RHAMNOLIPID AS ANTIFUNGAL AGENT AGAINST *Pyricularia oryzae* AND *Rhizoctonia solani* CAUSING BLAST AND SHEATH BLIGHT DISEASES OF PADDY

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by

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LIST OF SYMBOLS

˚C	Degree Celsius
˚E	East
g	Gravitational constant
<	Less than
×	Multiplication sign
˚N	North
p	P-value
%	Percentage
±	Plus minus
®	Registered trademark
V	Volt

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
bp	Base pair
cm	Centimetre
cm/day	Centimetre/day
cm^2	Square centimetre
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DS	Disease severity
$FeCl_3 \cdot 6H_2O$	Ferric chloride
g	Gram
g/L	Gram per litre
h	Hour
H_2SO_4	Sulphuric acid
ha	Hectare
IRRI	International Rice Research Institute
ITS	Internal transcribed spacer
K_2HPO_4	Dipotassium phosphate
KCl	Potassium chloride
kg	Kilogram
LSD	Least Significant Difference test
L	Litre
mA	Milliampere

MADA	Muda Agricultural Development Authority
MARDI	Malaysia Agricultural Research and Development Institute
$MgCl_2$	Magnesium chloride
$MgSO_4 \cdot 7H_2O$	Magnesium(II) sulphate
MIC	Minimum inhibitory concentration
min	Minute
mL	Millilitre
ML	Maximum likelihood
mM	Millimetre
$MnSO_4H_2O$	Manganese(II) sulphate
MR	Malaysia Rice
MSM	Mineral salt medium
$NaNO_3$	Sodium nitrate
NB	Nutrient broth
NCBI	National Center for Biotechnology Information
NJ	Neighbor Joining
nm	Nanometre
OD	Optical density
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PIDG	Percentage inhibition of diameter growth
PSI	Percentage of spore inhibition
PISC	Percentage inhibition of sclerotia number
ppm	Parts per million

RM	Malaysian Ringgit
rpm	Rotation per minute
s	Second
SDGs	Sustainable Development Goals
SEM	Scanning electron microscope
sp.	Species
spores/mL	Spores per millilitre
spp.	Species
TBE	Tris-Borate-EDTA
v/v	Volume per volume
µL	Mircrolitre
µm	Micrometre

LIST OF APPENDICES

RHAMNOLIPID SEBAGAI AGEN ANTIKULAT TERHADAP *Pyricularia oryzae* DAN *Rhizoctonia solani* PENYEBAB PENYAKIT KARAH DAN PENYAKIT HAWAR SELUDANG PADA PADI

ABSTRAK

Pyricularia oryzae dan *Rhizoctonia solani* ialah kulat yang menyebabkan penyakit karah dan penyakit hawar seludang pada padi (*Oryza sativa*) di Malaysia. Penggunaan varieti padi rintang terhadap penyakit dan racun kulat untuk mengawal kedua-dua penyakit tersebut boleh meningkatkan rintangan kulat dan pelepasan bahan kimia ke dalam saluran air. Penyelidikan ini menilai aktiviti antikulat rhamnolipid, biosurfaktan yang dihasilkan oleh *Pseudomonas aeruginosa* USM-AR2 yang lebih mampan berbanding racun kulat kimia. Tujuh pencilan kulat *Pyricularia* sp. dan lapan pencilan kulat *Rhizoctonia* sp. daripada tiga puluh pencilan kulat berjaya diperoleh daripada simptom karah dan hawar seludang dari sawah padi di Alor Setar, Kedah, Malaysia. Ujian kepatogenan mengesahkan semua pencilan *Pyricularia* sp. dan *Rhizoctonia* sp. adalah patogenik dengan masing-masing menunjukkan simptom tipikal jingga keperangan berbentuk berlian dan hawar seludang berbentuk tidak menentu seperti air terendam. Pencirian morfologi dan pengenalpastian molekul yang diwakili satu pencilan kulat daripada setiap kumpulan mengesahkan identiti sebagai *P. oryzae* USM-PD1 and *R. solani* USM-PD2. Selain itu, penilaian antikulat rhamnolipid bagi kedua-dua kulat menunjukkan perencatan miselium dengan lisis dan luka serta pertumbuhan perlahan pada plat agar. Peratus perencatan diameter pertumbuhan (PIDG) tertinggi bagi *P. oryzae* USM-PD1 dicapai pada 2000 ppm (48.30 ± 1.18%). Sementara itu, PIDG dan peratus perencatan bilangan sklerotia (PISC) bagi *R. solani* USM-PD2 masing-masing diperoleh pada 10 000 ppm (60.00 ±

2.35%) dan 800 ppm (100%). Pemerhatian di bawah mikroskop elektron imbasan mendedahkan lebih jelas pembentukan miselium dan spora yang tidak sihat apabila dirawat dengan rhamnolipid dengan morfologi sel kecut, nipis, dan terherot. Peratus perencatan spora tertinggi bagi *P. oryzae* USM-PD1 adalah 92.47 ± 4.04% pada 2000 ppm. Seterusnya, kajian *in vivo* pra-jangkitan dengan semburan rhamnolipid bagi varieti padi MR212, MR297, dan MR315 terhadap *P. oryzae* USM-PD1 masing-masing menunjukkan keterukan penyakit minimum sehingga 19.44 ± 5.94% (1000 ppm), 4.17 ± 5.75% (1000 ppm), dan 12.50 ± 2.57% (500 ppm). Manakala, bagi *R. solani* USM-PD2 sehingga 46.52 ± 8.37% (3000 ppm), 25.00 ± 6.64% (5000 ppm), dan 24.30 ± 8.36% (5000 ppm). Selain itu, jangkitan minimum pasca jangkitan dengan rawatan rhamnolipid terhadap *P. oryzae* USM-PD1 adalah 13.19 ± 4.13% (MR212), 45.83 ± 7.12% (MR297), dan 25.69 ± 4.13% (MR315) pada 1000 ppm. Manakala, bagi *R. solani* USM-PD2 pula diperoleh pada 3000 ppm dengan 30.56 ± 5.94% (MR212), 38.19 ± 9.12% (MR297), dan 15.28 ± 4.92% (MR315). Secara keseluruhannya, penyelidikan ini memberi sorotan potensi penggunaan rhamnolipid sebagai strategi pengurusan alternatif bagi kedua-dua penyakit dalam penanaman padi.

