

DETECTION OF GENES RESISTANT TO BACTERIAL LEAF BLIGHT IN RICE CULTIVARS FROM SITUBONDO AND JEMBER, INDONESIA

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ABSTRACT

Detection of genes resistant to bacterial leaf blight in rice cultivars from Situbondo and Jember, Indonesia. *Xanthomonas oryzae* pv. *oryzae* (Xoo), causing bacterial leaf blight, is a destructive pathogen that significantly affects rice production. The use of resistant varieties is the most effective and economical strategy to reduce the impact of the disease. This study aimed to analyze the genetic basis of resistance to Xoo in rice. The incidence and severity of bacterial leaf blight were assessed in the field through a diagonal random sampling method. PCR was used to detect resistant genes in rice with eight *Xa*-specific primers. The presence of *Xa* genes and environmental conditions were statistically analyzed to determine whether the disease incidence and disease severity were related to average temperature and rain intensity. The means for disease incidence and severity at the generative stage were higher than those at the vegetative stage. The 12 rice cultivars were categorized into four groups based on the presence of *Xa* gene sequences; most cultivars had *Xa10* and *Xa13*, and one cultivar had five *Xa* genes. Disease incidence and severity were weakly correlated to the presence of resistance genes, as well as to environmental factors. Most rice cultivars from the Regencies of Jember and Situbondo carry *Xa10* and *Xa13* resistance genes.

Key words: bacterial leaf blight, *Xa* genes, *Xa* specific primers, *Xanthomonas oryzae*

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important agricultural crops in the world. In Indonesia, rice is a main food crop, which has seen a continuous increase in production. However, plant pathogens, such as bacterial leaf blight, blast, tungro, and leafhopper (Almeida *et al.*, 2016), have a negative impact on rice production in Indonesia. One destructive disease that significantly affects rice production is bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae*. This pathogen can infect vegetative and generative phases of both resistant and susceptible cultivars of rice. However, infection by pathogen of BLB during vegetative stages may have a potentially more severe effect (Naqvi, 2019). The pathogen can cause yield loss, reaching 70–80%, depending on the resistance level of the rice cultivar, the growth stage, and environmental factors (Wahyudi *et al.*, 2011).

The control of BLB through the use of chemical pesticides, biological agents, plant extracts, and chitosan is still far from successful (Nisha *et al.*, 2012). In

addition, chemical pesticides continue to contaminate the environment, including the soil, water, turf, and other vegetation (Aktar *et al.*, 2009). It is imperative that alternative control strategies, including the use of resistant varieties, that are effective, efficient, and economical are considered. Some varieties show different levels of resistance against different pathotypes of BLB (Susanto & Sudir, 2012). The differences in resistance to the pathotypes are related to the presence and expression of resistance genes in rice plants (Niño-Liu *et al.*, 2006). About 42 *Xa* genes have been identified from rice cultivars, and are responsible for playing an essential role in the resistance against BLB (Arunakumari *et al.*, 2016).

In Indonesia, the development of resistant cultivars has significantly increased through conventional and molecular breeding. Selection of germplasm carrying resistant genes is the crucial step for preparing parental resources. The presence of resistance genes in rice can be determined by using polymerase chain reaction (PCR)-based detection. Homozygous and heterozygous pairings of different combinations of the BLB resistance

genes *Xa1*, *Xa2*, *Xa3/Xa26*, *Xa4*, *Xa5*, *Xa7*, *Xa10*, *xa13*, and *Xa21* have been used to develop new resistant rice cultivars (Perumalsamy *et al.*, 2010; Singh *et al.*, 2015). Recently, rice cultivars carrying 2–4 of the 42 *Xa* genes have been reported (Arunakumari *et al.*, 2016). Therefore, it is important to screen the genetic potential of local varieties of rice to identify superior candidates to provide sufficient data for producing resistant cultivars through molecular breeding. This study screened eight BLB resistance genes (*Xa1*, *Xa3*, *Xa4*, *Xa5*, *Xa7*, *Xa10*, *Xa13*, and *Xa21*) in several rice varieties cultivated in Situbondo and Jember Regencies of Indonesia. In addition, a correlation analysis was performed to understand which factors influenced the highest resistance in rice against *X. oryzae*.

