

## Donor parental determination for breeding the RD41 rice cultivar to improve bacterial blight resistance

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**ABSTRACT:** Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *Oryzae* (*Xoo*) is one of the most critical rice diseases in Southeast Asia. RD41 is one of the most popular rice varieties in Thailand's irrigation areas due to its early maturity and high productivity. However, it is highly susceptible to BB. To improve RD41's BB resistance, an experimental breeding program was conducted, in which ten rice varieties, five Thai recommended, three newly introduced varieties and two susceptible check were evaluated in order to identify a parental variety. Three *Xoo* isolates collected from Thailand's Northeast region were selected for artificial inoculation. Lesion length was observed at five days after inoculation (DAI). IR62266, one of the newly introduced varieties, was resistant to all *Xoo* isolates, and was greatest in broad-spectrum resistance (BSR); whereas the Thai varieties SPR1, SPR60, and SPR90 were susceptible with 0.00 BSR. KDML105, the susceptibility check variety demonstrated a high percentage severity (85%), whereas IR62266 was 35.5% at 9 DAI. Likewise, the areas under the disease development progress curve (AUDPC) was low for most of the proposed varieties. To confirm resistance, the F<sub>2</sub> population from a cross of RD41 X IR62266 illustrated a ratio of single dominant resistance. IR62266 has proven to have a high potential as a donor parent for breeding programs in which to achieve successful BB resistance.

**Keywords:** early maturity rice, disease resistance, backcross breeding.

### Introduction

Rice is a staple food for nearly half of the world's population (Thomas and Dobermann, 2002). Its production increases yearly (0.67 percent per year) to meet the enhanced demand of an ever-increasing population (Prasad *et al.*, 2017). In Thailand, rice is major food crop which occupies 11.53 million hectare of total planted area, that generated 4,358 million Baht in export value in 2018 (Office of Agricultural Economics, 2019). The Northeast region of Thailand is the largest agricultural sector representing 63.10 % of Thailand's total rice cultivation area. However, the rice yield in this area (354 kg/rai) is lower than that of Thailand's Central region (3.9 ton/ha), particularly the second rice crop, due to the poor resource base, such as infertile and sandy soil, the limited availability of surface water (Limpinuntana *et al.*, 1982), the undulating terrain, and limited irrigation (Tuyet and Patrick, 2018);

which can lead to drought stress, particularly in the second crops. Therefore, high yielding varieties with a short-duration to harvest must be utilized to increase yield productivity amid irrigation limitations. The RD41 rice variety has become popular due to high productivity, as well as its early maturity with 105 days to harvest, making it one of the best choices for second rice production in the Northeast region. However, the RD41 is susceptible to bacterial leaf blight (BB) (Rice Thailand, 2016) caused by *Xanthomonas oryzae* pv. *Oryzae* (*Xoo*), which is the most destructive disease affecting rice production and was first found in 1884 in Fukuoka, Japan (Ou, 1985). In Asia, it reduces grain quality, as well as up to fifty percent of total crop yield (Noh *et al.*, 2007), while in Thailand BB disease has spread to many rice growing regions, especially in irrigated and rainfed lowland ecosystems (Mew, 1987)

