Isoprothiolane resistance in the rice blast fungus in Indonesia: Field survey of its impact and molecular analysis of mutations

インドネシアにおけるイソプロチオラン耐性イネいもち病菌：その影響のフィールド調査および突然変異の分子解析

Hokkaido University Graduate School of Global Food Resources
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ABSTRACT

1 General Overview

Rice productivity in Indonesia is challenged by fluctuating production, harvest deterioration, climate instability, land conversion to urban centers, and disease outbreaks. Rice blast disease is caused by an ascomycete fungus, *Pyricularia oryzae*, which continues to become a major threat to rice production and global food security. Infection of leaf blast and neck blast both often lead to empty grains plant death resulting in annual 10% - 30% yield loss. Rice blast fungus (*P. oryzae*) exhibits a disease cycle both in vegetative and generative stages and during the off-season, this fungus survives in seeds and seedling debris and reentry the infection cycle by dissemination to healthy plants primarily by wind and raindrops. Efforts to control this disease are the use of resistant cultivars, biological control, habitat management unfavorable to the fungus development, as well as fungicide application. However, *P. oryzae* has high genetic variability and can adapt rapidly to various stressor stimuli, including mutation in sub-lethal doses of fungicide by altering the genes encoding fungicide targets, enabling this fungus to perform the continuous infection cycle and change the race dominance within a couple of growing seasons complicates the efforts to safeguard rice production. Fungicide resistance is a global issue and its discovery in Indonesia as an important rice-producing country is critical to assess.

2 Assessment of Blast Diseases in Three Regencies in West Java and Monitoring in An Endemic Area

P. oryzae will continuously perform the pathogenesis while compatibility presence of favorable environmental conditions, rice susceptibility, and virulence determined the degree of yield loss. A field survey in Bogor, Cianjur, Sukabumi Regencies in West Java, the second-largest rice-producing province in Indonesia was undertaken to investigate the pathological and socioeconomic aspects of blast disease endemic, dynamics of rice-fungus-fungicide, strategies, and challenges employed by farmers in controlling rice blast disease. The average temperature of 27 ºC, humidity >90%, and rainfall days reaching 25 days per month were recorded during the survey and were favorable for disease development. Cikembar District in Sukabumi Regency is an endemic area where farmers rely on isoprothiolane fungicide but reported that the efficacy has declined. In Cikembar, 67% of disease severity was developed while 35% of severity was maintained during dry season. *P. oryzae* isolates were collected and tested by a series of laboratory works to validate this anecdotal evidence. Acidic soil, seedlings, and fungicide cost, the diverse controlling strategy applied by farmers are among the current challenges making the integrated disease management effort of the farmers' group more complicated.

3 Sensitivity Assays of *Pyricularia oryzae* Isolates Collected From Three Regencies In West Java

A total of 14 Indonesian *P. oryzae* field isolates have white to gray colonies, slow-growing on potato dextrose agar (PDA) medium, and have the closest genetic relationship with isolates from India and Thailand. These isolates were tested for a sensitivity test in isoprothiolane (IPT)-amended PDA both commercial and reagent-grade IPT. Isoprothiolane is a systemic fungicide that targets Kennedy's pathway in *P. oryzae* choline biosynthesis. Ina-86 137, Japanese isolate was used as Control. Six isolates were indicated as mutants with the ability to grow at 20 mg L^{-1} IPT while maintaining radial growth and EC_{50} at 4-6 mg L^{-1}, 10-fold higher than the recommended concentration. At 0 mg L^{-1}, mutant colonies grow slower than the sensitive isolates. Colonies from the IPT-exposed PDA were subcultured to a fresh PDA medium and most of the isolates can regrow as their original colonies, while a few are not reversed expressed by irradiated growth and sector development, indicating a fitness cost. Mutants (isolates 1-6) were continued to mutation analysis on *Magnaporthe-oryzae* isoprothiolane resistance-related (MoIRR) gene.

4 Mutation Analysis of Isoprothiolane Resistance From Indonesian *Pyricularia oryzae* Field Isolates

Resistant *P. oryzae* mutants are reported to possess varying responses to an active ingredient and cross-resistance to different active ingredients compared to that of lab-generated mutants. Identification of the MoIRR gene revealed that bases shift (G→A changed Alaine and Threonine at codon 396 and T→A changed Asparagine to Lysine at codon 555) occurred in isolate 1 and 6, respectively, while isolate indicated an insertion of G base at codon 288 changed the subsequent Isoleucine to Asparagine followed by a stop codon. Isolates 2, 3, 5, and a sector that emerged from Ina-86 137 did not have a mutation in MoIRR. Identification of the MoVelB gene demonstrated in isolate 2, insertion of A at codon 437 changed Aspartic Acid to Arginine and repeated 2 times before a stop codon appeared, while still no mutation was identified in isolate 3, 5, and SectorIna. Mutation may occur in other unknown genes. *P. oryzae* easily mutates as confirmed by SectorIna with lesser sensitivity to IPT with EC_{50} 2 times lower than Ina-86 137. Sector Ina also has a fitness penalty while its growth is inhibited in the absence of IPT but higher growth, conidial formation, and viability in challenged growing resources.

5 General Discussion

Since its first appearance in dryland rice in 1985, rice blast (*P. oryzae*) has been the most important disease in irrigated rice in Indonesia with a hundred races where the dominance of its main races alternates over the decades. Its novel state of virulence has also emerged while tackling rice resistance, dramatic climate changes, and fungicides, like what has been observed during the field survey in West Java. Fungicide resistance has occurred in Indonesia with multiple mutation mechanisms in the MoIRR and MoVelB gene. A combination of IPT and IR64 or a mixture of rice cultivars is detected as promising for short-term disease control implying the variant sources of pressure to minimize a higher possibility of *P. oryzae* mutations. It is highly recommended to temporarily stop the use of IPT or substitute it with other active ingredients to delay the development of emerging *P. oryzae* mutants in the field.

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GENERAL OVERVIEW

1.1. Research Background

1.1.1. General Conditions of Rice Production in Indonesia

As the world's fourth most populous country, Indonesia's annual rice consumption totals 23 million tons. Annual rice production in Indonesia averages 30 million tons, and production in 2023 is the lowest in the recent decade, following a sharp fall in 2018 (Figure 1). In this condition, the rice production in Indonesia remains self-sufficient (Table 1). However, rice stocks for the following period rely on supply, harvest deterioration during processing and storage, continuous rice production conditions, and forecasts of climate instability, all of which threaten national rice sufficiency. This is worsened by the production trend, which has continued to decline over the last decade (Figure 1) (BPS 2024a).

Table 1 Rice production and consumption in Indonesia in 2019 – 2023

Year	Per capita needs / year (kg)	Total value (million tons)	
		Consumption[a]	Production[b]
2019	78.71	21.80	31.12
2020	78.75	21.82	31.15
2021	81.83	22.66	31.22
2022	81.35	22.53	31.02
2023	81.23	22.77	30.77

[a]Ministry of Agriculture (2024)

[b]BPS 'The Central Bureau of Statistics' (2024a)

Indonesia implemented a rice import strategy as an addition to its national production in order to provide enough rice for the 279,000,000 population, as well as a rice stockpile for the drier seasons. In 2023, Indonesia imported rice from eight major countries: Thailand (USD 800 million), Vietnam (USD 668 million), Pakistan (USD 182 million), Myanmar (USD 88 million), India (USD 35 million), Cambodia (USD 8.3), Japan (USD 155,000), and

China (USD 142,500), for a total import of 3 million tons, 600% higher than in 2022 (Figure 1) (BPS 2024b). Besides from the challenge of rice availability, rice production in Indonesia has been impacted by the conversion of rice fields into urban centers or other crop producing areas, floods and droughts, as well as pest and disease outbreaks. Rice diseases alone cause prevalent production losses, with yield losses ranging from 10% to 75% (Laha *et al.* 2017).

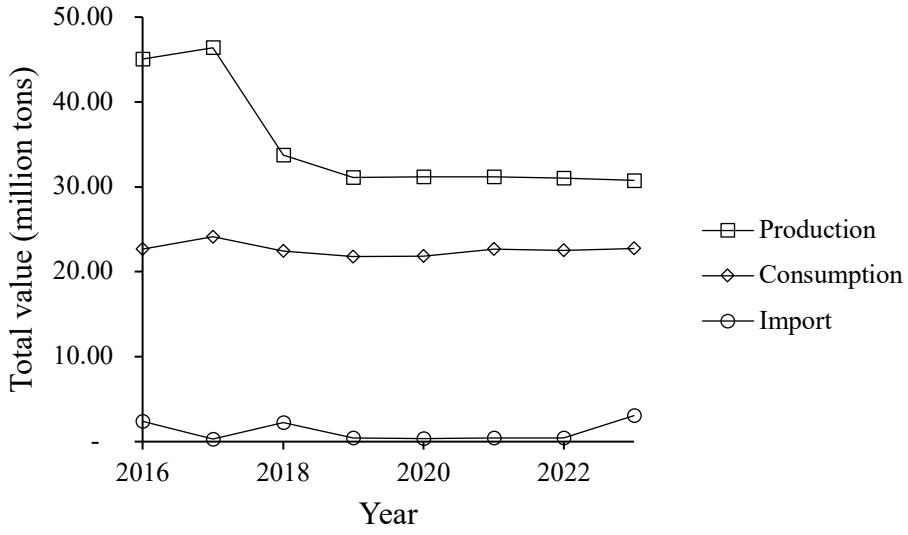

Figure 1 Trend of rice production, consumption, and import in Indonesia 2016 – 2023

Blast disease caused by *Pyricularia* oryzae is still a major problem in rice plants on a global scale with 10% - 30% yield loss every year, in addition to the main diseases in rice caused by various groups of microorganisms such as sheath blight fungi (*Rhizoctonia solani*), sheath rot (*Sarocladium oryzae*), bacterial leaf blight bacteria (*Xanthomonas oryzae* pv. *oryzae*), bacterial leaf streak (*X. oryzae* pv. *oryzicola*), tungro virus (Rice tungro bacilliform and spherical virus / RTBV, rice grassy stunt virus, nematodes *Aphelenchoides besseyi* (white tip disease) and *Meloidogyne graminicola* (rice root-knot disease). Since its first appearance in dryland in 1985, irrigated rice fields in Indonesia's three largest producing provinces (East Java, West Java, and Central Java) have been devastated by rice blast continuous infection. The fungi that spread in irrigated rice fields are believed to be novel races as a result of lowland rice-specific adaptations (Sudir *et al.* 2014). The significant increase in the prevalence and severity of blast diseases and infections across plant ages has garnered

increased attention, particularly in recent years. Outside of Java Island, Lampung Province is also known to be a rice-blast endemic area. In April 2024, 10 hectares of rice fields in Metro District, Lampung Province, were infected with blast disease while the plants were just a month old and failed the following planting stages and harvest.

1.1.2. Pyricularia oryzae as a Major Pathogenic Fungus in Rice

Rice blast fungus infects rice leaves, collars, nodes, and necks, resulting in empty grains and potentially premature death of plants (Elazegui & Islam 2013). The primary inoculum of this disease is the infected rice husk debris and seeds. The fungus, *P. oryzae*, can penetrate either passively through natural openings in leaves (e.g. stomata) and roots, or actively through the cuticle and epidermis using turgor pressure from the penetration peg at the tip of the appressorium (Figure 2). The mid-tillering stage is the most susceptible to the development of this disease. However, in endemic locations, plants might become infected with *P. oryzae* 14 days after planting. As a result, the health of the plant seeds utilized, as well as the genetic variety achieved through the use of multilines, have a significant impact on infection and disease development (Ishikawa *et al.* 2022). Environmental factors that are favorable for the spread and development of *P. oryzae* include humidity above 90% and temperatures above 25 ºC, with an optimum of 27 ºC. Crop growth stage and airborne conidia concentration are important factors in determining the risk of infection after early heading. The adaptability of *P. oryzae* is also influenced by dynamic environmental conditions; for example, a 1 ºC increase in average temperature is enough to increase the severity of leaf blast disease as reported by Castejón-Muñoz (2008).

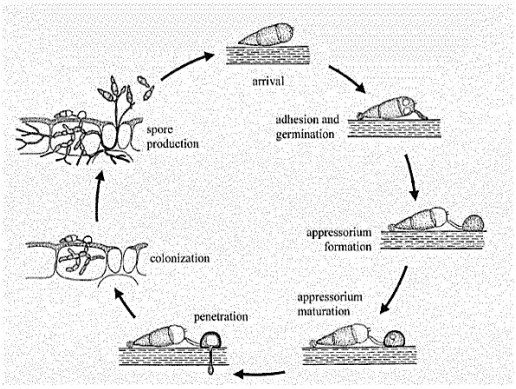

Figure 2 *Pyricularia oryzae* disease cycle (Abas 2011)

This pathogen is an Ascomycota fungus (teleomorph *Magnaporthe oryzae/Magnaporthe grisea*). Observation of colonies on agar media indicated the characteristics of colonies that developed relatively slowly, mycelium ranging in color from white to dark gray, and conidia that grew from 1, 2, or 3 cells at 6-18 hours. Conidia are pyriform, obclavate, with 2 septates. The species of *P. oryzae* infects a range of monocots, particularly those in the Poaceae family, and its host specificity is determined by the pathotype that infects poaceous grass and crop species, as well as the races that infect varieties of the same species. Experiments by Xiang *et al.* (2022) demonstrated that inoculation with *P. oryzae* and *P. grisea* can cause similar root browning, but the onset of symptoms on the leaves differs between the genera *Digitaria, Triticum* and *Oryza*. This distinction is directly related to the increase of pathogenesis-related genes and reactive oxygen species in response to fungal inoculation in the roots.

The fungus *P. oryzae* secretes effectors (*Avr* proteins) which facilitate the penetration and colonization of plant tissue while suppressing plant defense. Meanwhile, to combat a blast fungus capable of controlling the PAMP-triggered immunity (PTI), rice recognizes the effectors via nucleotide-binding site leucine-rich repeat (NLR) proteins (Figure 3). The *Avr* gene interacted with the rice plant's resistance gene (*Pi-ta*), resulting in rice resistance and susceptibility responses (Talbot 2003; Yin 2021). Sornkom *et al.* (2016) found that *Avr-Pia* can be produced before penetration and generate host resistance. Signal transduction is involved in the accumulation and signaling route of cAMP, which is critical in appressorium development (Prajanket *et al.* 2020). This interaction of genes results in new dynamics after an initial biotrophic phase, which is reflected in a variety of phenotypic symptoms.

Blast disease control often prioritizes the use of resistant cultivars. However, this control method must be reinforced with additional strategies due to the arms race between the agronomic resistance and genetic variability of *P. oryzae,* which causes specific cultivars to gradually lose resistance to new races of this pathogen. To suppress the production of primary inoculum for this disease, seeds can be treated with hot water immersion disinfection, silicic acid, deep-flood irrigation methods, nursery-blast eradication after transplanting, and rice husk debris management (Namai 2011). However, the typology of blast disease is polycyclic; in addition to the formation of the main inoculum, the secondary inoculum is

critical for the spread and persistence of the disease cycle. The *P. oryzae* fungus will continue to produce secondary inoculum as long as the crops are abundant and favorable environmental conditions are available.

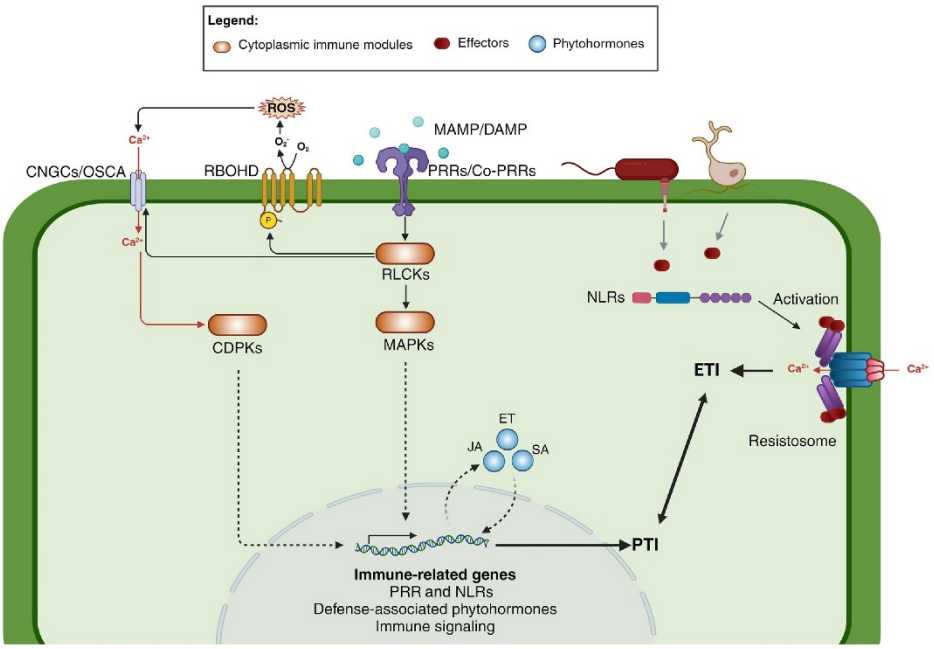

Figure 3 Simplified overview of the immune systems of plants (Roussin-Léveillée 2024)

1.1.3. Blast Disease Control, Global Climate Change, and the Risk of Resistance by Fungicides Continuous Application

Blast disease control strategies include cultivation techniques, the use of biological agents and synthetic chemicals. Pandit *et al.* (2024) investigated the use of biological agents *Trichoderma harzianum* and *T. viride*, as well as the fungicides tricyclazole, tebuconazole, and azoxystrobin, in seed treatment and foliar sprays. *In vitro* test findings for *T. harzianum* indicated suppression greater than 40%, while in field studies, fungicide treatment offered the greatest disease suppression (80%) and production savings of up to 29%. Horo & Gudisa (2021) and Kongcharoen *et al.* (2020) observed similar levels of disease control and the possibility of preventing yield losses by using different fungicide active ingredients. According to some surveys, fungicides are among the main methods used by growers for controlling blast disease in the field. In Indonesia, biological agents are prioritized for blast disease management. Nonetheless, fungicides are still used in the integrated control

component, with decisions highly influenced by farmers' socioeconomic conditions (Kihoro *et al.* 2013).

Global climate changes, such as increased air temperature, humidity, flooding, drought, salinity, and elevated CO_2, have been shown to have an impact on both plant health and the population and activity of pathogenic fungi. However, pathogenic microbes can adapt to the aforementioned stresses more easily rapidly, thereby increasing their new state of virulence (Roussin-Léveillée *et al.* 2024). The continued use of fungicides with the same active ingredients or mode of action contributes to dynamic changes in this disease triangle, increasing the risk of fungicide resistance emergence.

