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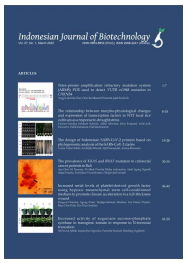

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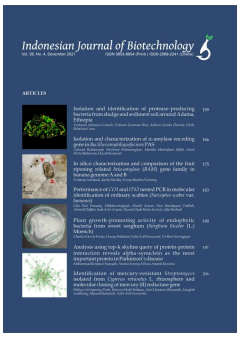

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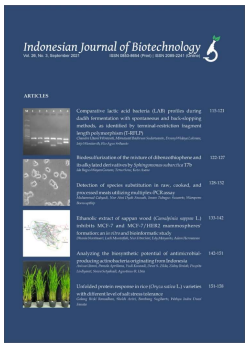

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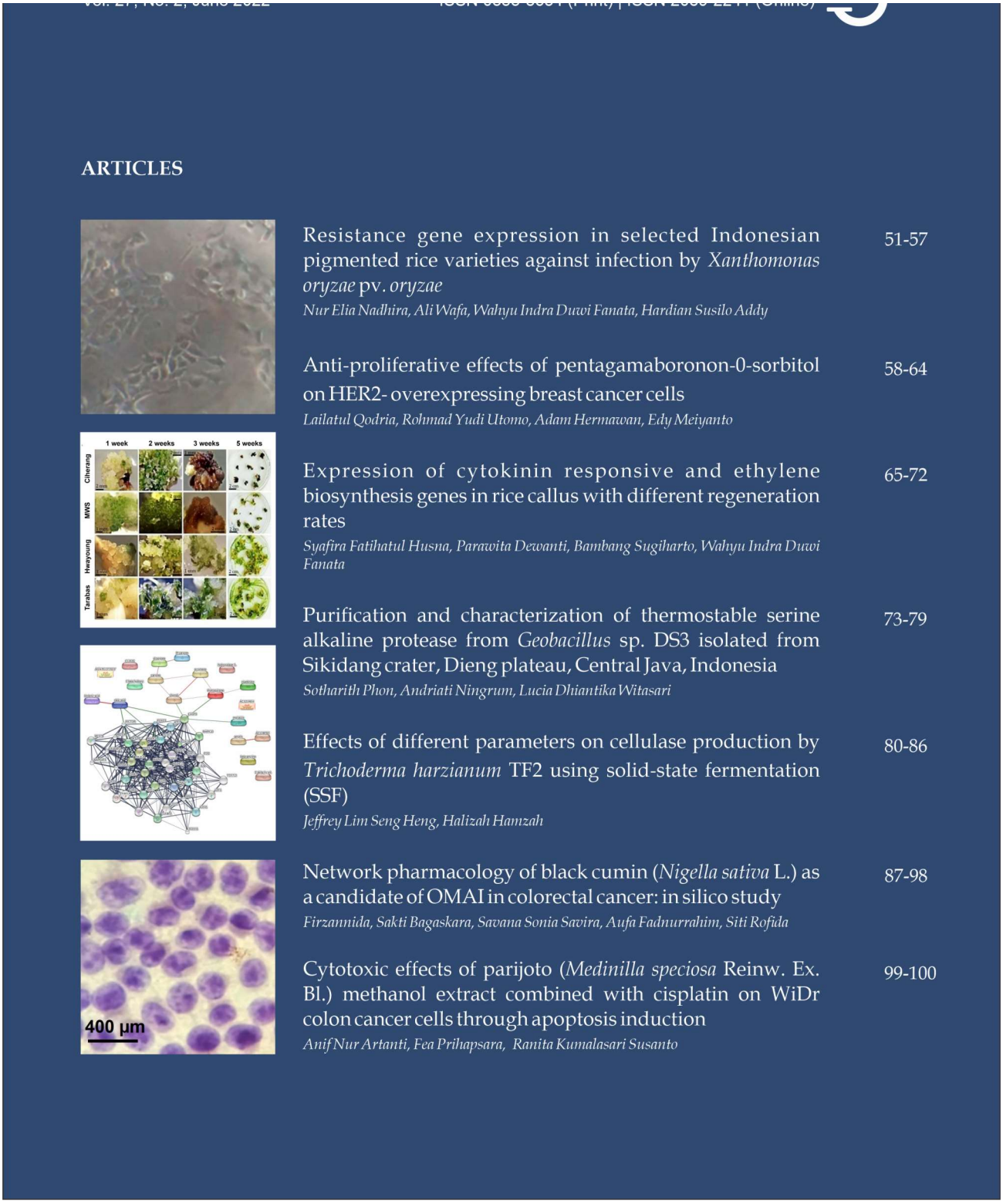