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To sustain productivity and prevent the outbreak of pathogens, the introgression of BB resistant genes into the RD41 through breeding programs is one possibility. However, a successful breeding program requires the determination of a genetic source of resistance. To date, more than 40 resistance genes have been identified, some of which have also been characterized (Nino-Liu *et al.*, 2006; Verdier *et al.*, 2011; Bhasin *et al.*, 2012; Zhang *et al.*, 2015; Kim *et al.*, 2015). Most of the genes were found in Asian rice accessions (*Oryza sativa* L.), of which 15 [*xa5*, *xa8*, *xa13*, *xa15*, *xa19*, *xa20*, *xa24*, *xa25*, *xa26b*, *xa28*, *xa31*, *xa32*, *xa33*, *xa34*, and *xa41(t)*] were recessive, whereas the remaining genes were dominant (Chen *et al.*, 2011; Liu *et al.*, 2011; Kim *et al.*, 2015; Hutin *et al.*, 2015). Several of the proposed varieties carrying major resistant genes, such as *Xa1*, *Xa4*, *xa5*, *Xa7*, *xa8*, *xa13*, *Xa21*, and *Xa27*; have been used to improve BB resistance in breeding programs (Rao *et al.*, 2002). The *xa5* gene, derived from the IR62266 variety, was reported as having broad-spectrum resistance against *Xoo* in central parts of Thailand (Pattawatang, 2005), as well as numerous Asian *Xoo* strains (Adhikari *et al.*, 1995). The *Xa21* dominant gene, a broad-spectrum BB resistant gene first found in the wild species *Oryza longistaminata* (Khush *et al.*, 1989), was then introgressed into the IRBB21 variety which proved effective against the *Xoo* race in Southeast Asia (Ronald, 1997). The *Xa4* resistance gene, found to have durable resistance in many commercial rice cultivars and present in 89.68% of Thai rice germplasm, contains cultivars carrying an allele similar to that of the *Xa4* resistance line, which demonstrated moderate resistance to at least one isolate test (Sombunjitt *et al.*, 2017). In addition to the proposed varieties, several Thai

recommended varieties for BB resistance were tested and approved under the Thai Ministry of Agriculture and Cooperatives (MOAC), including Suphan Buri 1, Suphan Buri 2, Suphan Buri 60, Suphan Buri 90, and RD31. As most of these varieties were developed from rice containing resistance genes, they may possibly be identified as a genetic source of BB resistance as well.

Our objective in this study was to identify resistant parental lines against the BB pathogen resulting in future RD41 rice cultivars as BB resistance genes for breeding programs within the Northeast region.

## Materials and methods

### *Varietal evaluation for resistance to bacterial blight*

The experiment was conducted in a greenhouse at the Agronomy Field Crop Station, Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand (16°26'48" N, 102°49'58" E, 167 m), in 2015. The trial was laid out using a completely randomized design (CRD) with four replications. Five Thai recommended varieties (SPR1, SPR2, SPR60, SPR90, and RD31), two susceptible check varieties (RD41 and KDML105) from the Ministry of Agriculture and Cooperatives (MOAC) (Rice Thailand, 2016) and three newly introduced varieties (IRBB5, IRBB21, and IR62266) with well-known resistance to BB (Blair and McCouch, 1997) (Ronald, 1997) from the International Rice Research Institute (IRRI) were evaluated against three *Xoo* isolates (BB2009-758 and BB2009-348, collected from Ubon Ratchathani; and BB2009-1066, from Udon Thani) as well as a mixture of the three isolates (**Table 1**).

**Table 1** Rice genotypes tested and *Xoo* isolates used for inoculation

Rice genotypes				<i>Xoo</i> isolates			
Origin	Name	Gene for resistance	Disease phenotype	Isolate	Cultivar isolated from	Province	Year of collection
IRRI	IRBB5	<i>xa5</i>	Resistant	BB2009-348	NA <sup>2</sup>	Ubon Ratchathani	2009
IRRI	IRBB21	<i>Xa21</i>	Resistant	BB2009-758	KDML105	Ubon Ratchathani	2009
IRRI	IR62266	<i>xa5</i>	Resistant	BB2009-1066	RD6	Udon Thani	2009
MOAC <sup>1</sup>	SPR1	<i>Unknow</i>	Resistant				
MOAC	SPR2	<i>Unknow</i>	Resistant				
MOAC	SPR60	<i>Unknow</i>	Resistant				
MOAC	SPR90	<i>Unknow</i>	Resistant				
MOAC	RD31	<i>Unknow</i>	Resistant				
MOAC	KDML105	-	Susceptible				
MOAC	RD41	-	Susceptible				

1/ Minister of Agriculture and Cooperatives (MOAC), Thailand

2/ Indicates that information is not available.

