pathogen populations present in Thailand.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7100375/s1, Figure S1: Multiple sequence alignments of rDNA-ITS regions in *Phytophthora palmivora* isolates analyzed in this study.

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