Fungicide-resistant fungus may emerge and change the race dominance within a couple of growing seasons. This shift in dominance complicates efforts to safeguard rice production. In the last decade, cases of resistance have been significantly documented in many fungal infections in various countries. For example, resistance to phenyl-pyrrole in *Botrytis cinerea* (Ziogas *et al.* 2005), fungicides from the QoI (quinone outside inhibitor) class in the cytochrome b (cyt b) gene in *Monilinia* species, *Alternaria* species, *Pyricularia* species; Mycosphaerella species, and the oomycete *Plasmopara viticola*, (D'Avila *et al.* 2021; Fernández-Ortuño 2008; Hily *et al.* 2010; Ishii 2006; Tenni *et al.* 2021).

As a pathogen with high genetic variability, *P. oryzae* fungicide resistance has been closely monitored for many years. The emergence of blast disease in the field caused growers in various areas of Japan to increase the frequency of kasugamycin application to two to three times the recommended level. The identical problem applies to organophosphorus fungicides, as well as cross-resistance between kasugamycin and other fungicides. This resistance can occur either through natural mutations (as in the example of antibiotic and organophosphorus fungicide resistance) or through experiments with continuous exposure and gradual increases in exposure concentrations (as in the case of copper sulfate resistance) (Yamaguchi 1988).

1.1.4. How Isoprothiolane Fungicide Works

Isoprothiolane (IPT), diisopropyl-1, 3-dithiolane-2-ydenemalonate, is used for controlling *P. oryzae* both preemptively and responsively. The mode of action was originally considered to interfere with protoplast cell wall regeneration (Ishizaki *et al.* 1985). At low doses (2-10 ppm), IPT reduces penetration and infective hyphal growth, as well as inhibits penetration peg formation. There was also a suppression of the incorporation of ^{14}C-sugar into the cell wall and ^{14}C-acetate into fatty acids and triglycerides. At 50 ppm, protoplasts were disrupted, resulting in a decrease in the number of protoplasts and a less well-organized condition. The damage to the protoplast additionally induces the fungus to fail to form its cell walls.

However, further research revealed that the effect of IPT on *P. oryzae* chitin biosynthesis, which impairs cell wall production, is simply a secondary effect, with the main effect being an alteration of the choline biosynthesis pathway (Uesugi 2001). This change begins with the instability of the fungal cell membrane, followed by cytoplasmic membrane leakage, leading to the conclusion that the disrupted metabolic system is phosphatidylcholine production. Choline is involved in conidial germination, germ tube elongation, mycelium development, infective hyphae activity, and virulence in *Botrytis cinerea*, *Aspergillus oryzae*, and other pathogenic fungi (Arya *et al.* 2021; Suzawa *et al.* 2024). Mutant isolates that perform the *choA* and *choC* genes absence in *Pestalotiopsis microspora* have swollen hyphal tips, enhanced hyphal branching, mycelial autolysis, impaired mycelia, conidia, and cell wall integrity. Moreover, less-choline *P. oryzae* mutant isolates exhibited higher stress to osmotic stress, cold, and metal ions (Akhberdi *et al.* 2018).

Phosphatidylcholine biosynthesis has two main pathways namely Greenberg's pathway and Kennedy's pathway (Figure 4). In Greenberg's pathway, transmethylation occurs at the final stage, while in Kennedy's pathway, transmethylation occurs at an early stage and produces choline (precursor to being incorporated into phosphatidylcholine). According to Yoshida (1984), Kennedy's pathway is the principal route for phosphatidylcholine biosynthesis in *P. oryzae*. Therefore, the fungicide isoprothiolane, is categorized as a choline biosynthesis inhibitor (CBI).

Other active compounds that function as CBI include iprobenfos and edifenphos. This CBI group can be used to control other pathogenic fungi, however its efficacy in inhibiting the development of pathogens other than *P. oryzae* is insufficient for preemptive control. The fungus *P. oryzae* has distinct physiological properties when it comes to CBI sensitivity. This is because of the phosphatidylcholine biosynthesis process in *P. oryzae*, which is carried out primarily by Kennedy's pathway, in contrast to other fungi which mainly use the Greenberg's pathway. Further research is needed to determine whether only *P. oryzae* has an enzyme mechanism for activating the response to isoprothiolane. When CBI fungicides are used repeatedly and solely in the field, *P. oryzae* develops resistance to them; however, when these fungicides are employed alternately, this mutation problem does not occur.

P. oryzae mutant isolates from the laboratory and the field have diverse reactions. Resistance in lab-generated mutants indicates susceptibility to phosphoramidates as a type of impaired detoxification. Meanwhile, most mutant isolates from the field did not exhibit negative cross-resistance to these phosphoramidates. Even though they have been used for decades, CBI fungicides still can be used to control blast disease, as long as efforts are made to delay the development of *P. oryzae* resistance.

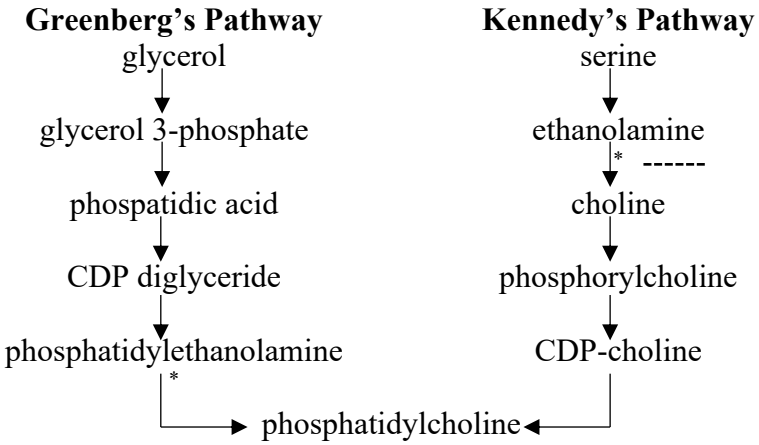

Figure 4 Choline biosynthesis pathway, transmethylation steps (*) and site of isoprothiolane of inhibition as CBI fungicide (---) (Uesugi 2001)

1.2. Objectives of the Research

This research was undertaken to explore the pathological, socioeconomical, and molecular aspects of rice blast disease status in three regencies in West Java, Indonesia, and the detection of natural mutation occurrence of *P. oryzae* to isoprothiolane. The results of this research are expected to reshape the understanding of the rice blast disease dynamics and help the farmers, academia, and policy-makers construct a strategy for long-term rice blast disease management.

2 ASSESSMENT OF BLAST DISEASES IN THREE REGENCIES IN WEST JAVA AND MONITORING IN AN ENDEMIC AREA

2.1. Introduction

For half of the world's population, rice constitutes a staple diet, and demand for it is rising in tandem with population growth (Khus 2005). Plant diseases, however, continue to be a significant constraint to rice productivity. There are nearly a hundred different pathogenic species that can infect rice, especially rice blast (*Pyricularia oryzae*), bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzicola*), white tip (*Aphelenchoides besseyi*), and tungro (Rice tungro spherical virus and Rice tungro baciliform virus). The range of diseases that can cause yield loss is 5%-85%, with rice blast disease continuing to be the most significant disease affecting rice plants (Nayak *et al.* 2021; Dean *et al.* 2012).

With yearly crop losses ranging from 10% to 30%, the rice blast fungus (*Pyricularia oryzae* Cav.; teleomorph *Magnaporthe grisea* (Hebert) Barr.) is the most destructive pathogen to rice productivity and food security worldwide (Talbot 2003). The symptoms are gray-brown neck rot and diamond-shaped lesions on rice leaves. In addition, brown blotches on grain may be an indication of this disease, particularly if the panicles, leaves, and neck have previously been affected. During the off-season, blast disease can infect rice seedlings and is primarily transmitted by wind and raindrops. In endemic locations and with favorable environmental circumstances, crop losses might be as high as 60%. The degree of yield loss is largely determined by the degree to which host susceptibility and pathogen virulence are compatible, as well as by the presence of favorable environmental conditions that support a perpetual cycle of pathogenesis (Agrios 2005).

The Asian continent, being the primary rice producer, is an important point for global rice supply and is critical for rice blast fungus persistence. This will depend on the accessible sources of disease inoculum, as well as the disease's potential to spread and persist across multiple growing seasons, particularly in tropical climates (Kirtphaiboon *et al.* 2021). Along with climatic change, changes in time, and farmer cultivation methods, disease fluctuations occur, resulting in diverse dynamics based on their agroecological origin (Asibi *et al.* 2019).

As a result, regular monitoring is required to determine the present blast endemic areas as well as farmer efforts to manage blast disease. Furthermore, early detection of possible disease outbreaks through the transmission of inoculum to other host plants and the emergence of fungicide resistance can be recognized through this routine monitoring, allowing disease development to be avoided more promptly.

As Indonesia's second-largest rice-producing province, the current and accurate condition of blast disease in blast endemic areas in West Java is critical to assess. We did a study on blast disease control utilizing synthetic chemical fungicides and their relationship with disease incidence and severity. Furthermore, the problem of blast disease and fungicide use is closely related to farmers' social and economic position. As a result, the difficulties farmers confront in controlling blast disease were also recorded in this study. The desired outcome of this research chapter is a better understanding of the situation with blast disease and its relationship to agro-climatic and socioeconomic factors. The benefit of this research is that it demonstrates the crucial spots for early detection of the emergence of fungicide resistance in the fungus that causes blast disease through field visits and reports from farmers.

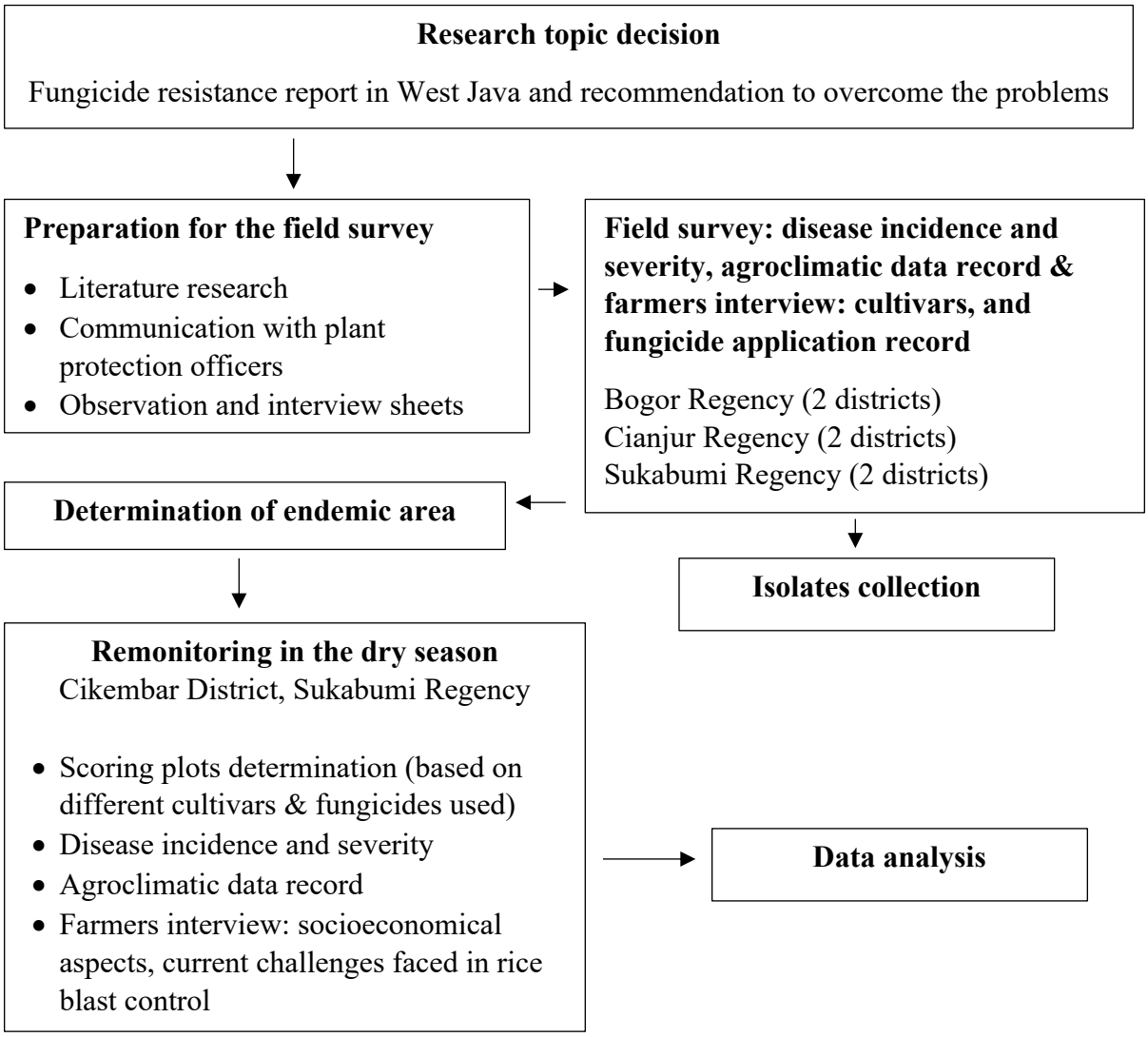

Figure 5 Research flow diagram of assessment of blast diseases in three regencies in West Java and monitoring in an endemic area

2.2. Materials and Methods

2.2.1. Disease incidence, disease severity, and fungicides used in three regencies in West Java and determination of endemic area

West Java is one of the primary rice producers which correlates positively with the possibility of blast endemic spots. Information on blast-affected areas was provided by literature searches and communication with plant protection officers. We used purposive sampling to measure the disease incidence and severity following the recommendation from the officers regarding the 2 most infected plots and continuously applied fungicides from each regency (Bogor, Cianjur, Sukabumi). Three plant rows are selected and 10 plants are scored resulting in 30 observation units from each plot (Figure 6). At the time of observation, the temperature and humidity were recorded. Farmers provided information on rice cultivars and fungicides used.

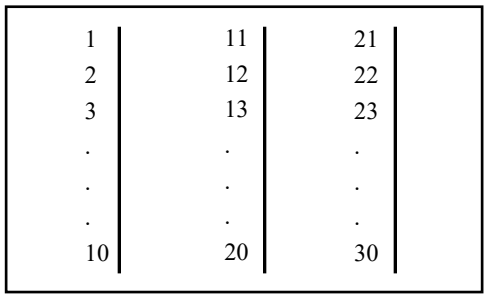

Figure 6 Schematic diagram of assessed plants inside a plot

Cooke's (1998) formula is used to assess the incidence and severity of blast disease, with IRRI (2014) scoring in Tables 2 and 3.

$$\text{Disease incidence (\%)} = \frac{n}{N} \text{ x } 100\%$$

n: number of infected plants; N: number of observed plants

$$\text{Disease severity (\%)} = \frac{\sum_{i=1}^{9} ni.vi}{N.Z} \text{ x } 100\%$$

ni: number of plants infected in a particular scale; vi: scale score; N: number of observed plants; Z: maximal disease scale

Analysis of variance (ANOVA) followed by a Tukey test (α 0.05) was performed to evaluate the disease incidence and severity among cultivars and fungicides used.

Table 2 Severity scale for leaf blast disease assessment

Scale	Symptoms description for affected leaf area
0	No lesion observed
1	Small brown specks of pinpoint size or larger brown specks without sporulating center
3	Small, roundish to slightly elongated necrotic sporulating spots, about 1-2 mm in diameter with a distinct brown
5	Narrow or slightly elliptical lesions, 1-2 mm in breadth, more than 3 mm long with a brown margin
7	Broad spindle-shaped lesion with yellow, brown, or purple margin
9	Rapidly coalescing small, whitish, grayish, or bluish lesions without distinct margins

Table 3 Severity scale for neck blast disease assessment

Scale	Symptoms description for affected neck area
0	No visible lesion or observed lesions on only a few pedicels
1	Lesions on several pedicels or secondary branches
3	Lesions on a few primary branches or the middle part of panicle axis
5	Lesion partially around the base (node) or the uppermost internode or the lower part of panicle axis near the base
7	Lesion completely around panicle base or uppermost internode3 or panicle axis near base with more than 30% of filled grains
9	Lesion completely around panicle base or uppermost internode or the panicle axis near the base with less than 30% of filled grains

2.2.2. Monitoring of disease intensity, fungicide used, and farmers' interview in Cikembar as an endemic area

The sub-district site with the highest infection rates in rice fields was determined based on the results of observations in sub-chapter 2.2.1. The disease severity was remonitored in five plots in the same site under varied agroclimatic circumstances (dry season) using the same procedures as previously assessed. The five rice plots were chosen based on differences in rice cultivars and fungicides used. At the time of observation, the temperature and humidity were recorded. Additionally, two observation points were taken from each plot to test soil acidity resulting in 10 observation plots in total. Based on farmers' experiences, interviews were undertaken to acquire information about rice planting and the development of blast disease from year to year. A total of 11 farmers were interviewed to gather information about cultivation techniques related to blast development. Questions included cultivation practices, production cost, and yearly and dramatic yield loss records.

2.3. Result

2.3.1. Description of survey area

The blast disease survey area in this study is located around Mount Gede Pangrango, West Java Province, with two rice fields each in Bogor (Situgede and Ciherang), Cianjur (Bojongpicung and Gekbrong), and Sukabumi (Kadudampit and Cikembar). The six rice fields observed were at an altitude of 180 – 900 masl (Figure 7). Both vegetative and generative stages rice plants were found in the same area, allowing the disease cycle to continuously occur alongside *P. oryzae* infection at different stages including rice seedlings (Figure 8A). Symptoms of leaf blast observed include chlorotic and necrotic areas in the form of brown spots, which then develop into a diamond shape with brown edges and gray centers (Figure 8B). On symptomatic leaves that are still infectious, aggregates of conidia are visible, indicated by white powder attached to the center of the symptoms, often found in multiple spots on a leaf (Figure 8C) and symptoms of neck blast disease in brown spots encircling the neck (Figure 8D). Plants infected with neck blast experience empty grains.

