

The Potential of Rhamnolipid as Biofungicide against *Rigidoporus microporus* Isolated from Rubber Tree (*Hevea brasiliensis*)

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ABSTRACT

Rigidoporus microporus is the main causal of white root disease (WRD) in rubber trees (*Hevea brasiliensis*). The present study investigates the use of rhamnolipid, a biosurfactant produced by *Pseudomonas aeruginosa* USM-AR2 against *R. microporus*. *In vitro* dose-responses towards rhamnolipid were determined on different isolates of *R. microporus* using the poisoned food technique (PFT). Inhibition of mycelial growth was found to be dose-dependent, with the highest inhibition of 76.74% at 200 ppm (pH 6.29) on SEG isolate. On the contrary, the lowest concentration of rhamnolipid applied at 10 ppm (pH 5.97) had effectively inhibited the growth of RL 19 to 34.36%. AM isolate was assumed to be the most aggressive pathogen due to the lowest inhibition recorded on all rhamnolipid concentrations tested. At the same time, RL 19 was the least aggressive pathogen compared to the other *R. microporus* isolates. The rhamnolipid concentrations (ppm), which reduced mycelial growth at 50% (EC₅₀), were recorded at 17.82 ppm for AM isolate, 12.52 ppm for RL 26, and 11.80 ppm for RL 19 isolate. This result indicated that rhamnolipid concentrations to inhibit 50% of mycelial growth might vary based on the aggressiveness and the virulence levels of different *R. microporus* isolates. It was found that pH changes after incorporating rhamnolipid into the PDA were not the main factor affecting the inhibition of *R. microporus* isolates. It is obvious that rhamnolipid had an inhibitory effect on fungal growth *in vitro*. It is the first report on rhamnolipid that has been shown to control *R. microporus* potentially.

Keywords: EC₅₀, inhibition, isolate, *in-vitro*

ARTICLE INFO

Article history:

Received: 07 October 2021

Accepted: 13 December 2021

Published: 10 February 2022

DOI: <https://doi.org/10.47836/pjtas.45.1.17>

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INTRODUCTION

White root disease, caused by the pathogenic fungus *Rigidoporus microporus*, is the most destructive root disease of *Hevea brasiliensis* Muell. Arg. worldwide (Oghenekaro et al., 2016). Rubber industries worldwide have been facing significant reductions in economic returns due to this disease since the infection kills rubber trees irrespective of age (Soytong & Kaewchai, 2014). *Hevea brasiliensis*, regardless of clone and age, are susceptible to white root disease (Farhana et al., 2017). This disease occurs in young rubber plantations, virgin jungles, and replanted areas. It has been determined to be more severe in young rubber plantations, especially after two years of planting (Prasetyo et al., 2009). In Malaysia, a survey of rubber diseases conducted by the Malaysian Rubber Board in 2012 revealed that the incidence of white root disease occurred in 10–15% of Peninsular Malaysia, 20–30% of Sabah, and 9–20% in Sarawak out of the total area of rubber plantations, which amounts to 1,065,630 hectares (Atan, 2015). Furthermore, white root disease has resulted in more tree losses than red or brown root disease, particularly between the first to fourth years after planting (Nicole & Benhamou, 1991; Wattanasilakorn et al., 2017). The application of sulfur as one of the standard preventive methods of white root disease during the early stage of rubber cultivation, has been adopted widely in rubber growing countries (Ismail & Azaldin, 1985; Rodesuchit et al., 2012; Satchuthananthavale & Halangoda, 1971).

In addition, drenching with triazole group of fungicide, propiconazole was believed to be an effective method to control *R. microporus* as the procedure was easy and fast (Hashim & Chew, 1997). Although effective, this fungicide has caused a variety of problems for natural environments, including a significant impact on soil microbial diversity and human health, as well as being quite expensive when it is required to be used consistently (Go et al., 2013; Jayasuriya & Thennakoon, 2007; Ogbebor et al., 2015; Satapute & Kaliwal, 2015). Moreover, the incidence of white root disease is still widespread, prompting immediate further investigation into safer sources of natural fungicides for the control of *R. microporus*. Monnier et al. (2020) mentioned that rhamnolipids (RLs) are natural glycolipids mainly produced by the bacteria *Pseudomonas aeruginosa*. The robust surface activity of dirhamnolipid could be related to antifungals produced by rhamnolipid that inhibit the growth modes of dimorphic fungi resulting in the disruption of the cell membrane spore (Sha & Meng, 2016). Aside from the recognized antifungal properties, rhamnolipids have never been studied for antifungal activities against *R. microporus*. RLs have a hydrophobic tail containing one or two fatty acids attached to the carboxyl end of one or two rhaminose molecules (Charles Oluwaseun et al., 2017). A few studies have shown a significant impact of rhamnolipid treatments in controlling plant diseases (Borah et al., 2016).