RHAMNOLIPID AS ANTIFUNGAL AGENT AGAINST *Pyricularia oryzae* AND *Rhizoctonia solani* CAUSING BLAST AND SHEATH BLIGHT DISEASES OF PADDY

ABSTRACT

Pyricularia oryzae and *Rhizoctonia solani* are fungi causing blast and sheath blight diseases of paddy (*Oryza sativa*) in Malaysia. The use of disease resistant rice varieties and fungicides to control both diseases might increase the fungal resistance and the discharge of chemicals into watercourses. This research evaluates the antifungal activity of rhamnolipid, a biosurfactant produced by *Pseudomonas aeruginosa* USM-AR2 which is more sustainable than chemical fungicides. Seven isolates of *Pyricularia* sp. and eight isolates of *Rhizoctonia* sp. from thirty isolates were successfully obtained from blast and sheath blight symptoms in a paddy field in Alor Setar, Kedah, Malaysia. Pathogenicity test confirmed that all *Pyricularia* sp. and *Rhizoctonia* sp. isolates were pathogenic by showing a typical blast as an orange brown edge diamond-shaped and sheath blight as an irregular water-soaked. Morphological characterisation and molecular identification of one representative isolate from each group confirmed the identity as *P. oryzae* USM-PD1 and *R. solani* USM-PD2. Moreover, antifungal evaluation of rhamnolipid against both fungi demonstrated retarded mycelia with lysis and lesion, as well as slow growth on plates. The highest percentage inhibition of diameter growth (PIDG) for *P. oryzae* USM-PD1 was achieved at 2000 ppm (48.30 ± 1.18%). Meanwhile, PIDG and percentage inhibition of sclerotia (PISC) number of *R. solani* USM-PD2 were obtained at 10 000 ppm (60.00 ± 2.35%) and 800 ppm (100%), respectively. Observation under scanning electron microscope revealed clearer formation of unhealthy mycelium and spore when treated with rhamnolipid with shrivelled, thinner, and distorted cellular morphology. The highest percentage of spore inhibition of *P. oryzae* USM-PD1 was 92.47 ± 4.04% at 2000 ppm. Subsequently, *in vivo* study of pre-infection spray with rhamnolipid for respective rice varieties of MR212, MR297, and MR315 against *P. oryzae* USM-PD1 exhibited the minimum disease severity of 19.44 ± 5.94% (1000 ppm), 4.17 ± 5.75% (1000 ppm), and 12.50 ± 2.57% (500 ppm). Whereas, for *R. solani* USM-PD2 at 46.52 ± 8.37% (3000 ppm), 25.00 ± 6.64% (5000 ppm), and 24.30 ± 8.36% (5000 ppm). Besides, the minimum infection for post-infection with rhamnolipid treatment against *P. oryzae* USM-PD1 at 13.19 ± 4.13% (MR212), 45.83 ± 7.12% (MR297), and 25.69 ± 4.13% (MR315) at 1000 ppm. Meanwhile, for *R. solani* USM-PD2 was at 3000 ppm with 30.56 ± 5.94% (MR212), 38.19 ± 9.12% (MR297), and 15.28 ± 4.92% (MR315). Overall, this research highlights the potential application of rhamnolipid as alternative management strategy of both diseases in paddy plantation.

CHAPTER 1

INTRODUCTION

1.1 Research background

Agricultural production and food supply must satisfy the expanding demands of the human population in the world (Hemathilake & Gunathilake, 2022). The agricultural sector is also known as the backbone of developing countries (Workie et al., 2020). Alongside, sustainability and food security challenges particularly in the development of agriculture, remain critical in the developed nation of Malaysia (Chamhuri Siwar et al., 2009; Islam & Siwar, 2012). Generally, plant diseases caused by phytopathogenic fungi reduce crop productivity in economically important agricultural crops. Recently, Malaysia's self-sufficiency program has focused on rice cultivation to sustain progressive and innovative efforts to support the development of the rice sector (NurulNahar et al., 2020).

Rice (*Oryza sativa*) is known as the country's primary staple food which influences the culture, cuisine, and economy of millions of people (Gnanamanickam et al., 2002). Rice contains a good source of vital nutrients, namely magnesium, phosphorus, manganese, selenium, iron, folic acid, thiamine, and niacin which benefit human health (Fukagawa & Ziska, 2019; Gnanamanickam et al., 2002). However, rice diseases are one of the challenges in rice cultivation. Common rice diseases afflicting rice crops in Malaysia and other rice-growing nations include blast, sheath blight, bacterial leaf blight, bacterial panicle blight, tungro, and bakanae (Habibuddin et al., 1997; Naqvi et al., 2019; NurulNahar et al., 2020; Senapati et al., 2022; Shahriar et al., 2020; Zhou, 2019). This decreases food availability by interfering crop yields, resulting in food insufficiency and reduced agricultural production quality and nutritional value. According to the Muda Agricultural Development Authority

(MADA) and the Malaysia Agricultural Research and Development Institute (MARDI), rice blast and rice sheath blight caused by phytopathogenic fungi, *Pyricularia oryzae* and *Rhizoctonia solani*, are two major diseases in paddy plantation in Malaysia.

Rhamnolipid, a glycolipid biosurfactant, is a promising antifungal agent for environmental-friendly agricultural practice. Rhamnolipid is mainly produced by *Pseudomonas aeruginosa* in the cultivation medium as a secondary metabolite (Sharma et al., 2018). *Pseudomonas aeruginosa* is a Gram-negative bacterium that can thrive in a wide range of environments, especially in oil-contaminated sites (Cheng et al., 2017; Deepika et al., 2017; Zhang et al., 2005). This bacterium secretes a high production of rhamnolipids when grown on oil substrate. Currently, waste cooking oil has been proven viable in producing good yields of rhamnolipid in *P. aeruginosa* USM-AR2 fermentation (Aggo et al., 2023; Md Noh et al., 2014).

Biosurfactants possess a hydrophilic-lipophilic balance, which describes the proportion of hydrophilic and hydrophobic constituents of the molecule (Desai & Banat, 1997). A review by de Souza et al. (2024) emphasized the advantages of biosurfactants over synthetic surfactants. In comparison to synthetic surfactants, biosurfactants are low toxicity, biodegradable, exhibit excellent surface activity, have high specificity, are effective in extreme conditions and can be produced from renewable sources (Lima et al., 2011; Thakur et al., 2021). Taking into account their versatile features, rhamnolipid biosurfactants have attracted interest in various industrial applications including environmental bioremediation and agriculture.

Nowadays, the commercialisation of rhamnolipid biosurfactant is increasing due to its diverse properties, notably in agriculture (Begum et al., 2023; Sachdev &

Cameotra, 2013). The best commercial producers of rhamnolipids from 2022 and 2023 include Evonik Industries (Germany), Jeniel Biotechnology (United States), AGAE Technologies (United States), Biotensidon GmbH (Germany), and Glycosurf (United States). In 2004, it was stated that around 0.2 million tons of surfactants were utilised in crop protection and pesticide formulations (Deleu & Paquot, 2004). The worldwide biosurfactant market is expected to increase from USD 4.07 billion in 2022 to USD 4.39 billion in 2023 at a compound annual growth rate (CAGR) of 7.8%, reaching USD 6 billion at a CAGR of 8.1% (Begum et al., 2023). According to Mordor Intelligence, the rhamnolipid market size is predicted to grow from USD 41.15 million in 2024 to USD 52.47 million with a CAGR of 4.98% during the forecast period (2024-2029) (Mordor Intelligence, 2024). In adhering to sustainable agricultural practices, renewable raw materials such as agricultural wastes can be used as feedstock for rhamnolipid production.