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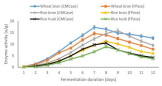

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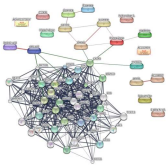

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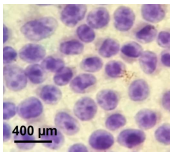

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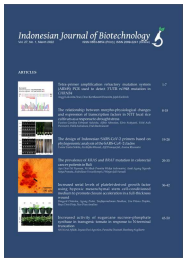

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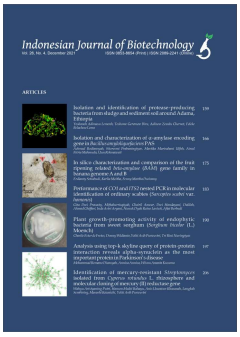

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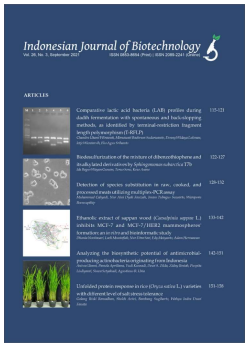

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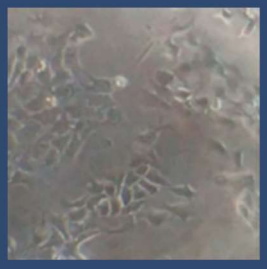

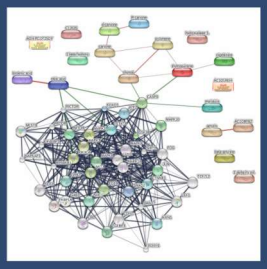

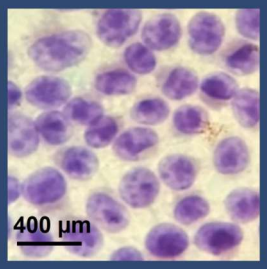

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VOLUME 27(2), 2022, 51-57 | RESEARCH ARTICLE

Resistance gene expression in selected Indonesian pigmented rice varieties against infection by *Xanthomonas oryzae* pv. *oryzae*

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ABSTRACT Rice (*Oryza sativa* L.) production is limited by bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). For decades, researchers have attempted to control this disease by growing plants with blight-resistant *Xa* genes. Genetic resources often vary between rice varieties, and there is little information about the genetic resources of the pigmented rice varieties widely grown in Indonesia and their resistance genes against *Xoo*. The purpose of this study was to determine the expression of *Xa* genes in pigmented rice such as Inpari 24 and Cempo Merah (red-pigmented) along with Hitam Bantul (black-pigmented) and white rice varieties IR64 and Ciherang, and to evaluate their resistance to BLB. All varieties carried the *Xa4*, *Xa10* and *xa13* genes but varied in the *Xa1*, *Xa7* and *Xa21* genes. The rice varieties expressed some of these genes only after inoculation with *Xoo*. Disease assessment categorised the three different pigmented rice varieties as resistant (Ciherang, Cempo Merah and Hitam Bantul), while IR64 (white) and Inpari 24 (red) were moderately resistant. There was no specific pattern of *Xa* genes possession, quality of expression or resistance level to *X. oryzae* pv. *oryzae*. Therefore, when breeding plants, the selection of parental variety must be considered in terms of the possession and expression of *Xa* genes such as *Xa10* as a molecular marker for resistance.

KEYWORDS *Xanthomonas oryzae*; *Xa* genes expression; bacterial leaf blight; pigmented rice

1. Introduction

Rice (*Oryza sativa* L.) is a vital crop consumed worldwide, particularly in Asia. Several biotic factors, such as pathogens, reduce the crop production by decreasing the yield (Arshad et al. 2013). Rice farmers consider bacterial leaf blight (BLB) caused by the bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Korinsak et al. 2021), the most destructive disease in rice. The bacterium can cause losses of up to 50% depending on multiple factors such as environmental conditions, growth stages, and rice varieties (Rasmiyana et al. 2019). The pathogen systemically spreads through the xylem tissue, causing wilting of the seedlings, yellowing, and leaf drying. These characteristics make this disease especially difficult to control conventionally.