The seeds of the ten varieties were soaked for 24 hours and moistened for three days. The pre-germinated seeds were sown in plastic trays with three plants per replication. Fertilizer (15-15-15) at the rate of 156.25 kg ha<sup>-1</sup> was applied at 15 days after sowing (DAS). All isolates were cultured on nutrient agar (NA) at 28-30°C for 72 hours. The cultured bacteria were harvested and diluted by sterile distilled water to obtain a suspension, adjusted to 0.6 of OD<sub>600</sub>. The uppermost fully expanded leaf of a given individual plant was inoculated through the clipping method (Kauffman *et al.*, 1973) at 21 DAS.

#### F<sub>2</sub> Observation for BB Resistance

Among the proposed varieties, IR62266 was used as the donor parent to cross with RD41 as the recurrent parents to develop a BB resistance segregated population. The observation of BB resistance was studied in the F<sub>2</sub> population, which consisted of 562 individual plants. The BB2009-1066 *Xoo* isolate was used for inoculation via the leaf clipping method. Five days after inoculation, lesion length (LL) was measured and classified. Lesion lengths shorter than 3 cm were deemed resistant, and those longer than 3 cm as susceptible (Chen *et al.*, 2001).

#### Data assessment

Data were collected five days after inocula-

tion (DAI). The lesion length (LL) was measured from the tip of the clipped surface. Means were calculated and used to classify the resistance reaction using the standards of the International Rice Research Institute (IRRI, 2013): resistant (R), moderately resistant (MR), moderately susceptible (MS), and susceptible (S); based on lesion lengths of 0.1-5 cm, 5-10 cm, 10-15 cm, and greater than 15 cm; respectively.

Disease reactions of bacterial blight were identified by severity index (SI) following the scale of the IRRI (2002), and were calculated through the following formula:

$$SI = \{(a_1N_1 + a_2N_2 + \dots + a_nN_n) / (\text{number of plants scored} \times 9)\} \times 100 \quad (1)$$

where, a is the score of each plant; N is the number of plants with a certain score.

The SI data were converted to the area under the disease progress curve (AUDPC) (Madden *et al.*, 2007) through the following formula:

$$AUDPC = \sum [x_i + x_{i+1}] / 2 (t_{i+1} - t_i) \quad (2)$$

Where x<sub>i</sub> represents the disease severity index at the *i*<sup>th</sup> observation, N = number of observations, and t<sub>i</sub> = time (days) of the *i*<sup>th</sup> observation.

Broad-spectrum resistance (BSR) was calculated following the method proposed by Ahn, 1994. Variance analysis and mean comparisons were then undertaken following Gomez and Gomez, 1984.

A chi-square test was used for determining the goodness of fit to the expected genetic ratios by using the formula:

Where: O = the observed frequency and E = the expected frequency.

$$\chi^2 = \sum_{i=1} \frac{(O-E)^2}{E}$$

### Results and Discussion

To identify resistant parental lines against the BB pathogen in the Northeast region that can be utilized to develop the RD41 rice cultivar, several varieties; including IR62266 (*xa5*), IRBB5 (*xa5*) (Blair and McCouch, 1997), and IRBB21 (*Xa21*) (Khush *et al.*, 1990) were evaluated together with five Thai recommended varieties that farmers usually cultivate for the prevention of BB disease. Artificial inoculation was performed with three *Xoo* isolates collected from widespread BB areas in the Northeast region. Five days after inoculation (DAI), disease response was observed by measuring the lesion length (LL) and classified. Kauffman, 1973; initially reported similar occurrences, reporting that disease symptoms first appeared 4-5 DAI in the form of curling near the cut-off portion.

