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ORIGINAL ARTICLE

Utilization of palm oil mill effluent as a novel substrate for the production of antifungal compounds by *Streptomyces philanthi* RM-1-138 and evaluation of its efficacy in suppression of three strains of oil palm pathogen

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Abstract

Aims: This study aimed to use palm oil mill effluent (POME) as a renewable resource for the production of antifungal compounds by *Streptomyces philanthi* RM-1-138 against *Ganoderma boninense*, *Ceratocystis paradoxa* and *Curvularia oryzae*.

Methods and results: The efficacy of antifungal compounds RM-1-138 against the three strains of fungal oil palm pathogen was evaluated both in vitro and on oil palm leaf segments. In vitro studies using confrontation tests on glucose yeast-malt extract (GYM) agar plates indicated that the strain RM-1-138 inhibited the growth of all three fungal pathogenic strains. The antifungal compounds produced in the GYM medium exhibited significantly higher inhibition (79%–100%) against the three fungal pathogens than using the diluted POME (50%) medium (80%–83% inhibition). The optimum condition for the production of antifungal compounds from the strain RM-1-138 was as following: POME of 47,966 mg L^{-1} chemical oxygen demand (COD), the initial pH at 7.0 and supplemented with yeast extract (0.4%). Meanwhile, severe morphological and internal abnormalities in *C. oryzae* hyphae were observed under a scanning electron microscope and transmission electron microscope. In vivo experiment on oil palm leaf segments indicated that the efficacy of the antifungal compounds RM-1-138 (DSI = 1.3) were not significantly difference in the suppression of *Curvularia* leaf spot compared with the two commercial chemical fungicides of mancozeb® (DSI = 1.0) and tetraconazole® (DSI = 1.3).

Conclusions: Antifungal compounds produced by *S. philanthi* RM-1-138 grown in POME have the potential to inhibit fungal pathogens.

Significance and impact of the study: The POME (about 47 mg L^{-1} COD) with the initial pH of 7.0 and supplementation of 0.4% nitrogen could be used as a culture medium for the growth and production of antifungal compounds of *S. philanthi* RL-1-138. In addition, the antifungal compound RM-1-138 could suppress the three strains of oil palm fungal pathogen tested on oil palm leaf segment.

KEYWORDS

antifungal compounds, oil palm fungal pathogen, palm oil mill effluent, *Streptomyces philanthi*

INTRODUCTION

Palm oil mill effluent (POME) is identified as the largest pollution load into environmental problems of the palm oil industry. POME is an acidic brownish colloidal suspension containing 86%–90% water, 0.8%–1.1% oil, 1.5–2.6 mg L^{-1} Fe^{2+}, 3.5%–4.2% total solids (TS), 0.85%–1.2% suspended solids, and having high chemical oxygen demand (COD) (45,200–56,300 mg L^{-1}), biochemical oxygen demand (12,000–24,300 mg L^{-1}) and temperature (70–80°C) (Mamimin et al., 2016; Prasertsan et al., 1990). POME cannot be discharged without treatment. The bioconversion of POME into high-value products has been recognized as an attractive alternative for waste management strategy. In bioconversion, the rich organic residue in POME is used as a substrate for specific microorganisms to grow and consume while concurrently producing biomass and the targeted products, for example, bioenergy and biochemicals. Most researcher's studies were reported using POME as a substrate for bioenergy (Cheng et al., 2019; Hamid et al., 2019; Krishnan et al., 2019; Mamimin et al., 2016; Mishra et al., 2019; Prasertsan et al., 2021; Rachmadona et al., 2021; Rosa et al., 2020; Zaied et al., 2020). Studies on ultrafiltered POME concentrates or retentates as growth media for *Penicillium chrysogenum* in the production of antibiotics have been conducted some time ago (Suwandi, 1991; Suwandi & Mohammad, 1984). However, there is no prior published research on POME as the substrate for the production of bioactive compounds of *Streptomyces* spp.

Actinomycetes are potent producers of wide variety of secondary metabolites with diverse biological activities, which includes therapeutically and agriculturally important compounds (Barka et al., 2016; El-Tarabily et al., 2020; El-Tarabily & Sivasithamparam, 2006). Actinomycetes effectively inhibit many soil fungi by degrading their chitinous cell walls (Olanrewaju & Babalola, 2019), and some of them are hyperparasites of fungal pathogens (El-Tarabily & Sivasithamparam, 2006). They have been evaluated as biocontrol agents against various plant pathogens (He et al., 2019; Li et al., 2020). Actinomycetes play a pivotal role in maintaining a satisfactory biological balance in soil, largely because of their ability to produce antibiotics and other secondary metabolites (El-Tarabily, 2008; El-Tarabily et al., 2019). Among actinomycetes, *Streptomyces* are potential biocontrol agents because they are ubiquitous in the environment, and many of them produce secondary metabolites such as enzyme inhibitors and antibiotics with diverse biological activities, including the ability to inhibit plant pathogenic fungi (Kamil et al., 2018; Olanrewaju & Babalola, 2019). Numerous studies on antimicrobial production by *Streptomyces* were reported using synthetic media (Duan et al., 2020; Kum & İnce, 2021; Li et al., 2011; Prabavathy et al., 2006; Shakeel et al., 2016; Sharma & Thakur, 2020; Xu et al., 2019), but it has never been reported using POME as the medium. In particular, the glucose yeast-malt extract (GYM) medium has shown to be a good medium for bacterial growth and antifungal production of *Streptomyces philanthi* RM-1-138 (Boukaew et al., 2011, 2013, 2017; Boukaew & Prasertsan, 2014). Antifungal compounds from *Streptomyces* have been used to control pathogenic fungi such as *Fusarium oxysporum* f. sp. *cubense* race 4 (Duan et al., 2020; Wei et al., 2020), *Colletotrichum fragariae* (Li et al., 2021), *Penicillium digitatum* (Boukaew et al., 2020a), *Ganoderma boninense* (Sujarit et al., 2020), *Curvularia lunata* (Wonglom et al., 2019) and *Curvularia oryzae* (Sunpapao et al., 2018).

Oil palm (*Elaeis guineensis* Jacq.) is a perennial economical crop cultivated especially in Thailand. During the last few decades, oil palm production has gradually decreased due to infection with various diseases such as basal stem rot caused by *G. boninense* and *Ceratocystis paradoxa*, and leaf spot caused by *C. oryzae*. In southern Thailand, leaf spot disease in oil palm seedlings is a very destructive disease, which causes severe problems in seedling stocks of private nurseries (Sunpapao et al., 2014). Therefore, the preventive measure by biocontrol using antagonistic *S. philanthi* RM-1-138 was proposed. The present study included the following objectives: (i) to use POME as a low-cost medium for the production of antifungal compounds by *S. philanthi* RM-1-138 compared with GYM medium, (ii) to optimize the concentration of POME, nitrogen sources and initial pH of POME for production of antifungal compounds from *S. philanthi* RM-1-138, (iii) to evaluate the efficacy of antifungal compounds from *S. philanthi* RM-1-138 against three strains of oil palm pathogen in vitro and (iv) to evaluate the efficacy of antifungal compounds from *S. philanthi* RM-1-138 against *Curvularia* leaf spot on oil palm leaf segments.