Pseudomonas aeruginosa USM-AR2, a hydrocarbon-utilizing bacterium, has been shown to secrete copious amounts of rhamnolipid when grown on water-immiscible substrates (Md Noh et al., 2014; Noh et al., 2012). Rhamnolipid has been explored as a potential antifungal agent for environmental-friendly agricultural practice. The concern lies in the repeated use of fungicides, which are harmful to the environment and increase the chemical resistance in the target organisms. Should rhamnolipid supplementation prove to be a viable alternative treatment for *R. microporus*, further study must be carried out to identify its mode of action and explore its potential in curbing the spread of white root disease. Hence, the current study has been intended to examine the ability of rhamnolipid to suppress *R. microporus* *in vitro* and its subsequent use to control white root disease of rubber *in vivo*.

MATERIALS AND METHODS

Source of Isolate

In this study, five isolates of *R. microporus* obtained from the Integrated and Disease Management Unit, Malaysian Rubber Board were RL 18, RL 19, RL 26, SEG, and AM. The locations of where the isolates were collected are presented in Table 1.

Table 1
List of *Rigidoporus microporus* isolates used in this study

Isolate	Locality of Collection
RL 18	Sungai Buloh, Selangor
RL 19	Seremban, Negeri Sembilan
RL 26	Kota Tinggi, Johor
SEG	Segamat, Johor
AM	Ayer Molek, Melaka

Production of Rhamnolipid

Rhamnolipid production was performed by cultivating *P. aeruginosa* USM-AR2 via a submerged batch fermentation. Cultivation was done in a 3.6 L stirred tank bioreactor (Labfors 4, INFORS HT, Switzerland) with a 1.5 L working volume at room temperature (27-30°C), agitated at 400 rpm aerated at an airflow rate of 0.5 vvm. The pH was left uncontrolled. A minimal salt medium (MSM) was used to cultivate *P. aeruginosa* USM-AR2 containing the ingredients as follows (per liter): sodium nitrate (NaNO₃) 8.25 g/L, magnesium sulfate heptahydrate (MgSO₄·7H₂O) 0.75 g/L, potassium chloride (KCl) 1.5 g/L, dipotassium hydrogen phosphate (K₂HPO₄) 0.45 g/L, waste cooking oil 5% (v/v). Seed culture was prepared in nutrient broth before inoculation into the production medium. A 2% (v/v) cell suspension of a 24-h culture (OD₅₄₀ = 2) was used as the inoculum.

Rhamnolipid Recovery

The fermentation broth was centrifuged (Hettich Zentrifugen, Universal-320R, Germany) at 8000 × g for 20 minutes to separate the supernatant containing rhamnolipid from the cells. Subsequently, the supernatant was transferred into a 500 ml shake flask and added ethyl acetate at a 1:1 volume ratio. The supernatant-solvent mixture was shaken at 200 rpm for 1 hour in an orbital shaker (Thermo Scientific, MaxQ-4000, USA). It was subsequently transferred into a separating funnel, where it was left to stand overnight to form separate layers. The upper aqueous layer containing rhamnolipid

was carefully transferred into a glass Petri dish and dried overnight in a fume hood chamber. Dried crude rhamnolipid was scraped from the surface of the glass Petri dish and stored at 4°C for further use.