One of the strategies to control the disease is to use the resistant plant varieties produced by breeding two or more different germplasm lines (Ke et al. 2017; Tekete et al. 2020). This technique is widely used to promote the sharing of beneficial genetic resources between germplasms, especially those conferring resistance against BLB disease resistance. This beneficial genetic resource sharing is accomplished through molecular breeding, such as molecular assisted selection and genetic engineering (Chukwu et al. 2019; Mukhtar and Hasnain 2017). Indonesia possesses over 17,000 rice germplasms, including wild and pigmented rice (Maulana et al. 2014; Mau et al. 2017), demonstrating diverse morphology, biochemical properties, and genetic characteristics. These germplasms provide genetic resources for rice breeding to create new varieties with specific characteristics.

Molecular genetic studies have revealed that approximately 44 rice genes confer resistance to various strains of *Xoo* (He et al. 2006; Sombunjitt et al. 2017; Neelam et al. 2020). Gene *Xa1, Xa3, Xa4, Xa5, Xa7, Xa10, xa13* and *Xa21* have been widely investigating and hypothesizing as genes that strongly involved with the resistance of rice

against *Xoo* (Neelam et al. 2020). Most *O. sativa* subsp. *japonica* and *O. sativa* subsp. *indica* rice varieties possess these genes related to resistance, and approximately 14 genes are recessive (Neelam et al. 2020). However, although a variety may possess *Xa* genes, that variety may not express the gene when challenged with *Xoo*. For example, both IRBB1 and IR24 rice varieties carry gene *Xa1*, but that expression was detectable only in IRBB1 at five days post-inoculation with strain T7174, a representative strain of Japanese *Xoo* race 1 (Yoshimura et al. 1998). Furthermore, both IRBB5 and IRBB54 varieties carry *Xa5* and *Xa21* but demonstrate different expression levels (Gao et al. 2018). These results showed that varieties carrying resistance gene sequences present different expression levels that correlate to the resistance level in BLB and disease development.

Studies in Indonesia have reported that pigmented rice (red or black) had better resistance than white rice inoculated with *Xoo*. However, no data have demonstrated the expression of *Xa* genes in pigmented rice. Therefore, the aim of this study was to determine the expression level of *Xa* genes in pigmented rice varieties and evaluate their resistance against BLB.

2. Materials and Methods

2.1. Growing pigmented rice seedlings

The five Indonesian rice varieties used in this study included white rice (IR64 and Ciherang), red-pigmented rice (Cempo Merah and Inpari 24), and black-pigmented rice (Hitam Bantul) (Figure 1). In this study, Hitam Bantul was selected to represent the black-pigmented rice with the same germination and growth rate as well as white and red-pigmented rice compared to another black-pigmented rice (Ketan Hitam). The seeds from the collection of Center of Excellence on Crop Industrial Biotechnology, University of Jember, Indonesia were treated with 10% sodium hypochlorite and rinsed twice with sterile distilled water before imbibition by deeping in sterile distillated water for one week. Seedlings were grown in a container containing rice field soil in the greenhouse for three weeks before genetic analysis and *Xoo* inoculation experiments. Each container was planted with 6 germinated seeds.

2.2. DNA genome isolation

Genomic DNA isolation was performed according to Sambrook and Russell (2001) with some modifications. Briefly, approximately 2 g of 4-week-old rice seeds were ground into a powdered in liquid nitrogen. The sample was transferred and mixed with 800 µL of lysis buffer (1 M of Tris-HCl [pH 8.0], 0.5 M of EDTA, and 10% of SDS) in 1.5 ml centrifuge tube, and the mixture was heated for 10 min at 65 °C. After centrifugation for 10 min at 8,000 × g and 20 °C, the supernatant (700 µL) was transferred into a new 1.5 mL tube, mixed with the equal volume of phenol-chloroform-isoamyl alcohol (PCI), and centrifuged for 10 min at 8,000 × g at 20 °C. The upper layer (approximately 500 µL) was mixed with 0.1 volumes of 3 M of sodium acetate and 2.5 volumes of absolute ethanol. After 1 hour of incubation at -20 °C, the mixture was centrifuged for 10 min at 10,000 × g. The precipitate was washed with 70% ethanol and dissolved in 70 µL of 1×TE buffer (pH 8.0).