Rhamnolipid offer applications in agriculture such as biological control, improving agricultural soil quality, enhancing biodegradation of pollutants, and increasing plant-microbe interactions (Sachdev & Cameotra, 2013). Previous reports documented that rhamnolipid as an antifungal agent has been studied against phytopathogenic fungi such as *Alternaria alternata, Botrytis cinerea, Colletotrichum falcatum,* and *Fusarium verticillioides,* isolated from different types of host plants. Several studies reported the mechanisms of action are via the destruction of the cell membranes and reduction of spore movement that may lead to spore collapse (Borah et al., 2016; Goswami et al., 2015; Sha et al., 2011; Yan et al., 2015). These significant qualities demonstrate the potential of rhamnolipid as an antifungal agent in agriculture for plant disease management.

1.2 Problem statement

Phytopathogenic fungi, *P. oryzae* and *R. solani* cause blast and sheath blight diseases in rice, reducing crop yields globally. Moreover, outbreaks of rice blast and sheath blight in paddy plantation have been major and recurring concerns. To date, various management strategies have been taken to control both diseases such as breeding rice with improved resistance, using chemical control, cultural practices, and nutrient management. However, one of the main challenges in producing new varieties of rice is the need to increase resistance towards *P. oryzae* and *R. solani.*

It is a common and standard practice to apply chemical fungicides to control plant diseases and to increase crop productivity in agriculture. Nevertheless, the excessive and repeated use of synthetic fungicides has resulted in several worrisome consequences, including the release of chemical fungicide into water bodies, disruption of the ecological balance of natural soil microbes, fungal resistance, and increased health risks to humans. Although conventional chemical fungicides inhibit hyphal growth and enzyme activity in microorganisms, long-term use of fungicides may result in the development of phytopathogenic fungi strains tolerant or resistant to antifungal drugs.

In light of this, by having a good natural producer of rhamnolipid, along with the motivation to address food security and sustainable development goals (SDGs) through agriculture, rhamnolipid offers a sustainable, safer, and environmental-friendly alternative compared to commercial chemical fungicides. Thus far, there are no reports of rhamnolipid against *P. oryzae* and *R. solani* causing blast and sheath blight diseases of paddy, respectively.

1.3 Hypothesis

Rhamnolipid produced by *P. aeruginosa* USM-AR2 possesses antifungal efficacy as a biofungicide against phytopathogenic fungi, *P. oryzae* and *R. solani*, to control rice blast and rice sheath blight.

1.4 Objectives

The objectives of this research are:

i. To screen the fungal isolates associated with blast and sheath blight of paddy.
ii. To characterise *P. oryzae* and *R. solani* based on morphological, pathogenicity, and molecular analysis.
iii. To evaluate the effect of rhamnolipid against *P. oryzae* and *R. solani* on mycelial growth, spore and sclerotia number, and cellular morphology.
iv. To assess the effect of rhamnolipid against *P. oryzae* and *R. solani* conducted *in vivo* on rice seed and rice plant.

CHAPTER 2

LITERATURE REVIEW

2.1 Food security and agricultural production

Food is one of the most important fundamental needs for human survival. “Safe food” is defined as high quality, ecologically friendly, free of genetically modified organisms (GMOs), and free from potentially dangerous food-related factors such as pesticides (Umarjonovna & Gulomjonovna, 2022; Zibaee, 2013). According to the World Health Organization (WHO), food security exists when everyone in the country has sufficient access to safe and nutritious food that satisfies their dietary needs and food preferences for an active and healthy lifestyle (World Health Organization, 2023). It is impacted by food availability, access, usage, and supply stability, and is connected to livelihood security (de Pee., 2012). This vision necessitates efforts across the food, health, social protection, trade, and education sectors, as well as effective food system governance, to guarantee that the urgent and long-term food and nutrition requirements of the population are satisfied.

Alongside, food demand increases along with increases in population variation and demographic changes, including population growth, changes in population structure, consumption growth, and urbanization. Challenges in food security also necessitate more than merely inventing to raise yields; it necessitates changes in agricultural systems to encourage sustainable and resilient food production to suit the nutritional needs of local and global populations (Benton, 2016). According to the latest Global Report on the Food Crises 2023 Mid-Year Update, 238 million people across 48 countries face severe food insecurity and malnutrition. The three key drivers of food insecurity in 2023 include conflict, economic issues, and climate-induced extreme weather. This challenge brings all countries together to ensure the long-term viability of food, particularly in agriculture.

The issues of sustainability and food security remain essential to Malaysia's vision of becoming a developed nation, especially in agricultural development. This is in parallel to the sustainable development goals (SDGs) that require Member States to "end hunger, achieve food security, improve nutrition, and promote sustainable agriculture" (Jones & Ejeta, 2016). Agriculture is particularly important in the economy since it is recognized as the backbone of the economic system in developing countries (Azam & Shafique, 2017; Basiron, 2007). Agriculture has the potential to alleviate poverty, increase income, and enhance food security for 80% of the world's poor, especially those living in rural regions (Basiron, 2007).

2.1.1 Rice production in Malaysia

Increasing agricultural production to fulfil the food demand of the growing human population is a major challenge for all countries (Sachdev & Cameotra, 2013). Malaysia's self-sufficiency programme has been built on rice and rice farming, the country's principal staple food crop. This is the primary reason why paddy plantation has received more attention in recent years. Three food security policy objectives have been developed since the 1970s: to provide a high price to paddy farmers who produce rice, to attain a specified level of rice self-sufficiency (SSL), and to assure consistent and high-quality rice to consumers (Bala et al., 2014). To address food security issues, the government adopted the National Food Security Policy Action Plan 2021-2025 and the National Agro-Food Policy 2021-2030 (DAN 2.0).

Along with this endeavour, Malaysia has maintained progressive and innovative efforts to encourage the development of the paddy and rice sector (Firdaus et al., 2020). An important goal of the Ministry of Agriculture and Food Security (MAFS) is to raise the self-sufficiency level (SSL) and industrial revenue. According to NurulNahar et al. (2020), SSL accounts for around 72% of Malaysia's entire demand. The SSL national ideas involve the capacity to produce adequate food locally, receive enough healthy food, and sustain it easily. Imports from other nations are required to compensate for the insufficient rice supply in the country.