Agar Preparation

Different rhamnolipid concentrations were screened against *R. microporus* isolates while commercial triazole group fungicide, propiconazole was tested against the most aggressive isolate of *R. microporus* using poisoned food technique (PFT) (Balamurugan, 2014; Durgeshlal et al., 2019) with slight modification. Firstly, 3,000 ppm of propiconazole and rhamnolipid were prepared as a stock of treatments and autoclaved at 121°C, 1.05 kg/cm² for 21 minutes. Then, a specific amount of propiconazole and rhamnolipid stocks was incorporated into autoclaved potato dextrose agar (PDA) to achieve the desired concentration of 10, 25, 50, 100, and 200 ppm. The Petri dishes were shaken gently and laterally to allow propiconazole and rhamnolipid to distribute evenly in the PDA medium. PDA without rhamnolipid served as a negative control, while PDA amended with propiconazole serves as a positive control. The pH values of the prepared medium were recorded using Delta 320 pH meter (Mettler Toledo Instruments (Shanghai) Co. Ltd, China). The pH value of unamended PDA was recorded to be 6.0. Additionally, the pH values recorded for propiconazole and rhamnolipid ranged between 5.45 to 5.91 and 5.39 to 6.29, respectively, with the different concentrations applied into

amended PDA. Subsequently, the agar solutions were decanted into Petri dishes and left to set before being incubated for two days to ensure no contamination.

Antifungal Activity Assay

A five mm diametric mycelial plug of 7 days old culture of *R. microporus* was placed at the center of the Petri dishes. The Petri dishes were then sealed with parafilm (Pechiney, USA) and incubated at room temperature. Data on the radial colony diameter were recorded seven days after incubation or when the growth of the control treatment completely covered the Petri dishes. In addition, a colony radius on PDA for each rhamnolipid concentration was measured from the bottom side of the Petri dishes. The mycelium was observed in-situ under an Olympus CX41 light compound microscope (Olympus Optical Co. Ltd. Tokyo, Japan) with a magnification of 10x and images recorded using an XCAM-a camera (The Imaging Source GmbH, Germany). Percentage inhibition of radial growth (PIRG) was calculated using the following formula developed by Skidmore and Dickinson (1976):

$$\text{PIRG (\%)} = \left(\frac{\text{fungal growth} - \text{control growth}}{\text{control growth}} \right) \times 100\%$$

Experimental Design and Data Analysis

The experiments were conducted in a completely randomized design (CRD) with three replications. An analysis of variance (ANOVA) was performed using SPSS®

24.0 for the Windows program. Factorial ANOVA analyses and Duncan's multiple range test (DMRT) were used to detect significant differences between treatments, and differences were considered significant when $p \leq 0.05$. All data were expressed as mean \pm standard error. EC_{50} represents the concentration at which a rhamnolipid exerts half of its maximal response was analyzed using EC_{50} calculator software (AAT Bioquest Inc, n.d.).

RESULTS AND DISCUSSION

The ability of *P. aeruginosa* culture to produce rhamnolipid when grown on oil substrates has been widely reported (Ndlovu et al., 2017; Shi et al., 2021; Vanavil & Seshagiri, 2018). In this study, *P. aeruginosa* USM-AR2 was grown in a medium supplemented with waste cooking oil. Many studies have shown waste cooking oil as a renewable and low-cost substrate for rhamnolipid production (Chen et al., 2018; Radzuan et al., 2017). Waste cooking oil contains palm oil and other nutrient-rich compounds primarily to support microbial growth. Palm oil in

waste cooking oil consists of saturated and unsaturated fats, made up of triglycerides, diglycerides, monoglycerides, and free fatty acids.

After five days of fermentation, *P. aeruginosa* USM-AR2 produced the rhamnolipid concentration of 3.0 g/L. Rhamnolipid is a secondary metabolite characterized by its production during the stationary phase. Towards the end of fermentation, an increase in rhamnolipid production was observed after microbial growth had ceased (Md Noh et al., 2014). Rhamnolipid is an extracellular metabolite; hence, it is secreted into the culture broth. It was harvested and recovered from the culture supernatant. Subsequently, the crude rhamnolipid suspension was used to screen antifungal activity against *R. microporus*.

It was found that rhamnolipid could inhibit the growth of *R. microporus* (RL 19 isolate) to 34.36% at the lowest concentration of rhamnolipid (10 ppm) used. In comparison, the highest concentration (200 ppm) of rhamnolipid had inhibited 76.74% growth of SEG isolate of *R. microporus* (Table 2). In addition, three

Table 2

Mean inhibition of *Rigidoporus microporus* isolates at different rhamnolipid concentrations in amended PDA