2.3. Amplification of Xa genes DNA fragments

Genomic DNA fragments of eight resistance genes were detected by PCR using specific pair primers. PCR amplification was performed using 80 nanograms of DNA sample in a 20-µL total reaction volume, including the MyTaq HS Red Mix (Bioline, Nottingham, UK) mixture. The thirty-five reaction cycles were pre-denaturation at 94 °C for 3 min, denaturation at 94 °C for 1 min, annealing for 1 min at a specified temperature (Table 1), elongation at 72 °C for 1 min, and final elongation at 72 °C for 10 min. The result was analyzed using a 1% agarose gel electrophoresis and was visualized under a UV transilluminator in the gel documentation CCD image system (Major Science, Saratoga, CA, USA).

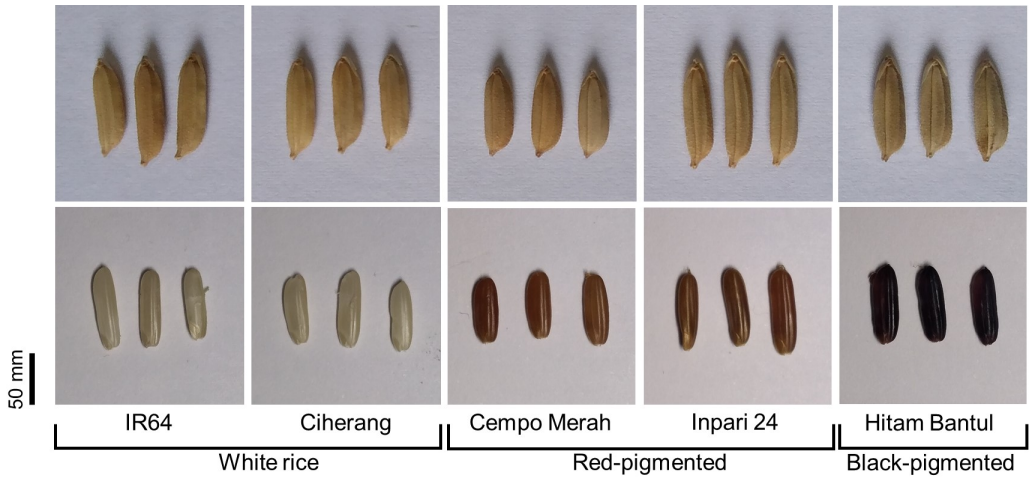

FIGURE 1 Grain morphology of selected Indonesian rice varieties

TABLE 1 PCR variables for amplifying target resistance genes

Target *Xa* genes	Oligonucleotides (5' to 3')	Annealing temperature (°C)	Product size	Refs.
Xa1	F: ACTGCCCTCTTGCACACGCCTTTGG R: CCGGTACATCAGTATTGTCCATCGG	66	447	Yoshimura et al. (1998)
Xa4	F: ATCGATCGATCTTCACGAGG R: TGCTATAAAAGGCATTCGG	53	150	Chen et al. (2000)
Xa7	F: CGATCTTACTGGCTCTGCAACTCTGT R: GCATGTCTGTGTCGATTCGTCCGTACGA	65	294	Porter et al. (2003)
Xa10	F: CAACGCCTATCTTCTGCATTTC R: GTGACCCTAGTTTCTGGTTATG	53	604	Gu et al. (2008)
xa13	F: GGCCATGGCTCAGTGTTTAT R: GAGCTCCAGCTCTCCAAATG	55	400	Hajira et al. (2016)
Xa21	F: GATCGGTATAACAGCAAAAC R: ATAGCAACTGATTGCTTGG	50	1400	Wang et al. (1996)
β-actin	F: TGTATGCCAGTGGTCGTACCA R: CCAGCAAGGTCGAGACGAA	56	121	Chu et al. (2006b)