To attain national food security, Malaysia must achieve both sufficient rice production and consumption. This is consistent with the 2024 budget, which also focuses on the rice and paddy sector including efforts to invest RM 3 billion to develop irrigation infrastructure in the Muda Agricultural Development Authority (MADA) districts in Perlis and Kedah, to increase rice production (Astro Awani, 2023). This effort is in line with the National Agricultural Policy, which attempts to intervene strategically in the paddy and rice sector while ensuring the country's food security.

2.2 Rice diseases in Malaysia

In Malaysia, rice diseases such as blast, sheath blight, bacterial leaf blight (BLB), and tungro affect the rice productivity system (Shakirin et al., 2019). Blast disease is caused by *P. oryzae*, and the first case was reported in Peninsular Malaysia in 1945 (Habibuddin, 2012). According to NurulNahar et al. (2020), the national yield loss in Jaya, a susceptible rice variety, was estimated to be over 70%. Moreover, sheath blight of rice caused by *R. solani*, was reported to exceed 45% (Margani et al., 2018). Other than fungi, bacterial and viral infections also affect rice productivity. Bacterial leaf blight infected by *Xanthomonas oryzae* pv. *oryzae* also affects rice yields (Chukwu et al., 2019). About 30 to 50% of yield reduction is due to BLB, and the most severe BLB outbreak for about 50 to 70% loss of output in the previous 30 years occurred in paddy fields located in Sekinchan, Selangor (Shamsudin et al., 2019; Toh et al., 2019). Bacterial panicle blight caused by *Burkholderia glumae* results in 50% yield loss (Dorairaj & Govender, 2023; Urakami et al., 1994). Moreover, leafhoppers transmit rice tungro disease caused by a combination of two viruses, rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV) (Dorairaj & Govender, 2023; Habibuddin et al., 1997). In addition, brown spot disease is reported to be infected by *Bipolaris oryzae* (Dorairaj & Govender, 2023). Rice diseases and their symptoms are shown in Table 2.1 and Figure 2.1.

Table 2.1 The list of rice diseases, pathogens, and symptoms observed on rice crops (*O. sativa*)

Plant diseases	Pathogens	Symptoms	References
Blast (Bl)	*Pyricularia oryzae*	Diamond-shaped symptomatic leaves with a greyish center and brown edge	Hajano et al. (2011), NurulNahar et al. (2020), Shahriar et al. (2020)
Sheath blight (ShB)	*Rhizoctonia solani*	Circular or oval with water-soaked spots and greenish-grey in colour	Margani et al. (2018), Senapati et al. (2022)
Bacterial leaf blight (BLB)	*Xanthomonas oryzae* pv. *oryzae*	Irregular water-soaked lesions later turned to yellowish white in colour	Chukwu et al. (2019), Mizukami & Wakimoto (1969), Naqvi et al. (2019)
Bacterial panicle blight	*Burkholderia glumae*	Discoloration of grain and panicle blanking	Dorairaj & Govender (2023), Urakami et al. (1994), Zhou. (2019)
Rice tungro disease	Rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV)	Discoloration, stunted, fewer tillers, and infertile-filled grains	Azzam & Chancellor (2002), Habibuddin (2012)
Brown spot (BS)	*Bipolaris oryzae*	Lesions with a pale reddish-brown core surrounded by a dark brown edge	Ashfaq et al. (2021), Dorairaj & Govender (2023)
Bakanae disease (Bak)	*Fusarium fujikuroi*	Infected seedlings are taller, slender, and slightly chlorotic	Wan Nur Ain et al. (2015)
False smut (FSm)	*Ustilaginoidea virens*	Orange-coloured spore ball, and transforms into olive-coloured spore ball when mature	Khanal et al. (2023)

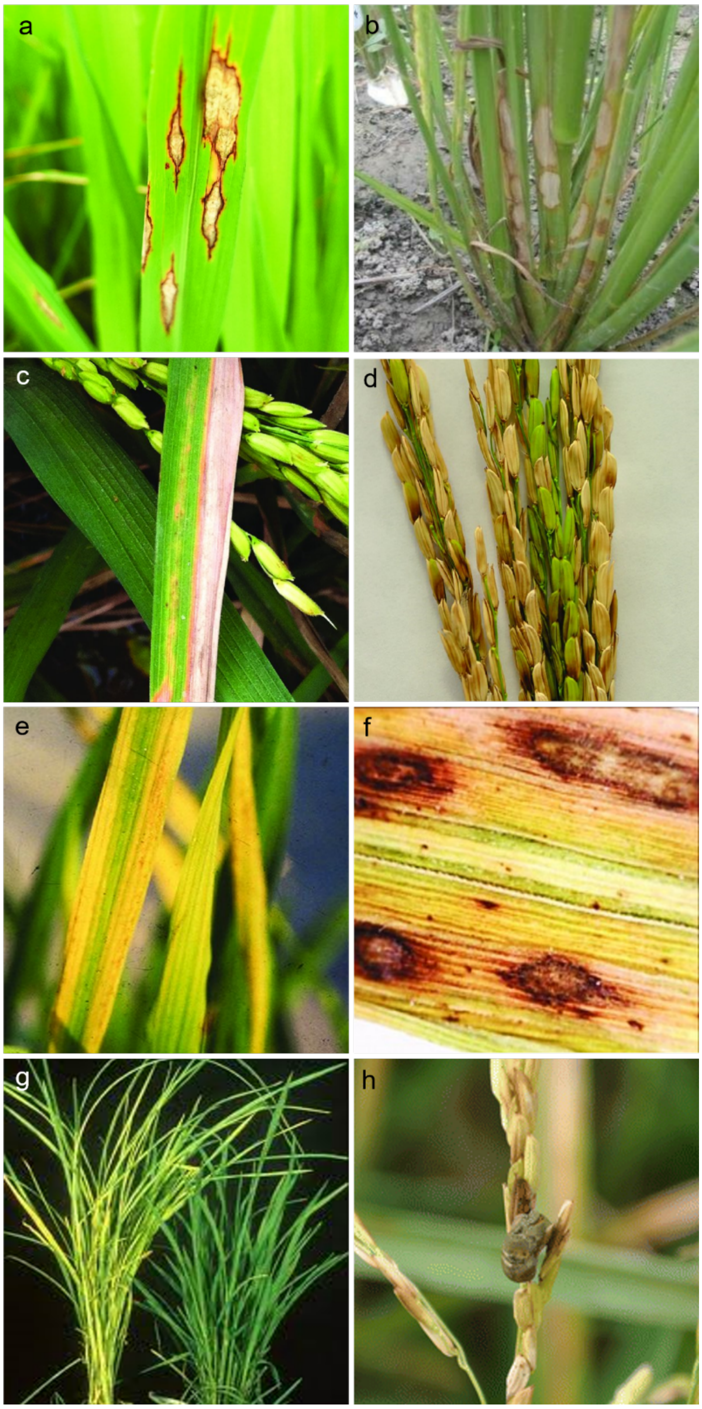

Figure 2.1 The symptoms of various rice diseases. (a) blast caused by *Pyricularia oryzae*; (b) sheath blight by *Rhizoctonia solani*; (c) bacterial leaf blight by *Xanthomonas oryzae* pv. *oryzae*; (d) bacterial panicle blight by *Burkholderia glumae*; (e) rice tungro disease by rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV); (f) brown spot by *Bipolaris oryzae*; (g) bakanae disease by *Fusarium fujikuroi*; (h) false smut by *Ustilaginoidea* virens (Abrol et al., 2022; Faizal Azizi & Lau, 2022; Khanal et al., 2023; Rice Knowledge Bank, 2023a; Zhou, 2019)