MATERIALS AND METHODS

Research Site. Twelve rice varieties, six each from different geographical locations in each of Jember and Situbondo Regencies of Indonesia (Figure 1), were collected in March 2018. The rice varietal name, developmental stage of the plant, and field location were recorded for each rice variety (Table 1). All samples were collected, placed in plastic bags and stored at -20 °C until further use.

Assessment of Disease Incidence and Severity. Disease incidence and severity of BLB were assessed in the field through a diagonal random sampling method with 15–20 clumps per location. Disease incidence was calculated by the equation:

$$DI = \frac{n}{N} \times 100\%$$

DI = disease incidence,
n = number of symptomatic plants,
N = the total number of plants observed.

In addition, disease severity was calculated at the time of observation using the equation:

$$DS = \frac{\sum ni \times vi}{V \times Z} \times 100\%$$

DS = disease severity (%),
ni = number of clumps with disease scale value i,
vi = value of disease scale i,
V = highest scale value,
Z = number of clumps observed.

The disease scale was scored by Mew & Misra (1994): 0 = no symptoms; 1 = 1–5% blight; 3 = 5–12% blight; 5 = 12–25% blight; 7 = 25–50% blight; 9 = 50–100% blight.

In addition, the resistance of rice to BLB was categorized by the incidence of the disease as follows: resistant (0–35% incidence); moderately resistant (36–70% incidence); and susceptible (>70% incidence) (Ilaio & Lastimosa, 1985). The category of resistance was based on severity, in accordance with the standard evaluation system, as follows: resistant (1–5%); moderately resistant (>5–12%); moderately susceptible (>12–25%); susceptible (>25–50%); and very susceptible (>50%) (Mew & Misra, 1994).



Figure 1. Locations of sample collection in two Regencies, Jember and Situbondo, of East Java, Indonesia. Asterisks (*) represent sampling areas.

Isolation of Plant DNA. Plant DNA was isolated from 0.5 g of rice leaves by grinding in liquid nitrogen, and dissolving in 500 µl extraction buffer (containing 0.8 M guanidine thiocyanate, 10 mM EDTA, 5% Tween 20, 0.5% Triton X-100, 50 mM HEPES-acid), 25 µl of 20% sodium dodecyl sulfate, and 1.25 µl of β -mercaptoethanol. The sample was extracted by following the standard cetyl trimethylammonium bromide (CTAB) protocol, and purified by following the phenol

chloroform isoamyl-alcohol (PCI) protocol (Sambrook & Russell, 2001). The extracted DNA was precipitated in ethanol and washed with 70% ethanol before dissolving in 30 µL of TE buffer (10 mM Tris-HCL pH 8.0, 1 mM EDTA pH 8.0), to which 1 µL of RNase A (20–40 mg/mL; Sigma, USA) was added, and kept at -20 °C until use. The DNA quantity was measured using NanoVue™ Plus Spectrophotometer (GE Healthcare, UK).

Table 1. Sample codes, area of sampling, rice variety, growth stage, average temperature, and annual rainfall intensity

Codes	Area	Varieties	Plant growth stage	Average temperature (°C)*	Annual rainfall intensity (mm)*
R-S2	Panji	Inpari 13	Vegetative	25.9	1,200
R-S3	Jangkar	Ciherang	Vegetative	26.4	975
R-S5	Banyuputih	Local variety (a)	Vegetative	22.8	2,092
R-S6	Kendit	Ciherang	Vegetative	26.4	1,230
R-J1	Mayang	Cibogo	Vegetative	no data	no data
R-J2	Silo	Sidenuk	Vegetative	26.5	1,450
R-J3	Kalisat	Inpari 30	Generative	24.5	2,163
R-S4	Arjasa	Local variety (b)	Generative	26.5	1,450
R-S7	Kapongan	Local variety (c)	Generative	26.4	1,077
R-J4	Tempurejo	Cibogo	Generative	no data	no data
R-J5	Ajung	Patiwi	Generative	26	2,143
R-J6	Jenggawah	Aris	Generative	26	1,952