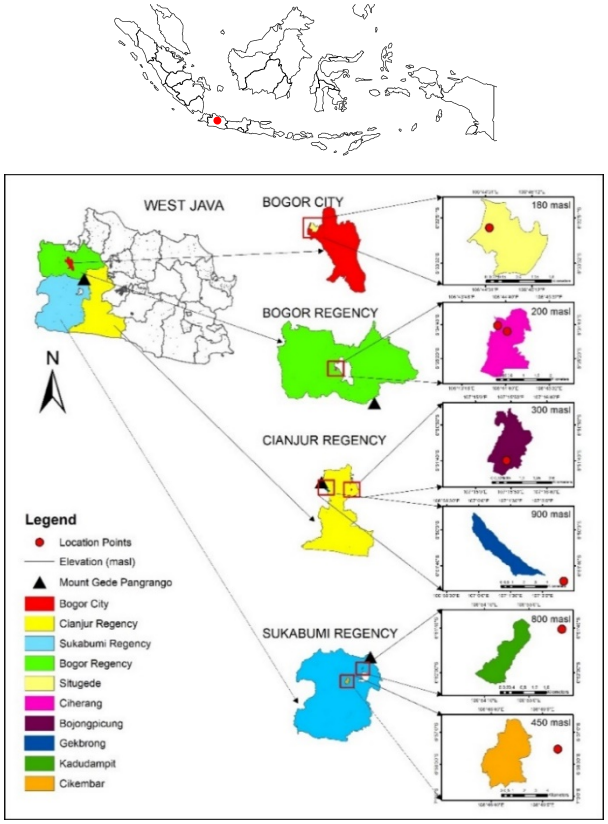

Figure 7 Maps of rice blast disease survey area in 2021

Figure 8 Symptoms of blast disease observed in West Java including leaf blast in 2-weeks-old seedlings (A), light and severe leaf infection (B-C), and neck blast (D)

2.3.2. Current situation and dynamic of blast disease

In December 2021, the three monitored regencies were experiencing the rainy season, with the number of rainy days reaching 25 days/month. The average temperature fluctuates between 24 and 27 ºC, with humidity reaching 98% (Table 4). These conditions are ideal for the dispersal of blast disease in the field. We conducted an initial mapping of the sites of blast disease and efforts undertaken by the farmers to control the disease. Farmers generally plant Ciherang and Inpari cultivars three times a year and apply various fungicides to control blast disease five to six times during each planting season. Despite the continuous and widespread use of these rice cultivars and intensive application of fungicides, as well as the favorable environmental conditions mentioned above, the disease intensity remained varied. The incidence of blast disease ranged from 53% to 100%, with severity ranging from 8% to 67% (Table 5).

Compared to Bogor and Cianjur, Sukabumi Regency had the highest incidence of blast diseases which is heavily influenced by the high rainfall rate (545 mm) and high temperature (27 ºC) (Table 4). In addition, all plots observed in Sukabumi have both leaf and neck blast. Cikembar District in Sukabumi Regency had the highest disease incidence and severity, with values of 100% and 67%, respectively, and clear symptoms could have been noticed in 2-

week-old seedlings (Figure 8A). Disease dynamics in Cikembar are interesting to be investigated more thoroughly.

Table 4 Average temperature, relative humidity, rainfall days, and rainfall rate in Bogor, Cianjur, and Sukabumi Regency in December 2021

Climatic factors	Bogor	Cianjur	Sukabumi
Temperature (°C)	20 – 33 (av. 26)	19 – 31 (av. 24)	19 – 28 (av. 27)
Relative humidity (%)	54% - 98%	76% - 97%	67% - 98%
Rainfall days (days/month)	20	23	25
Rainfall rate (mm)	279	353	545

Table 5 Disease incidence and severity in six fields in Bogor, Cianjur, and Sukabumi

Districts	Rice cultivars	Type of blast		Fungicide	Incidence (%)	Severity (%)
		Leaf	Panicle			
Situgede	Ciherang	o	×	Azoxystrobin	70.0a	20.7a
Ciherang	Inpari 32	o	o	Propiconazole	53.3a	8.1a
Bojongpicung	Inpari 32	o	×	Difenoconazole	73.3a	16.3a
Gekbrong	Inpari 33	o	o	Thiophanate-methyl	86.7a	31.1ab
Kadudampit	Inpari 32	o	o	Tricyclazole	76.7a	21.8a
Cikembar	Ciherang	o	o	Isoprothiolane	100.0a	67.4b

The persistent availability of *P. oryzae* fungal inoculum both during and outside of the growing season is one of several variables that contribute to extremely high levels of infection in Cikembar. Associated with fungicide usage, isoprothiolane (IPT) is the current commonly used fungicide by farmers in Cikembar District to control blast disease, as it remains the most effective fungicide among several alternative fungicide options. Farmers, on the other hand, report that the efficacy of isoprothiolane is declining.

Farmers who were usually able to harvest 6 tonnes/ha have only harvested 5 tonnes/ha after using IPT continuously in the past three years. This 16% decline caused uncertainty, and farmers believe that the effectiveness of IPT has been lowered, prompting them to begin

looking for substitutes. However, genetic alterations in *P. oryzae* that allow it to become less sensitive to IPT may be the source of decreasing control performance, rather than the fungicide no longer being effective. This concern requires additional confirmation by laboratory tests of isolates from field collections that have been extensively exposed to IPT.

Given that farmers have reported that their predominant fungicide has reduced performance, as demonstrated by the high disease severity, it is thought that the *P. oryzae* population in this area was subjected to a mutation. Isoprothiolane is a systemic fungicide and has already been reported to generate resistance to *P. oryzae* overseas, thus comparable examples of resistance reports have probably evolved in Indonesia requiring further research. Aside from the indispensable of *P. oryzae* genetic variability reflected in disease dynamics and geographical conditions supporting the disease development, farmers face some other challenges when managing blast disease. Acidic soil (pH 4.2 ± 0.2) is a current challenge that farmers also encounter in Cikembar District (Figure 9).

Figure 9 Device used by farmers to measure the soil pH showing low acidity (pH 4.1)

The recurrence of blast disease in Cikembar during the dry season has raised concern about whether the infection cycle could be highly suppressed. At the time of observation, the air temperature ranged between 25 and 32 ºC, with a humidity of 75%. The number of rainy days is only about 7 - 11 days per month, with a rainfall rate of less than 100 mm/day. This is heavily influenced by the length of the dry season in 2023. This dramatic change in climate conditions was predicted significantly inhibiting for *P. oryzae*. However, *P. oryzae* noted a higher adaptability in facing climate change expressed by high disease reaching 90% with moderate disease severity up to 36% (Table 6). Given that five observed plots were in the

same location and had similar sources and amounts of inoculum, the IR64 cultivar had the lowest disease severity (20%) and undeveloped symptoms confirmed by *halo* encircling most of the small spots (Table 6; Figure 10C).

Table 6 Disease incidence and severity in five plots with different cultivars and fungicides used in Cikembar, West Java (November 2023)

Plot	Rice cultivars	Fungicide	Incidence (%)	Severity (%)
1	Mixture	Isoprothiolane	90 ± 8.2	28.5 ± 6.8
2	Ciherang	Carbendazim	100 ± 0.0	33.3 ± 10.1
3	IR64	Tricyclazole + Azoxystrobin	97 ± 4.7	20.4 ± 2.6
4	Ciherang	Isoprothiolane	90 ± 8.2	30.7 ± 8.6
5	Local	Tricyclazole	100 ± 0.0	35.6 ± 6.3

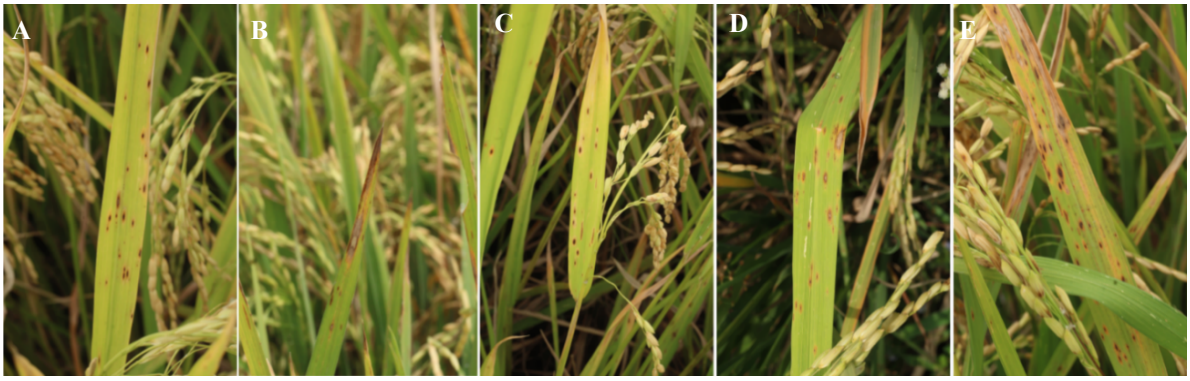

Figure 10 Common leaf blast appearance observed in the combination of cultivars and fungicide usage in Cikembar District including Mixture – isoprothiolane (A), Ciherang – carbendazim (B), IR64 – tricyclazole + azoxystrobin (C),Ciherang – isoprothiolane (D), and Local – tricyclazole (E)

The use of isoprothiolane on mixed cultivars yielded intriguing results. The plant's response to this practice resembles that of IR64 with undeveloped multiple spots (Figure 10A). This is thought to be the result of varying resistance degrees provided by the multiline.

Amid the complexity of blast disease control, farmers rely on resistant cultivars that exhibit varying levels of resistance, as well as fungicides, to mitigate yield losses caused by rice blast.

2.3.3. Socioeconomic information on farmers' cultivation strategy to control blast disease

All interviewed farmers had experienced 10%-15% production loss per year owing to blast, with the worst condition ever being 55%, thus they designed several strategies to mitigate this threat in the future. Farmers continue to prefer the Ciherang cultivar due to its high yield potential and social acceptability but also plant the IR64 cultivar considering lower production loss when the rice blast outbreaks due to IR64's higher performance of resistance. Other challenges in integrated rice blast management are seedlings and fungicide costs which reach 24% of total production expenditures. To cut production expenses, some farmers discontinue using fungicides or limit their frequency of use, especially if the yield that can be saved is predicted not to cover the whole production cost.

According to Mr. Ferry (41), the head of the Cikembar District farmers group, the fluctuation in blast disease occurs every year. Leaf blast disease symptoms emerge 14-30 days after planting and progress for up to 60 days. Some plants are infected with panicle blast, whereas others are not. Farmers utilize a cultivar of fungicide active ingredients to control blast disease and vary the active ingredients when the fungicide's effectiveness is compromised. Farmers typically apply fungicide three times throughout the growing season (25 and 45 days after planting and when panicles emerge). Farmers might increase the spraying frequency to 5-6 times if the infection is severe.

Differences in farmer blast control tactics are related to decision-making during observation and fungicide cost consideration. Farmers frequently discover a latent period, where symptoms are less likely to appear, after 50 days and some farmers decide not to spray anymore. However, farmers need to continue to spray considering the possibility of neck blast infection, as halting spraying increases the rapid development of neck blast to other rice fields, and in severe infection blast severe infection, resulting in empty grains, significant yield loss, and novel higher status of primary and secondary inoculum sources.

These different strategies are heavily influenced by the land ownership status and size of the land tenure system. Some farmers work as cultivators rather than landowners. The

typical size of land possessed by landowners is 1-2 hectares, but farmers will not earn a sufficient amount of capital to cover the high production costs of a small land area. At least, a minimum of 8 hectares of land is required to provide farmers with a reasonable calculation for yielding revenue despite all the challenges mentioned above. Currently, aside from fungicides, farmers are waiting for the introduction of new blast-resistant lineages/rice varieties as one component of integrated blast disease control.

2.4. Discussion

During the field observations, the environmental factors (high temperature, humidity, and rainfall) were favorable for rice blast development in the rice fields in West Java, Indonesia. Rice blast disease spreads quickly at temperatures ranging from 25 to 29 ºC, humidity levels ranging from 60% to 95%, and soil temperatures ranging from 28 to 31 ºC (Kirtphaiboon *et al.* 2021). Rising atmospheric CO_2 levels also create climatic circumstances favorable to the growth of *P. oryzae* (Luck *et al.* 2011). Cikembar District, Sukabumi Regency has the highest disease incidence and severity and is determined as the endemic area. Aside from the persistence of *P. oryzae* inoculum availability and survivability along multiple growing seasons, this high disease intensity is also supported by the geomorphology of Sukabumi, which appears as a terraced valley, providing for continual trapping of high humidity (Hardini *et al.* 2019; Jamil *et al.* 2022). Besides the rice blast disease, acidic soil (pH 4-5) has recently been a new difficulty for farmers in Cikembar District. This acidic state is favorable for pathogen development. This acidic soil is favorable for pathogen development, especially soil-borne disease, but is less favorable for plant growth in nutrient absorption, particularly Na, Ca, Mg, and Cl (Thakur *et al.* 2021). Farmers anticipate these disadvantages by regularly measuring the soil acidity and then applying calcium carbonate and dolomite.

A moderate *El Nino* has been recorded in West Java Province since mid-2023 (BMKG 2023). This climate anomaly causes a long dry season (more than 60 days) on Java Island, with 84% of days without rain. However, this dry season in 2023 was still enabling the high disease incidence while moderate disease severity in Cikembar. Although the daily humidity is lower, the presence of dew in the morning provides adequate water for conidial germination (Everts 1990). In November 2023, all plants were in the generative phase, with an average plant age of 80 days where the density of tillers increases microhumidity and contributes to the emergence of panicle blast while maintaining the leaf blast development.

Varying response of resistance is demonstrated by the use of different rice cultivars and fungicides in Cikembar. IR64 cultivar showed the lowest disease severity (20%) among different cultivars planted and various fungicides employed. According to Santoso *et al.* (2021), different rice cultivars performed a diverse host response against 200 Indonesian

strains of *P. oryzae* and its main races. IR64 is known to include 11 blast resistance genes that are effective in blast control tactics, particularly in tropical countries (Fukuta *et al.* 2022). Jamaloddin *et al.* (2021) explained resistance rice cultivars are capable of exhibiting multiple pathogen races (vertical resistance) or numerous diseases simultaneously (horizontal resistance).

Following IR64, the use of mixed cultivars combined with isoprothiolane exhibited a moderately low severity (below 30%). In addition to horizontal resistance demonstrated by the use of multiline, isoprothiolane performed a relatively high activity conveying fungal cell membrane disorganization in the choline biosynthesis pathway (Uesugi 2001). Suganda *et al.* (2016) indicated that isoprothiolane fungicide could reduce potential yield losses by 28% when compared to untreated plots in the Ciherang variety. On one side from host plant resistance, pathogen virulence, and natural environmental circumstances, cultivation parameters such as balanced fertilization, the use of healthy seeds, and the use of fungicides are all strongly related to the prevalence of blast disease in the field. According to Zulaika *et al.* (2018), nitrogen fertilization intensity raises the likelihood of disease severity by 8.4%. Because a 1% rise in contaminated seeds causes a 2% increase in disease severity, using healthy seeds corresponds with the risk of disease breakouts in the field.

Uncertain climatic conditions necessitate a more constructive strategy for rice blast disease control, including the monitoring of mutation occurrence. Sukarta *et al.* (2018) model that increased rainfall in West Java predicts an outbreak of blast diseases between 2030 and 2036. Despite intensive regular application and reports of declining performance by farmers as well as recent updates of fungicide resistance overseas, resistance of *P. oryzae* to isoprothiolane probably has probably emerged in Indonesia. It will be of interest to understand the novel dynamics of rice-*P.oryzae*-fungicide due to pathogen mutation and how this information translates to possible efforts to have better strategies in long-term rice blast control.

3 SENSITIVITY ASSAYS OF *Pyricularia oryzae* ISOLATES COLLECTED FROM THREE REGENCIES IN WEST JAVA

3.1. Introduction

With a 30% contribution to rice yield loss worldwide, rice blast disease caused by *Pyricularia oryzae* still poses the most serious threat to rice productivity (Wilson & Talbot 2009). This ascomycetes fungus infects both vegetative and generative stages of plants with major symptoms on leaves and panicles. Broad-spectrum resistance varieties have been studied as most approaches in blast disease control (Rosas *et al.* 2020; Younas *et al.* 2023). However, several dynamics appear between rice cultivars and *P. oryzae* leading to the disease management strategies being more difficult. This fungus has a complex infection cycle both for navigating through plant tissue invasion using mechanical and chemical properties and for avoiding rice immunity systems (Cruz-Mirelez *et al.* 2021). Regarding the interspecies development, *P. oryzae* from rice performed a high incidence and severity in several Poaceous crops and grasses with high virulence implicating the issue of novel alternative hosts and a higher risk of cereal crop production (Pak *et al*. 2021). Understanding the fungus side needs to receive special attention since it contributed one-third to the amount of disease (Agrios 2005).

To directly target the fungi, synthetic chemical fungicide is still considered more practical by damaging fungal cell membranes, interfering with fungal energy production, and working either before or after the infection and symptoms (Caffi & Rossi 2018; McGrath 2004). During field observations for this study, farmers continue to rely on isoprothiolane fungicide due to its excellent track record of efficacy. Isoprothiolane is a systemic fungicide that disrupts Kennedy's pathway in phosphatidylcholine production, causing cytoplasmic chemicals to flow out and the fungal cell membrane to become disorganized (Uesugi 2001). However, continuous use of one specific fungicide kind may lead to a reduced sensitivity of the fungal isolate because of genetic pressure when the fungus adapts to severe environmental stressors. This resistance could be expressed by lower conidia production, delayed colony growth, and poor ecological fitness (Ziogas *et al.* 2005).

The occurrence of fungicide resistance in Indonesia raises high concern since abundance reports have been published in other countries (Ishii 2006). Extensive research has been conducted on *P. oryzae*'s resistance to azoxystrobin and azole groups, as well as how to manage this resistance. (D'Avila 2021; Kimura & Fukuchi 2018; Dorigan *et al.* 2019). Reports of *P. oryzae* resistance to isoprothiolane are still restricted to mutant isolates generated in the lab, given the fact that it has been produced and used since 1975 (Wang *et al.* 2018). As West Java is Indonesia's second-largest rice-producing region, it is necessary to determine the current state of sensitivity as well as the potential for the emergence of isolates that have mutated naturally in the field to become isoprothiolane-resistant isolates.