Concentration of rhamnolipid (ppm)	Percentage inhibition of radial growth (%)				
	RL 18	RL 19	RL 26	SEG	AM
0	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a
10	30.39 \pm 1.71 ^b	34.37 \pm 1.83 ^b	29.52 \pm 3.35 ^b	25.64 \pm 2.37 ^b	24.66 \pm 2.47 ^b
25	57.06 \pm 0.34 ^c	56.51 \pm 0.99 ^c	55.02 \pm 1.12 ^c	57.26 \pm 0.25 ^c	47.26 \pm 3.89 ^c
50	65.29 \pm 0.59 ^d	67.98 \pm 0.58 ^d	60.24 \pm 1.20 ^d	64.41 \pm 1.45 ^d	59.81 \pm 1.65 ^d
100	69.22 \pm 1.09 ^e	71.53 \pm 1.00 ^e	64.66 \pm 0.20 ^d	69.98 \pm 0.34 ^e	64.38 \pm 0.68 ^d
200	75.10 \pm 0.85 ^f	76.47 \pm 0.90 ^f	73.89 \pm 0.72 ^e	76.74 \pm 0.38 ^f	74.66 \pm 5.14 ^e

Note. Data are means of three replicates \pm SE. Different letters within each column indicate significantly different values ($p \leq 0.05$) according to Duncan's multiple range test (DMRT)

isolates of *R. microporus* known as RL 19, RL 26, and AM collected from different locations in Peninsular Malaysia (Table 1) showed significant differences after exposure against rhamnolipid (Table 2 and Figure 1). Indeed, the effectiveness of rhamnolipid in promoting plant growth and controlling plant diseases has been discussed in many studies (Goswami et al., 2015; Jishma et al., 2021; Monnier et al., 2020).

The effectiveness of rhamnolipid in reducing the mycelial growth of the *R. microporus* depends highly on its concentration. It showed a significant difference among the total concentrations (ppm) applied compared to control (without rhamnolipid addition) (Figures 2 and 3). The results obtained were similar to Deepika et al. (2015), who claimed a significant reduction in tomato disease severity with the increased concentration of

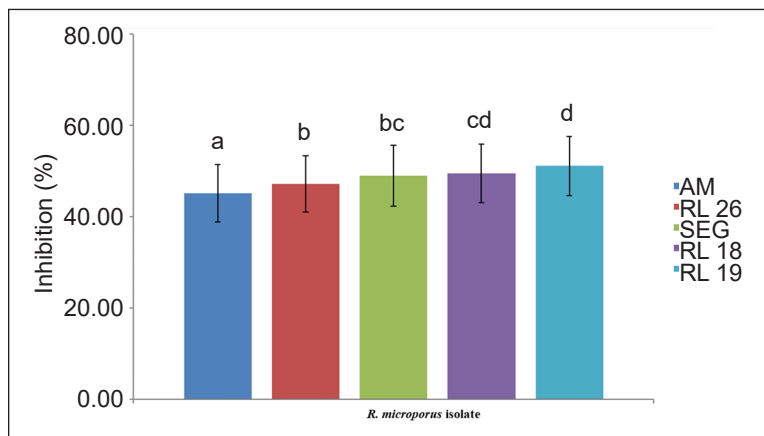


Figure 1. Inhibitory effect of rhamnolipid on mycelial growth of five (5) *Rigidoporus microporus* isolates on amended PDA with rhamnolipid. Error bars represent standard error (SE). Different letters indicate significantly different values at $p \leq 0.05$ according to Duncan's multiple range test (DMRT)

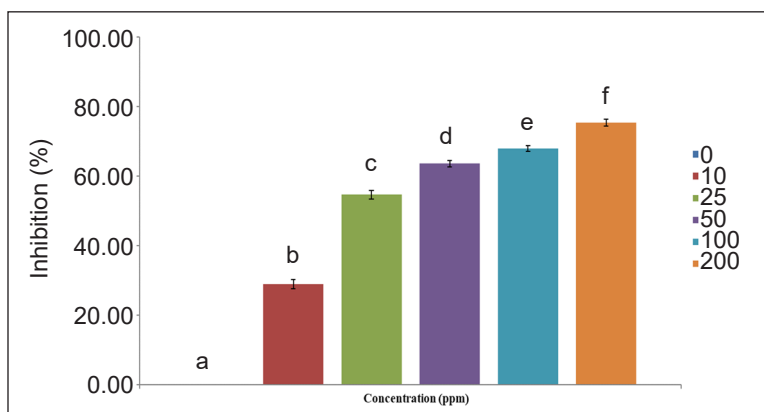


Figure 2. Inhibitory effect of different rhamnolipid concentrations on mycelial growth of *Rigidoporus microporus* isolates on PDA. Error bars represent standard error (SE). Different letters indicate significantly different values at $p \leq 0.05$ according to Duncan's multiple range test (DMRT)