* Source: <https://en.climate-data.org/>

Table 2. Target resistance genes, primer sequences, annealing temperatures, and product sizes

No.	Target genes	Primer sequences	Annealing temperature (°C)	Product (bp)
1	<i>Xa1</i>	F: 5'-ACTGCCCTCTTGACACACGCCTTTGG-3' R: 5'-CCGGTACATCAGTATTGTCCATCGG-3'	66	447
2	<i>Xa3</i>	F: 5'-CCACAATGCCATGTCAGGTGGCATCCCTGCA-3' R: 5'-AGGTGTTGGAGGATTGGCAT-3'	67	255
3	<i>Xa4</i>	F: 5'-ATCGATCGATCTTCACGAGG3' R: 5'-TGCTATAAAAAGGCATTCGG-3'	53	150
4	<i>Xa7</i>	F: 5'-CGATCTTACTGGCTCTGCAACTCTGT-3' R: 5'-GCATGTCTGTGTCGATTCGTCCGTACGA-3'	65	1,170 or 294
5	<i>Xa10</i>	F: 5'-CAACGCCTATCTTCTGCATTTTC-3' R: 5'-GTGACCCTAGTTTCTGGTTATG-3'	53	604
6	<i>Xa5</i>	F: 5'-AGCTCGCCATTCAAGTTCTTGAG-3' R: 5'-TGACTIONTCTCCAAGGCTT-3'	57	200, 300, or 400
7	<i>Xa13</i>	F: 5'-GGCCATGGCTCAGTGTATAT-3' R: 5'-GAGCTCCAGCTCTCCAAATG-3'	55	400 or 200
8	<i>Xa21</i>	F: 5'-CGATCGGTATAACAGCAAAC-3' R: 5'-ATAGCAACTGATTGCTTGG-3'	50	1400

Detection of *Xa* Genes. *Xa* gene sequences in rice were detected through polymerase chain reaction (PCR) using specific gene primers (Singh *et al.*, 2015). Eight specific primers pairs were employed to amplify particular *Xa* sequences from the DNA template in the GoTaq® Green Master Mix (Promega, USA) at different annealing temperatures (Table 2). The PCR was performed in 35 cycles with initial denaturation at 94 °C for five minutes, denaturation at 94 °C for one minute, annealing for one minute, extension at 72 °C for one minute, and the final extension at 72 °C for 10 minutes (based on the Promega protocol). The PCR products were separated in agarose gel (1.5% w/v in TAE buffer pH 8.0 (containing 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) and visualized under UV light after staining with ethidium bromide (10 mg/L) for 10 minutes.

Statistical Analysis. The analysis of variance and Pearson correlation coefficient analysis were performed

using Microsoft Excel 2016 for Windows to evaluate whether disease incidence and severity were related to plant growth stages, the presence of resistance genes, average temperature, and rainfall intensity. Results were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Disease Incidence and Severity. Disease incidence and severity observations were grouped based on the developmental stage of the plant-vegetative or generative. Overall, the mean values of disease incidence and severity in the vegetative stage (Figure 2A) were lower than those in the generative stage (Figure 2B). According to the data for disease incidence and severity, in relation to the resistance status of rice against BLB, the results showed that all varieties were in the same category of resistance, with a disease incidence of >70% and disease severity of 25–50%.