MATERIALS AND METHODS

Microorganisms

S. philanthi RM-1-138 was previously isolated and characterized as described by Boukaew et al. (2011) and grown on GYM agar at room temperature (28 ± 2°C) for 10 days before use.

The pathogenic fungi of oil palm; *C. oryzae* was isolated at different times from naturally infected oil palm plants in the field. It was obtained from the Suratthani Oil Palm Research Center, Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand. *C. paradoxa* and *G. boninense* were obtained from the Biohythane Laboratory at Prince of Songkla University, Thailand. All fungal strains were grown on potato dextrose agar (PDA) slants at room temperature (28 ± 2°C) for 3 days and

kept at 4℃. They were subcultured freshly for use in each experiment.

Palm oil mill effluent and its characteristics

POME was kindly provided by Laff Tavee Palm Co., Ltd., and kept at −20°C until use. Characteristics of POME were determined for COD, TS, total suspended solids, volatile solids (VS), volatile suspended solids, total phosphorous and phosphate following the standard method (APHA, 1999). Total Kjeldahl nitrogen was analysed using Kjeldahl method (Singh & Pradhan, 1981). Temperature and pH were measured using thermometer and pH meter, respectively. Glucose, xylose, fructose and arabinose were determined using high-performance liquid chromatography (Eyéghé-Bickong et al., 2012).

Antagonism of *Streptomyces philanthi* RM-1-138 against oil palm pathogens

S. philanthi RM-1-138 was evaluated for their antagonistic properties against the three strains of oil palm pathogens (*C. oryzae*, *G. boninense* and *C. paradoxa*) using a dual-culture technique (Boukaew et al., 2011). A streak of spore suspension (10^7 spores ml^{-1}) of *S. philanthi* RM-1-138 was deposited on one side of a GYM agar medium in Petri dishes. Plates were then incubated in a growth chamber for 10 days at 28 ± 2℃ in the dark.

A 5-mm-diameter mycelial plug, excised from a 3-day-old of each oil palm pathogen colony, was transferred to the centre of each plate. As a control, a mycelial plug of oil palm pathogen was placed on a GYM plate without *S. philanthi* RM-1-138. The dual-culture plates were further incubated in a growth chamber (28℃) for 2–7 days, after which the radial mycelial growth of the oil palm pathogen was measured and compared with that of the control. Three replicates were conducted for each *Streptomyces*—and oil palm pathogen strain combination. The colony size in each treatment was recorded, and the percentage inhibition of hyphal growth was calculated using the equation: Percentage of inhibition = [{(Control − Treatment)/Control} × 100].

Comparison of the efficacy of the antifungal compounds RM-1-138 against oil palm pathogens

Comparison of the efficacy of antifungal compounds from *S. philanthi* RM-1-138 produced in synthetic medium (GYM) and wastewater (POME) against three strains of pathogenic fungi was investigated. An antifungal supernatant was prepared by submerged cultivation of *S. philanthi* RM-1-138 at room temperature (28 ± 2℃) on a rotary shaker (150 rpm) in a 250-ml flask containing 100 ml of GYM medium with the pH adjusted to 7.0 using 5 M NaOH before autoclaving. After 3 days of cultivation, 5-ml aliquots of this seed culture was transferred into 100 ml of GYM, undiluted POME (100%) and diluted POME (50% with distilled water) with pH adjusted to 7.0 and incubated for 10 days under the same conditions. The culture broth was centrifuged (at 8880 *g* for 20 min) and then filtered through a 0.45-mm Millipore membrane. The supernatant was used for antifungal assay.

Antifungal assay

The antifungal supernatant from each treatment (1 ml) was mixed with 9-ml melted sterile PDA and poured onto a 9-cm diameter culture plate, while distilled sterilized water (1 ml) was mixed with 9-ml melted sterile PDA at an equivalent amount was used as a control. A 5-mm-diameter plug of mycelia was cut from a 3-day old in each oil palm pathogen colony and transferred onto the centre of the test agar plates. The cultures were further incubated at room temperature (28 ± 2°C) for 2 days of *C. paradoxa*, 4 days of *C. oryzae* and 7 days of *G. boninense*.

The experiment was conducted in three replications. The colony size of each treatment was recorded, and the percentage inhibition of hyphal growth was calculated as described above.

Optimization of POME medium and testing for efficacy of the antifungal compounds RM-1-138

Effect of COD concentration of POME on the antifungal compounds production

Five-ml aliquot seed culture of *S. philanthi* RM-1-138 was transferred into 100 ml of sterilized (121℃/15 min) undiluted POME, POME diluted in the ratio of 1:1, 1:3, 1:6 or 1:9 (v/v) with distilled water. The initial pH was adjusted to 7.0 using 5 M NaOH and incubated on a rotary shaker (150 rpm) at room temperature (28 ± 2°C) for 10 days. Then, the culture broth was centrifuged (at 8880 *g* for 20 min) and filtered through a 0.45-mm Millipore membrane. The antifungal supernatant of each treatment was tested for its efficacy against the three fungal oil palm pathogens using antifungal assay (as described above).

Effects of nitrogen sources on the antifungal compounds production

The effect of nitrogen sources (yeast extract, malt extract and ammonium nitrate at 0.4% (v/v)) for production of antifungal compounds by *S. philanthi* RM-1-138. Five-ml aliquot seed culture of the strain RM-1-138 was transferred into 100 ml of sterilized (121℃/15 min) optimum POME medium (result from the previous experiment) (with distilled water; pH adjusted to 7.0) and incubated on a rotary shaker (150 rpm) at room temperature (28 ± 2°C) for 10 days. Then, the culture broth was centrifuged (at 8880 *g* for 20 min) and then filtered through a 0.45-mm Millipore membrane. The antifungal supernatant of each treatment was tested for its efficacy against the three fungal oil palm pathogens using antifungal assay (as described above). No added nitrogen sources were used as control.

Effect of initial pH on the antifungal compound production

The effect of initial pH (6.0, 6.5, 7.0, 7.5, and 8.0) for the production of antifungal compounds by *S. philanthi* RM-1-138. Five-ml aliquot seed culture of the strain RM-1-138 was transferred into 100 ml of sterilized (121℃/15 min) optimum POME medium (result from the previous experiment) and incubated on a rotary shaker (150 rpm) at room temperature (28 ± 2°C) for 10 days. Then, the culture broth was centrifuged (at 8880 *g* for 20 min) and then filtered through a 0.45-mm Millipore membrane. The antifungal supernatant of each treatment was tested for its efficacy against the three fungal oil palm pathogens using antifungal assay (as described above).