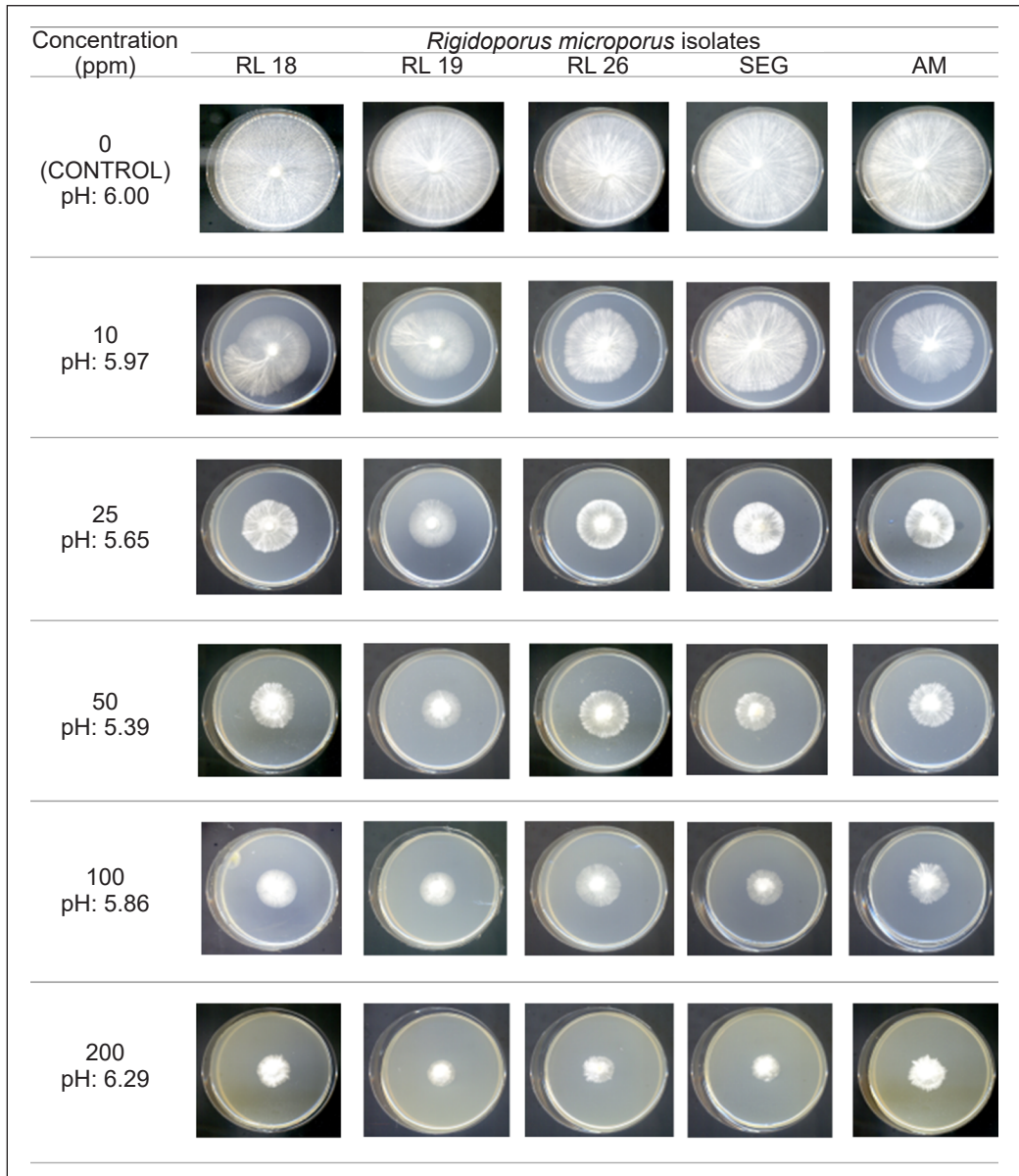


Figure 3. Cultures of *Rigidoporus microporus* in control and different concentrations of rhamnolipid incorporated into the PDA after 7 days of incubation

rhamnolipids used as treatment. Goswami et al. (2015) mentioned that more than 60 % reduction occurred on spore germination of *Colletotrichum falcatum* after exposure to the rhamnolipid at the concentration of 50 μgml^{-1} . Similarly, Sha and Meng (2016)