The results showing the lesion lengths of the ten different varieties against four *Xoo* isolates are presented in Table 2, in which the three isolates, as well as the mixed isolate, were significant. The BB2009-758 showed the most significant damage range of 1.8-12.6 cm. The resistant variety, IRBB5, produced the shortest lesion length of 1.8 cm and was classified as resistant (1.0-5.0 cm.); whereas the SPR1 (Thai recommended variety) resulted in a lesion length of 11.4 cm, similar to that of the two susceptible checks (RD41 and KDML105). Likewise, the BB2009-348 isolate and IRBB5 variety also classified as resistant, whereas all four Thai recommended varieties (SPR1, SPR2, SPR60, and SPR90) proved moderately resistant. Interestingly, the proposed IR62266 variety expressed a strong reaction with 4.3 cm of LL and was also classified as resistant; whereas KDML105, RD41, SPR60, and SPR90 were found to be moderately susceptible, with 10.8, 11.1, 10.1, and 10.4 LL; respectively. These results imply

that the newly introduced varieties possess a greater potential against each of the *Xoo* isolates than the Thai recommended varieties. Although the Thai recommended varieties were designated as BB prone, the pathogens found in Central Thailand's rice growing area are different from those present in the Northeast region (Rice Thailand, 2016). In contrast, the IRBB5 and IR62266 varieties were developed from the same rice cultivar (DZ192) which carries the *xa5* gene, known to be an important recessive bacterial blight resistant gene, proven to be effective in several rice breeding programs throughout Asia (Naveed *et al.*, 2010). Our results also demonstrated the high average BSR value of these two varieties. Particularly, the IR62266 variety had highest the highest BSR value of 0.75, while the two susceptible checks, and even the Thai recommended varieties (SPR1, SPR60, SPR90) showed a 0.00 BSR value. The greater resistance of the varieties containing the *xa5* gene against multiple *Xoo* isolates was also reported by Saengchai, 2009; in which the *xa5* gene was highly resistant to the most aggressive pathogen testing, producing the broadest spectrum (BSR = 1) of BB resistance against 78 *Xoo* isolates collected from Thailand's Northeast region. These findings indicate the potential of the *xa5* gene as a source of resistance for BB outbreak.

These finding indicated that all varieties carrying *xa5* gene is high potential to be resistant source of BB outbreak. Even though, both of IR62266 and IRBB5 variety can also carry *xa5* gene, the BSR of IR62266 is higher than IRBB5 (0.50), indicating that IR62266 is better in resistance against several pathogen than IRBB5. These finding with result reported by Sripakhon (2009) which found that IR62266 was greater resistant against several isolates than IRBB5 variety. This revealed that IR62266 might have additional effect beside *xa5* gene Pattawatang (2005) reported the BB resistance of IR62266 was contributed by *xa5* gene and additional effect located on chromosome 4. It was concluded that IR62266 variety should be considered than IRBB5 variety for donor determination.

**Table 2** Mean comparisons of lesion lengths and reactions of ten varieties in response to the *Xoo* isolates

Varieties	Isolates												BSR
	BB2009-758			BB2009-348			BB2009-1066			MIXED			
	LL (cm.)	Reaction		LL (cm.)	Reaction		LL (cm.)	Reaction		LL (cm.)	Reaction		
SPR1	11.4	a	MS	9.4	abc	MR	8.8	abc	MR	6.7	bc	MR	0.00
SPR2	4.4	de	R	5.2	cd	MR	8.1	bcd	MR	8.3	ab	MR	0.25
SPR60	10.6	ab	MS	5.9	bcd	MR	10.1	ab	MS	8.6	ab	MR	0.00
SPR90	8	bc	MR	5.8	bcd	MS	10.4	ab	MS	6.5	bc	MR	0.00
RD31	4.1	de	R	4.6	d	R	6.4	cde	MR	7.8	ab	MR	0.50
IR62266	3.3	de	R	5.1	d	MR	4.3	e	R	4.3	c	R	0.75
IRBB21	5.2	cd	MR	6.9	bcd	MR	5.7	de	MR	7.3	b	MR	0.00
IRBB5	1.8	e	R	4.3	d	R	8.4	bcd	MR	6.3	bc	MR	0.50
KDML105	10.4	ab	MS	9.9	ab	MR	10.8	ab	MS	10.6	a	MS	0.00
RD41	12.6	a	MS	8.5	abcd	MR	11.1	a	MS	7.6	ab	MR	0.00
F-Test	**			*			**			*			
C.V. (%)	32.9			42.01			22.37			28.01			