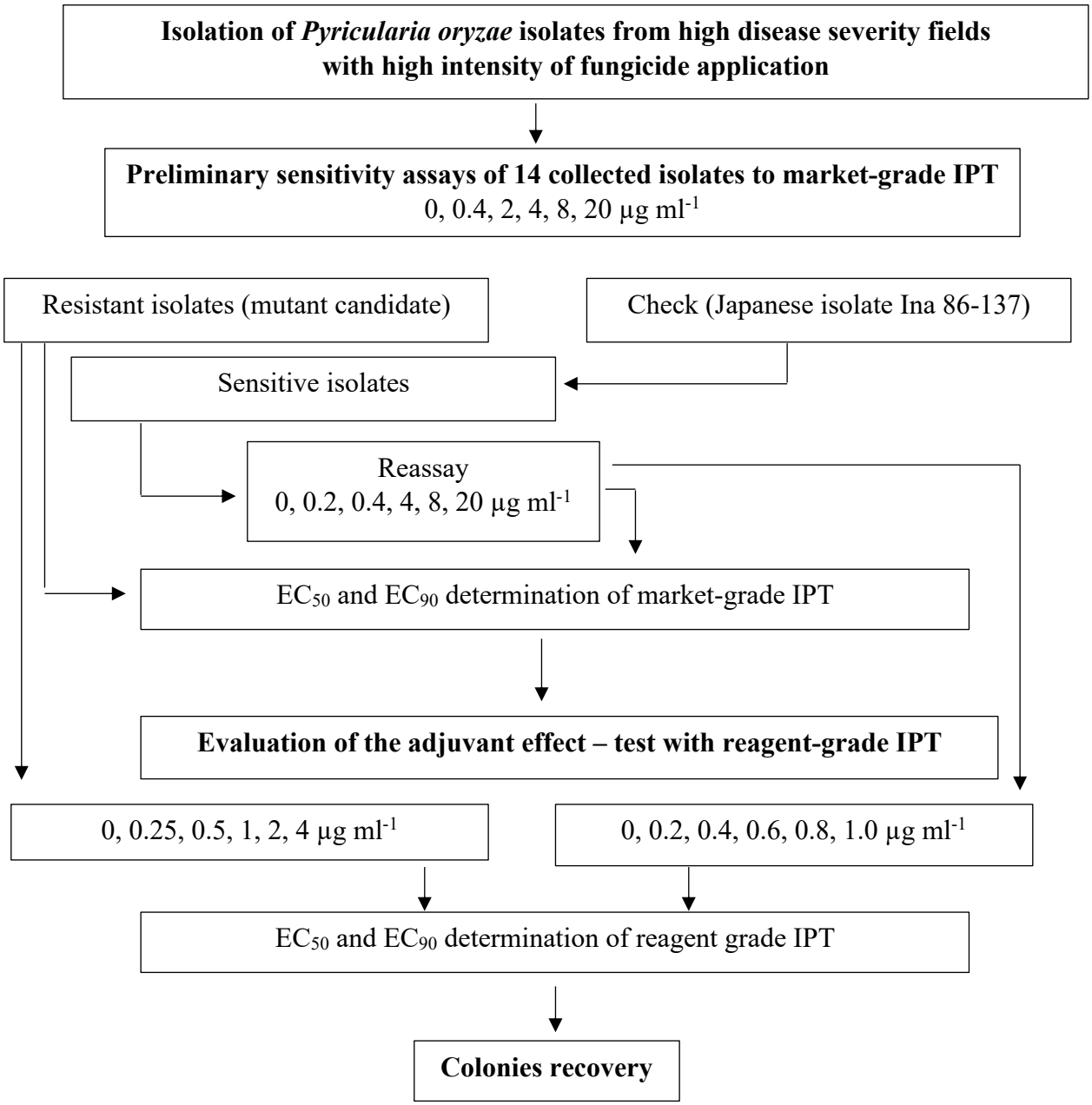

Figure 11 Research flow diagram of sensitivity assays of *Pyricularia oryzae* isolates collected from three regencies in West Java, Indonesia

3.2. Methods

3.2.1. Pathogen isolation and morphological identification

Purposive sampling was used to isolate *Pyricularia oryzae* fungus isolates from rice plantations in Bogor, Cianjur, and Sukabumi districts, particularly based on the disease severity and the intensity of fungicide application. Samples of infected leaves and panicles were cut in a 1 cm^2 at the borderline between the infected and healthy areas. The samples were surface sterilized with 1 minute of sterile water, 1 minute of NaOCl, and 3 times rinsed with sterile water. The samples were then dried on a paper towel for 5 minutes to remove excess water.

Each of the four sample pieces was placed on potato dextrose agar (PDA) and water agar (WA) media containing chloramphenicol antibiotic (500 mg L^{-1}) and incubated at room temperature for 2 days (PDA) and 4 days (WA) (Fig. 12). Colonies with white or gray symptoms, strong adhesion on media, and slow growth are subcultured onto a fresh PDA media and incubated for 7 days. Morphological identification was undertaken by macroscopic and microscopic characterization of *P. oryzae* referred to by Barnett & Hunter (1998).

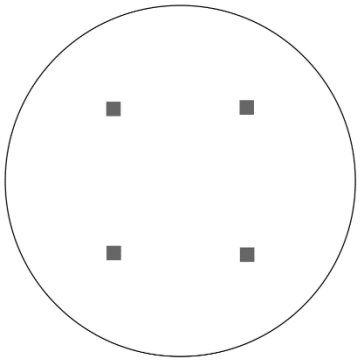

Figure 12 Placement of infected leaf or panicle on PDA and WA medium

3.2.2. Molecular identification of P. oryzae for 5.8 sDNA coding sequence

To confirm the identity of *P. oryzae*, molecular characterization was performed on the fungal colonies after morphological confirmation. DNA isolation was carried out using the Abd-Elsalam *et al.* (2003) protocols, with modifications to the precipitation steps based on Gupta *et al.* (2013). Excess ethanol is completely removed via centrifugation and pipetting

to optimize the DNA purity (A260/280). Polymerase chain reaction (PCR) was used to analyze the 5.8-coding sequence using primers ITS1F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4F (5'-TCCTCCGCTTATTGA TATGC-3'), which are specific for fungal groups (Martin & Rygiewicz 2005). A total volume of 25 µl (Green PCR MasterMix 12.5 µl, 2.5 µl of ITS1F 0.5 µM, 2.5 µl of ITS4R 0.5 µM, 1 µl of DNA template 50 ng/µl, and 6.5 µl nuclease-free water) is used as the PCR mixture. The PCR program is described in Table 7.

The PCR results were resolved on a 2% agarose gel (1st BASE, SG) with Gel-RedTM Nucleic Acid Gel Stain (Biotium, US) loading buffer that does not require EtBr staining and a 1 kb marker (Geneaid Biotech, TW) was used as a ladder. PCR products with DNA bands were delivered to the 1st BASE Laboratory (MY) for sequencing. Bioedit and MEGA X were used for analysis of the sequence and alignment to the database in GenBank and to construct the phylogenetic tree.

Table 7 PCR program for the analysis of ITS region

Stage	Temperature (°C)	Time (min.)
Initial denaturation	94	02:00
35 cycles		
• Denaturation	94	00:50
• Annealing	55	01:00
• Extension	72	01:00
Final extension	72	05:00
Hold	4	∞

3.2.3. Preliminary in vitro *sensitivity assays to isoprothiolane (Fujiwan 400 EC) and EC value determination*

Sensitivity tests to 14 *P. oryzae* isolates were carried out using a commercial Fujiwan 400 EC, a commercial isoprothiolane (IPT) with IPT-amended PDA methods. A 5-mm mycelial plug was inoculated onto a medium containing IPT at concentrations of 0, 0.4, 1, 2, 10, 20, and 50 µl ml^{-1} PDA (Wang *et al.* 2018, modified). After converting the formulation code and concentration formulation, the isoprothiolane test concentrations above were 0, 0.4,

2, 4, 8, dan 20 mg L^{-1} and incubated within 9 days at 27 ºC. A Japanese *P. oryzae* isolate (Ina 86-137) was used as a control (CK). Each concentration contains 5 replications.

Observations were conducted by measuring the colony diameter and then calculating the relative inhibition rate (IR) with the formula:

$$\text{IR (\%)} = \frac{\text{Diameter on Control} - \text{Diameter on Treatment}}{\text{Diameter on Control}} \text{ x } 100\%$$

The IR data was plotted in a line graph (Ms. Excel), and the visualization of *P. oryzae* colonies growth at 9 dpi was shown in image form (Ms. Publisher). The decimal values in the IR results are rounded and organized in Notepad with n: maximum possible inhibition rate (100%) (example in Fig. 13). Probit analysis was used to determine effective concentrations (EC_{50} to EC_{90}) using Polo Plus LeOra Software 1.0 (2002-2004).

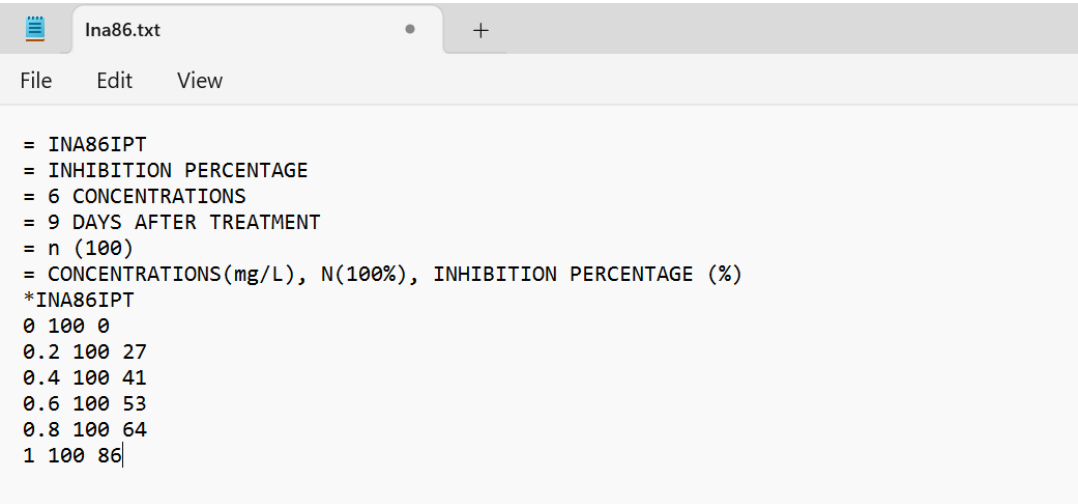

Figure 13 Example command writing in Notepad for PoloPlus probit analysis

Highly sensitive *P. oryzae* isolates that EC values could not be determined in the initial concentrations above were reassayed with lower concentrations of 0, 0.2, 0.4, 0.6, and 1.0 mg L^{-1} with five replications and incubated for nine days. The previously indicated methods were employed for both observation and data analysis.

3.2.4. Confirmation of adjuvant effect by in vitro *sensitivity assays to reagent-grade isoprothiolane*

A reagent-grade isoprothiolane was used in sensitivity assays to evaluate the effects of adjuvants included in the Fujiwan 400 EC formulation. Two concentration groups were used for the testing based on the result of the preliminary test. The concentrations employed for isolates that were reported to be resistant were 0, 0.25, 0.5, 1, 2, and 4 mg L^{-1}. Meanwhile, the concentrations used in isolates that have been identified as sensitive are 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg L^{-1}. Ina 86-137 collection was used as a sensitive control. Each treatment has 5 replications. The same techniques that had been addressed previously were applied for observation and data analysis.

3.2.5. Prediction of resistance dynamics in P. oryzae *isolate*

The fungal colonies that maintained growing at the maximum concentration in previous sensitivity assays (20 mg L^{-1} for resistant isolates or 1 mg L^{-1} for sensitive isolates) were subcultured on fresh PDA media and incubated for 9 days at 27 ^{o}C to recover the colonies. Any fungal visual abnormalities were recorded. This recovery technique will determine whether or not Fujiwan 400 EC is still required for application in the field based on the alterations that occurred in the *P. oryzae* populations.

3.3. Results

3.3.1. Pyricularia oryzae colonies description

As many as 37 fungi were isolated from the fields in Bogor, Cianjur, and Sukabumi(leaf and neck blast). Distinguished from other fungi, *P. oryzae* on potato dextrose agar (PDA) medium has slow growth (±5 mm per day) at room temperature, grew firstly as white colonies, then developed to grey, concentrical radial growth (more clearly seen from the colony's reverse), and slightly flat elevation. Sukabumi which has the highest disease intensity provides more isolates than Bogor and Cianjur. All these 10 isolates are light brown to dark brown reverse colony color. These isolates have light-colored conidia, 3 cells (2-septate), solitary, obclavate, with a size of about 10 µm (Fig. 14). 14 colonies with relatively rapid growth were used for the sensitivity assay.

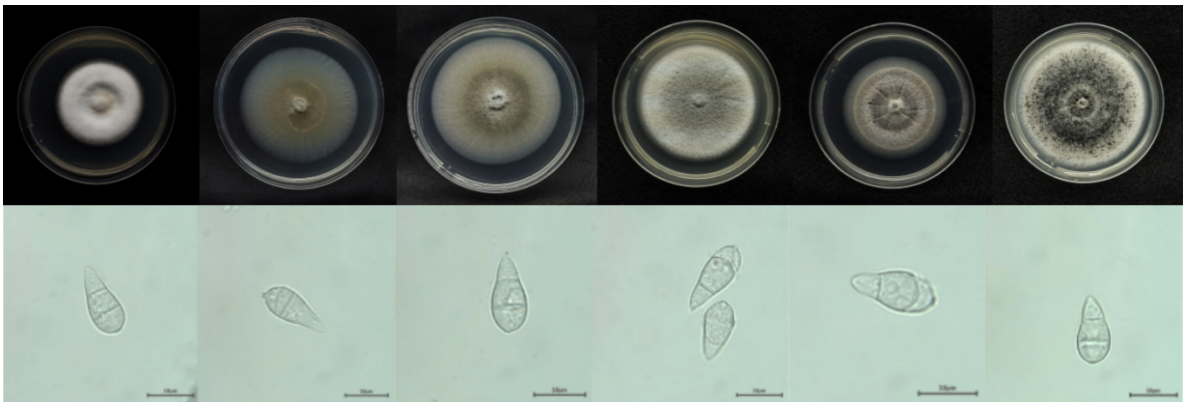

Figure 14 *Pyricularia oryzae* isolates collected from three regencies in West Java in diverse colonies appearances on PDA medium (above) and conidia in 1000 optical zoom (below)

3.3.2. Identification of 5.8S-coding sequence in ITS region

The 14 isolates detected the 5.8S-coding sequence at ±500 bp and identified as *Pyricularia oryzae* and have a high query cover (81% - 99%) and percent identity (92.86% – 99.81%) with the isolates, particularly on accession number LK932250.1 from India and OR492538.1 from Thailand. The genetic group among the 14 isolates is displayed in Fig. 15.

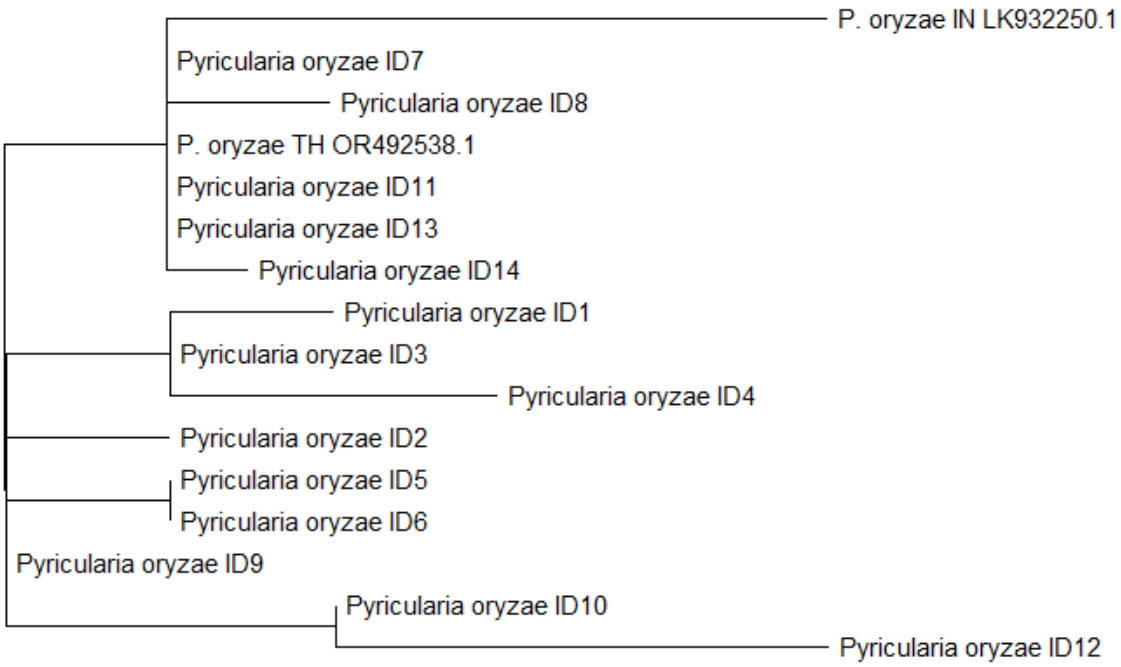

Figure 15 Phylogenetic tree of 14 *Pyricularia oryzae* isolates

3.3.3. P. oryzae colonies growth after being subjected to isoprothiolane (Fujiwan 400 EC)

Preliminary assay grouped two levels of *P. oryzae* resistance to Fujiwan 400 EC (market-grade isoprothiolane) into 6 resistant isolates and 8 sensitive isolates (Fig. 16-17). Ina-86 13 (CK) is classified as a sensitive isolate. The IR value in sensitive isolates moved stagnant and there was almost no growth starting at a concentration of 2 mg L^{-1}. Meanwhile, an exponential inhibition pattern was shown in resistant isolates. The six resistant isolates showed the ability to grow up to 20 mg L^{-1} while maintaining normal radial growth until 4 mg L^{-1}. Isolates 1, 2, 3, 5 had an IR below 80% at a concentration of 20 mg L^{-1}. Resistant isolates begin to lose gray pigment at high concentrations so that the colonies become white (Fig. 17). The EC_{50} and EC_{90} values for these resistant isolates were 2-4 mg L^{-1} and 15-59 mg L^{-1} respectively (Table 8). This EC_{50} value is the basis for determining the concentration of reagent-grade isoprothiolane.

Eight susceptible isolates failed to generate the EC_{50} or EC_{90} values in the preliminary assay. This is due to the Polo Plus system's inability to interpret the data since concentrations

ranging from 2 to 20 mg L^{-1} did not produce an IR pattern. As a result, testing was repeated for these eight isolates using a smaller concentration range. At a concentration of 0.8–1.0 mg L^{-1}, an IR value of 60%–80% was achieved (Fig. 16B). In isolate 10, a new colony known as a sector appears as a result of *P. oryzae* adapting to a harsh environment. The CK isolate also showed sectors (Fig. 18). These highly sensitive isolates have EC_{50} and EC_{90} values of 0.1-0.7 mg L^{-1} and 1.2-3.3 mg L^{-1}, respectively.

The sensitive isolate grows more quickly than the resistant isolate when the controls for each isolate are compared (0 mg L^{-1}) (Fig. 17). This is believed to be the result of a fitness penalty that *P. oryzae* in this study encountered. Resistance fungal isolates naturally experience fitness costs in reaction to shifts in the population's degree of dominance.