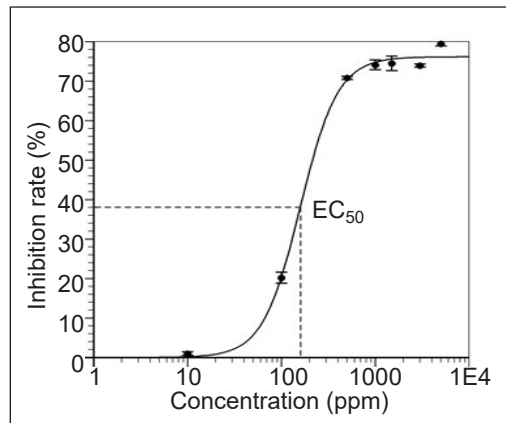
found that the application of 60 μgml^{-1} rhamnolipids had inhibited the colony growth of *Verticillium dahliae* ATCC 7611 up to 73%.

In addition, rhamnolipid biosurfactant was observed to be highly effective not

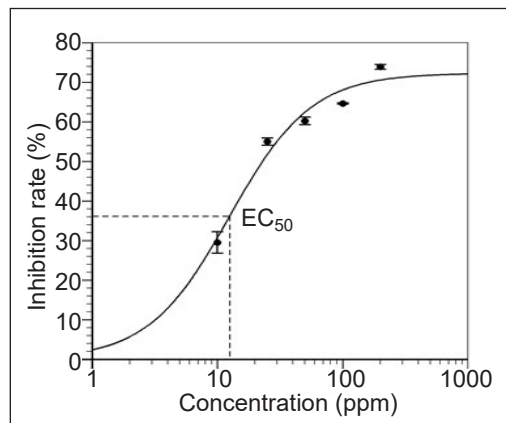
only in completely inhibiting the eggplant disease severity caused by *Fusarium oxysporum* f. sp. *melongenae* (Fomg) was also environmentally friendly (Nalini & Parthasarathi, 2018). On the contrary, Yan et al. (2014) revealed that rhamnolipid alone was not practical to control the growth of *Alternaria alternata* infection in cherry tomato fruit *in vivo*, but it was more efficient when combined with *Rhodotorula glutinis*. This finding was contended by Borah et al. (2016), who found the complete inhibition of stalk and ear rot disease in maize cv. PAC740 in a single application of rhamnolipid at 50 mg l⁻¹.

On average, AM isolate exhibited the lowest inhibition rate (%) compared to RL 19 isolate after being treated with different rhamnolipid concentrations (ppm). It is speculated that this is due to the different levels of virulence among isolates. Based on phylogenetic analysis of the β -tubulin gene region, it was discovered that *R. microporus* isolates collected from various areas in Malaysia using cultural and molecular characteristics had a distinct geographical origin among 27 local isolates (Andrew et al., 2021). Additionally, Siddiqui et al. (2017) claimed that AM isolate had been classified as an aggressive pathogen due to the expression of a higher number of proteins in AM-infected samples.

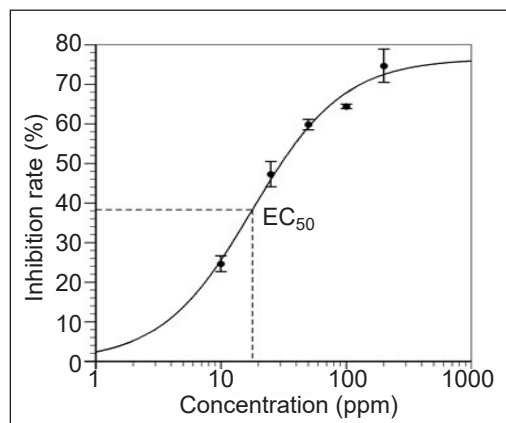
It was observed that the concentrations of rhamnolipid required to reduce mycelial growth at 50 % (EC₅₀) on RL 19, RL 26, and AM were recorded at 11.80 ppm, 12.52 ppm, and 17.81 ppm, respectively (Figure 4). The minimal concentration of



(a)



(b)



(c)

Figure 4. EC₅₀ values of rhamnolipid concentration (ppm) on mycelium growth of three (3) isolates of *Rigidoporus microporus* on PDA. (a) EC₅₀ of RL 19 = 11.80 ppm; (b) EC₅₀ of RL 26 = 12.52 ppm; (c) EC₅₀ of AM = 17.81 ppm

rhamnolipid needed to reduce 50% of the *R. microporus* growth implies the efficacy of rhamnolipid as an alternative method to control the incidence of white root disease in rubber. In comparison to propiconazole, the efficacy of rhamnolipid requires a bit higher concentration to reduce 50% of fungal growth. The results were similar to the study on the effect of rhamnolipid towards *A. alternata* on cherry tomato fruit, where higher rhamnolipid concentration was required to control the pathogen compared to synthetic fungicides (Yan et al. 2014).