2.4. Inoculum preparation and inoculation

The BLB pathogen, *Xanthomonas oryzae* pv. *oryzae* strain *XooJ2* was grown at 28 °C in a yeast extract dextrose medium for 24 h (Rejeki et al. 2021). The inoculum was prepared from a 24-hour culture of *X. oryzae* and adjusted to a density of 10^8 CFU per milliliter. Pathogen inoculation was performed by the leaf clipping method, as previously described (Ke et al. 2017). Briefly, sterile scissors were dipped in inoculum suspension and used to cut the leaves of 3-week seedlings 2-3 cm away from the tip. Plants were grown and maintained at 28–32 °C in the greenhouse for symptom observation and disease assessment.

2.5. Total RNA isolation

Leaves were harvested three days after pathogen inoculation and used for RNA isolation. The 65 mg of frozen leaves were ground into a powder in a pre-cooled mortar with liquid nitrogen. Total RNA was extracted and purified using the RNAprep pure Kit (TIANGEN, Beijing, China) according to the manufacturer's directions. The resulting total RNAs were confirmed by 1% agarose gel electrophoresis using 1× tris boric acid EDTA (TBE) buffer (pH 8.0). Total RNA quantification was performed using a NanoVue Plus spectrophotometer (Biochrom, Holliston, MA, USA). Purified total RNA was then stored at -80 °C until further analysis.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Since this study is earlier step on rice parental screening for molecular breeding, then we qualitatively analyzed the *Xa* genes expression through RT-PCR. Briefly, the first-strand complementary DNA (cDNA) was synthesized from 100 ng of total RNA in 50-µL total reaction volume using reverse transcriptase master mix (ReverTra Ace, TOYOBO, Osaka, Japan). The resulting cDNA was used as a template for PCR amplification using a specific targeted-gene primer (Table 1) in the 20-µL volume of MyTaq HS Red Mix (Bioline, Nottingham, UK). Thirty-five cycles for targeted DNA amplification were set for denaturation at 98 °C for 5 min, elongation at 72 °C for 1 min, while the annealing temperature was based on the specific primer used for the target gene (Table 1). PCR products were analyzed on 1% agarose gel with TBE buffer and were stained then visualized using a gel documentation CCD image system.

2.7. Bacterial leaf blight disease assessment

BLB leaf lesions were assessed at 0, 7, and 14 days after inoculation. Disease evaluation was performed using the standard evaluation system (SES) recommended by the International Rice Research Institute (IRRI 2013) by mea-

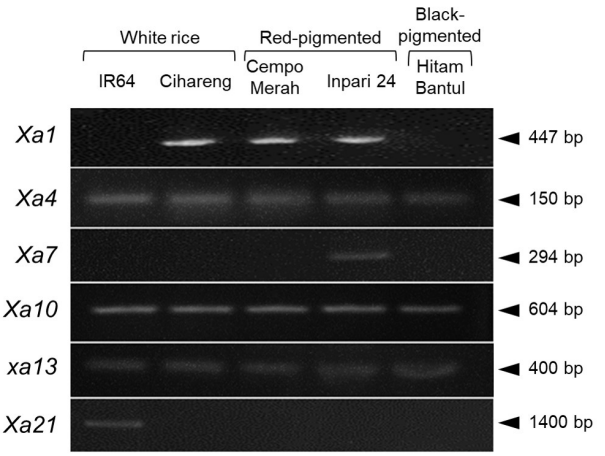

FIGURE 2 Agarose gel electrophoresis of PCR products from the genome of five rice varieties to detect *X. oryzae* pv. *oryzae* resistance-related genes using specific *Xa*-gene primers (listed in Table 1).

suring the length of the lesion. Plants were considered resistant if the average lesion length was ≤3.0 cm, moderately resistant if the average lesion length was 3.0–6.0 cm, moderately susceptible if the average lesion length was 6.0–9.0 cm, and susceptible if the average lesion length was >9.0 cm. The disease incidence was calculated as a percentage of the number of symptomatic plants per total number of observed plants (Rasmiyana et al. 2019).