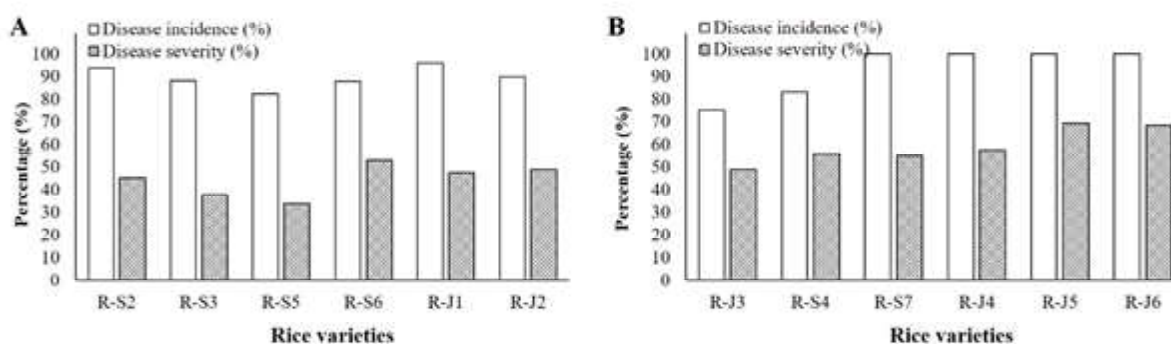


Figure 2. Disease incidence and severity data for all rice varieties at the vegetative (A) and generative (B) stages. The codes for the rice samples are the same as those listed in Table 1

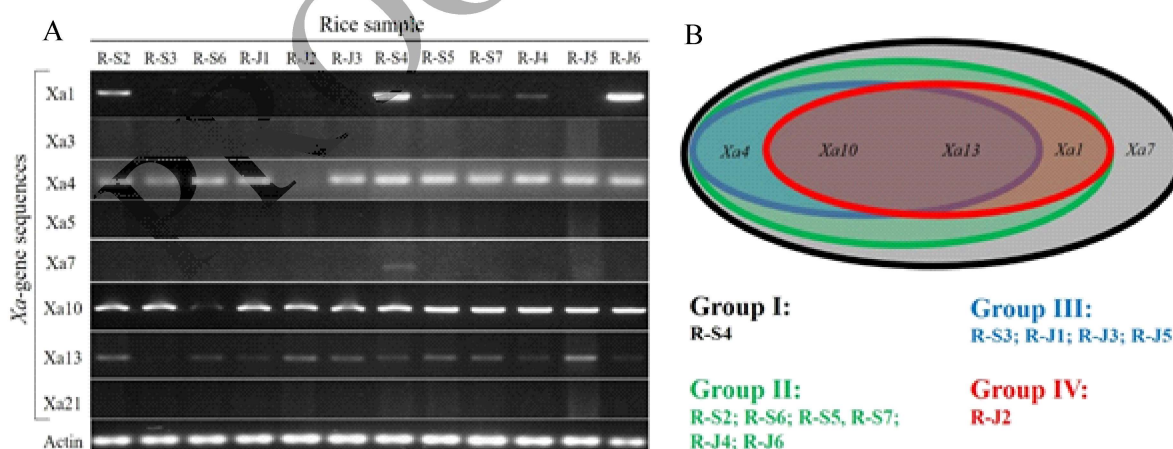


Figure 3. Rice sample codes represent the varieties given in Table 1. *Xa* gene sequences were amplified using primer pairs listed in Table 2. Actin was used as a control primer for all samples (A). All rice varieties are categorized into four groups according to the number of *Xa* gene sequences detected in the genome (B). Each group is represented by a different color according to the *Xa* gene composition (enclosed within the colored circles).

Detection of *Xa* Resistance Genes in Rice. The PCR assay to detect the presence of particular *Xa* gene sequences showed that at least five *Xa* genes were present in most varieties, except for *Xa3*, *Xa5*, and *Xa21* (Figure 3A). Accordingly, the rice varieties were categorized into four groups. Group I included the rice varieties containing the most number of resistance genes (all five genes); only one local variety (R-S4) collected from Arjasa, Situbondo Regency was in this group. Group II consisted of all the rice varieties carrying the four *Xa* gene sequences, *Xa1*, *Xa4*, *Xa10*, and *Xa14*; most of the varieties (six varieties) belonged to this group. Group III and Group IV included rice varieties that had the same number of *Xa* gene sequences but in different combinations (Figure 3B).