According to the Muda Agricultural Development Authority (MADA) and the Malaysian Agricultural Research and Development Institute (MARDI), rice blast and rice sheath blight diseases are among the major plant diseases that infected rice crops that are speculated to be worse, hence reducing yield production in Malaysia. Therefore, an attempt was carried out through this study to evaluate the antifungal properties of rhamnolipid biosurfactant to control both blast and sheath blight caused by *P. oryzae* and *R. solani*, respectively.

2.3 Biosurfactants

Biosurfactants, also known as 'green' surfactants, are a structurally diverse family of surface-active compounds produced by living organisms, most notably bacteria and yeasts (Desai & Banat, 1997; Sachdev & Cameotra, 2013). They possess hydrophilic lipophilic balance which specifies the proportion of hydrophilic molecules and hydrophobic constituents (Banat et al., 2010; Desai & Banat, 1997; Saimmai et al., 2012). Their hydrophilic group consists of monosaccharides, oligosaccharides or polysaccharides, peptides or proteins, whereas their hydrophobic group consists of saturated, unsaturated, or hydroxylated fatty acids or fatty alcohol. These structures have a variety of features, including the capacity to reduce liquid surface and interfacial tension, to create micelles and microemulsions between two distinct phases (Banat et al., 2010). Microbes capable of producing biosurfactants include *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Rhodococcus*, and *Thiobacillus* (Dias & Nitschke, 2023; Geetha et al., 2018).

Biosurfactants are good emulsifiers, foaming agents, and dispersants. Their unique features, such as low toxicity, high biodegradability, stability, environmental compatibility, high selectivity, and specific activity at extremes of temperature, pH, and salinity piqued the curiosity of many potential uses for these surface-active biomolecules (Desai & Banat, 1997; Georgiou et al., 1992; Kretschmer et al., 1982; Razafindralambo et al., 1996). Based on their chemical composition, biosurfactants can be grouped as glycolipids, lipopeptides, neutral lipids, polymeric biosurfactants, fatty acids, and phospholipids (Cameotra et al., 2010). Glycolipids and lipopeptides are among promising biosurfactants (Adu et al., 2020; Shu et al., 2021; Thakur et al., 2021). The most well-known glycolipids are rhamnolipid, trehalolipid, and sophorolipid (Chrzanowski et al., 2012). Figure 2.2 shows a general biosurfactant molecule consisting of a hydrophilic head and a hydrophobic tail, as well as micelle formation.

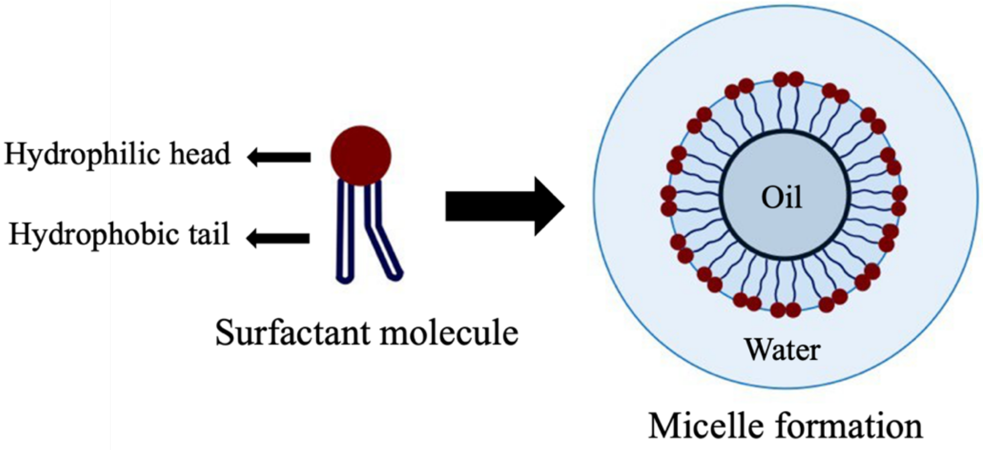

Figure 2.2 Biosurfactant and micelle formation structure

2.3.1 Rhamnolipid properties and applications

Rhamnolipid, mainly produced by *Pseudomonas aeruginosa*, is the most studied glycolipid biosurfactant (Kim et al., 2011; Sodagari et al., 2013; Zhou et al., 2019). *Pseudomonas aeruginosa* is a common opportunistic pathogenic bacterium that can be isolated from a variety of environments including water, soil, and plants (Soberón-Chávez et al., 2021; Toribio et al., 2010). Rhamnolipid is hypothesised to be produced by hydrocarbon-degrading microbes due to their amphipathic properties to facilitate uptake of hydrophobic carbon source (Chong & Li, 2017; Toribio et al., 2010). Most *P. aeruginosa* strains capable of producing rhamnolipid have been isolated from crude oil polluted sites (Cheng et al., 2017; Deepika et al., 2017; Zhang et al., 2005). Specifically, *P. aeruginosa* USM-AR2 used in this study was isolated from a crude oil sample and has been reported to produce high yield of rhamnolipid (Md Noh et al., 2014).

Generally, *P. aeruginosa* strains grown in liquid cultures produce two types of rhamnolipid congeners: mono-rhamnolipid (one rhamnose moiety and a fatty acid dimer) and di-rhamnolipid (two rhamnose moieties and a fatty acid dimer) (Soberón-Chávez et al., 2021) (Figure 2.3). Sugar (dTDP-L-rhamnose) and hydrophobic moieties such as 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA) are the precursors of rhamnolipid formation (Déziel et al., 2003). The sugar moiety may be produced from D-glucose, but the hydrophobic moiety can be produced via the fatty acid synthesis route, beginning with two-carbon units (Abdel-Mawgoud et al., 2014).