\*\* = significant at 0.01 level \* = significant at 0.05 level

1/ mean in the same column followed by the same letter(s) are not significantly different at  $P < 0.01$  by Least Significant Difference (LSD)

The infection rate was monitored via the severity index (SI), in which the severity of leaf infection (as a percentage) was observed at 5, 7, and 9 DAI (**Figure 1**). As our results indicate that differences in resistance of Thai recommended and introduced varieties to *Xoo* were related to disease development over days after inoculation. Our results indicated that the disease resistance of the proposed and Thai recommended varieties varied in development over time. Disease severity typically began at lower levels and gradually increased over time. In the susceptible KDML105 variety, disease development was higher than that of the resistant varieties, such as IR62266, which expressed strong resistance to low severity percentages of all *Xoo* isolates. Particularly within the mixed isolate at 9 DAI (**Figure 1D**), KDML105 showed a high development of severity (85.8%) in comparison to IR62266 (35.5%). The BB2009-348 isolate (**Figure 1B**), among the three proposed varieties (IRBB5, IR62266, and IRBB21) expressed low percentages of severity at 9 DAI, at 28.1%, 29.9%, and 31.3%; respectively; which were in contrast to both the SPR2 (39.5%) and RD41 (51.4%). These findings indi-

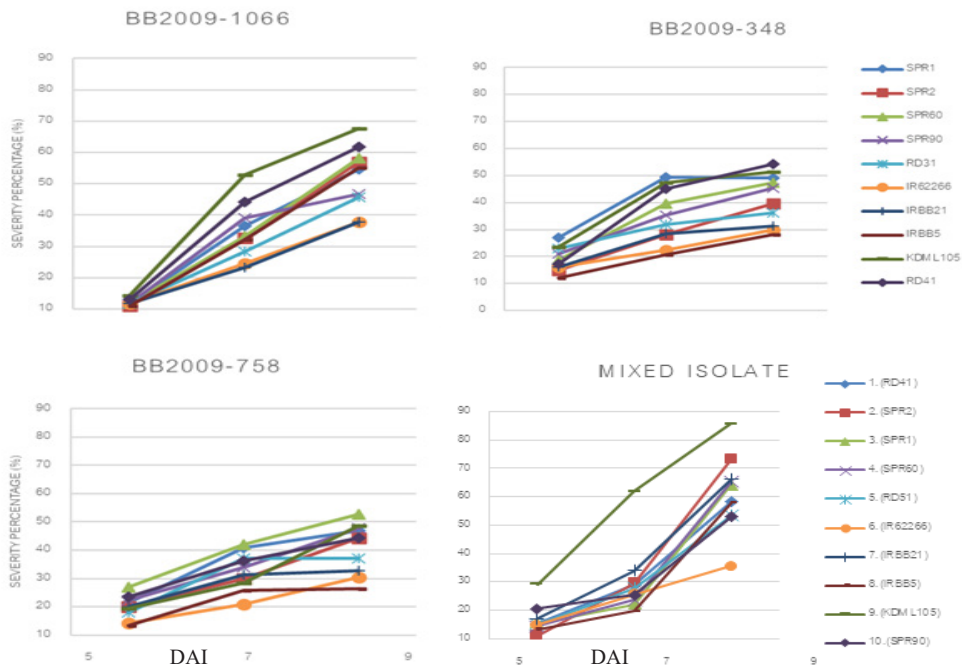
cate that SPR2 can decrease *Xoo* infection rates just as those introduced varieties that are known to contain the BB resistance gene. As a result, the SPR2 was cross-pollinated with RD23 and IR60, and further developed under the Suphanburi Rice Research Center. Sombunjitt *et al.*, 2017; reported that RD23 proved resistant to all *Xoo* test isolates, as well as carrying an allele found in the resistance line of *Xa4*. Although the *Xa4* gene has been used for more than 30 years and has introgressed in several high yielding varieties throughout Asia, the widespread cultivation of varieties has led to the predominance of *Xoo* races capable of overcoming the *Xa4* gene (Mew *et al.*, 1992). Therefore, the continuation of major BB resistant genes, such as *xa5*, *xa13*, and *Xa21* have become more appropriate for utilization in current breeding programs.