Analysis of Factors Related to Disease Incidence and Severity. Results showed that disease incidence and severity were related to plant growth stage, the presence of resistance genes, and average temperature and rainfall intensity. Plant growth stage (vegetative or generative) had a significant effect on disease severity

(F -value = 11.266), but not on disease incidence (F -value = 0.517; Table 3).

In addition, there was a positive correlation between the presence of *Xa* genes and disease incidence or disease severity (Table 4). However, the Pearson correlation coefficients were small ($R = 0.02$ for disease incidence and $R = 0.15$ for disease severity), indicating that the relationship between the number of *Xa* genes and disease incidence or disease severity is weak.

The correlation between environmental factors (annual rainfall intensity and average temperature) and disease incidence or severity was also weak (Table 5). Only average temperature exhibited a strong correlation to disease incidence ($R = 0.528$; $p < 0.05$); the correlation coefficient for this relationship was higher than that for the relationship with disease severity, indicating that the average temperature was more influential for disease incidence than for disease severity. In addition, the results strongly imply that disease severity is influenced by disease incidence since there was a strong positive correlation between the two ($R = 0.602$, $p < 0.05$; Table 5).

Table 3. Analysis of variance between plant growth stages (vegetative or generative) against disease incidence and disease severity

Source of variation	Statistical information			Annotation
	F -value	p -value	F -critical	
Disease incidence	0.517	0.489	4.965	ns
Disease severity	11.266	0.007	4.965	*

ns = not significant; * = significant.

Table 4. Pearson's correlation coefficient analysis between the number of *Xa* genes against disease incidence and disease severity

Source of relationship	Statistical information			Annotation
	R^2	R	P -value	
Disease incidence	0.0003	0.02	0.96	weak
Disease severity	0.0213	0.15	0.65	weak

Table 5. Comparison of the Pearson's correlation coefficient between average temperature and annual rain intensity against disease incidence and disease severity

	Annual rainfall intensity	Average temperature	Disease incidence	Disease severity
Annual rainfall intensity	1			
Average temperature	-0.692 ^b	1		
Disease incidence	-0.183 ^a	0.528 ^b	1	
Disease severity	0.288 ^a	0.408 ^a	0.602 ^b	1

a = weak correlation; b = strong correlation.

BLB, caused by *X. oryzae* pv. *oryzae*, depends on the interactions between the three disease triangle components (pathogen, host, and environmental conditions), especially when the interactions promote the development of the pathogen or disease (Agrios, 2005). The results of this study indicated that the growth stage of rice significantly influenced the severity, but not the incidence of BLB (Table 3). In addition, disease severity at the generative stage was higher than the vegetative stage, with the pathogen continuing to grow and the disease continuing to increase in severity from the vegetative stage (Figure 2A) to the generative stage (Figure 2B). This probably because rice is more susceptible to *X. oryzae* infection at the vegetative stage rather than at the generative stage, and by the time plants reach the generative stage, the disease has become more severe (Khaeruni *et al.*, 2014). Furthermore, the impact of disease on yield is theoretically influenced by several factors, such as the resistance of the cultivar and environmental factors (Yoshimura *et al.*, 1998).

Plant resistance is genetically dependent on resistant genes (*R* genes) that interact with pathogens, triggering resistance mechanisms (Vikal & Bhatia, 2017). In rice, about 42 *Xa* resistance genes have been identified and studied (Webb *et al.*, 2010). However, this study showed that the presence of resistance genes only had a weak effect on disease incidence and severity in rice (Table 4). Nevertheless, the genetic analysis showed that all rice cultivars in Jember and Situbondo possess more than three *Xa* genes (Figure 3A); they were categorized accordingly into four groups depending on the *Xa* gene composition, with most rice cultivars carrying the *Xa10* and *Xa13* genes (Figure 3B).