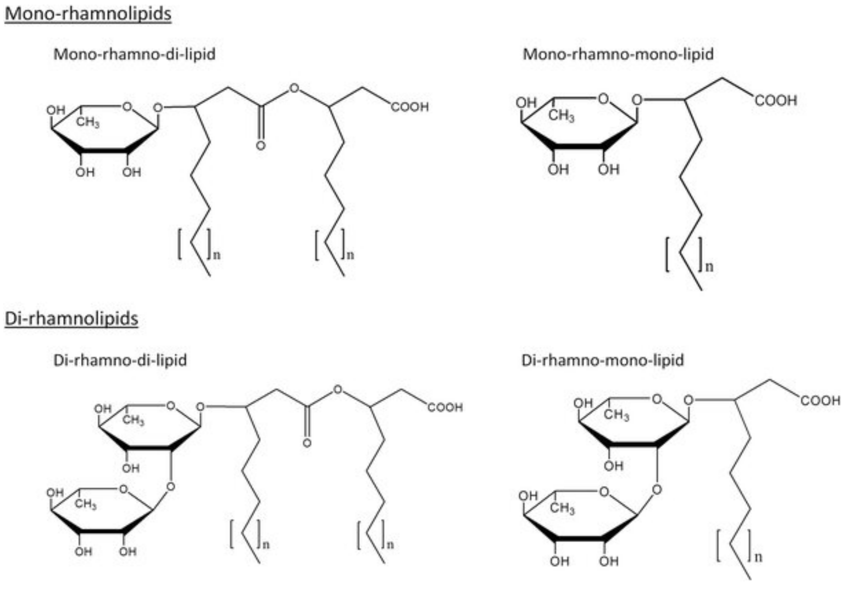

Figure 2.3 The chemical structures of mono- and di-rhamnolipids (Wittgens et al., 2018)

Rhamnolipid is produced as a secondary metabolite, extracted at the end of exponential phase and the onset of stationary phase of microbial growth (Déziel et al., 1996). The downstream process involves the recovery, concentration, and product purification. According to Mukherjee et al. (2006), the three major problems hindering the commercialisation of biosurfactants include expensive cost of raw materials, high recovery and purification expenses, as well as low yields in production procedures. The suitable downstream processing strategy is determined by the nature of the substrates, the fermentation methodology as well as the type and physiochemical characteristics of the resulting biosurfactants (Sinumvayo, 2015). Therefore, the utilisation of cheaper raw materials, along with the optimisation and enhancement of the bioprocesses, may lead to the successful commercialisation of these beneficial and versatile products in the near future.

Researchers are trying to enhance rhamnolipid production in response to the present challenges and issues (Eslami et al., 2020). Considering sustainable agriculture, renewable raw materials such as agricultural and domestic waste, including water-immiscible substrates can be used as a carbon source in rhamnolipid production. Rhamnolipid production by *P. aeruginosa* utilises a low-cost and waste raw material as carbon sources such as glucose, glycerol, corn steep liquor, molasses, and waste frying oil to enhance the production of rhamnolipid (Geys et al., 2014; Gudiña et al., 2015; Haba et al., 2000; Rahman et al., 2002; Saharan et al., 2012). Specifically, hydrophobic or insoluble substrates have been documented to produce a high yield of rhamnolipid by *P. aeruginosa* USM-AR2 in scale-up production (Aggo et al., 2023; Md Noh et al., 2014, Nasir et al., 2024).

In addition, rhamnolipid produced by *P. aeruginosa* have excellent physiological properties that can be employed in a variety of industrial applications (Randhawa & Rahman, 2014; Soberón-Chávez et al., 2021). Rhamnolipid is in high demand and have enormous application potential in agricultural production (Borah et al., 2016; Goswami et al., 2015), bioremediation (Mulligan, 2009; Sun et al., 2019), oil exploitation (Liu et al., 2018; Zhao et al., 2015), pharmaceutical industry (Chen et al., 2017; Gaur et al., 2019; Yi et al., 2019), food processing (Nitschke & Costa, 2007), detergent industry (Khaje Bafghi & Fazaelipoor, 2012), and cosmetic industry (Vecino et al., 2017) (Figure 2.4).

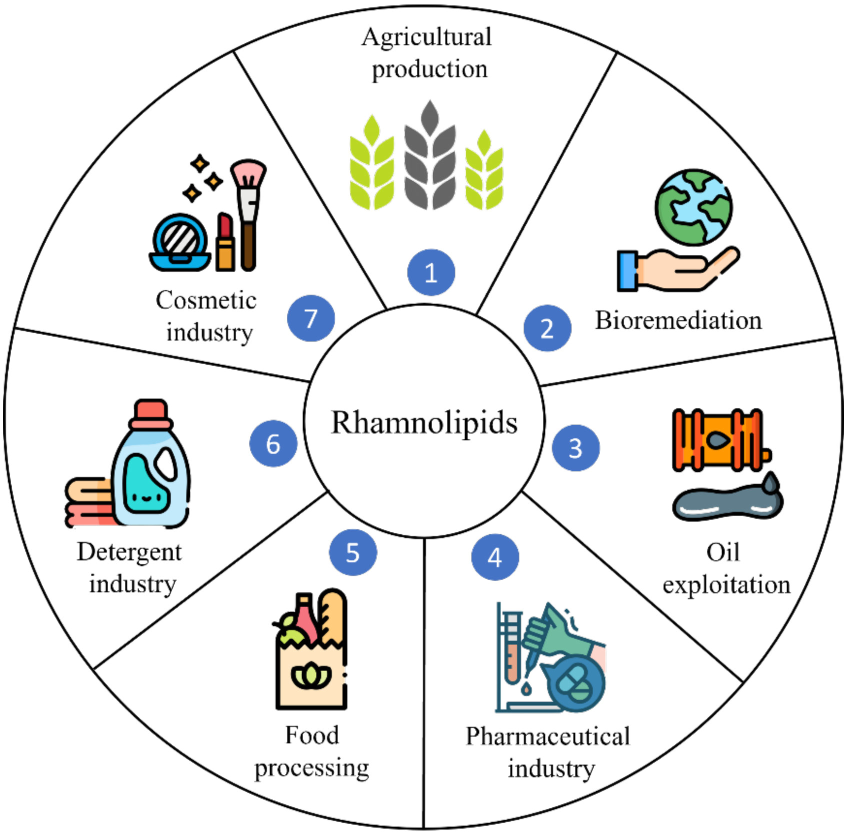

Figure 2.4 Rhamnolipid potential applications

2.3.2 Rhamnolipid in agriculture

Agricultural products meet the demands of the world's growing population (Sachdev & Cameotra, 2013; Sinumvayo, 2015). Considering food security issue and the diverse benefits of rhamnolipid, this 'green' surfactants offers promising potential, particularly in agriculture. Moreover, this biologically produced surfactant has several benefits over chemically manufactured surfactants (Singh et al., 2007). Sachdev & Cameotra (2013) documented that this biosurfactant can be widely used in agriculture for biological control, to improve the agricultural soil quality and pollutant biodegradation, as well as increase plant-microbe interaction. Its use in agriculture not only enhances soil and water quality, but also reduces plant toxicity, and eventually, human health as well as improves the socioeconomic conditions of marginal farmers (Kumar & Das, 2018).