Effects of the antifungal compounds of *Streptomyces philanthi* RM-1-138 on the ultrastructure of *Curvularia oryzae*

Any effect of the antifungal compounds from *S. philanthi* RM-1-138 on the microscopic characteristics of the treated (with the antifungal compounds of *S. philanthi* RM-1-138) and nontreated (control) mycelium of *C. oryzae* (result from the previous experiment) was detected by using the scanning electron microscope (SEM) and transmission electron microscope (TEM). The mycelium affected by the antifungal compounds of *S. philanthi* RM-1-138 was transferred to a glass cover slip, then fixed with 1.5% glutaraldehyde and dehydrated with a graded series of ethanol washes followed by drying in a desiccator (Walter & Crawford, 1995). Samples were affixed to SEM stubs using carbon tape followed by a thin coating with gold and examined under the SEM (FEI Quanta 400, SEM-Quanta). For the TEM, the hyphae of *C. oryzae* treated (with the antifungal compounds of *S. philanthi* RM-1-1-38) and nontreated (control) were resuspended in 2.5% glutaraldehyde, fixed overnight at 4℃, rinsed in phosphate buffer (50 mM, pH 6.8), postfixed for 2 h at 4℃ with 1% (w/v) osmium tetroxide in the same buffer, dehydrated in an ethanol series and infiltrated by Jembed 812 resin following the procedure described by Kang et al. (2001). Thin sections (90 nm) were cut with a diamond knife for TEM, mounted on nickel grids, stained with uranyl acetate and lead citrate and examined with a TEM (JEM-2010, JEOL).

Efficacy of the antifungal compounds RM-1-138 against *Curvularia* leaf spot

Oil palm leaves (*E. guineensis*) were obtained from the Faculty of Natural Resources, Prince of Songkla University (PSU). A detached leaf assay on the pathogenicity of *Curvularia* leaf spot after treatment with the antifungal compounds of *S. philanthi* RM-1-138 and the two chemical fungicides, mancozeb® and tetraconazole®, was tested. Healthy leaves from oil palm were collected, cut into 6-cm-length segments, surface-sterilized using 2% sodium hypochlorite and washed with sterile distilled water. Ten millilitre of antifungal compounds of *S. philanthi* RM-1-138 and the two chemical fungicides at concentration of 100 μg ml^{-1} were sprayed on the leaf segments. When the leaves were dried (between 30 and 60 min after treatment), they were removed, and three leaflets were placed in a transparent plastic box with humid absorbent paper to maintain high relative humidity (close to 80%–90%). A spore suspension of *C. oryzae* at the concentration of 10^7 spores ml^{-1} was sprayed onto the leaflets of the oil palm segments. Leaves removed from nontreated plants were inoculated as a control. For each treatment, six leaflets of oil palm leaf segments (three plastic boxes) were inoculated. To evaluate disease severity, an interval scale was used: 0 = no spots, 1 = spot area 0%–20%, 2 = 20%–40%, 3 = 40%–60%, 4 = 60%–80% and spots coalesced by about 50% and 5 = 80%–100% and spots coalesced by more than 75%. Disease severity scores were converted to a disease severity index (DSI) as described by Promwee et al. (2017).

Statistical analysis

The data were analysed for variance using Statistical Package for the Social Sciences (SPSS) ver. 26 (IBM Corp). A p-value <0.05 was considered significant.

RESULTS

Characteristics of palm oil mill effluent

Raw POME had a pH of 4.5, a brown colour and a temperature of 70–80℃. The characteristics of POME (Table 1) indicated that the high values of organic matter (95,933 mg L^{-1}, COD) showed the possibility to be used as a carbon source for the growth of microorganisms, TS (75,333 mg L^{-1}) with about 80% of its value as VS (62,666 mg L^{-1}). It had acidic pH (4.65), nitrogen content (TKN) of 1050 mg L^{-1} and ammonium-nitrogen (NH_4-N) of 175.7 mg L^{-1}. The glucose, xylose, fructose and arabinose concentrations were 3.34, 1.99, 1.71 and 0.61 g L^{-1}, respectively.

Antagonism of *Streptomyces philanthi* RM-1-138 against oil palm pathogens

The in vitro dual-culture assay showed that *S. philanthi* RM-1-138 inhibited the mycelial growth of the three strains of oil palm pathogens (*C. oryzae*, *G. boninense* and *C. paradoxa*) in the range of 96%–100% inhibition on GYM medium after 2–7 days of incubation at 28 ± 2℃, compared with the control treatment without *S. philanthi* RM-1-138 (Figure 1). Significant differences ($p < 0.05$) in sensitivity of *S. philanthi* RM-1-138 to the three strains of oil palm pathogens were observed. The most pronounced inhibitory effect (100%) was against *C. oryzae* and *G. boninense*, while the least inhibition was against *C. paradoxa* (95.9%) (Table 2).

TABLE 1 Characteristics and chemical composition of palm oil mill effluent for bioactive compounds production from *Streptomyces philanthi* RM-1-138

Component	Unit	Content
Temperature	°C	85–90
pH		4.65
Total Kjeldahl nitrogen	mg L^{-1}	1050 ± 7.1
Ammonium-nitrogen (NH_4-N)	mg L^{-1}	175.7 ± 5.7
Total phosphorous	mg L^{-1}	332 ± 4.5
Phosphate	mg L^{-1}	124.7 ± 7.3
Chemical oxygen demand	mg L^{-1}	95,933.3 ± 289.6
Total solids	mg L^{-1}	75,333.3 ± 30.5
Total suspended solids	mg L^{-1}	42,666.7 ± 11.5
Volatile solids	mg L^{-1}	62,666.7 ± 5.4
Volatile suspended solids	mg L^{-1}	38,666.7 ± 15.4
Glucose	g L^{-1}	3.34 ± 0.15
Xylose	g L^{-1}	1.99 ± 0.10
Fructose	g L^{-1}	1.71 ± 0.13
Arabinose	g L^{-1}	0.61 ± 0.09

Note: Data are the mean of three replicates ± standard deviation (SD).

Comparison of the efficacy of the antifungal compounds RM-1-138 against oil palm pathogens

Comparison of the efficacy of antifungal compounds of *S. philanthi* RM-1-138 produced in GYM and POME medium against *C. paradoxa*, *C. oryzae* and *G. boninense* is shown in Table 3. The antifungal compounds produced in the GYM medium exhibited significantly ($p < 0.05$) higher inhibition in the range of 79.0%–100.0% against the three strains of the fungal pathogen than using diluted POME (50% dilution) medium (80.0%–83.0% inhibition). No antifungal activity was detected from the undiluted POME (100%) medium. The strong antifungal activity of bioactive compounds of *S. philanthi* RM-1-138 against the three strains of the fungal pathogen was confirmed on a PDA agar plate as illustrated in Figure 2.

Optimization of pome medium and testing for efficacy of the antifungal compounds RM-1-138

POME is highly polluting wastewater with high COD. The effect of POME concentration (undiluted POME (95,933 mg L^{-1}, COD), diluted POME in the ratio 1:1, 1:3, 1:6 and 1:9) on the production of antifungal compounds against *C. paradoxa*, *C. oryzae* and *G. boninense* is illustrated in Figure 3a. Results in the POME experiment showed that there were significant differences ($p < 0.05$) among different POME concentrations on the antifungal activity of *S. philanthi* RM-1-138. Increasing the dilution of the POME showed lower antifungal activity of the isolated RM-1-138. The highest antifungal activity (80–83% inhibition) on the three strains of oil palm pathogen was achieved at 1:1 dilution (47,966 mg L^{-1}, COD).