3. Results and Discussion

3.1. The presence of Xa genes in pigmented rice

Xa-related DNA fragment analysis for two of white rice varieties (IR64 and Ciherang), two of red rice varieties (Cempo Merah and Inpari 24), and one of black rice varieties (Hitam Bantul) showed that only Inpari 24 carried *Xa*-related DNA fragments for *Xa1, Xa4, Xa7, Xa10, and xa13*. All varieties contained specific DNA fragments for *Xa4*, *Xa10*, and *xa13*. However, DNA fragments for *Xa7* and *Xa21* were produced only by Inpari 24 and IR64, respectively (Figure 2).

3.2. The expression of Xa genes

RT-PCR analysis of six *Xa* resistance genes in five rice varieties showed that specific *Xa* genes were detected in *Xoo*-inoculated plants but not in non-*Xoo*-inoculated. Genes detected in rice varieties after inoculation with the pathogen included *Xa7*, *Xa10*, *xa13*, and *Xa21*. Notably, only Inpari 24 exhibited the band representing *Xa1* after inoculation with the pathogen. In addition, *Xa4* expression was lower in inoculated plants and was absent in all Cempo Merah both of inoculated and non-inoculated plants (Figure 3).

3.3. Disease assessment of pigmented rice

Although all varieties showed BLB symptoms, the incidence varied. The highest incidence was 61.4% on IR64 and the lowest in another white rice (Ciherang), 22.7%. Blight incidence on red and black rice was moderate, 42.5–50%. Analysis of disease severity, determined by measuring the lesion length from the leaf tip (Figure 4a), was highest for IR64 and the lowest for Hitam Bantul (Figure 4b). Consistently, the lesion length significantly increased between 7 and 14 days post-inoculation on IR64 by approximately 59%.

3.4. Discussion

Resistance genes (*R* genes) play an important role in inhibiting disease development in rice and resistance to *X. oryzae* pv. *oryzae* is associated with over 44 *Xa* genes (Neelam et al. 2020). The presence of these genes become an important source of genetic information when producing a new variety of rice, either through molecular plant breeding methods including marker-assisted selection or genetic engineering (Das et al. 2017). However, the presence and expression of these resistance genes highly depend on the rice varieties (Fatimah et al. 2014).

Most of the rice varieties (white, pigmented-red, and pigmented-black) possessed multiple genes for resistance to *Xoo* (Figure 2). However, possessing these genes does not confer specific resistance against BLB as well as the data of lesion length and disease incidence (Figure 4). Pathotypes, environmental conditions, and gene expres-