3.3.4. Evaluation of adjuvant effect by in vitro *sensitivity assays to reagent-grade isoprothiolane*

Some adjuvants are commonly included in a fungicide formulation to make fungicide applications more effective. However, adjuvants have the potential to influence the results of EC analysis in sensitivity assays, making it unclear whether the efficacy observed is only due to the fungicide's active ingredient. Sensitivity assays were employed to evaluate the effects of reagent-grade isoprothiolane on the EC value generated.

The EC_{50} value from the preliminary assay was used to determine the maximum concentration in this test, which was 4 mg L^{-1}. Furthermore, this concentration can still support the growth of *P. oryzae* colonies with only minor visual defects, according to preliminary results. The IR generated by reagent-grade IPT at 4 mg L^{-1} for the six isolates ranged from 50% to 70% (Fig. 19A and Fig. 20). This result differs slightly from the wider preliminary assay's IR at 4 mg L^{-1} in that multiple isolates had an IR of 90%. According to Table 9, the test yielded EC_{50} and EC_{90} values of 2–6 mg L^{-1} and 9–20 mg L^{-1}, respectively. This demonstrates that the adjuvant in Fujiwan 400 EC has a slight effect on the inhibition activities. The EC_{50} value in this test is roughly the same as the EC value in the preliminary assay, but the EC_{90} value drops. This is believed to be the case because the mode of action activity is more concentrated on going straight after the fungus's target site. In conclusion,

both commercial and commercial IPT are reliable in distinguishing resistant isolates from the sensitive ones.

For the sensitive isolates, reagent-grade IPT showed a wider sensitivity window. Certain isolates have an IR of 50% at a concentration of 1 mg L^{-1}, while other isolates have IR values reaching 100%. At 0.8 mg L^{-1}, isolate 13 was no longer able to develop, demonstrating the isolate's extreme sensitivity even at low concentrations (Fig. 19B). According to Table 3, the test yielded EC_{50} and EC_{90} values of 0.37–1.27 mg L^{-1} and 1.2–4.8 mg L^{-1}, respectively. These sensitive isolates have more dynamic EC_{50} values than resistant isolates, with the EC_{90} matching the results of the preliminary assay. In the meantime, this sensitive isolate's growth was severely inhibited by commercial isoprothiolane, resulting in EC_{50} values that were all below 1 mg L^{-1} (Table 9).

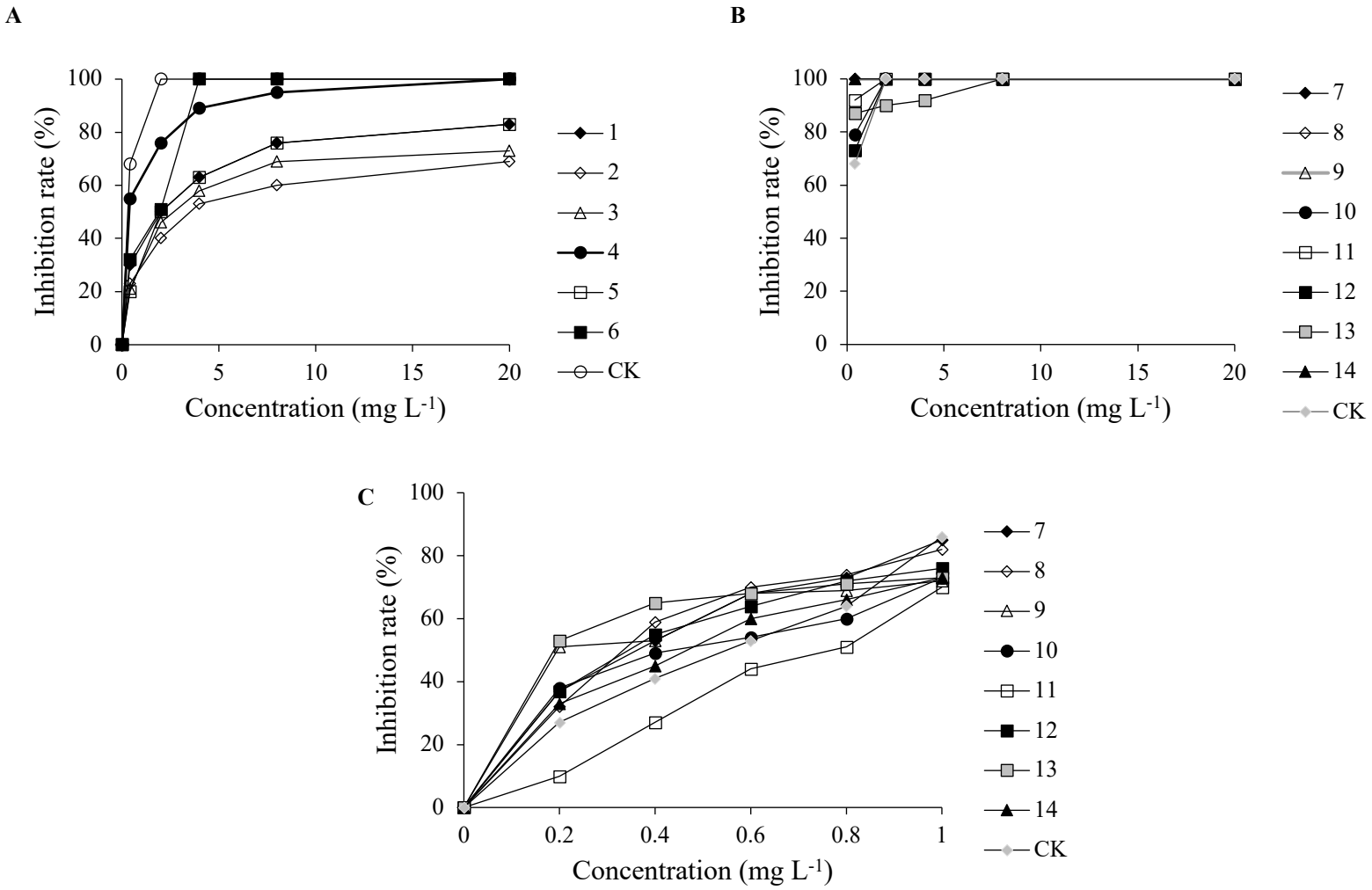

Figure 16 Inhibition rate of isoprothiolane (Fujiwan 400 EC) in preliminary evaluation to *Pyricularia oryzae* in resistant isolates (A), sensitive isolates (B), and reassay with lower concentrations for sensitive isolates (C)

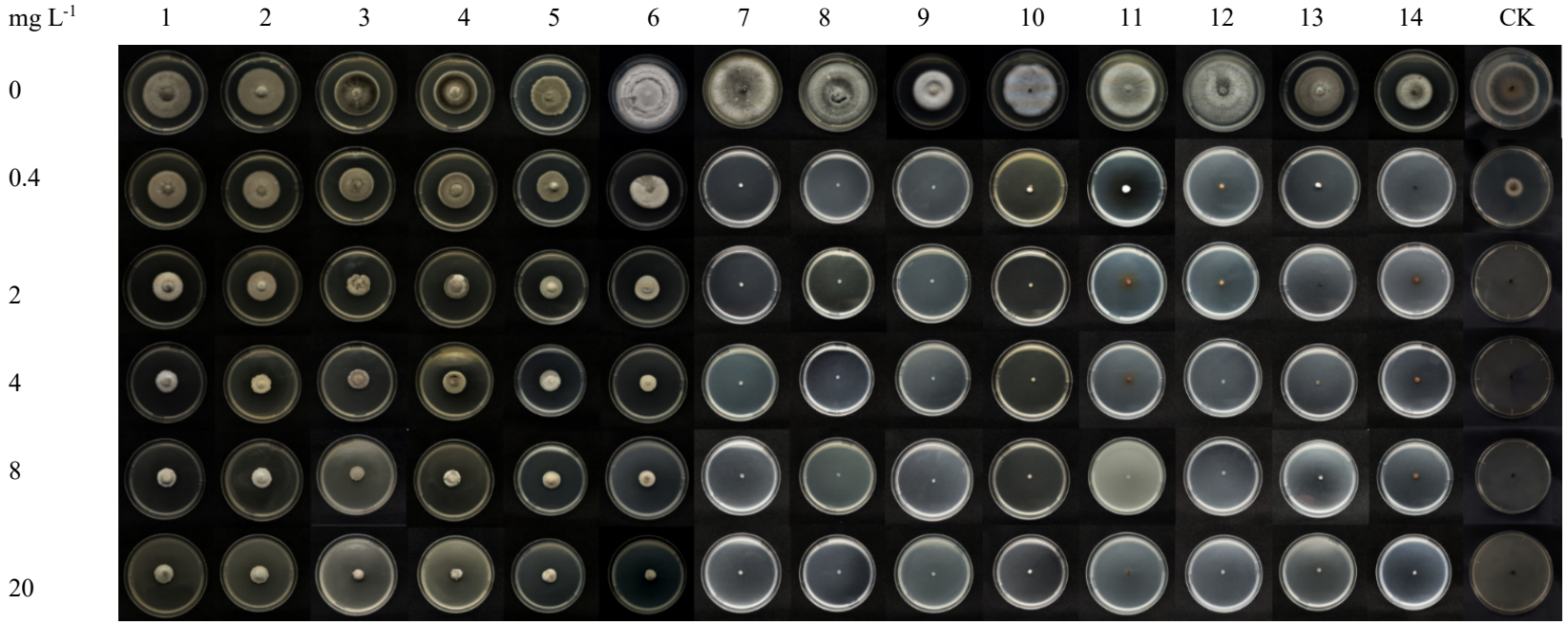

Figure 17 Visualization of *Pyricularia oryzae* colonies growth on PDA medium amended with different isoprothiolane concentrations in the preliminary evaluation

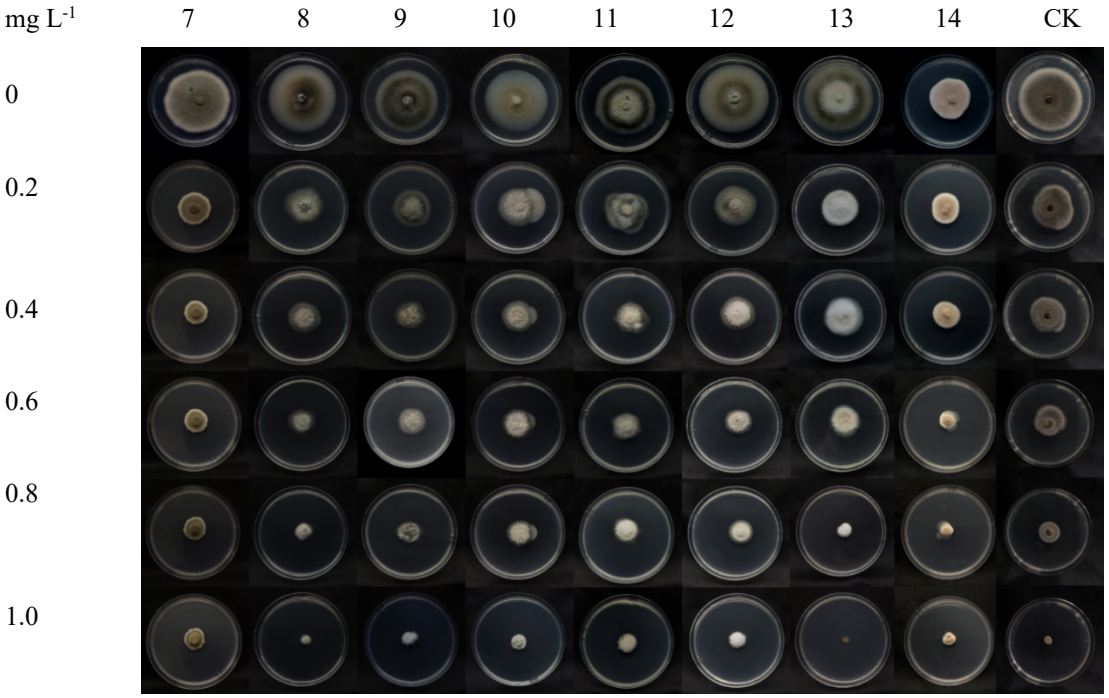

Figure 18 Visualization of nine *Pyricularia oryzae* colonies growth on PDA medium amended with lower isoprothiolane concentrations

Table 8 Effective concentrations and categorization of *Pyricularia oryzae* isolates to isoprothiolane (Fujiwan 400 EC)

Isolate	EC_{50} (mg L^{-1})	EC_{90} (mg L^{-1})	Sensitivity group
1	3.08	23.6	Resistant
2	3.67	59.6	Resistant
3	2.53	26.2	Resistant
4	2.97	22.4	Resistant
5	2.04	15.9	Resistant
6	2.55	46.3	Resistant
7	0.17	2.05	Sensitive
8	0.33	1.40	Sensitive
9	0.33	1.19	Sensitive
10	0.44	2.36	Sensitive
11	0.75	1.97	Sensitive
12	0.33	1.56	Sensitive
13	0.15	2.26	Sensitive
14	0.43	1.95	Sensitive

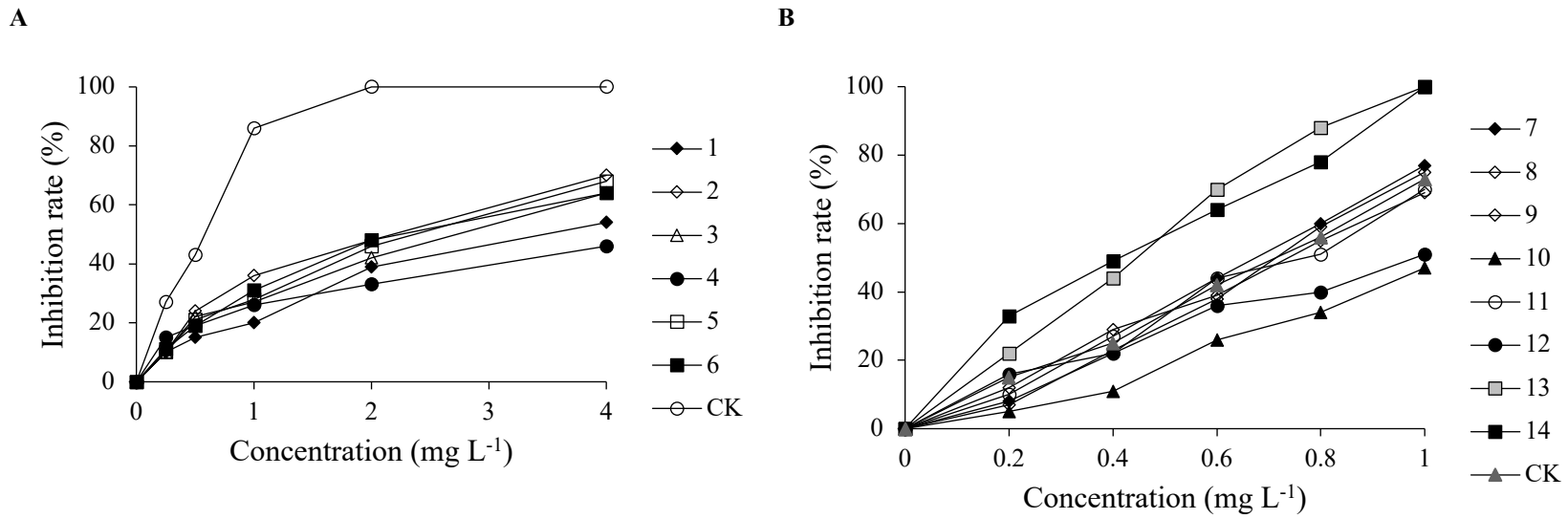

Figure 19 Inhibition rate of reagent-grade isoprothiolane in the evaluation of adjuvant effect to *Pyricularia oryzae* in higher concentrations for resistant isolates (A) and lower concentrations for sensitive isolates (B)

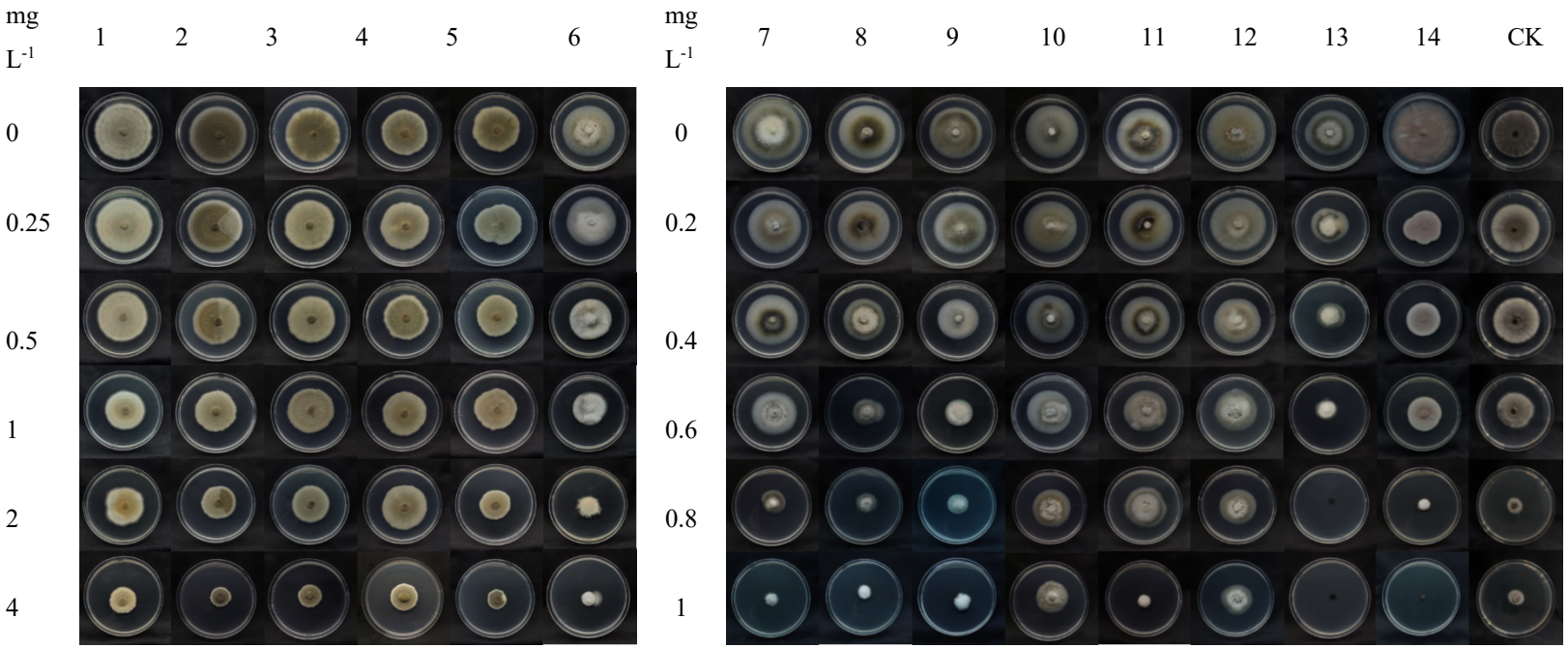

Figure 20 Visualization of *Pyricularia oryzae* colonies growth on PDA medium amended reagent-grade isoprothiolane

Table 9 EC and categorization of *Pyricularia oryzae* isolates to reagent-grade isoprothiolane

Isolate	EC_{50} (mg L^{-1})	EC_{90} (mg L^{-1})	Sensitivity group
1	4.24	20.5	Resistant
2	2.01	9.7	Resistant
3	3.11	16.6	Resistant
4	6.47	14.3	Resistant
5	2.54	12.2	Resistant
6	2.67	10.5	Resistant
7	1.27	4.8	Less sensitive
8	0.71	1.6	Sensitive
9	0.88	2.1	Sensitive
10	1.24	4.7	Less sensitive
11	0.75	1.9	Sensitive
12	1.25	4.7	Less sensitive
13	0.39	0.7	Sensitive
14	0.37	1.2	Sensitive
CK	0.74	3.2	Sensitive

3.3.5. Prediction of resistance dynamics in Pyricularia oryzae isolates

The colonies of *P. oryzae* that were treated to high fungicide concentrations and then regrew on fresh PDA media displayed colony regeneration (Fig. 21). After being treated with fungicide, colonies regained their natural appearance (Fig. 21A). Certain colonies exhibit some abnormalities, including clumped and compacted colonies, irregularly growing colonies that lose pigment, and rapidly growing colonies that are noticeably thinner than the original colony (Fig. 21B-D). These findings suggest that there are wide regrowth variations in the way fungi respond to their surroundings. Not all fungi are able to perform colonies that resemble their initial colonies, even when they are subcultured on a fresh PDA medium might indicate the fungal colonies experiencing a fitness cost.