Theoretically, each *Xa* gene has specific function and role in the defense mechanism against the BLB pathogen (Vikal & Bhatia, 2017). In particular, the *Xa1* gene detected in cultivar Groups I, II, and IV (Figure 3B) play a role in recognizing pathogens and increasing the efficiency of interaction with the *avr* gene (Yoshimura *et al.*, 1998). In contrast to the *Xa1* gene, the *Xa4* gene detected in cultivars in Groups I, II, and III has a function in increasing the number of incompatible reactions between pathogens and hosts (Vikal & Bhatia, 2017). Uniquely, the *Xa7* gene present (together with *Xa1*, *Xa4*, *Xa10*, and *Xa13*; Figure 3A) only in the local variety R-S4 from Situbondo provides a mechanism for resistance through the biosynthesis of abscisic acid (Cohen *et al.*, 2017). Although all cultivars carry *Xa* genes, all are susceptible to *X. oryzae* (Figure 2). Interestingly, rice varieties with *Xa10* and *Xa13* genes

(known to encode, respectively, the executor of R protein in triggering a hypersensitive response and activating resistance, and transcription activator-like effectors), are more susceptible to BLB (Antony *et al.*, 2010; Jeung *et al.*, 2006; Wang *et al.*, 2017). It is noteworthy that *Xa10* and *Xa13* are in all cultivars included in the susceptible resistance category in this study.

Environmental factors, such as wind, rain, irrigation, contact between plants, and others, may influence the spread of pathogens (Agrios, 2005). This study suggests that BLB incidence is influenced by the average temperature of both Regencies (Table 5). Optimally, the BLB pathogen grows at a temperature range between 25 °C and 30 °C (Niño-Liu *et al.*, 2006). The average temperature observed in the rice fields of both Regencies, 22.8–26.5 °C, is well within the optimal range for the pathogen. Therefore, temperature may represent the most probable factor influencing BLB incidence and severity in Jember and Situbondo. This is supported by our finding that temperature has a strong impact on disease incidence. In addition, disease incidence and severity are strongly correlated due to the favorable conditions for the pathogen (Niño-Liu *et al.*, 2006). In summary, the temperature in rice fields promotes infection of rice by *X. oryzae*, resulting in a higher incidence of disease, especially at the generative stage of rice; disease severity is simultaneously higher due to survival and establishment of the pathogen, which infects rice at the vegetative stage (Khaeruni *et al.*, 2014).

Another factor influencing the development of BLB is the difference of pathogen types (pathotypes), with different pathogenic ability or virulence against host plants (Suparyono *et al.*, 2004). Interestingly, *X. oryzae* strains are grouped into 12 pathotypes based on virulence against different rice varieties, and are widespread in Java and also found in lowland areas (Suryadi *et al.*, 2016). Further identification of the pathotypes that infect rice cultivars in Jember and Situbondo is needed to obtain comprehensive information on the resistance status of all cultivars grown in these areas. This information will be useful for new rice cultivar development.

CONCLUSION

In conclusion, the results show that *Xa10* and *Xa13* genes are the most prevalent resistant genes, which are carried by all rice varieties in this study. One rice cultivar carries all five *Xa* genes (*Xa1*, *Xa4*, *Xa7*, *Xa10*,

and *Xa13*). However, the presence of resistance genes, plant growth stage, and rainfall intensity have no strong correlation with disease incidence or severity. However, temperature has a strong influence on BLB disease incidence.

ACKNOWLEDGMENTS

The authors would like to thank the Ministry of Research, Technology, and Higher Education, the Republic of Indonesia (through the University of Jember) for funding this research through the Fundamental Research Grant Scheme (no. 061/SP2H/LT/DRPM/2019). Data collection was partially supported under the Hibah Pendukung IDB 2018 (contract no. 2944/UN25.3.1/LT/2018).

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