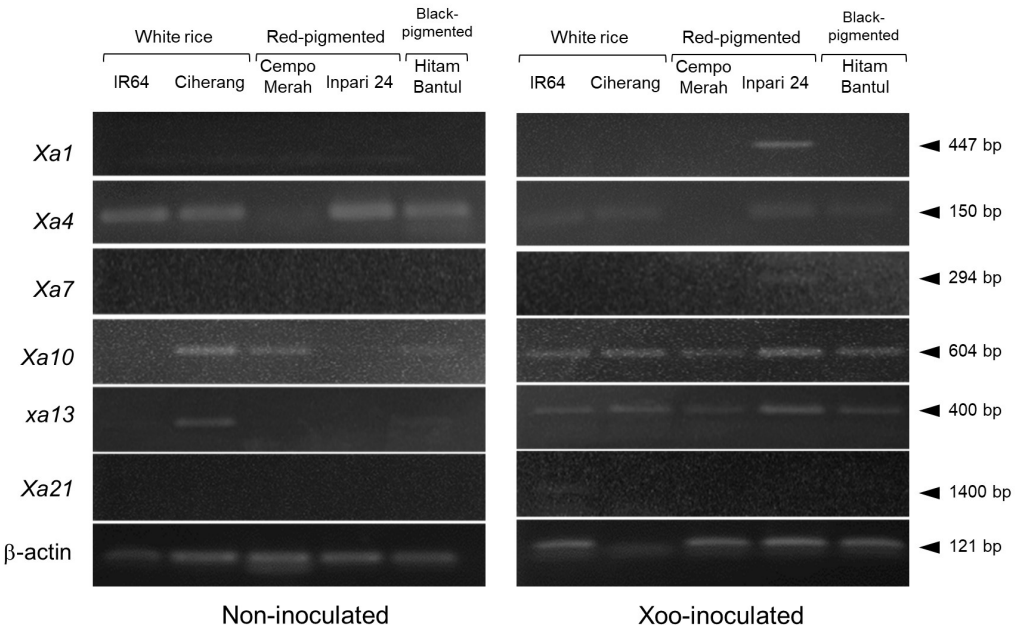

FIGURE 3 Agarose gel electrophoresis of RT-PCR products from the total RNA of five (white, red-pigmented, and black-pigmented) rice varieties to detect the qualitative expression of *X. oryzae* pv. *oryzae* resistance-related genes three days after *Xoo* inoculation using specific *Xa* genes primers (listed in Table 1). White rice varieties used in this study were IR64 (lane 1) and Ciherang (lane 2); red rice, Cempo Merah (lane 3) and Inpari 24 (lane 4); black rice, Hitam Bantul (lane 5). The non-inoculated leaf treatment was sterile water.

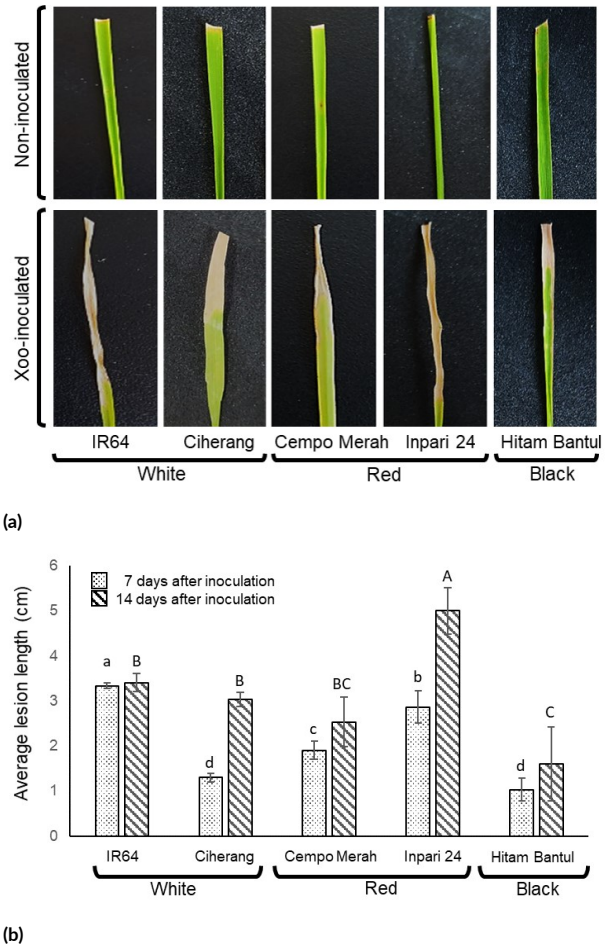

FIGURE 4 Disease symptom of the five rice varieties at 14 days post-inoculation with *X. oryzae* pv. *oryzae* (a) The lesion lengths were measured at 7 and 14 days post-inoculation (b) The error bars represent the standard deviation of the lesion lengths from 10 leaves. Not significantly different (Duncan Multiple Range Test) among the same observation indicated by the notation above the same shaded-bar indicated.

sion levels are the key factors of plant disease development. Rasmiyana et al. (2019) reported that despite the presence of several resistance genes, temperature plays the most influential role in disease incidence, and subsequently, disease severity. Moreover, Sahu et al. (2020) reported that rice variety IR24 showed different genes and pathways regulation at lower temperature of 21 to 29 °C compared to higher temperature of 31 to 35 °C. Notwithstanding, the expression level of resistance genes in rice such as *Xa1*, *Xa7*, *Xa10*, and *Xa21* has a role in inhibiting the development of BLB in rice varieties (Yoshimura et al. 1998; Ronald et al. 2008; Wang et al. 2017, 2021).