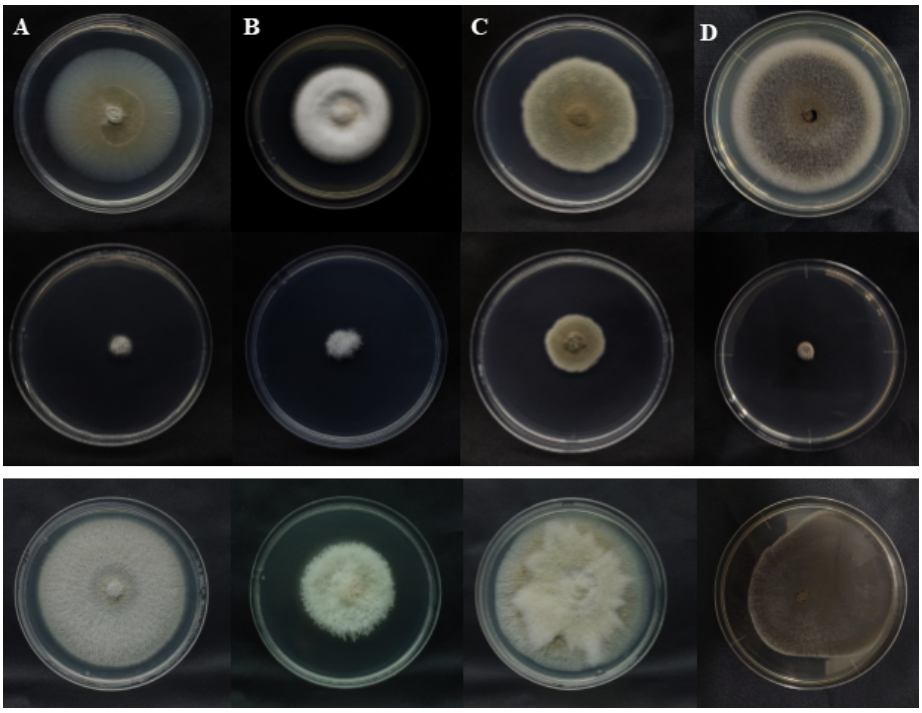

Figure 21 Samples of *Pyricularia oryzae* colonies change (below) after being subcultured from the highest concentration tested (above, 2nd row) to a new PDA medium with the visual return to the original colonies' appearance (A), colonies crawl and compact (B), colonies lose grey pigment and produce sectors (C), and colonies to grow irradiate and consume the media highly rapidly (D)

3.4. Discussion

Six resistant isolates that were thought to have spontaneous mutation were found in this research. These six isolates may still grow at 20 mg L^{-1} and maintain radial growth at 4 mg L^{-1} which is 10 times the recommended concentration (0.4 mg L^{-1}). In this *in vitro* test, the mutant candidate exhibited an EC_{50} value 6–8 times higher than that of the sensitive control (Ina-86 137). For practical purposes, in the mutant candidate isolates, the concentration needs to be increased by more than five times to maintain population control at the same inhibition rate. The multiplication EC_{50} value is 11–17 times even when the isolates from both market-grade and reagent-grade isoprothiolane tests with the most resistant isolates and the most sensitive isolates are compared. To figure out the variety that emerges at the genotype level, these six mutant candidate isolates must be validated by molecular investigation.

When it comes to managing *P. oryzae*, isoprothiolane exhibits both dynamics of the EC value and dynamics of the sensitivity window between market-grade and reagent-grade. This indicates that the adjuvant used in the Fujiwan 400 EC isoprothiolane formulation shares a slight effect to the EC value generated. Adjuvants, according to Bhuiyan *et al.* (2024), improve the fungicide sprays' dispersion, decreasing the rate and intensity of the active ingredients' evaporation, enhancing the fungicide's capacity to be absorbed by plants, and extending the interval between fungicide applications. Accroding to the EC value, both commercial and reagent-grade IPT fungicides are still reliable for classifying the mutant candidates from isolates that were collected from the field.

At a concentration of 0 mg L^{-1}, a comparison of colonies for each isolate revealed that the sensitive isolate grew more quickly than the resistant candidate isolate indicating the mutant candidates expressing. The fitness penalty, as defined by Hawkins and Fraaije (2018), is the state in which resistant isolates lose fitness relative to sensitive isolates in the absence of fungicide exposure. To counteract the dominance of resistance isolates in the field, it is still required to use fungicides sparingly, as evidenced by data on the diversity of fungal appearances from recovery test results. To continue reducing the severity of the disease in the field and to slow down the rate at which other isolates of the disease are forming mutations, the use of isoprothiolane must be integrated with other management approaches.

4 MUTATION ANALYSIS OF ISOPROTHIOLANE RESISTANCE FROM INDONESIAN *Pyricularia oryzae* FIELD ISOLATES

4.1. Introduction

Staple food production, including rice, is performed by compound interest, whereas human population growth follows exponential interest, necessitating enormous productivity. Due to this need, the release of high-yielding and blast-resistant cultivars becomes the primary focus of improving output. However, in order to tackle rice resistance, novel mechanisms of adaptive evolution in virulence effector and telomere-to-telomere gapless genome of *P. oryzae* has been described explaining an exit strategy performed by this fungus to the host adaptation, resulting in cultivar resistance breakdown (Le-Naour *et al.* 2023; Li *et al.* 2024). Perspective of disease control and rice productivity often place the fungus's side lower than the spotlight to the rice genetics especially the relations to chemical substances in terms of fungicide application. Whereas, continuous pressure on the same target site by a single fungicide active ingredients leads to genetic stress experiences by the fungus and in order to adaptation, fungus could exhibit high genetic variability including mutations.

Over the previous decade, rice blast research in Indonesia has focused on epidemiology, yield loss estimation, breeding line evaluation, and biological control. There has been limited research into fungicide resistance. However, the occurrence of mutation leading to fungicide resistance raises a high concern (Ishii 2006). Published reports in *Pyricularia* species including *P. oryzae* resistance to fungicide were extensively studied recently on azoxystrobin, triazole, triazole, and tricylazole (Bezerra *et al.* 2021; D'Avila *et al.* 2021; Dorigan *et al*. 2019; Tenni *et al.* 2021). However, studies on isoprothiolane (IPT) resistance are still limited.

Research on the *P. oryzae* resistance to IPT has been monitored since its first release on 1975 since the Kennedy's pathway which isoprothiolane interupts is highly related to the specific response activated by *P. oryzae* in the choline biosynthesis pathway. Reports on the resistance of *P. oryzae* to IPT especially investigating MoIRR and MoVelB gene as resistance elemets are still limited in a few countries. Hu *et al.* (2014) explored the mechanisms of isoprothiolane resistance by identifying *PEAMT, CHO3*, and *OPI3* genes and reported that these genes are not responsible for isoprothiolane resistance. Wang *et al.* (2018) then discovered that *Magnaporthe oryzae*

resistance-related (MoIRR) gene is the core element then followed by attempts to manage the emerging resistant populations and *Magnaporthe oryzae* Velvet Family B (MoVelB) as another gene responsible to unstable IPT resistance (Wang *et al.* 2022; Meng *et al*. 2022). Since isoprothiolane is frequently used by farmers in Indonesia, particularly in blast-endemic areas, and *in vitro* assays have indicated the presence of mutation, the mechanisms of Indonesian field isolate resistance encourage more thorough verification through mutation analysis.

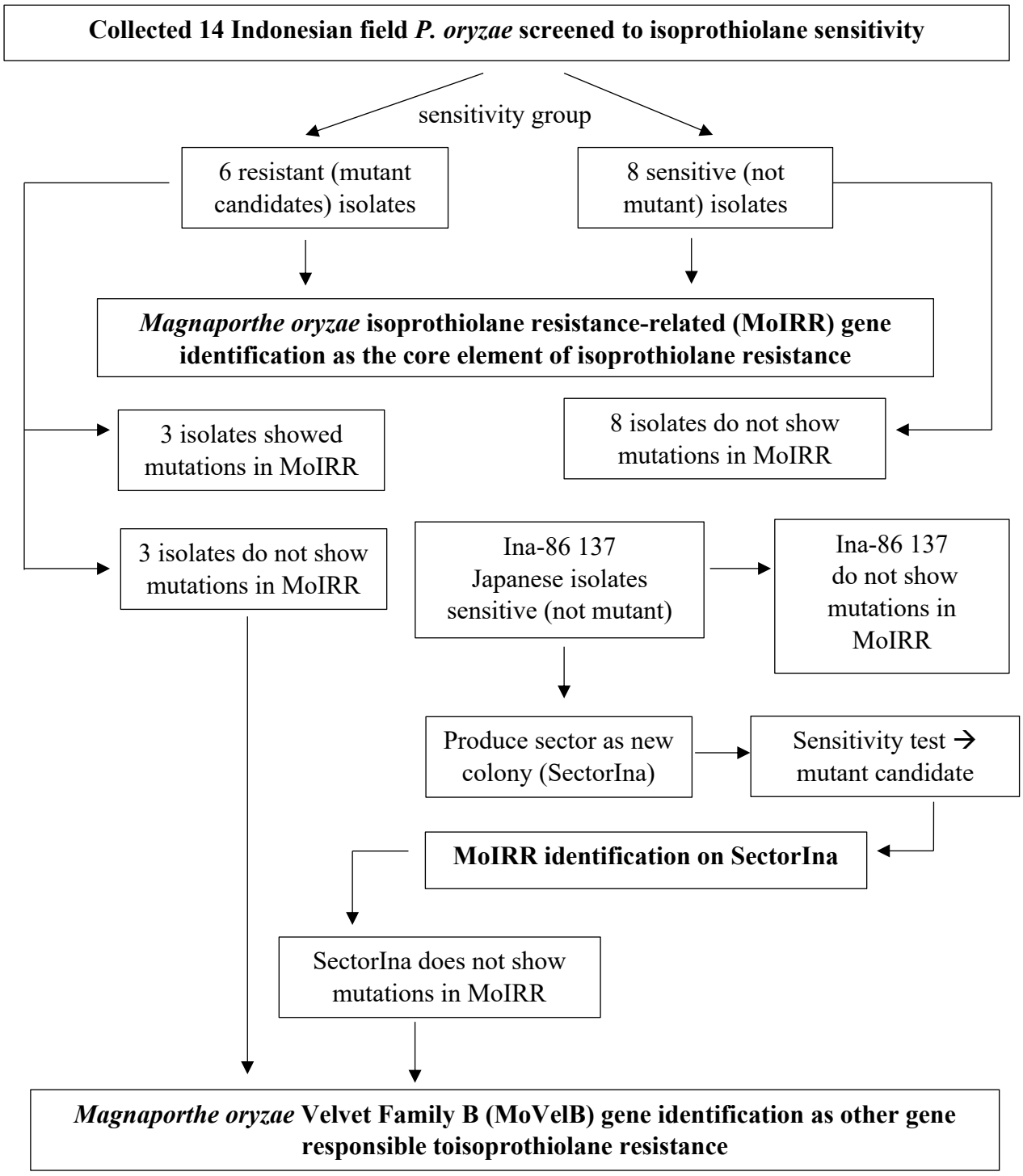

Figure 22 Research flow diagram of mutation analysis of isoprothiolane resistance from Indonesian *Pyricularia oryzae* field isolates

4.2. Methods

4.2.1. Amplification and sequencing of MoIRR gene in 14 Indonesian P. oryzae field isolates

Characterization of the MoIRR gene from these isolates was conducted by amplification using primers (forward 5'-CCTGGAACTAGACAGCTCAGCC-3' and 5'-CCTACG GGCGGGTCCAGTAGTCG-3') yielding amplicon at the size of 3.6 kb. A total volume of 25 µl (Green PCR MasterMix 12.5 µl, 2.5 µl of MoIRR-F 0.5 µM, 2.5 µl of MoIRR-R 0.5 µM, 1 µl of DNA template 50 ng/µl, and 6.5 µl nuclease-free water) is used as the PCR mixture. The PCR program is described in Table 10.

Table 10 PCR program for the analysis of MoIRR region

Stage	Temperature (°C)	Time (min.)
Initial denaturation	94	02:00
35 cycles		
• Denaturation	94	00:30
• Annealing	60	01:30
• Extension	72	03:00
Final extension	72	07:00
Hold	4	∞

PCR products were resolved in 1% agarose gel (1 µl of loading buffer and 5 µl of sample) with 1 kb and 100 bp marker, electrophoresis at 50 V for 30 min then stained by ethidium bromide (EtBr) for 20 min. Purification stages are undertaken using a Macherey-Nagel (FR) NucleoSpin Gel and PCR Clean-Up kit followed by a preparation for the BigDye (JP) direct Sanger sequencing reaction after splicing 6 pairs of primers for each 500 bp in C-Primer software (Table 11). A mixture of 20 µl per sample (2 µl of ready reaction mix, 3 µl of BigDye sequencing buffer, x µl of 20 ng/µl DNA template, and 14-x µl of water) was used as a mixture for sequencing with BigDye methods and perform the program as displayed in Table 12.

Table 11 Spliced primers in addition to MoIRR-F and MoIRR-R used for Sanger sequencing

Regions	Primer code	Sequencing primers
1-500	1F	TGC TAA CTC TCG CCT TTC CC
	6R	ACA AGG CCA GAA ACG TCC TG
501-1000	2F	GGG CAC ATC TTC CGC TTG
	5R	GTC CCA ATC TGC CCC GTC
1001-1500	4F	GAC TGC TCT TAT GTC CGG CC
	3R	AAC CTT GCA AGA GTT CCG ATA GC
1501-2000	4F	GCT ATC GGA ACT CTT GCA AGG TT
	3R	AGG CCG GAC ATA AGA GCA GTC
2001-2500	5F	GAC GGG GCA GAT TGG GAC
	2R	CAA GCG GAA GAT GTG CCC
2501-3000	6F	CAC AAC GGC ACC AAT ACA GC
	1R	CGG GAA AGG CGA GAG TTA GC

Table 12 Cycling conditions for sequencing

Stages	Temperature (ºC)	t (min)
Hold	96	01:00
25 cycles	96	00:10
	50	00:05
	60	04:00
Hold	4	∞

Following the thermal cycling, purification was undertaken using protocols by Macherey-Nagel (FR) NucleSpin Gel and PCR Clean-Up to remove the BigDye, primers, buffer, nucleotide, dNTPs, salts, and other low molecular weight materials for sequencing. Genome assembly was conducted using ATSQ software and then aligned to the Benchling website. MGG_04843 is used as the negative control for MoIRR sequence analysis.

4.2.2. Performance evaluation and MoIRR identification of sector emerged from Ina-86 137

A sector emerged in *in vitro* sensitivity assays that began at 0.6 mg/l IPT-amended PDA from wild-type (WT) Ina-86 137. This sector is subsequently mentioned as SectorIna/Mut. SectorIna indicated the fungus' effort to avoid toxicants in the media. Monoconidial purification on water agar (WA) was performed to isolate Mut from WT. The growth performance of Mut was evaluated on PDA as a favorable medium and the fungus culture was then incubated at 27 ^{o}C and colony diameter measurement and appearance were observed at 5, 8, and 11 days post-inoculation (dpi). To validate the performance of Mut under unfavorable conditions, a similar test was undertaken using WA represents a medium with poor growing resources.

We investigate the conidiation ability of Ina-86 137 (WT) and Mut under fungicide stressors by directly contacting the 10 µl conidial suspension (10^4 conidia/ml) with 10 µl of 1 mg/l IPT incubated at 27 ^{o}C for 24 hr to see the immediate effect of conidial formation inhibition and the effect represented by the conidia, which eliminated the avoidance mechanisms observed in the IPT-amended PDA. A viability test was carried out to determine whether IPT-exposed conidia were still viable. 1 ml of conidial suspension (concentration 10^3 ml^{-1}) was placed into 9 ml of potato dextrose broth (PDB) containing 1 mg L^{-1} IPT and incubated on an orbital shaker (125 rpm) at 27 ^{o}C for 24 hours. Subsequently, 100 µl of the exposed conidia were then transferred to a fresh PDA medium and cultured at 27 ^{o}C for 3 days.

Further sensitivity assays in sectors that emerged are used to forecast the dynamics of possible resistance that may arise in *P. oryzae* isolates. Following nine days of incubation, Mut were exposed to sensitivity tests at concentrations of 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg L^{-1} with three replications. Following the calculation of the IR values for the new sectors, the

values of the existing colony's EC_{50} and EC_{90} were compared and the MoIRR gene locus in Mut was identified with methods similar to 4.2.1. This finding is used to forecast the emergence of resistance isolates with continuous application of the isoprothiolane.