Rhamnolipid is widely employed in agriculture to boost the antagonistic action of microorganisms and microbial products. Sha et al. (2011) demonstrated that crude rhamnolipid have potent antifungal action against *Phytophthora infestans, P. capsici, Botrytis cinerea, Fusarium graminearum,* and *F. oxysporum*. Moreover, rhamnolipid was reported to inhibit the mycelia and zoospores of *Phytophthora sojae* (Soltani Dashtbozorg et al., 2015). Other than that, rhamnolipid can reduce mycelial development in *Pythium myriotylum* and *B. cinerea* (Perneel et al., 2008; Varnier et al., 2009). According to Vatsa et al. (2010), rhamnolipid also can increase plant immunity. Several *in vitro* and *in vivo* studies have shown that rhamnolipid can improve insecticidal activity. Kim et al. (2011) proved that rhamnolipid from the *Pseudomonas* strain possesses insecticidal efficacy against green peach aphids (*Myzus persicae*). In addition, the rhamnolipid produced by *P. aeruginosa* USM-AR2 showed efficacy as a bioinsecticide when mixed with neem extract against the bagworm, *Metisa plana* (Zaludin et al., 2021).

Other than biocontrol agents, rhamnolipid has been used for soil remediation to improve soil quality (Randhawa & Rahman, 2014; Sachdev & Cameotra, 2013). Rhamnolipid supplementation increase soil development, reduce salt stress in the rhizosphere, alter the rhizosphere community, drive nutrient cycles, and improve microbial collaboration (Chen et al., 2024). Moreover, rhamnolipid has been reported to be effective in the removal of polyaromatic hydrocarbons and pentachlorophenol from soil (Mulligan & Eftekhari, 2003; Poggi-Varaldo & Rinderknecht-Seijas, 2003). Dittmann et al. (2023) also reported the use of rhamnolipid for soil washing in boosting the release of hydrocarbons from soil.

There is heavy metal contamination in agricultural fields caused by excessive use of metal salt-based fungicides, sewage and sludge additions. These heavy metals are essential micronutrients for plants, nevertheless, excessive use can impair plant development by inducing root necrosis and leaf purpling (Sachdev & Cameotra, 2013). Rhamnolipid has been shown to remove toxic metals such as cadmium, lead, nickel, and zinc from soil as reported by Dahrazma & Mulligan (2007) and Herman et al. (1995). Furthermore, rhamnolipid aids in the regulation of intercellular quorum sensing by influencing microbial motility, signaling, and differentiation, as well as biofilm formation (Ron & Rosenberg, 2001; Van Hamme et al., 2006). In addition, Sachdev & Cameotra (2013) also documented that rhamnolipid improve soil wettability and enable the appropriate dispersion of chemical fertilisers in the soil, hence promoting plant development. The beneficial roles of rhamnolipid in agriculture are summarised in Figure 2.5.

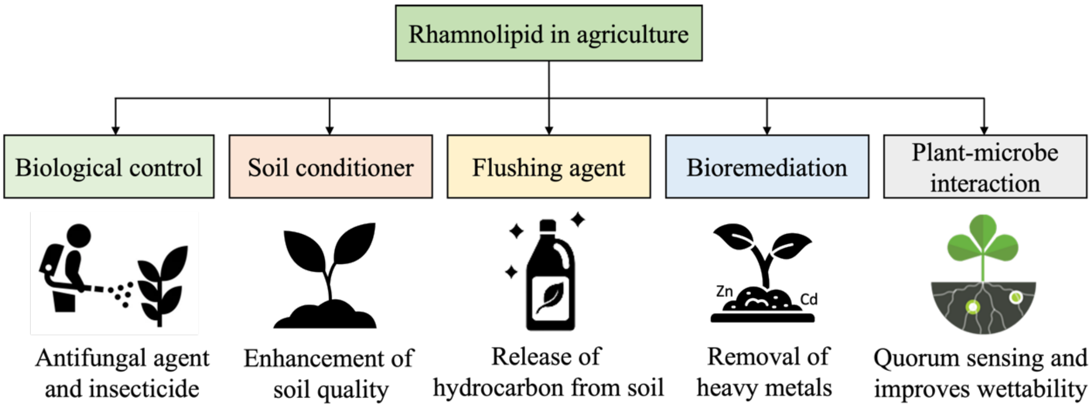

Figure 2.5 Rhamnolipid use in agriculture

2.4 Escalating microbial resistance on chemical fungicides

Excessive use of chemical fungicides causes adverse effects such watercourses contamination, lingering toxicity, reduced soil quality, and ecological degradation (Goswami et al., 2018; Law et al., 2017; Mohiddin et al., 2021). Hazardous residue may enter the food chain, endangering human health and other living organisms (Senapati et al., 2022). A new chemical must strike a balance between disease control and environmental safety (Reddy, 2014). Effective fungicides that do not fulfill the requirements established by the representative organisation may be prohibited from usage entirely. Furthermore, new pesticides should be constantly reassessed and re-registered, and standards for the application procedures and residual levels changed (Knight et al., 1997; Reddy, 2014). Tricyclazole was reported as the most effective fungicide used in blast management. However, it has been banned due to the increasing concerns of its hazardous effects on human health, particularly in India (Mohiddin et al., 2021). The applications of non-chemical control methods such as biology, culture, and the use of resistant cultivars, provide a feasible alternatives to both blast and sheath blight management.

Although blast and sheath blight can be controlled by using resistant cultivars, the pathogens have effectively overcome single-gene resistance in a short period of time, rendering some previously resistant varieties vulnerable to the diseases (Mohiddin et al., 2021). The use of a single chemical with the same mode of administration over a long period also causes the fungus to evolve resistance (Uppala & Zhou, 2018). Moreover, finding resistance genes may need a large amount of human resources, finance, and time. New virulent pathotypes also have the potential to break down resistance varieties. Thus, new plant breeding strategies must investigate the combination of several race-specific resistance genes in a single genotype to strengthen the rice plants (NurulNahar et al., 2020). This issue is a challenge in the fields of medicine and agriculture (Al-Hatmi et al., 2016).

Clearly, the discovery of new antifungal compounds must take into account the toxicity and adverse effects of existing fungicides. In contrast, this is not a concern in the application of rhamnolipid biosurfactants as biofungicides that offer a sustainable, safe, and environmentally friendly alternative to conventional chemical fungicides.