Nitrogen sources (yeast extract, malt extract and ammonium nitrate (0.4%)) were added in the diluted POME (1:1 ratio) for the production of the antifungal compounds by *S. philanthi* RM-1-138 compared with the control (only the diluted POME) was investigated (Figure 3b). Results indicated that adding yeast extract and malt extract in the diluted POME showed higher antifungal activity of the strain RM-1-138 against the three strains of oil palm pathogen than no added nitrogen (76%–80% inhibition). Among the three nitrogen sources, yeast extract gave the highest inhibition (96%–100% inhibition) followed by

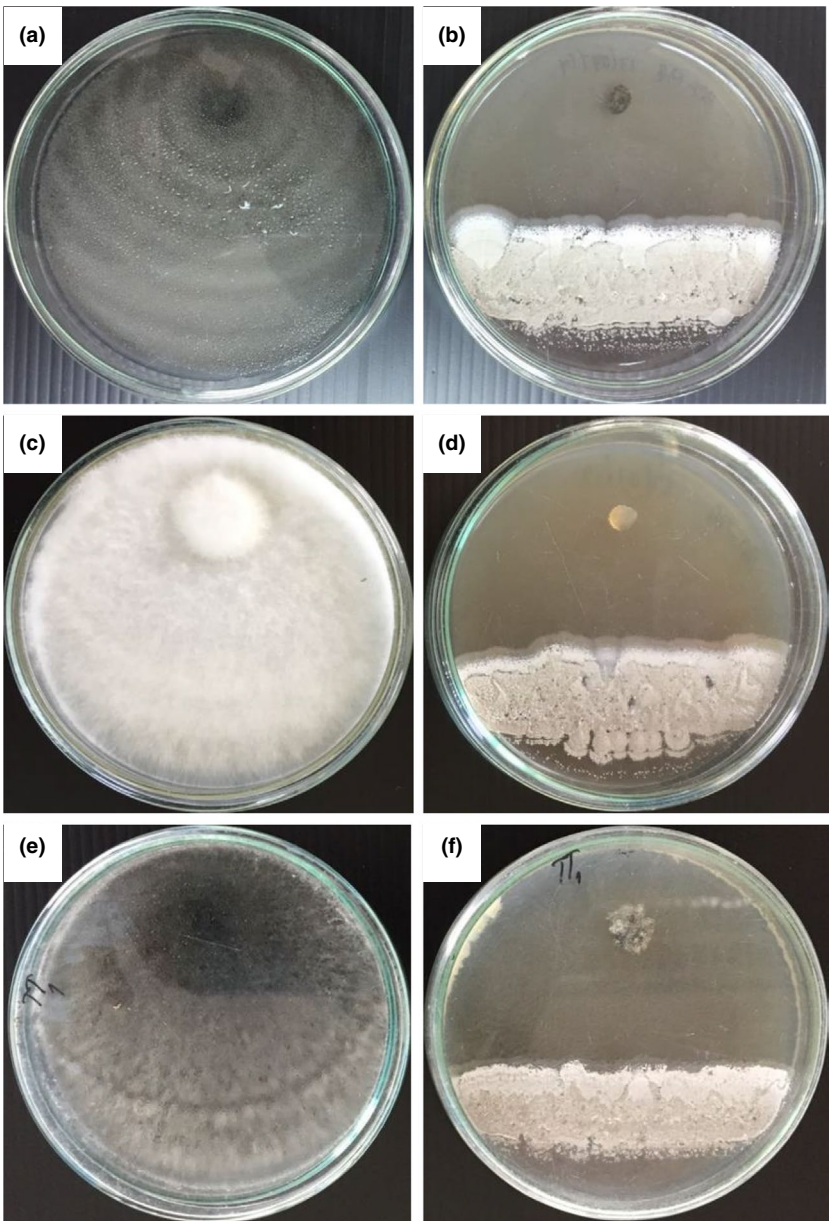

FIGURE 1 Antifungal activity of *Streptomyces philanthi* RM-1-138 against three strains of the oil palm pathogen by using dual-culture technique on glucose yeast-malt agar (GYM) medium and incubated at 28 ± 2℃ for 2–7 days. (a) *Curvularia oryzae* (control), (b) *C. oryzae* + *S. philanthi* RM-1-138, (c) *Ganoderma boninense* (control), (d) *G. boninense* + *S. philanthi* RM-1-138, (e) *Cyanophora paradoxa* (control) and (f) *C. paradoxa* + *S. philanthi* RM-1-138

TABLE 2 The inhibition percentages of *Streptomyces philanthi* RM-1-138 against three oil palm pathogenic fungi mycelial growth on glucose yeast-malt extract agar by using dual-culture technique after 2–7 days

Plant pathogenic fungi	Host of origin	Location (Province)	Inhibition percentage
Curvularia oryzae	Oil palm (*Elaeis guineensis*)	Suratthani	100 ± 0^{a}
Ganoderma boninense	Oil palm (*E. guineensis*)	Suratthani	100 ± 0^{a}
Ceratocystis paradoxa	Oil palm (*E. guineensis*)	Songkhla	95.9 ± 1.3^{b}

Note: Data are the mean of three replicates ± standard deviation (SD). Data followed by same letter within each column are not significantly different using ANOVA after Duncan multiple range test at $p < 0.05$.

malt extract (92%–93% inhibition) and ammonium nitrate (77%–79% inhibition), respectively.

The effect of the initial pH (6–8) of the optimum diluted POME supplemented with yeast extract (0.4%) for the production of antifungal compounds from *S. philanthi* RM-1-138 against the three strains of oil palm pathogen is illustrated in Figure 3c. The optimum pH at 7.0 was found to have the highest antifungal activity (95%–100% inhibition).

TABLE 3 Comparison on the efficacy of antifungal compounds of *Streptomyces philanthi* RM-1-138 produced in glucose yeast-malt extract medium and palm oil mill effluent against three strains of the fungal pathogen and incubated at 28 ± 2℃ for 2 days of *Ceratocystis paradoxa*, 4 days of *Curvularia oryzae* and 7 days of *Ganoderma boninense*

	Inhibition percentage		
Sources of antifungal compounds RM-1-138	***Ceratocystis paradoxa***	***Curvularia oryzae***	***Ganoderma boninense***
In GYM medium	79.53 ± 1.5[a]	86.46 ± 3.64[a]	100.0 ± 1.5[a]
In diluted POME (50%) medium	80.38 ± 0.88[a]	82.50 ± 1.88[b]	83.75 ± 2.17[b]
In undiluted POME (100%) medium	0.00 ± 0[b]	0.00 ± 0[c]	0.00 ± 0[c]

Note: Data are the mean of three replicates ± standard deviation (SD). Data followed by same letter within each column are not significantly different using ANOVA after Duncan multiple-range test at $p < 0.05$.