BB disease symptoms developed in all tested varieties and within the susceptible check varieties five-days post inoculation, however, the resistant plants exhibited lower disease progress than the susceptible varieties at the final data assessment 9 DAI (**Figure 1**). In earlier research, Iyer-Pascuzzi *et al.*, 2008; hypothesized that



the difference in disease progression, due to the movement of bacteria within the plant after infection, is different between resistant and susceptible varieties. **Figure 2** depicts the phenotypes of the five Thai recommended varieties (SPR1, SPR2, SPR60, SPR90, and RD31), the three proposed resistant varieties (IRBB5, IRBB21, IR62266), and the two susceptible check varieties (RD41, KDML105). The infected leaves of the KDML105 varieties turned grayish and

rolled up upon disease progression at 9 DAI, whereas the BB resistant varieties, particularly IR62266 and IRBB21, developed yellow lesions on the clipped surface, notably less than the margins of the two susceptible varieties. Adhikari and Mew, 1994; also reported that differences in *Xoo* resistance in rice were related to disease developments over time, which were more rapid in the susceptible varieties.



**Figure 1** Severity index of the 10 rice varieties during 5, 7 and 9 days after inoculation (DAI) against BB2009-758 (A), BB2009-348 (B), BB2009-1066 (C) and Mixed isolated (D)



**Figure 2** Phenotypes of the five Thai recommended varieties (SPR1, SPR2, SPR60, SPR90, RD31), three introduced resistant varieties (IRBB5, IRBB21, IR62266), and two susceptible check varieties (RD41, KDML105), after 9 DAI under the BB2009-1066 isolate

To understand disease progression, areas under the disease progress curve (AUDPC) were developed by plotting the severity index (%) over time (**Figure 3**). While the progression values of each variety varied slightly, lower disease progress was found mostly in the introduced varieties, the IR62266 variety in particular, in which the AUDPC value was below 138.46 in all isolates. Higher values were found in the two susceptible varieties, SPR1 in the BB2009-348 isolate, and SPR60 in the BB2009-758 isolate, at 199.52 and 206.59, respectively. These results indicated that the varieties containing the *xa5* gene generated less disease development (**Figure 3**). Iyer-Pascuzzi *et al.*, 2008; demonstrated that a model of *xa5*-mediated recessive resistance was able to delay the movement of bacteria, resulting in the reduction of disease development. We determined this gene to be highly efficient in reducing disease infection at the initial growth stage.

IRBB21, which possesses the *Xa21* gene, exhibited significant disease progress similar to