On the contrary, the application of the triazole group of fungicide, propiconazole, showed the highest inhibition growth of the *R. microporus* even at the minimal concentration of 10 ppm (76.27%) (Table 3). Although effective in inhibiting the *R. microporus* growth, propiconazole could be detrimental to the environment and human health. Knebel et al. (2018) as well as Satapute and Kaliwal (2015) revealed that propiconazole was discovered to be cytotoxic to human cancer cell line, exhibits an anticancer property, and is toxic to the liver. Furthermore, the Risk Assessment Committee (RAC) of the European Chemicals Agency (ECHA) has proposed that propiconazole be classified as toxic for

reproduction category 1B, in accordance with the provisions of Regulation (EC) No 1272/2008, due to the toxic effects on the endocrine organs and the contamination of groundwater (Arena et al., 2017).

Although there is a concern on cost-effectiveness to produce a higher yield of rhamnolipid, Nalini and Parthasarathi (2018) suggested the application of solid-state fermentation, which was more efficient and cheaper in the production of biosurfactant compared to the conventional method by using submerged fermentation. Furthermore, they mentioned that rhamnolipid is a better alternative to chemical surfactants because of its low toxicity, greater biodegradability, environmentally friendly, and ability to reduce agrochemicals. Besides, rhamnolipid could also be applied directly to the diseased plant to control the fungal growth *in vivo* (Monnier et al., 2020).

Presumably, rhamnolipid incorporated into the PDA had not caused any significant changes in the pH values compared to the control (Figure 3). According to de Freitas Ferreira et al. (2019), rhamnolipid increased the antimicrobial activity in acidic conditions, and it can be classified as a pH-dependent biosurfactant. This finding seems contradictory with the result in this

Table 3
Mean inhibition of *Rigidoporus microporus* (AM isolate) at different propiconazole concentrations in amended PDA

Percentage inhibition of radial growth (%)	Concentration of propiconazole (ppm)					
	0	10	25	50	100	200
AM	0.00 ± 0.00 ^a	76.27 ± 0.90 ^b	100.00 ± 0.00 ^c	100.00 ± 0.00 ^c	100.00 ± 0.00 ^c	100.00 ± 0.00 ^c

Note. Data are means of three replicates ± SE. Different letters within each column indicate significantly different values ($p \leq 0.05$) according to Duncan's multiple range test (DMRT)

study that showed the highest inhibition (>70%) of *R. microporus* isolates at the pH 6.29 (neutral condition) compared to a low inhibition (%) at a lower pH (more acidic condition). In the other study, Hadi et al. (2021) revealed that the higher pH of PDA after amended with soluble silicon did affect the growth of *R. microporus* (AM isolate), whereas no inhibition of *R. microporus* isolates was recorded in amended PDA without soluble silicon at higher pH. This finding was supported by the other study that mentioned the increase in pH values after incorporating soluble silicon into PDA did not solely cause the inhibition of mycelial growth of *Phytophthora cinnamomi* as the comparison test by increasing the pH values of PDA by using potassium hydroxide (KOH) had not given a significant reduction in the growth of *P. cinnamomi* mycelial (Kaiser et al., 2005). On the other hand, Prasetyo et al. (2009) mentioned that the growth of *R. microporus* in the rubber plantation was enhanced in the porous soil with neutral soil pH approximately between 6 to 7. Thus, the pH values can be ruled out as the main cause of the *R. microporus* growth inhibition.