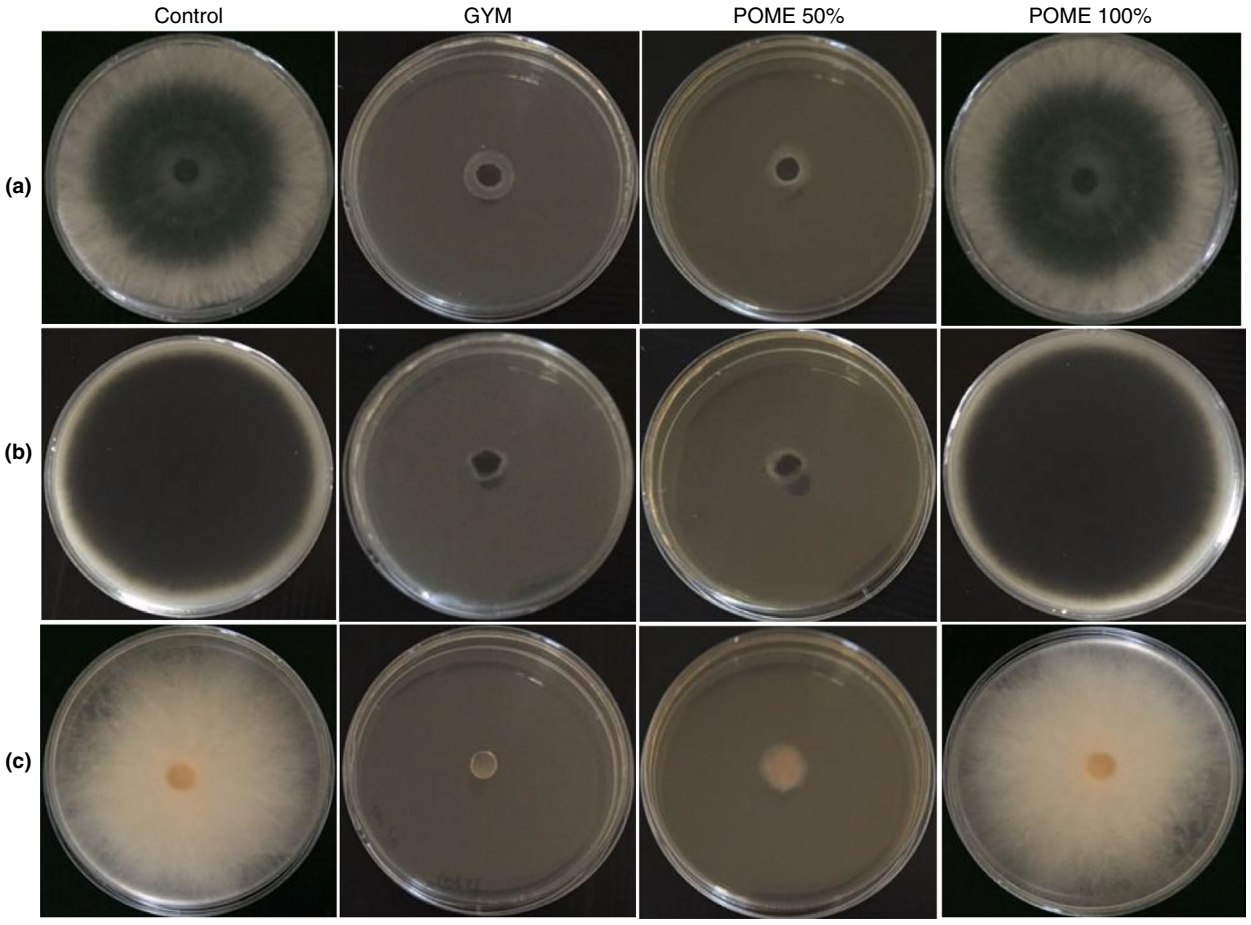

FIGURE 2 Comparison on the efficacy of antifungal compounds of *S. philanthi* RM-1-138 produced in a glucose yeast-malt extract (GYM) agar medium and palm oil mill effluent (POME) against three strains of the oil palm pathogen and incubated at 28 ± 2℃ for 2 days of *C. paradoxa* (a), 4 days of *C. oryzae* (b), and 7 days of *Ganoderma boninense* (c)

Effects of the antifungal compounds of *S. philanthi* RM-1-138 on the ultrastructure of *C. oryzae*

The morphological changes in 4-day-old *C. oryzae* exposed to the antifungal compounds of *S. philanthi* RM-1-138 produced in the optimum POME medium were observed by SEM and TEM. The SEM observation showed that untreated *C. oryzae* hyphae retained complete tubular shapes with the normal appearance of a barrel-like formation and smooth cell walls (Figure 4a,b). After being exposed to antifungal compounds from *S. philanthi* RM-1-138, the aberrant and distorted morphologies of the fungal hyphae were distinctively observed. Markedly shrivelled, and crinkled cell walls, and flattened hyphae were the evidences from the SEM (Figure 4c,d).

The many destructive sites of fungi revealed by TEM supported that multiple compounds contributed to the

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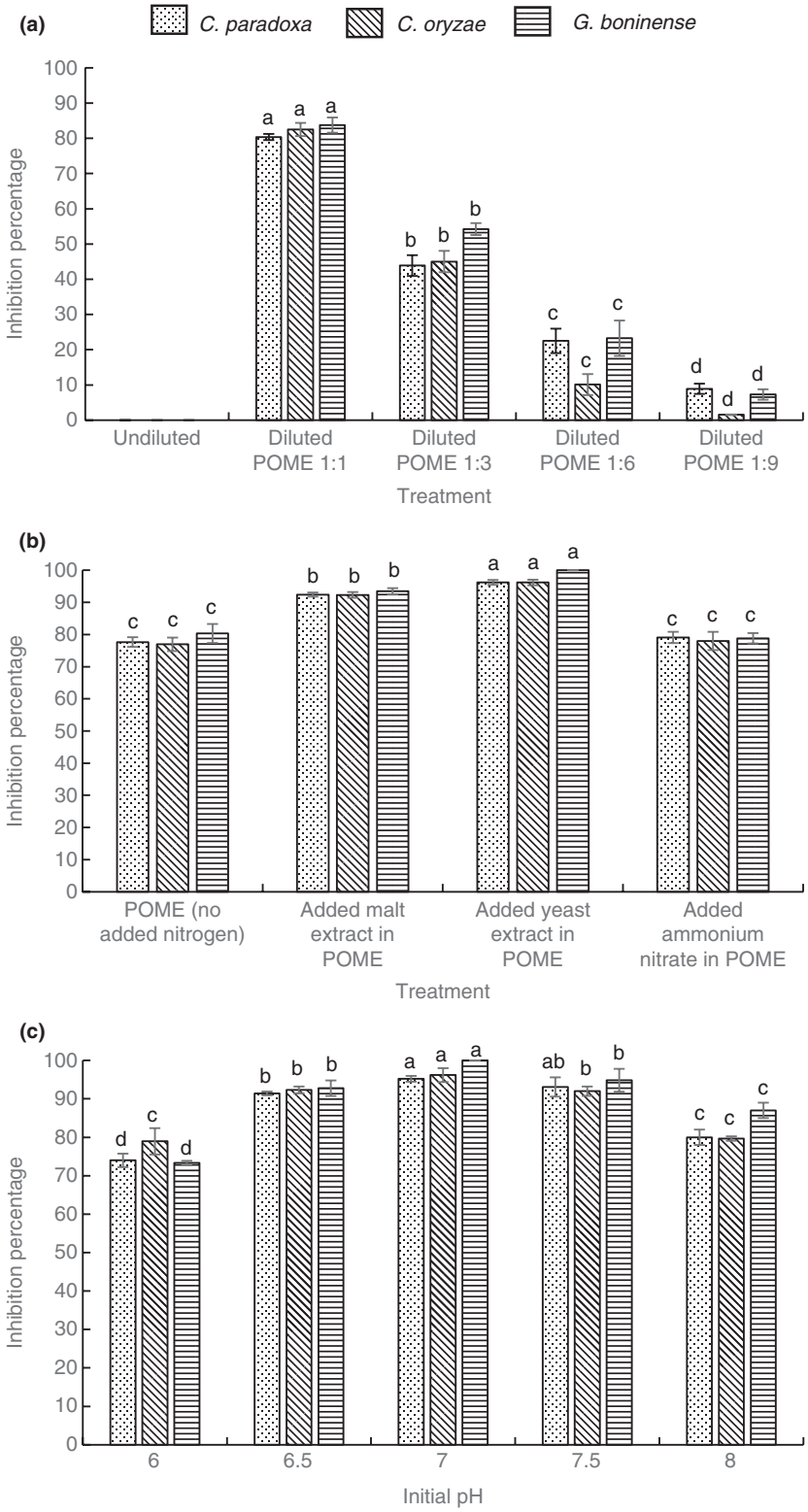