4.2.3. Investigation of the MoVelB gene for mutants without mutations in the MoIRR gene

Three Indonesian *P. oryzae* isolates 2, 3, and 5 are considered mutants as they could grow in high IPT-amended PDA concentrations. However, no mutations in the MoIRR gene were identified in these 3 isolates, as well as SectorIna which emerged from IPT-exposed Ina-86 137, indicating a mutation occurred in another responsible gene, i.e. MoVelB (Meng *et al.* 2023). Characterization of the MoVelB gene was performed using primers MoVelB-F (5'-GTCGTCGCCACTTTTTTCACC-3') and MoVelB-R (5'- CATGATTTAATGC GATATGCAATCAA-3') yielding amplicon at the size of 1.7 kb. A total volume of 25 µl (Green PCR MasterMix 12.5 µl, 2.5 µl of MoVelB-F 0.5 µM, 2.5 µl of MoVelB-R 0.5 µM, 1 µl of DNA template 50 ng/µl, and 6.5 µl nuclease-free water) is used as the PCR mixture. The PCR program is described in Table 13 and followed by sequencing. Genome assembly used MGG_01620 gene as negative control in Benchling.

Table 13 PCR program for the analysis of MoIRR region

Stage	Temperature (°C)	Time (min.)
Initial denaturation	94	02:00
35 cycles		
• Denaturation	94	00:30
• Annealing	56	01:30
• Extension	68	01:00
Final extension	68	07:00
Hold	4	∞

4.3. Results

4.3.1. Amplification and sequencing of MoIRR gene in 14 Indonesian P. oryzae field isolates

The MoIRR gene in 14 Indonesian *P. oryzae* and Ina-86 137 (CK) isolates were successfully amplified at ±3,600 bp (Fig. 22). Following sequencing and genome assembly, multiple mechanisms are demonstrated among the 6 resistant isolates. Resistant isolates 1 and 6 showed a shift (G→A changed Alanine to Threonine at codon 396 and T→A changed Asparagine to Lysine at codon 555, respectively), while isolate 4 indicated an insertion of G base at codon 288 changed the subsequent Isoleucine to Asparagine followed by stop codon) and truncated the protein (Fig. 23).

Figure 23 MoIRR gene amplification of 14 Indonesian *Pyricularia oryzae* isolates compared to that of Ina-86 137 (CK) showing bands at ±3,600 bp (marker: 1 kbp)

However, resistant isolates 2, 3, and 5 did not have a mutation in the MoIRR gene locus, as evidenced by either no shift detected or a change in a base that did not result in a change in amino acid (Fig. 24) It is highly predicted that resistant isolates 2, 3, and 5 contain mutations in other unknown responsible genes.

Isolates	EC_{50} (mg/L)	68	288	383	396	528	555	
MGG_04843	-	LWQV	ESPGIEP	ESVE	SCKVAYLW	QLSVSG	SRINL	[-ca]
Isolate 1	3.08	LWQV	ESPGIEP	ESVE	SCKV**T**YLW	QLSVSG	SRINL	[-ca]
Isolate 2	3.28	LWQV	ESPGIEP	ESVE	SCKVAYLW	QLSVSG	SRINL	[-ca]
Isolate 3	2.52	LWQV	ESPGIEP	ESVE	SCKVAYLW	QLSVSG	SRINL	[cca]
Isolate 4	2.96	LWQV	ESPG**N**EP	ESVE	SCKVAYLW	QLSVSG	SRINL	[-ca]
Isolate 5	2.04	LWQV	ESPGIEP	ESVE	SCKVAYLW	QL**S**VSG	SRINL	[-ca]
Isolate 6	2.55	LWQV	ESPGIEP	ESVE	SCKVAYLW	QLSVSG	SRI**K**L	[-ct]
Isolate 7	0.17	LWQV	ESPGIEP	ESVE	SCKVAYLW	QLSVSG	SRINL	[-ca]
Isolate 8	0.33	LWQV	ESPGIEP	ESVE	SCKVAYLW	QLSVSG	SRINL	[-ca]
Isolate 9	0.33	LWQV	ESPGIEP	ESVE	SCKVAYLW	QLSVSG	SRINL	[-ca]
Isolate 10	0.44	LWQV	ESPGIEP	ESVE	SCKVAYLW	QLSVSG	SRINL	[-ca]
Isolate 11	0.75	LWQ**V**	ESPGIEP	ESVE	SCKVAYLW	QLSVSG	SRINL	[-ca]
Isolate 12	0.33	LWQV	ESPGIEP	ESV**E**	SCKVAYLW	QLSVSG	SRINL	[-ca]
Isolate 13	0.15	LWQV	ESPGIEP	ESVE	SCKVAYLW	QLSVSG	SRINL	[-ta]
Isolate 14	0.43	LWQV	ESPGIEP	ESVE	SCKVAYLW	QLSVSG	SRINL	[-ta]
Ina-86 137	0.51	LWQV	ESPGIEP	ESVE	SCKVAYLW	QLSVSG	SRINL	[-ca]

Figure 24 MoIRR gene locus displays isolates 1-6 (resistant) showed multiple mechanisms of mutation (red amino acid code) or mutation undetected (red isolate number). Isolates 7-14 (sensitive) did not show mutation or change in a base without change in amino acid translated (green amino acid codes) Ina-86 137 is used as a wild-type.

4.3.2. Performance evaluation and MoIRR identification of sector emerged from Ina-86 137

Sector (SectorIna) from Ina-86 137 (WT) emerged with a group of colonies that appeared either as a new colony or a whitish colony on the top of WT (Fig 25). When compared to Ina-86 137, this SectorIna grows more rapidly on the same IPT-amended medium showing an ability to adapt to challenged environmental circumstances

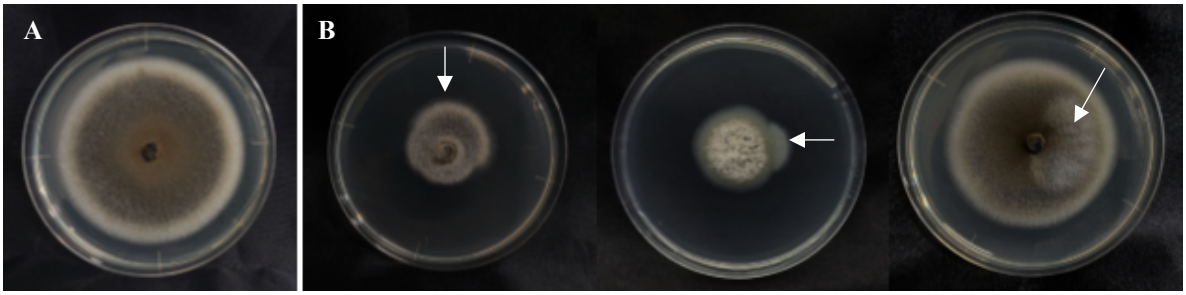

Figure 25 The original colony of Ina-86 137 (WT) (A) and sector emerged (SectorIna/Mut) on IPT-amended PDA medium (B)

Purified Mut exhibits lower radial growth compared to WT at 5, 8, and 11 dpi (Fig. 26A-B_{1-3} and Fig. 27A). Not only the less-performed mycelial development, but Mut also demonstrated lower pigmentation compared to that of WT (Fig. 26A-B_4). This indicates that the ability of Mut to perform optimal growth in favorable conditions is lesser than WT. Challenges of poor nutrition medium in WA showed that Mut overlapped the growth of WT at 8 dpi (Fig. 27B), indicating a risk of fitness cost. We hypothesized that distinct physiological features explain the abovementioned Mut performance. Furthermore, a test was made to determine the optimal observation time for WT and Mut in terms of conidial production. It was found that 18 hpi was the best observation time to compare the radial growth of WT and Mut since at 24 hpi on water agar, most of the conidia had been released from the conidiophore and were being flattened (Fig. 28).

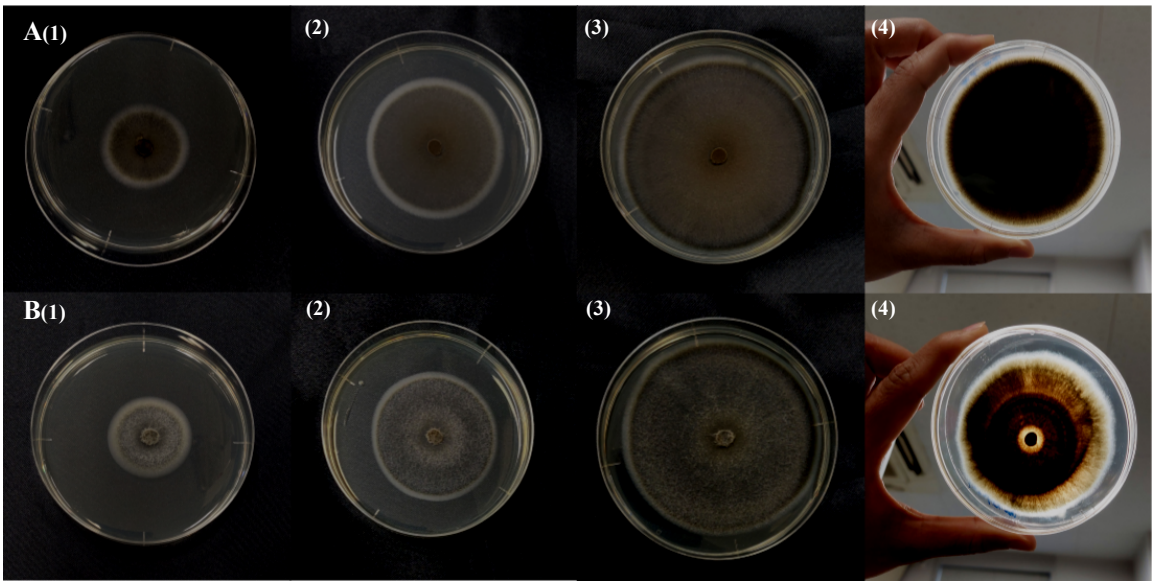

Figure 26 Visualization of Ina-86 137 and Sector Ina on PDA 5, 8, 11 dpi (obverse) (A-B 1-3) and 11 dpi reverse (A-B 4)

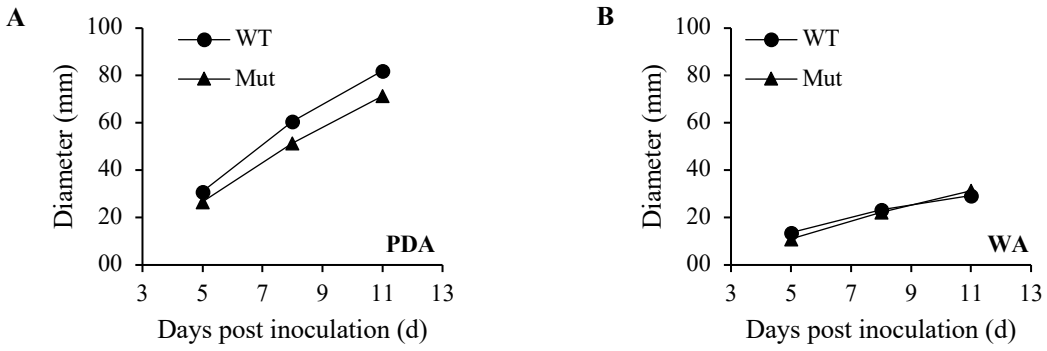

Figure 27 Growth of Ina-86 137 (WT) and SectorIna (Mut) on PDA (A) and WA (B) medium 5, 8, and 11 days post-inoculation

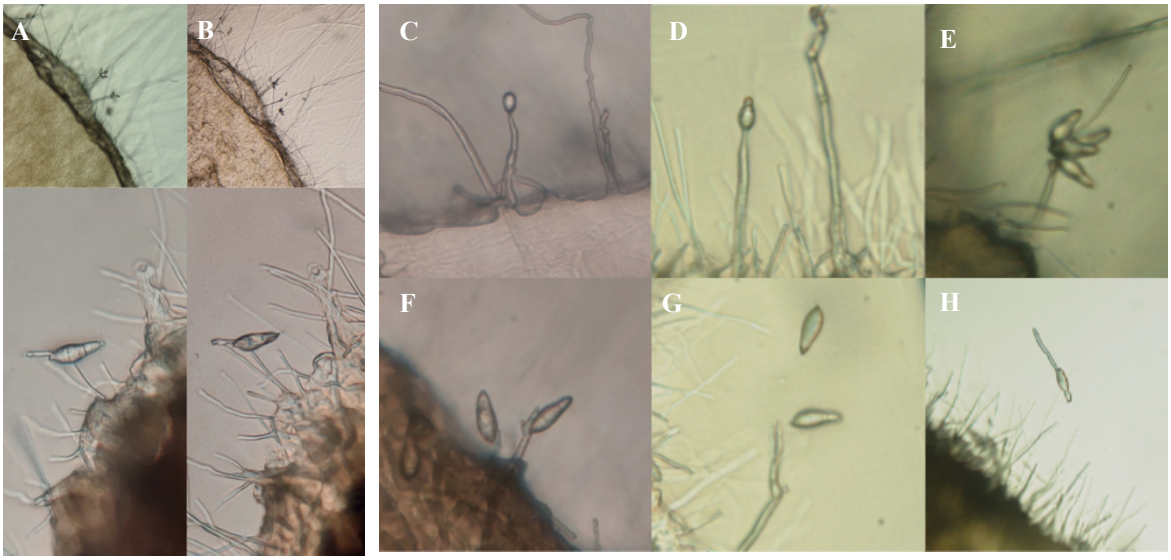

Figure 28 Determination of optimal time for conidial observation on water agar with no fungicide exposure, conidiophore and conidia at 18 hours post inoculation (hpi) (A), 24 hpi (B), and optimal growth of conidiophore at 16-18 hpi showing conidiophore with 1 cells conidia (C) 2-cell conidia attached on conidiophore (D), 3-cell conidia (E) 3-cell conidia is ready to release from conidiophore (F), released conidia (G), and germinated conidia (H)

At 18 hpi on WA, Mut produces abundant of conidia while WT produces less. In contrast, Mut has only a few mycelial masses compared to WT (Fig. 29). This series of

phenomena is thought that Mut expresses a survival behavior as a result of previous memory of toxicant presence in the growing media, therefore mycelial production falls lower than conidial production. The treatment of IPT exposure directly to the conidial suspension on a slide revealed that in untreated conditions, the conidial formation of WT is higher than Mut, but after exposure, the conidial formation of WT is significantly inhibited compared to Mut inhibition (75% and 35%, respectively) (Fig. 29C).

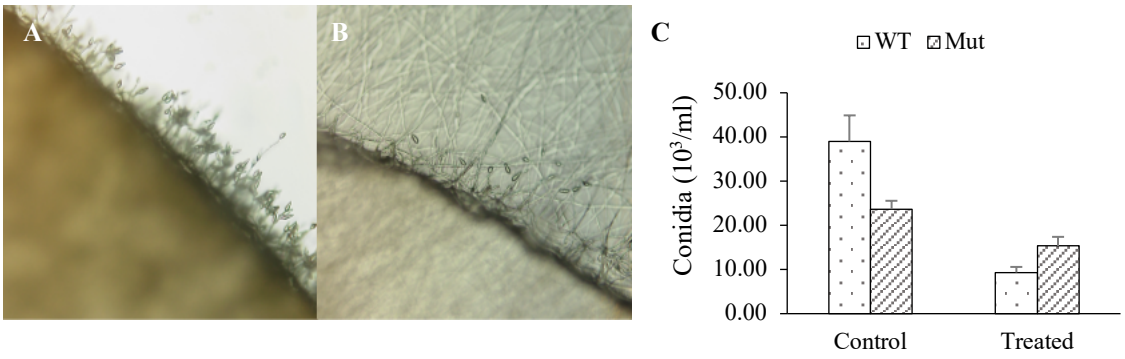

Figure 29 Comparison of mycelial and conidial production on SectorIna (Mut) showing abundant conidia and a few mycelia (A) compared to a few conidia and abundant mycelia on Ina-86 137 (WT) (B) at 18 hpi on WA medium. Under no IPT exposure, conidial production of Mut is lower than WT while under IPT exposure, conidial production of Mut is significantly higher than WT (C)

Aside from the number of affected conidia, Mut had more conidial defects than WT, including flattened, wrinkled, greasy, cracked, deflated, swelled, and abnormal germ tube formation, demonstrating Mut's encounter to reproduce asexually despite poor conidial appearance (Fig. 30). Furthermore, Mut has twice as many viable conidia as WT (3.2×10^4 and 1.6×10^4 conidia/ml, respectively) resulting in 29% of viable conidia of Mut which is considerably greater than the WT's 9% (Fig. 31). The growth performance confirmed that the population of Mut is highly affected by the presence of external stressor stimuli while the population will be controlled in the absence of fungicide. This could be a basis for decision-making of isoprothiolane application in the future to anticipate the mutant development.

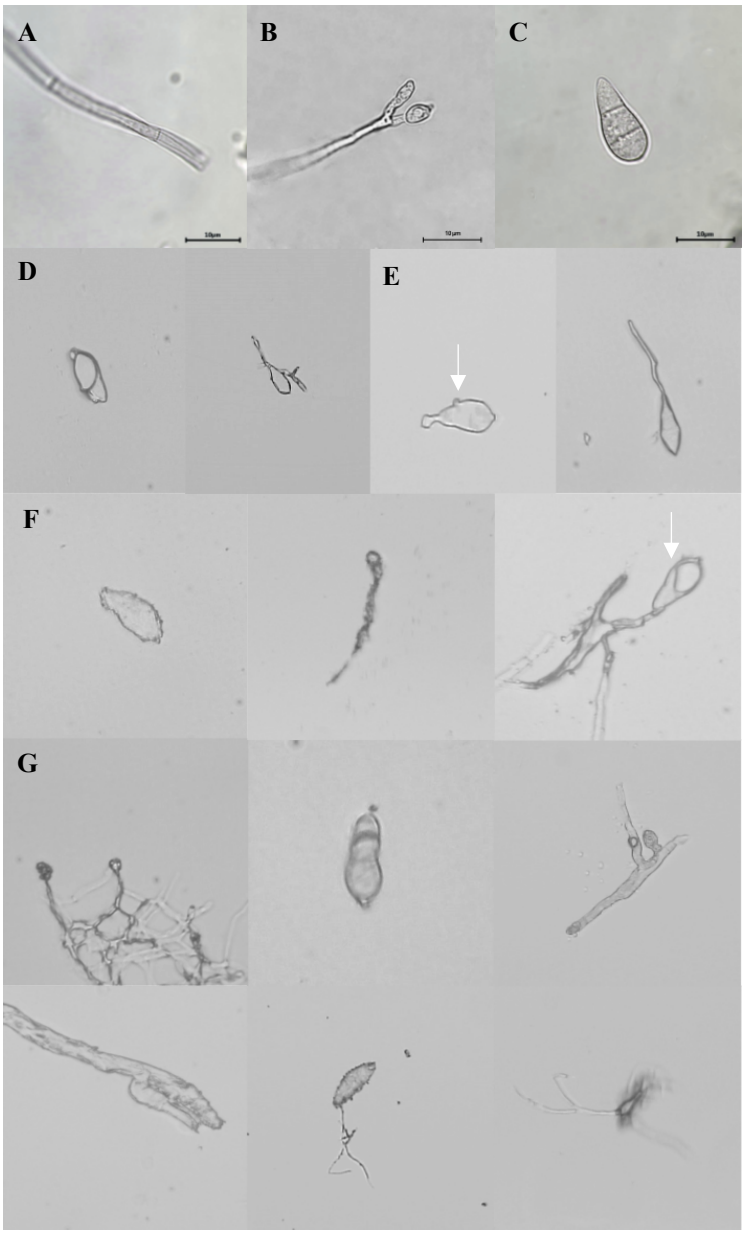

Figure 30 Abnormalities of conidia exposed to IPT compared to normal hyphae (A), conidiophore (B), and conidia (C). Common abnormalities include bulged/knotted and flattened conidia on Ina-86 137 (WT) (D) and SectorIna (Mut) (E). Other abnormalities were expressed on WT i.e. serrated, greasy, and swollen conidia on WT (F) while more varied abnormalities on Mut i.e. curved conidiophore, crooked conidia, enlarged, leaked, lytic hyphae, and swelled conidia (G).