2.4.1 Rhamnolipid as antifungal agent

Rhamnolipid as an antifungal agent produced by *Pseudomonas* is increasing in the literature (Borah et al., 2016; Goswami et al., 2015; Kim et al., 2000). A large number of studies have characterised their antifungal action against plant pathogens even though no strong evidence for antibacterial or antiviral activity of rhamnolipids against plant diseases has been identified (Crouzet et al., 2020). These actions mostly target fungi and oomycetes such as *Alternaria* sp., *Aspergillus* sp., *Botrytis* sp., *Colletotrichum* sp., *Fusarium* sp., *Pythium* sp., and *Phytophthora* sp. (Table 2.2). Rhamnolipid has been shown to have antifungal actions, including zoospore lysis, spore and mycelial growth inhibition, as well as showing low minimum inhibitory concentration (MIC).

Table 2.2 Antifungal effects of rhamnolipid

Phytopathogens infected	Effects	References
Alternaria alternata	Spore germination and mycelial growth inhibition	Yan et al. (2015)
Aspergillus carbonarius	Mycelial growth inhibition	Rodrigues et al. (2017)
Botrytis cinerea	Spore germination and mycelial growth inhibition	Varnier et al. (2009)
Cercospora kikuchii, *Cladosporium cucumerinum,* *Colletotrichum orbiculare,* *Cylindrocarpon destructans,* *Magnaporthe grisea,* *Phytophthora capsici*	Zoospore lysis, spore germination, and mycelial growth inhibition	Andrieu et al. (2000)
Colletotrichum falcatum	Spore number and mycelial growth inhibition	Goswami et al. (2015)
Fusarium oxysporum	Mycelial growth inhibition	Borah et al. (2015)
Fusarium oxysporum	Mycelial growth and biomass accumulation inhibition	Deepika et al. (2015)
Fusarium sacchari	Mycelial growth inhibition	Goswami et al. (2014)
Fusarium verticillioides	Spore number and mycelial growth inhibition	Borah et al. (2016)
Pythium myriotylum	Mycelial growth inhibition	Perneel et al. (2008)
Phytophthora infestans, *Phytophthora capsici,* *Botrytis cinerea,* *Fusarium graminearum,* *Fusarium oxysporum*	Mycelial growth inhibition	Sha et al. (2011)

Overall, taking into account the severity of blast and sheath blight caused by *P. oryzae* and *R. solani*, respectively, this study was performed to assess antifungal activity of rhamnolipid to control both diseases. The observation on fungal morphology when exposed to rhamnolipid was evaluated *in vitro* and *in vivo*.

2.5 Rice and its importance

Rice (*Oryza sativa*) is the most important staple food crop in Asia influencing the culture, cuisine, and economy of millions of people (Gnanamanickam et al., 2002; Gul et al., 2015). It is the primary energy source for more than half of the world, as well as a substantial energy source with various functional qualities. This cereal food crop belongs to the grass family (Poaceae) of the plant kingdom (Palisoc et al., 2020). It is also known as the queen of cereal crops due to its high nutritional content, including carbohydrates, fat, fiber, protein, vitamins, dietary energy, mineral profile, and fatty acids (Verma & Srivastav, 2020). Rice grain carbohydrates are mostly starch (75 to 90%) consisting of amylose and amylopectin, with 12% water and 7% protein. There are other minerals such as magnesium, phosphorus, calcium, and trace amounts of iron, zinc, copper, and manganese (Kowsalya et al., 2022; Verma & Srivastav, 2017). Umadevi et al. (2012) reported that rice contains low fat, low salt, low sugar, no gluten, and no addition of additives and preservatives.

Rice was produced 10,000 years ago in the river valleys of South and Southeast Asia and China as it is the most essential food for humans. Although Asia is the primary region of rice production, it is also cultivated in other continents such as Latin America, Europe, some areas in Africa, and even the United States (Zibaee, 2013). According to Gnanamanickam et al. (2002), paddy plantation is believed to have begun concurrently in numerous regions about 6500 years ago. The earliest crops were recorded in China and also Thailand, later expanding to other countries. The origins of rice have long been contested, but the plant is so ancient that the specific time and location of its earliest development may never be discovered. However, the domestication of rice is unquestionably one of the most significant advances in history, as rice is the oldest cultivated cereal crop in the world.

Rice grains undergo various processes before they can be used as food, including cleaning, hulling, and post-hulling (whitening, polishing, and grading) (Gul et al., 2015). Rice constituents or rice bran have been found to consist of various pharmacological and phytochemical activities with numerous biological functions, including antioxidant, anticancer, antidiabetic, anti-inflammatory, immunomodulatory, cardiovascular protective, antihyperlipidemic, hepatoprotective, nephroprotective, and antimicrobial properties. These properties make them potential components to be used as a therapeutic nutraceutical (Kowsalya et al., 2022; Sen et al., 2020).

In the Philippines, rice bran is used as a good source of vitamin B to prevent and treat beriberi. In our country, apart from being as a staple food, the Medicinal Book of Malayan Medicine advocates using cooked rice as an eye ointment and for treating acute inflammation of the interior body tissues, as well as applying a combination of dried and powdered rice to specific skin disorders. Moreover, the Pharmacopoeia of India recommends rice water as an ointment to treat irritated skin (Umadevi et al., 2012). Furthermore, according to Chinese royal physicians, rice is used for healing purposes, toning muscles, and relieving digestion (Jamil & Anwar, 2016).

However, rice diseases are one of the challenges in maintaining rice productivity in Malaysia and other countries. According to NurulNahar et al. (2020), the existence of pests and diseases may result in major economic losses and declines in national SSL. Our government has made enormous efforts to ensure a consistent supply of rice and treat rice crop diseases. The use of resistant cultivars, chemical fungicides, biology, cultural techniques, and a variety of other practices are mainly aimed at controlling rice diseases.

2.5.1 Growth stages of rice

Paddy or rice plant is an annual grass with spherical, hollow, jointed culms, rather flat, sessile leaf blades, and a terminal panicle (Nordström & Glass, 2002). During germination of seed, the coleorhiza emerges with the radicle. As progressed, the primary leaf pushed out from and secondary leaf differentiates into sheath, blade, ligule, and auricle (Mew et al., 1994). A cultivated rice plant may reach a height of 120 cm. The branches and leaves are hollow, and the leaves are long and flat. The fibrous root system can be vast and pervasive at times. Flowers are produced in a panicle, which produces fruit or grain (Chang & Bardenas, 1965).

According to Gnanamanickam et al. (2002), rice plant contains a main stem and several tillers that may grow to a height of 6 cm. The tiller has a ramified panicle that is 20 to 30 cm in width. Moreover, each grain is formed by 50 to 300 florets or spikelets on the plant. There are considerable differences in panicle size, shape, and weight, as well as production volume amongst varieties. The plant may live more than a year in optimal conditions (Chang & Bardenas, 1965). Figure 2.6 shows the illustration of morphology of seed and rice plant.