FIGURE 3 Effect of diluted of palm oil mill effluent (POME) medium (a), nitrogen source (0.4%) (b) and initial pH (c) on production of antifungal compounds of *Streptomyces philanthi* RM-1-138 against *Cyanophora paradoxa*, *Curvularia oryzae* and *Ganoderma boninense*. Data are the mean of three replicates ± standard deviation (SD). Values with the same letter are not significantly different (ANOVA, $p < 0.05$; Duncan multiple range test)

antifungal activity of *S. philanthi* RM-1-138. The TEM observations clearly showed that the nontreated sample is uniform and protected thoroughly by an intact fibrillar layer. The plasma membrane was unfolded with a uniform shape. The septum and all organelles including mitochondria, vacuole and nucleus had normal appearances (Figure 5a), whereas in the cells treated with antifungal compounds of *S. philanthi* RM-1-138, the plasma membrane seemed to be the primary target. The complete detachment from the cell wall and lysis or disruption and disappearance of the plasma membranes were obvious, leading to a marked depletion of the cytoplasm content

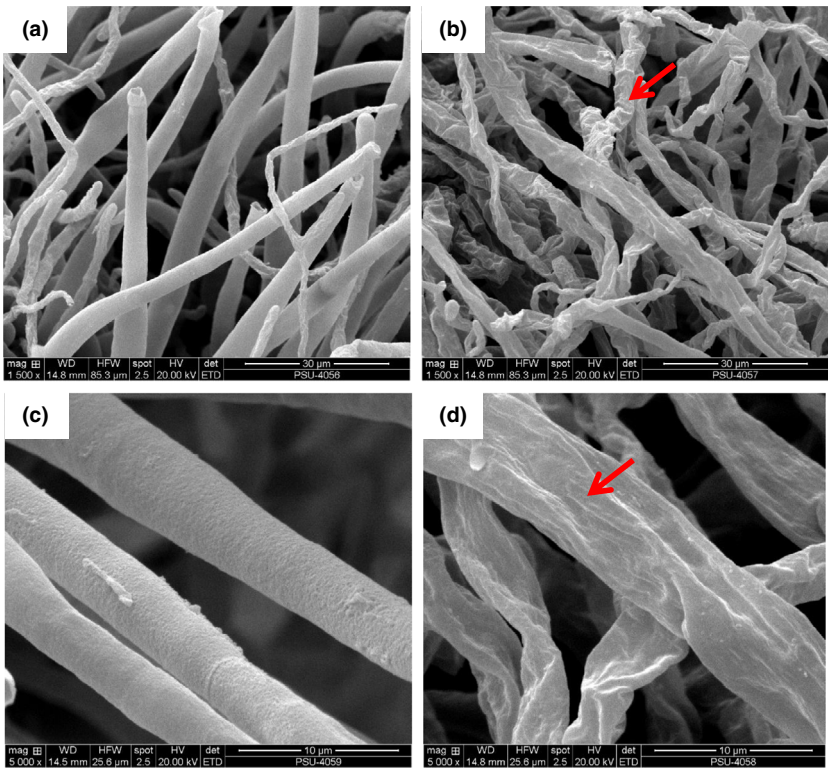

FIGURE 4 Scanning electronic micrographs (SEM) of *Curvularia oryzae* growing on potato dextrose agar (PDA) in the absence (control) (a and b) or presence (c and d) of antifungal compounds of *Streptomyces philanthi* RM-1-138 after incubation at 28 ± 2°C for 4 days. Red arrows indicate *C. oryzae* hyphal cell wall damaged by antifungal compounds of *S. philanthi* RM-1-138 at 1500× (c) and 5000× (d)

with the formation of small lomasomes and membrane-bound vesicles. The disruption and degeneration of various fungal organelles, such as the loss of mitochondrial cristae and fusion of the nucleus are clearly illustrated in Figure 5b–d. The diminution and disorganization of the plasma membrane as well as the disintegration and disruption of the membranous organelles seemed to be responsible for cell death. The SEM and TEM showed that antifungal compounds from *S. philanthi* RM-1-138 targeted multiple sites of *C. oryzae*, particularly the lipid bilayer and cell wall.

Efficacy of the antifungal compounds RM-1-138 against *Curvularia* leaf spot

The oil palm leaf segments inoculated with the spore suspension of *C. oryzae* (positive control) showed the highest DSI = 2.67, whereas those inoculated with distilled water (negative control) had no disease symptoms (DSI = 0) (Figure 6). The oil palm leaf segments treated with antifungal compounds of *S. philanthi* RM-1-138 (DSI = 1.33) showed no significantly difference ($p > 0.05$) in the suppression of *Curvularia* leaf spot compared with the others treated with two commercial chemical fungicides of mancozeb® (DSI = 1.0) and tetraconazole® (DSI = 1.33). Therefore, the efficacy of the antifungal compounds of *S. philanthi* RM-1-138 was equal to the tetraconazole® and slightly better than the mancozeb®.