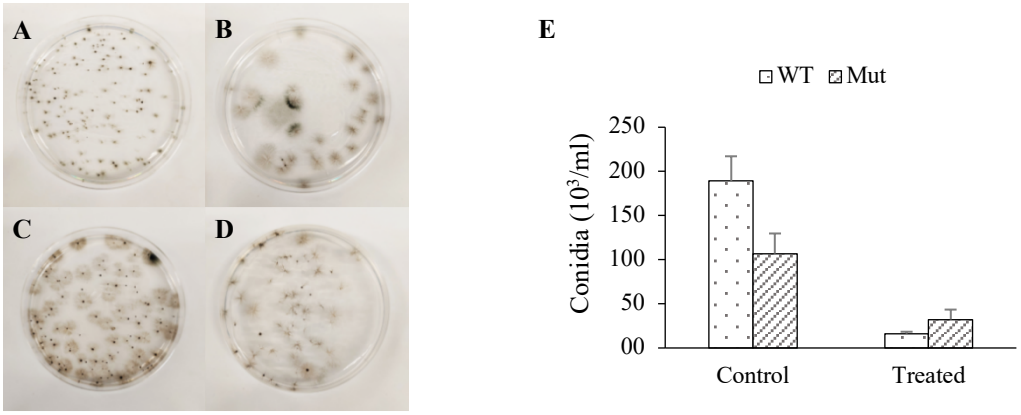

Figure 31 Viability test of IPT-exposed conidia on PDA medium on Ina-86 147 (A→B), SectorIna (Mut) (C→D) confirmed by measurement of conidial number (F) revealed that after exposure, more Mut conidia are still viable demonstrating Mut resilience during a challenged environment

The sensitivity test showed that SectorIna has a lesser sensitivity, as seen by a lower inhibition rate (Fig. 32A-B). At 1 mg/l, IPT inhibits 54% of SectorIna while inhibiting 86% of WT growth, resulting in increased EC_{50} and EC_{90} for SectorIna, which are 2-3 times higher than WT (Table 15). This demonstrates that if the same fungicide is used repeatedly, sensitive isolates may eventually become less sensitive. Although Mut exhibited resilience in response to IPT *in vitro*, detection and identification of Mut showed no mutations were found in MoIRR sequence (Fig. 33-34).

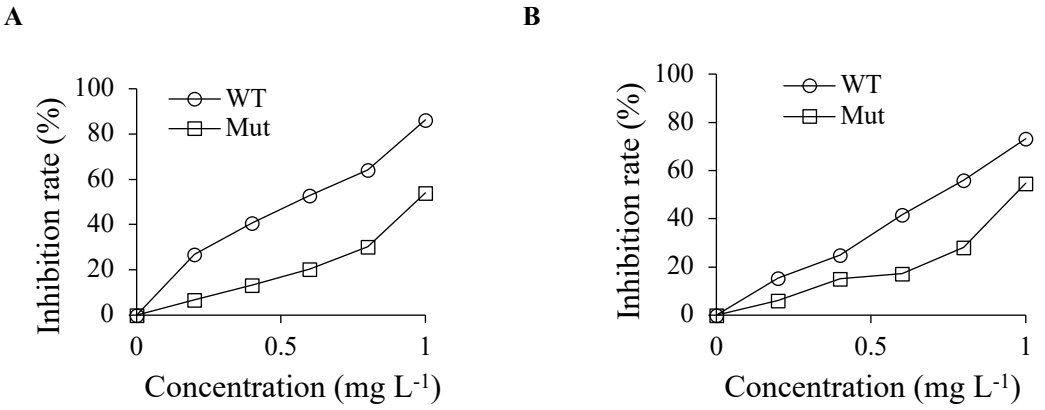

Figure 32 Decrease of the inhibition rate of *Pyricularia oryzae* Ina-86 137 (WT) and SectorIna (Mut) against market-grade (A) and reagent-grade isoprothiolane (B)

Table 15 Change in response of SectorIna (Mut) on IPT-amended PDA colony of *Pyricularia oryzae* compared to that of Ina-86 137 (WT)

Isolates	Commercial IPT (Fujiwan 400 EC)		Reagent-grade IPT	
	WT	Mut	WT	Mut
Inhibition rate at 1 mg L^{-1} (%)	86	54	73	55
EC_{50} (mg L^{-1})	0.51	1.09	0.74	1.11
EC_{90} (mg L^{-1})	3.29	10.83	3.20	14.90

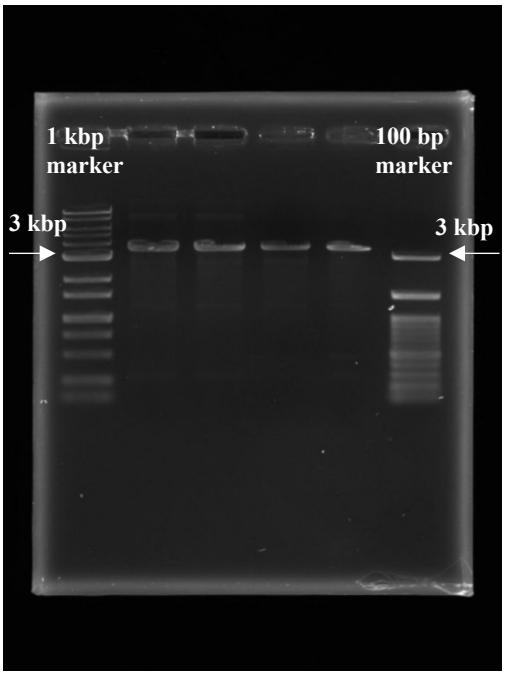

Figure 33 MoIRR amplification of SectorIna (Mut) showing bands at ±3,600 bp (marker: 1 kbp and 100 bp)

```
Isolates  EC50(mg/L)  68        288       383     396       528       555
MGG_04843    -     LWQV...ESPGIEP..ESVE.SCKVAYLW...QLSVSG.SRINL...[-ca]
Ina-86 137 0.51    LWQV...ESPGIEP..ESVE.SCKVAYLW...QLSVSG.SRINL...[-ca]
SectorIna  1.09    LWQV...ESPGIEP..ESVE.SCKVAYLW...QLSVSG.SRINL...[-ca]
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Figure 34 MoIRR identification showed no mutation in SectorIna (Mut) compared to that of Ina-86 137 (WT)

4.3.3. MoVelB gene locus identification of 3 Indonesian mutant isolates and SectorIna

The MoVelB gene of mutant isolates 2, 3, 5, and SectorIna (Mut) was amplified at ±1,700 bp (Fig. 35). Sequencing analysis of the MoVelB gene in these isolates revealed that in isolate 2, an A insertion at codon 437 lead to an amino acid change of Aspartic Acid (D) to Arginine (R) repeated two times then followed by a stop codon (UGA). Meanwhile, no mutation in MoVelB gene was identified in isolates 3, 5, and SectorIna (Fig. 36)

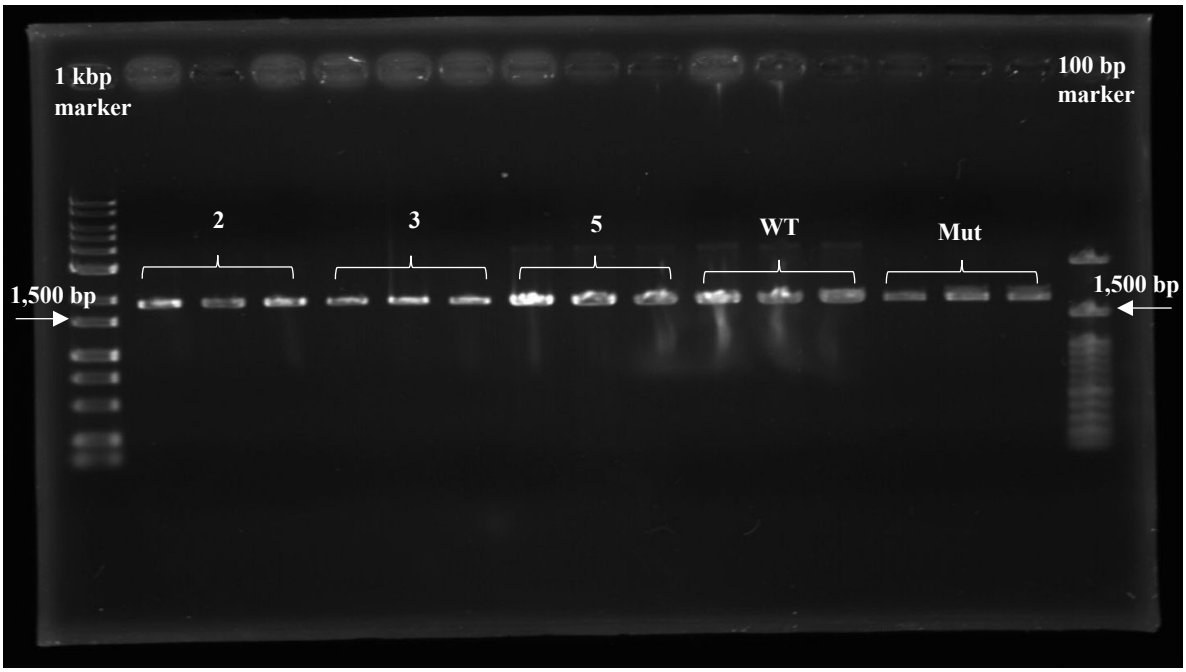

Figure 35 MoVelB gene amplification of mutant strains 2, 3, 5, and Mut *Pyricularia oryzae* isolates compared to that of Ina-86 137 (Mut) showing bands at ±1,600 bp (marker: 1 kbp and 100 bp)

Strains	EC_{50} (mg/L)	244	354	437
MGG_01620	-	VGPAYGMVATYGGQG...	LRFSYNVGAPTR...	IRKAEGGGKNNDDDDDY
Isolate 2	3.28	VGPAYGMVATYGGQG...	LRFSYNVGAPTR...	IRKAEGGGKNNDRRstop
Isolate 3	2.52	VGPAYGMVATYGGQG...	LRFSYNVGAPTR...	IRKAEGGGKNNDDDDDY
Isolate 5	2.04	VGPAYGMVATYGGQG...	LRFSYNVGAPTR...	IRKAEGGGKNNDDDDDY
Ina86-137	0.51	VGPAYGMVATYGGQG...	LRFSYNVGAPTR...	IRKAEGGGKNNDDDDDY
SectorIna	1.09	VGPAYGMVATYGGQG...	LRFSYNVGAPTR...	IRKAEGGGKNNDDDDDY

Figure 36 MoVelB gene identification showed a change in the amino acid (Aspartic Acid to Arginine) as a result of A insertion at codon 437 in Indonesian isolate 2

4.4. Discussion

The MoIRR and MoVelB genes are useful in rapidly detecting mutations in rice blast fungus to isoprothiolane from the field. Frameshifts appear as a common resistance mechanism of *Pyricularia oryzae* to isoprothiolane, followed by insertions. Based on the MoIRR and MoVelB identification, spontaneous mutation to isoprothiolane has occurred in rice fields in West Java Province, Indonesia. However, not all resistance strains show a mutation in both MoIRR and MoVelB, as well as not all sensitive strains containing MoIRR and MoVelB, indicating that other transcription factors might be responsible for *P. oryzae* resistance to isoprothiolane and require gene-expression levels experiment. Mutation could occur in other genes, such as MoICl1 as recently reported in the other role of this secondary metabolites-producing gene to reduce the toxicity effect of IPT (Bi *et al.* 2023).

Various conidial abnormalities exhibited by SectorIna (Mut) could represent the fungus adaptation to ensure the life cycle inheritance despite the abnormal phenotypic appearance of the parental conidia. According to Habig (2021), when the micro and macro environment changes dramatically, the fungus may adapt better by increasing the mutation rate, which is governed by histone marks. Sequences of findings of SectorIna (Mut) show the presence of fitness penalty since Mut grows slower than WT in favorable conditions on Mut but with higher resilience in response to external stressor stimuli.

No mutation in either MoIRR orMoVelB gene in Mut after being exposed once to IPT demonstrated two possibilities as well as explaining phenomena in isolates 3 and 5: 1) mutation occurred in a gene other than MoVelB and MoIRR or 2) the mutation on the MoIRR and MoVelB genes is the result of multiple and continuous exposure in the fields. Disease control will become more challenging when new isolates with novel traits emerge and develop in the field. Therefore, it is recommended to temporarily stop or substitute isoprothiolane to delay the rapid development of Mut and similar mechanisms to Mut.

5 GENERAL DISCUSSION

Blast disease has been a serious disease in many rice production centers in Indonesia since its first appearance in upland rice in 1985 (Sudir 2014). Disease dynamics as presented in Chapter 2 showed the direct impact of global climate changes on both plant health and the activity of pathogenic fungi performing a novel state of disease triangle including the rice–*P. oryzae* pathosystem. Not only affect fungal development, but drought stress also causes less sensitivity to phytohormone activities and several downregulations of defense-related transcripts making the plants more susceptible to new infection. According to Roussin-Léveillée *et al.* 2024, elevated temperatures decrease salicylic acid (SA) production while increasing jasmonic acid (JA) resulting in an SA/JA antagonism and imbalance of defense-related phytohormone. High relative humidity decreases the ACC deaminase enzyme which acts as an ethylene suppressor and alters the responsiveness of stomatal guard cells leading to a decrease in the response for PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). In other words, climate change impacts both plants and pathogens, but this fungus can immediately adapt to become more resilient and virulent. Research in Chapter 2 concludes that the use of isoprothiolane is still considerable, with the precautions taken against fungicide resistance.

A mutation stimulated by continuous isoprothiolane application is highly hypothesized been occur in Cikembar since this endemic area exhibits high disease intensity on both favorable and unfavorable conditions for disease development. Mutation from fungicide resistance can result from alterations to genes that encode fungicide targets or from a wide range of mechanisms triggered by sub-lethal fungicide exposures (Deising *et al.* 2008).The series of laboratory works in Chapter 3 and 4 revealed that a mutation triggered by isoprothiolane fungicide has emerged in *P. oryzae* isolates from Cikembar, the rice-blast endemic area in West Java. The response of *P. oryzae* to isoprothiolane *in vitro* has reduced as the EC_{50} of mutant isolates is above 4 mg L^{-1}, 10-fold higher than the recommended concentrations (1 ml L^{-1} or 0.4 mg L^{-1}). Three isolates of Indonesian field *P. oryzae* have mutations in the MoIRR gene including shift and insertion leading to stop codon. Wang *et al.* (2018) generated lab mutants that have EC_{50} up to 20 mg L^{-1} and the knockout

transformants have recovered sensitivity as the parental isolates. MoIRR gene has been considered as the core element of isoprothiolane resistance. However, not all six Indonesian *P. oryzae* mutants have mutations in the MoIRR gene.

Meng *et al.* (2022) reported that besides the MoIRR gene, the MoVelB gene is also responsible for isoprothiolane resistance, especially the lower resistance and unstable responsiveness from field isolates. Our findings conclude MoVelb gene has shown mutations in isolate 2, which does not possess mutations in the MoIRR gene. One-time exposure of Ina-86 137 generating SectorIna with lesser sensitivity, varied conidial abnormalities, and high resilience as a survival strategy on IPT-exposed media did not successfully identify mutation in the MoIRR and MoVelB gene. Neither identification in the MoIRR and MoVelB genes identified in isolates 3, 5, and sector emerged from Ina-86 137 reflects that spontaneous mutation in the Indonesian field *P. oryzae* mutants is the result of mutation in other genes or the effect of simultaneous exposure to isoprothiolane. Therefore, isoprothiolane use in Indonesia has to be managed more wisely.

Based on the findings in this research, isoprothiolane are still essential for practical application, but efforts have to be implemented to prevent the rapid development and domination of mutants in the fields. To continue reducing the severity of the disease in the field and slowing the rate at which other strains of the disease form mutations, the use of isoprothiolane must be combined with other management approaches. One promising strategy found from this research is to combine isoprothiolane with IR64 or mixed varieties to perform the highest efficacy completed with varying degrees of resistance from the host Possible attempts to integrate rice blast disease management include the combination of cultivation of blast-resistant rice, fludioxonil, melatonin, *Bacillus*, iron, and balanced micronutrients (Bi *et al.* 2023; Li *et al.* 2022; Sanchez-Sanuy *et al.* 2022 Wang *et al.* 2022). The other attempt is to replace isoprothiolane with fungicides with different active ingredients for a temporary growing season to eliminate the mutants from the fields. In the future, an *ad planta* inoculation test might be performed to evaluate the fitness penalty among the mutant isolates and enhance the basis for *P. oryzae* investigations for the updated dominant races of Indonesian isolates.

In conclusion, mutations of *Pyricularia oryzae* has been occurred in Indonesia resulting in the resistance of field isolates to isoprothiolane. Indonesian field *P. oryzae* mutant isolates exhibit multiple mutation mechanisms, including the insertion and shift in the MoIRR or MoVelB gene locus. Mutant candidates that have no evidence of a mutation in the MoIRR or MoVelB genes are strongly predicted to have other pathways with the responsible gene unknown but easily mutate and gain lower responsiveness to IPT-exposed media, including SectorIna. SectorIna is known to have higher resilience in IPT-exposed environment but has low growth performance in the absence of IPT. Based on this, it is important to temporarily stop the use of isoprothiolane, or substitute with other active ingredients, to inhibit the development of resilient mutant candidates. It is of interest to translate this recommendation by development delay of emerging IPT-resistant *P. oryzae* populations. To the best of our knowledge, this is the first report of a spontaneous mutation of *P. oryzae* to isoprothiolane from Indonesia.

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