Although Ciherang and the two red rice varieties, Cempo Merah and Inpari 24, possessed *Xa1*, only Inpari 24 expressed the gene, but with a greater mean lesion length at 14 days after pathogen inoculation (Figure 3). The difference of expression level for each cultivar was highly influenced by the ineffectiveness of resistance gene expression to inhibit the development of *Xoo* that may be due to pathogen race differences. The *Xa1* gene confers resistance specifically against race 1 strains of *Xoo* originating from Japan (Yoshimura et al. 1998), while the *XooJ2* strain is a species isolated from Indonesia (Rasmiyana et al. 2019). Similar to the expression of *Xa1*, a resistance gene associated with transmembrane protein synthesis (Yoshimura et al. 1998), *Xa21* is also responsible for a transmembrane protein specific for *Xoo* isolates from race 6 strains originating from the Philippines (Ronald et al. 2008). Although the expression in IR64 increased after *Xoo* inoculation, it did not significantly inhibit the mean lesion length progression (Figure 3). Yoshimura et al. (1998) reported that *Xa21* could be ineffective against *Xoo* if protein fail to recognize specific pathogen ligands for defense response activation.

Although IR64 and Inpari 24 had almost the same gene expression pattern, they differed in average lesion length (Figure 4). This result is likely due to *Xa7*, which is only present and expressed in Inpari 24 (Figure 3). *Xa7* plays a role in encoding orphans that trigger programmed cell death in rice and lead to the enduring resistance of rice to *X. oryzae* pv. *oryzae* (Wang et al. 2021). This gene is prevalent in *O. sativa* subsp. *indica* and inhibits the exploitation of the sucrose transporter protein (sugars will eventually be exported by Transporter-14 [SWEET14]) by the pathogen through effector AvrXa7. The effector induces the synthesis of the SWEET14 protein required for the rapid multiplication of *Xoo* in plants. The inhibition of SWEET14 production by the *Xa7* gene product causes the infected rice to be more resistant to the pathogen (Luo et al. 2021).

Xa4 expression in inoculated rice decreased in quality, indicating loss of expression (Figure 3). This result indicates that weakening the cell wall was occurring after pathogen inoculation. The *Xa4* is a gene associated with cell wall synthesis, so its expression will strengthen cell walls (Hu et al. 2017). González et al. (2012) reported that *Xoo* breaks the integrity of the cell wall of rice by producing enzymes via the type two secretion system (T2SS) resulting in the disease symptom on leaves. Decreasing the quality of *Xa4* expression may cause disease incidence in all varieties (Table 2). In Ciherang and Hitam Bantul, both had similar gene expression but the lesion length (Figure 4) and disease incidence (Table 2) results were slightly different from Cempo Merah. This is probably caused by the simultaneous expression of *Xa4* and *xa13* genes both before and after inoculation. Chu et al. (2006a) observed that the expression of the recessive *xa13* gene with

TABLE 2 Disease incidence of five rice varieties at 14 days post-inoculation with *X. oryzae*

Rice group	Variety	Disease incidence (%)
White	IR64	61.4 d
	Ciherang	22.7 a
Red-pigmented	Cempo Merah	42.3 b
	Inpari 24	42.9 b
Black-pigmented	Hitam Bantul	50.0 c

Xa4 results in less resistance than in rice plants that have these genes separately. Interestingly, only these three varieties (Ciherang, Cempo Merah, and Hitam Bantul) started to express *Xa10* before pathogen inoculation (Figure 4). *Xa10* is the gene that encodes the transcription activator-like (TAL) effector-dependent responsible for pathogen effector recognition (Wang et al. 2017). Recognition of the pathogen effector triggers the plant resistance mechanism (Zeng et al. 2015). This may affect that these varieties were less lesion than in IR64 and Inpari 24 that expressed *Xa10* only after pathogen inoculation.

This study suggests that the possession and expression of *Xa* genes play an important role in rice resistance. However, the expression of *Xa* genes, particularly *Xa10* is suggested as the most considered resistance genes from among five varieties for further selection for molecular rice breeding.

4. Conclusions

Our results showed that the selection of source resistance is not pigment-based but it depends on the composition and expression of the resistance genes. The results also showed that possessing resistance genes does not guarantee the resistance of a rice variety to *Xoo*. Notably, possessing several *Xa* genes, particularly recessive genes, can trigger the susceptibility of rice to *Xoo*. Therefore, the accurate selection of gene source and type of *Xa* gene is essential for producing new rice varieties resistant to *Xoo*.

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Authors' contributions

NEN performing experiments and collecting data. WIDF analyzing the data. AW conducting experimental design. HSA conducting experimental design and data analysis. NEN, WIDF, AW, HSA wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors have declared that they have no competing interests.

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