DISCUSSION

The antifungal metabolites produced in the synthetic medium of *Streptomyces* species are known to be efficient against oil palm pathogenic fungi (Nur Azura et al., 2016; Saeed et al., 2017; Shariffah-Muzaimah et al., 2018, 2020; Sujarit et al., 2020; Sunpapao et al., 2018). However, knowledge about the antifungal metabolites produced in POME of *S. philanthi* RM-1-138 against *C. oryzae*, *G. boninense* and *C. paradoxa* is limited. Therefore, this paper provides updated information relating to the efficacy of antifungal bioactive compounds produced by the strain RM-1-138 grown on POME against the three strains of oil palm fungal pathogen in vitro and leaf segments. The undiluted POEM was a high content of phenolic compounds (Azimatun Nur et al., 2020). It is possible that the presence of the phenolic compound in undiluted POME, which had inhibited the growth of *S. philanthi* RM-1-138 for the production of bioactive compounds. The results showed that *S. philanthi* RM-1-138 grown in the diluted POME (50%) could produce bioactive compounds against all three fungal pathogens with equal efficacy to those produced in the synthetic medium of GYM.

Based on the dual-culture technique bioassay, the presence of *S. philanthi* RM-1-138 had a significant ($p < 0.05$) effect on inhibition of the mycelial growth of the three oil palm pathogenic strains grown on GYM agar plates, suggesting a strong direct antifungal effect. The antifungal activity was most pronounced against *C. oryzae* and *G. boninense* (100% inhibition). The evidence of the ability

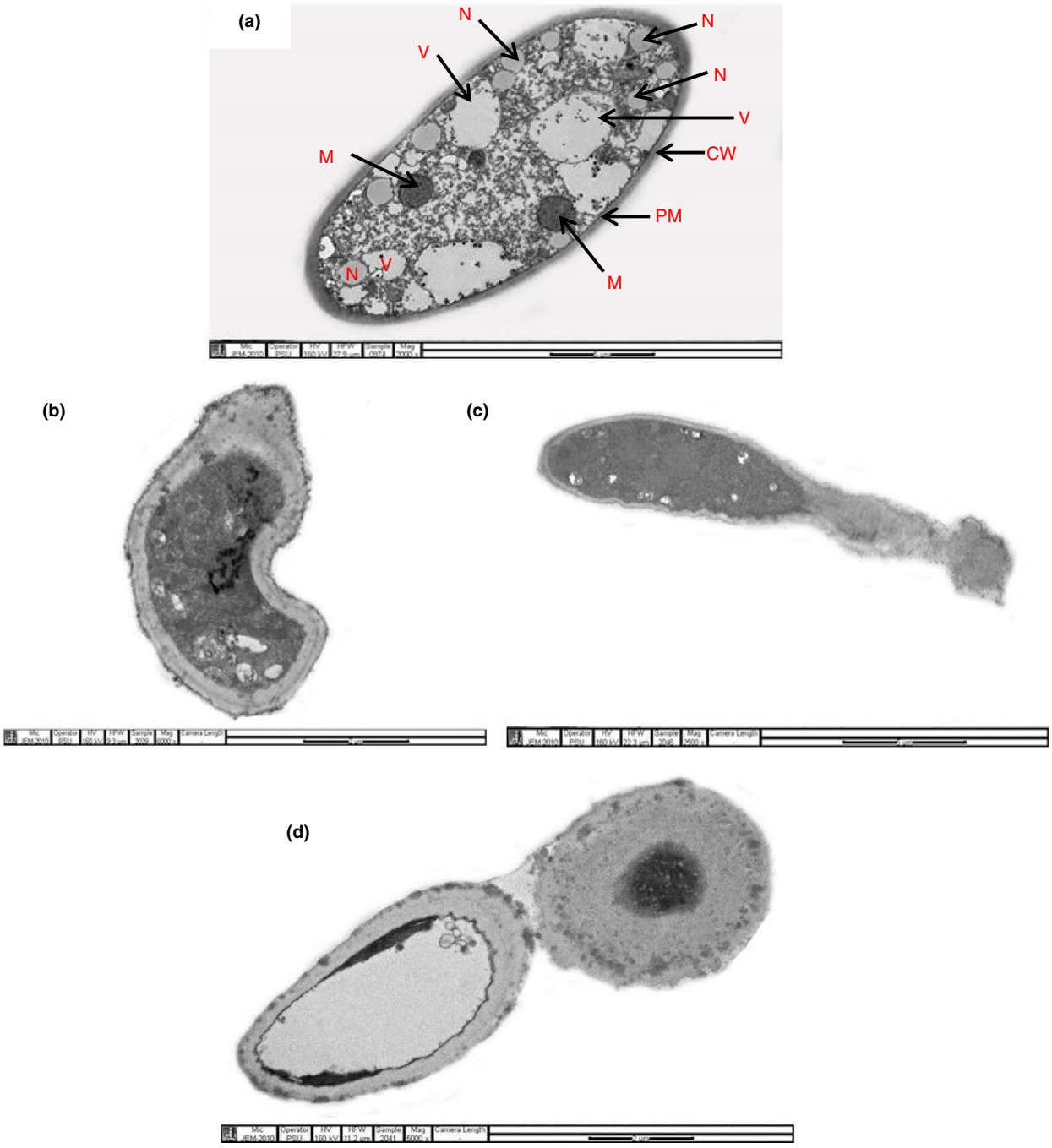

FIGURE 5 Transmission electron micrographs (TEM) of *Curvularia oryzae* growing on potato dextrose agar (PDA) in the absence or presence of antifungal compounds of *Streptomyces philanthi* RM-1-138 after incubation at 28 ± 2°C for 4 days. (a) Control mycelia are homogenous and the fibrillar layer (FL), cell wall (CW), plasma membrane (PM) and intracellular organelles including mitochondria (M), vesicles (V) and nucleus (N) have a uniform and normal structures. (b, c, d) *C. oryzae* exposed to antifungal compounds of *S. philanthi* RM-1-138. PM is changed progressively and detached from the cell wall

of *Streptomyces* to produce antifungal substances and secreted into the medium was supported by many reports (Duan et al., 2020; Nur Azura et al., 2016; Sunpapao et al., 2018; Wonglom et al., 2019). In addition, the *Streptomyces* are also a major source of innumerable antibiotic agents (Lee & Hwang, 2002; Mahadevan & Crawford, 1997; Neeno et al., 2001).