The observation of *R. microporus* mycelial under a light compound microscope revealed the morphological changes of the mycelial structure comparing the untreated mycelial of *R. microporus* with the treated mycelial in amended PDA with 10 ppm and 200 ppm of rhamnolipid (Figure 5). The untreated mycelial were long, even, and the round hyphal with a smooth surface (Figure 5a). However, it was observed that the treated mycelial of *R. microporus* with rhamnolipid had shown sparse, asymmetric, curling, and twisting mycelium (Figure 5b); thinner and distorted newborn hyphae (Figure 5c). This result is similar to the scanning electron microscope observations of Borah et al. (2016), who found that the mycelial of *Fusarium verticillioides* FS7 was exhibited an irregular shape with an uneven surface, severely reduced thickness, and breakage after treated with rhamnolipid at the concentration of 200 mg l⁻¹ in potato dextrose broth as compared to untreated mycelial. A fungus contains the vegetative structure known as mycelium that plays a crucial role in asexual reproduction and disease progression. As a result, any disruption to the mycelial integrity of a

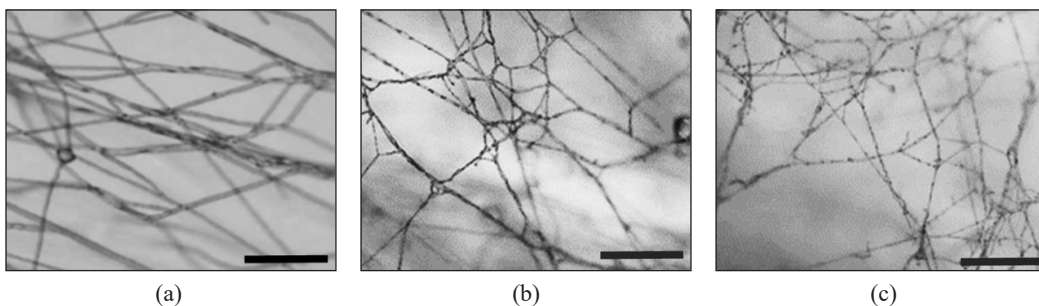


Figure 5. Olympus CX41 light compound microscope images of (a) the untreated mycelial of *Rigidoporus microporus* (AM isolate); (b) mycelial treated with 10 ppm rhamnolipid; (c) mycelial treated with 200 ppm of rhamnolipid (scale bar: 200 μ m; magnification at 10 \times)

fungus may have a detrimental effect on the pathogenicity of the fungus.

In this study, the light microscope was used to assess the effect of rhamnolipid on the mycelia of *R. microporus*. The findings demonstrated that rhamnolipid, a biosurfactant generated by *P. aeruginosa* USM-AR2, could severely modify the morphology of fungal mycelia. It has previously been observed that rhamnolipid influences the mycelial structure of fungi (Borah et al., 2016; Yan et al., 2015). The damage could be caused by the rhamnolipid's surfactant activity, which causes the breakdown of the phospholipid bilayer of the cell membrane, resulting in the leakage of electrolytes, proteins, and DNA (Bharali et al., 2013; Yan et al., 2015). According to Sotirova et al. (2012) and Monnier et al. (2019), the ability of rhamnolipid to inhibit fungal mycelial growth was attributed to the degradation of the cell membrane. Based on these results, early predictions can be made where rhamnolipid could have an inhibitory effect on fungal growth *in vitro*, mostly fungicidal.

CONCLUSION

The results of the present investigation reveal that the rhamnolipid biosurfactant produced by the bacterial strain *Pseudomonas aeruginosa* USM-AR2 has strong antifungal activity against *Rigidoporus microporus*, which may offer the possibility of its application as an alternative fungicide. In addition, the production of rhamnolipid from recycled material, such as waste

cooking oil, is expected to be more economical and environmentally friendly than a current commercial fungicide. However, the concentration at which complete suppression of a particular fungus occurs varies and must be determined *in vitro* before *in vivo* investigations are initiated. Therefore, pot and field trials are suggested to be carried out in the near future to confirm the efficacy of rhamnolipid against *R. microporus in vivo*.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ACKNOWLEDGEMENTS

The authors would like to thank the researchers from the Integrated Disease and Plant Management Unit of the Malaysian Rubber Board for kindly supplying the isolates of *R. microporus* as well as for technical support and help in the experiments. We also thank the Malaysian Rubber Board for the academic scholarship. This research is funded by the Fundamental Research Grant Scheme: FRGS/1/2020/WAB04/USM/02/2 from the Ministry of Higher Education Malaysia. Furthermore, rhamnolipid production was supported by Universiti Sains Malaysia through Research University Individual (RUI) Grant (1001/PBIOLOGI/8011090).

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