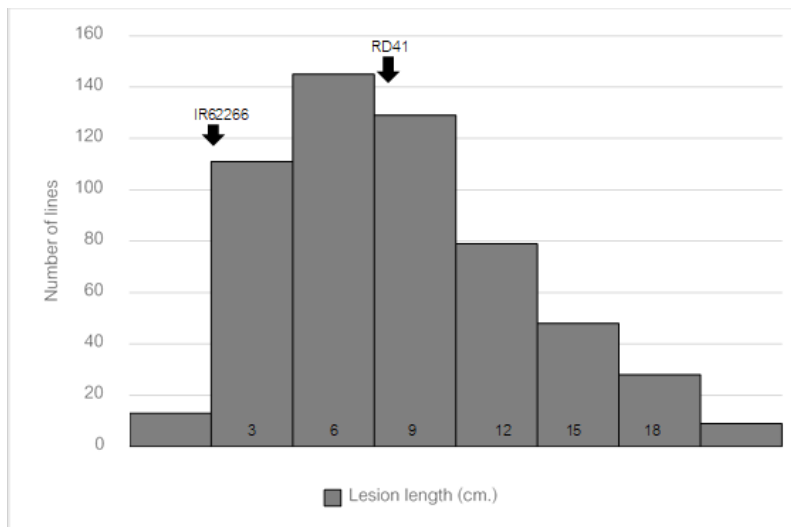
that of the two susceptible checks, particularly with the mixed isolate, presenting an AUDPC value of up to 193.70. Normally, rice resistance genes are capable of expressing higher levels of disease resistance and higher AUDPC values only within the adult stage. In contrast, the *Xa21*-mediated resistance increased progressively in the susceptible varieties at the juvenile stage, to full resistance at the later adult stage. (Mazzola *et al.* 1994; Win *et al.* 2012). This explains why the IRBB21 variety lacks fully resistant capabilities within our study. However, future pyramiding of gene combinations with varied modes of action can produce durable and broad-spectrum resistance in future breeding programs (Pradhan *et al.*, 2015). These finding is the reason why IRBB21 variety unable to fully resistance in this study. However, the pyramiding in the future of gene combination with IRBB21, where *Xa21* contributes the largest portion of resistance will be most effective (Sabar *et al.*, 2016)



**Figure 3** The area under the disease progress curve (AUDPC) of 10 varieties responded to BB2009-758 (A), BB2009-348 (B), BB2009-1066 (C), 348 758 and mixed isolate (D)

According to Supakin, 2007; pathogen isolates from Northeastern Thailand possess the genetic diversity to increase the efficiency of BB resistance, and may be distributed to all parts of the region. Supakin reported that the IR62266 variety, with the highest BSR value of 0.75, was strong enough to enhance the BB resistance of the RD41. To further ensure the development of BB resistance, the  $F_2$  population was derived from a cross between the RD41 and IR62266 varieties. Continuous distributions of LL were observed within the  $F_2$  Population (Figure 4), where the average lesion lengths of the RD41 and IR62266 varieties were 7.1-8.0 and 1.1-2.0 cm, respectively; whereas most of the  $F_2$  progenies were skewed toward susceptible. However,

determinations based on lesion length can cut-off the number of resistant and susceptible lines within the  $F_2$  population at 124 and 438 lines, respectively. These segregation ratios fit well within the expected 1R:3S at  $\chi^2 = 2.58$ ,  $P=0.11$ . The results further confirm that BB resistance in the IR62266 variety is governed by a single recessive gene. We may also note that the RD41 progenies carrying homozygous IR62266 alleles (*xa5/xa5*) exhibited a similar high resistance to that of the donor parent, the IR62266. We, therefore, determined that selection of the IR62266 as a donor parent for the RD41 will offer improved resistance to bacterial blight disease.



**Figure 4.** The distribution of lesion lengths after isolate infection of the BB2009-1066 in a sample containing 562 random individuals from the  $F_2$  population, derived from a cross between the RD41 and IR62266. The average lesion lengths of the RD41 and IR62266 were 7.1-8.0 and 1.1-2.0 cm, respectively.



## Conclusion

Our work herein has attempted to improve the progress of existing rice breeding programs against bacterial blight disease in Northeast Thailand. Among the varieties introduced, the IR62266 carrying the *xa5* gene presented the broadest spectrum of disease resistance though the accompanying isolates. We have therefore concluded that the RD41 variety bearing the resistant gene will provide effective resistance to BB disease, suitable for development, particularly in second rice production in Thailand's Northeast region.

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