Our previous study indicated that the antifungal metabolites of *S. philanthi* RM-1-138 produced in GYM could significantly inhibit the growth of rice sheath blight in vitro and in vivo, implying that the strain RM-1-138 could secrete highly active compounds (Boukaew & Prasertsan, 2014). Thus, the antifungal metabolites RM-1-138 was used to examine the suppressive effect against the three

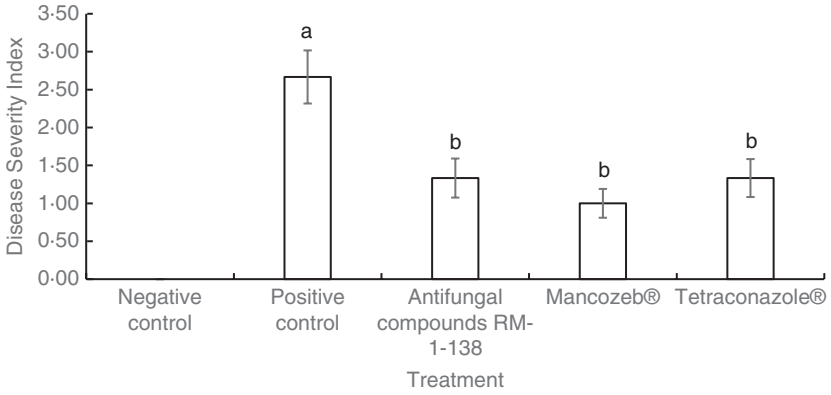

FIGURE 6 Mean disease severity index in oil palm leaf segments at 10 days, after treatments with distilled water (positive control), antifungal compounds of *S. philanthi* RM-1-138, and the two commercial chemical fungicides of mancozeb® and tetraconazole®. Data are the mean of three replicates and five oil palm leaf segments/treatment ± standard deviation (SD). Values with the same letter are not significantly different (ANOVA, $p < 0.05$; Duncan multiple-range test)

strains of oil palm pathogen in this study. The high inhibitory levels of the antifungal metabolites produced in GYM (79.0%–100% inhibition) and diluted POME (50%) (80.0%–83.0% inhibition) medium were evidence while there was no inhibition in the undiluted POME medium. This was because there was no growth of *S. philanthi* RM-1-138 in the undiluted POME medium due to the high organic load (95,933 mg L^{-1} COD) of the raw POME. This agreed with the result of Louhasakul et al. (2016) who reported that the two-fold dilution POME (using distilled water) gave higher biomass and lipase activity of *Yarrowia lipolytica* than using the undiluted POME. Furthermore, the diluted POME of 10% (v/v) was found to be a good substrate for growth and bioflocculant production by *Aspergilluis niger* (Aljuboori et al., 2014).

An optimization process was employed to determine the best combination of parameters such as COD concentration of POME, nitrogen source (0.4%), and initial pH for production of bioactive compounds from *S. philanthi* RM-1-138 prior to the testing for antifungal activity against the three strains of pathogenic fungi. POME contains high concentrations of carbon and nutrients. The highest antifungal activity (80.0%–83.0% inhibition) on the three strains of oil palm pathogenic fungi was achieved at 1:1 dilution (47,966 mg L^{-1}, COD). Increasing dilution of POME from 1:3 to 1:9 or undiluted POME showed lower antifungal activity of *S. philanthi* RM-1-138. The data clearly indicated the variation of COD concentration gave variables antifungal activity. The antifungal metabolites of *S. philanthi* RM-1-138 were significantly ($p < 0.05$) enhanced when supplemented with either yeast extract or malt extract. It is therefore suggested that nitrogen sources need to be included in the diluted POME. These results are in agreement with the studies of Boukaew et al. (2016) where malt extract supplementation in a basal medium gave the maximum chitinase (0.53 U ml^{-1}) and β-1,3 glucanase (8.79 U ml^{-1}) production by *S. philanthi* RM-1-138. Gethins et al. (2015) also reported that yeast extract had pronounced effects on the production of volatile fragrance and flavour metabolites by the yeast *Kluyveromyces marxianus*. The pH of the medium is a very critical parameter for secondary metabolites particularly in submerged fermentation (Al-Kordy et al., 2021; Selvakumar & Sivashanmugam, 2017). The variation in pH is directly associated with the change in antifungal activity, and this could be explained based on the fact that biological processes require optimum physiological pH (Moeini et al., 2018). Interestingly, the antifungal activity against all of these fungi changed when the pH values of the POME media were increased from 6.0 to 8.0. The high antifungal activity of *S. philanthi* RM-1-138 was recorded at pH 7.0 that the maximum production of antifungal compounds from *Streptomyces* sp. was at pH 7.0 (Kavitha & Vijayalakshmi, 2011; Tanabe et al., 2000; Yano et al., 2008).

The effect of the antifungal compounds from *S. philanthi* RM-1-138 produced in the optimal POME medium on the ultrastructure of *C. oryzae* was visualized by SEM and TEM. In SEM images using the untreated *C. oryzae* hyphae displaying a smooth surface as a control, the antifungal substances treated could alter the *C. oryzae* hyphal morphology and led to the gradual destruction of mycelia and finally cell death due to cytoplasmic extrusions. TEM images of the control *C. oryzae* revealed a clear picture of the cell wall, nuclear envelope and cellular organelles, while the cell wall and membrane of hyphae treated with antifungal substances were obviously wrinkled and vacuolated, the space between the cell wall and membrane widened, and the cell organelles and septum were much less distinct than those in the control hyphae. These results indicated that the antagonistic metabolites penetrated into the cytoplasm and damaged the organelles. It has been

reported that antifungal compounds, as well as fungal cell wall lytic enzymes from *Streptomyces* antagonists, could induce various morphological alterations, such as distortion and swelling in the mycelial and conidial structure of pathogenic fungi (Lu et al., 2018; Manhas & Kaur, 2016; Xu et al., 2019). *S. philanthi* RM-1-138 could produce extracellular chitinase and β-1,3-glucanase (Boukaew et al., 2016) as well as secondary antifungal metabolites (Boukaew & Prasertsan, 2014), that could damage the fungal cell wall. Therefore, the main action of antifungal substances from *S. philanthi* RM-1-138 was on the destruction of the cell wall of *C. oryzae*.

The *Curvularia* leaf spot is the main pathogen of oil palm seedlings, which causes severe problems in seedling stocks of private nurseries (Sunpapao et al., 2014). Oil palm leaf segments were tested, it was of much interest that, the antifungal metabolites (DSI = 1.33) of *S. philanthi* RM-1-138 effectively reduced *Curvularia* leaf spot and this effect was comparable with the commonly used fungicide, tetraconazole® (DSI = 1.33) and mancozeb® (DSI = 1.0). It was reported that *S. hygroscopicus* OsiSh-2 antifungal metabolites treated rice against rice blast pathogen caused by *Magnaporthe oryzae* showed fewer and smaller lesions in rice seedlings with a disease index of 41.3% (Xu et al., 2019). Treatment of antifungal metabolites produced by *S. globisporus* JK-1 on rice seedlings in the greenhouse before *M. oryzae* inoculation exhibited 89.1% control efficiency for rice blast, whereas the unfiltered culture broth of JK-1 showed 100% disease control (Li et al., 2011). Moreover, treatment with the culture filtrate at 50 ml of *S. cinnamonensis* strain KPP02129 producing the streptavidins completely controlled (100%) *Fusarium* wilt of tomato plants (Jeon et al., 2021). The mycelial growth of *Aspergillus parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 was completely inhibited (100%) by 10% (v/v) culture filtrate concentration, whereas aflatoxin B_1 (AFB_1) production was completely inhibited at 5% (v/v) (Boukaew et al., 2020b).

In conclusion, the results obtained in this study indicated that POME medium was developed and used for the growth and production of antifungal compounds of *S. philanthi* RM-1-138. The antifungal compounds have the high efficacy against all three fungal oil palm pathogenic strains. Further development of this work is to enhance and confirm its efficacy in both greenhouse and field conditions.

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CONFLICT OF INTEREST

No conflict of interest was declared.

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